

**Studies on soil microbial diversity of Darjeeling
hills and their evaluation for utilization in the
improvement of crop health**

Thesis submitted to the
University of North Bengal
For the award of Doctor of Philosophy
in
BOTANY

by

Kiran Sunar

Supervisor

Professor B.N. Chakraborty

Co-Supervisor

Professor U. Chakraborty

Immuno-Phytopathology Laboratory

Department of Botany

University of North Bengal

Raja Rammohunpur, Siliguri

AUGUST, 2013

DECLARATION

I declare that the thesis entitled “Studies on soil microbial diversity of Darjeeling hills and their evaluation for utilization in the improvement of crop health” has been prepared by me under the guidance of Professor B.N. Chakraborty and Professor U. Chakraborty, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.



(Kiran Sunar)

Department of Botany
University of North Bengal
Raja Rammohunpur, P.O.- NBU
Dist.- Darjeeling, Pin- 734013

DATE: 19-08-2013



UNIVERSITY OF NORTH BENGAL
DEPARTMENT OF BOTANY
IMMUNO-PHYTOPATHOLOGY LABORATORY



August 05, 2013

CERTIFICATE

We certify that Mr. Kiran Sunar has prepared the thesis entitled “Studies on soil microbial diversity of Darjeeling hills and their evaluation for utilization in the improvement of crop health”, for the award of Ph.D degree of the University of North Bengal, under our guidance. He has carried out the work at the Department of Botany, University of North Bengal.

(Professor B.N. Chakraborty)

(Professor B. N. Chakraborty)
Department of Botany
University of North Bengal

Supervisor

(Professor U. Chakraborty)

Professor U. Chakraborty
Department of Botany
University of North Bengal

Co-Supervisor

ABSTRACT

The present study on microbial diversity of Darjeeling Hills and their evaluation for utilization for improvement of crop health was carried out with an objective of isolation of microorganisms from rhizosphere of forests, agriculture fields as well as from riverine soils of major river basins of Darjeeling hills, their characterization and identification followed by screening for important characters like phosphate solubilization, chitin, cellulose and lignin degradation and utilization of potential isolates as bio control agents against fungal pathogens. The next phase was to evaluate the selected microorganism for plant growth promoting and disease suppressing activities and finally to analyze the molecular diversity of the selected isolates using relevant tools. In view of this object of study a review of literature has been presented which focuses on the current perspectives of Agriculturally Important Microorganisms (AIMs) in relation to plant health improvement with diverse mechanisms like soil phosphate mobilization and induction of resistance in host plant either directly or with the help of secondary metabolites.

Accordingly suitable methods were employed to isolate, characterize and identify soil microorganisms. Field, pot and nursery experiments were carried out in randomized block designs in the experimental fields and glass house of Immuno-phytopathology Laboratory, Department of Botany, University of North Bengal. Statistical analyses were conducted whenever necessary.

The results obtained on the basis of experiments conducted revealed that microbial population in soils ranged between 4×10^3 - 6×10^4 cfu in case of fungi and 5×10^6 cfu- 6×10^6 cfu in case of bacteria. A total of 637 fungal isolates were obtained from the major forest, agricultural fields and river basins of Darjeeling hills. Out of the total collection, 205 isolates were obtained from forest, 373 from agricultural and 59 isolates were obtained from river basins. Similarly, a total of 135 bacterial isolates were obtained from various sources. Among them 39 were obtained from forest soil, 73 from rhizosphere of agricultural crops and 23 from riverine soil.

On the basis of microscopical characterization and morphological studies it was found that the dominant fungal isolates belonged to the genera *Absidia*, *Acremonium*, *Alternaria*, *Aspergillus*, *Byasiochlamus*, *Colletotrichum*, *Drechslera*, *Emericella*, *Fusarium*, *Curvularia*, *Gonronella*, *Macrophomina*, *Noesertoria*, *Paecilomyces*, *Penicellium*, *Pseudoeutatum*, *Rhizoctonia*, *Rhizopus*, *Sclerotianum*, *Sporotrichum*, *Syncephalastrum*, *Talaromyces*, *Thanetophorus* and *Trichoderma*. Whereas the most common and abundant bacterial species were *Bacillus sp.*, *Micrococcus sp.*, *Coryneform sp.*, *Staphylococcus sp.*, *Serratiasp.*, *Paenibacillus sp.*, *Pseudomonas sp.*, *Enterobacter sp.* well as *Burkholderia sp.*

All the fungal and bacterial isolates were characterized for their agriculturally important properties *in vitro*. Out of a total of 637 fungal isolates 150 fungal isolates showed phosphate solubilizing activity as detected on Pikovskaya's (PVK) agar medium. After screening on solid medium, their phosphate solubilizing potential was quantified in liquid medium, Among them, isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromyces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) were found to solubilize rock phosphate and tricalcium phosphate more efficiently than rest of the others. One of the interesting findings of the present study was isolation of one potential fungal isolate *Talaromyces flavus* RHS/P-51/NAIMCC-F-01948, which has been reported as a potential phosphate solubilizers for the first time in this study.

Among the bacterial isolates a total of 48 bacterial isolates were found to solubilize phosphate when screened on solid medium. For quantification of phosphate solubilization in liquid medium, all the isolates were grown in modified PKV broth medium supplemented with Rock phosphate and Tricalcium Phosphate. The results revealed that isolate *Bacillus altitudinis* BRHS/S-73 could solubilize maximum amount of rock and tricalcium phosphate followed by *B. pumilus*, BRHS/C-1, *Enterobacter cloacae*, BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* BRHS/P-92 and *B. aerophilus* BRHS/B-104. All these

potential isolates were found to produce IAA, siderophore, HCN as well as a considerable amount of ACC deaminase *in vitro*.

Apart from the phosphate solubilizers, a large number of *Trichoderma* isolates were also obtained from various sources. A total of 26 isolates of *T. harzianum* 10 isolates of *T. viride*, 13 isolates of *T. asperellum* and 6 isolates of *T. erinaceium* were obtained from various sources and were tested for their ability to produce Chitinase *in vitro*. The net exo and endo chitinase activities of the isolates were determined spectrophotometrically. *T. harzianum* RHS/S-559 and RHS/S-560 obtained from the rhizosphere of *Sechium edule*, *T. viride* isolate RHS/G 251, *T. asperellum* and *T. erinaceium* RHS/Rd-551 showed maximum endo and exo chitinase activities.

In vitro tests for cellulose activities of fungal isolates were conducted and results showed that, isolates of *A. niger* (FS/L-04, FS/L-40, FS/C-140, RS/P/14, FS/Td-173 and RHS/T-198), *A. melleus* (FS/L-13, FS/L-17, FS/L-18, RHS/R-12 and RS/P-05), *A. fumigates* (FS/R-263), *A. clavatus* (RHS/P-38, RHS/T-99, and RHS/P-114), *P. digitatum* (RHS/T-455 and RHS/C-338), *P. italicum* (RHS/M-403 and RHS/P-414), *P. crysogenum* (RHS/T-269), *T. flavus* (RHS/P-54, RHS/P-51, RHS/P-50 and RHS/P-120), *T. harzianum* (RHS/S-559 and RHS/S-560), *T. viride* (RHS/B-245 and RHS/G-251), *T. asperellum* (RHS/S-561, RHS/Cd-601 and FS/L-188) and *T. erinacium* (RHS/T-626 and FS/Td-166) had comparatively higher exo and endo cellulase activities.

Both the potential bacterial and fungal isolates were tested for their antagonistic effect against the fungal pathogens. Isolates of *T. harzianum* (RHS/S-559/NAIMCC-F-01968 and RHS/S-560/NAIMCC-F-01966), *T. asperellum* (RHS/S-561/NAIMCC-F-01967), *T. erinaceium* (RHS/T-474/NAIMCC-01960) and *T. viride* (FS/L-186) showed maximum inhibitory activities against *S. rolfsii* and *T. cucumeris*. The SEM micrographs revealed that the *Trichoderma* mycelium profusely parasitized the pathogen mycelium and inhibited its growth. On the later stage of growth the pathogen was completely overgrown by the antagonists. On the other hand, *T. flavus* which showed highest phosphate solubilizing abilities *in vitro* could inhibit mycelial growth and development of *S. rolfsii* in dual culture. Sclerotial germination of *S. rolfsii* with cell free culture

filtrates of *T. flavus* showed 90-95 % inhibition in comparison to control. Similarly among the bacterial isolates viz *Bacillus pumilus*, *Enterobacter cloacae*, *Paenibacillus polymyxa*, *B. altitudinis*, *B. methylotrophicus*, *Burkholderia symbiont* and *B. aerophilus* that showed positive result for all the tested PGP characteristics were tested for their antifungal activities against the fungal pathogens *Sclerotium rolfsii*, *Thanatophorous cucumeris*, *Rhizoctonia solani* and *Macrophomina phaseolina*. All these bacterial isolates were found to inhibit the test pathogens where the average inhibition percentage ranged from 60- 80%.

Most of the commonly occurring fungal isolates, potential PSFs as well as *Trichoderma* isolates have been deposited to the National Agriculturally Important Microorganisms Culture Collection (NAUMCC) centre of National Bureau of Agriculturally Important Microorganisms (NBAIM), ICAR and accession numbers have been provided to them.

Another phase of study was the analysis of diversity among the beneficial group of microorganisms with the help of relevant tools. Among the total collection of phosphate solubilizers genetic relatedness among four isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) was carried out using decamer primers. Out of the 30 loci scored only 12 (40 %) were polymorphic. Highest level of polymorphism was recorded in primer OPD-5 (75.00 %) followed by OPB-2 (62.50 %), OPD-6 (40.00 %) and AA-5 (26%). The degree of similarity between *T. flavus* and *Aspergillus* isolates ranged from 14.00 % to 22 % (Moderate dissimilar values). PCA of the similarity coefficient values further revealed that each group of phosphate solubilizers was grouped in separate clades. Among the Biocontrol agents, isolates *T. harzianum* (RHS/S- 559, RHS/S 560, RHS/M 501, RHS/M 511) and *T. asperellum* (RHS/S 561, RHS/M 512, RHS/M 517) were found to show maximum inhibitory effect against fungal pathogens *in vitro*. Out of the 17 loci scored only 10 (58.82 %) were polymorphic and the highest level of polymorphism was recorded in primer AA-05 (62.50 %) followed by AA-11

(55.55 %) and overall the degree of similarity between *T. harzianum* and *T. asperellum* isolates ranged from 28.00 % to 71.00 % (Moderate dissimilar values). Since all the bacterial isolates were identical in their function and biochemical analysis, genetic relatedness among all the 135 bacterial isolates were carried out using decamer primers. The average number of polymorphic bands produced by the primer OPD-05 was 7 and the highest degree of polymorphism recorded was 63.63 % followed by OPD-02 (57.10 %), AA-11 (40.00%), OPD-06 (37.50 %), AA-05 (33.33%), OPA-04 (28.57 %). Similarly, Similarity co-efficient reveals that most of the bacterial isolates belonging to the same genera and species showed highest degree of similarity. Overall all the bacterial isolates were separated into four major clusters irrespective of their origin and biochemical similarities. PCA analysis of the similarity coefficient values revealed that all the bacterial isolates exhibited a wide degree of genetic diversity which has been represented with a number of dispersed points distributed in the plot area.

A second level of genetic relatedness study was conducted to analyze a specific gene sequence of selected and closely related group of microorganisms to draw variations in their genetic makeup. This was achieved with sequence data from the ITS 1 region of the ribosomal gene complex. In general, sequence data from the ITS 1 region of the selected isolates were tested which was distinguished by Denature Gradient Gel Electrophoresis (DGGE). The results supported the RAPD analysis up to a certain extent that there was a considerable amount of variability among the organisms even belonging the same genera. The DGGE analysis was also helpful to draw similarities between identified and unidentified isolates.

On the basis of *in vitro* analysis a large number of potential PGPF, BCA and PGPR isolates were obtained. The identities of all the selected microorganisms were confirmed on the basis of rDNA sequences. The rDNA sequences were amplified using universal primer pairs (both fungal 18S rDNA and bacterial 16S rDNA), sequenced and submitted to NCBI-Genbank Database where an accession number for each gene sequence has been provided.

Among all the phosphate solubilizing fungal isolates, isolate RHS-P-51 was found to be an efficient phosphate solubilizer whose identity was confirmed as *Talaromyces flavus*, the accession number for isolate *T. flavus* RHS/P-51, provided by NCBI is GU324073. Similarly, identities of BCA isolates were confirmed as *Trichoderma erinaceum* (FS/L-20, FS/S-474 FS/S-475, FS/S-478) *Trichoderma harzianum* (RHS/S-559, RHS/S-560) and *Trichoderma asperellum* (RHS/S-561). The accession number for all these isolates, provided by NCBI are HM107419, GU187915, GU191829, HM117841, HQ334995, HQ334997 and HQ334996 respectively. Among the selected potential PGPR isolates, isolate BRHS/C-1, BRHS/P-22, BRHS/R-71, BRHS/R-72, BRHS/S-73, BRHS/P-91, BRHS/P-92 and BRHS/B-104 were identified as *Bacillus pumilus*, *Bacillus altitudinis*, *Enterobacter cloacae*, *Paenibacillus polymyxa*, *Bacillus altitudinis*, *Bacillus methylotrophicus*, *Burkholderia sp.* and *Bacillus aerophilus* respectively. The NCBI Accession numbers for each isolate is JF836847, HQ849482, KC703974, KC703775, JF899300, JQ765577, JQ765578 and KC603894 respectively.

Series of *in vivo* experiments were carried out next with the selected phosphate solubilizing fungi, Biocontrol agents and PGPR isolates to determine their plant growth promoting activity in the field and potted conditions. On the basis of initial screening of fungal isolates for phosphate solubilization, *A. niger* FS/L-04, *A. melleus* FS/L-17, *A. clavatus* RHS/P-38 and *T. flavus* RHS/P-51 were found to be most efficient phosphate solubilizers. Evaluation of these isolates for enhancement of growth of six different crop plants *viz.* *Phaseolus vulgaris*, *Glycine max*, *Cicer arietinum*, *Vigna radiata*, *Pisum sativum* and *Oryza sativa* in green house condition was carried out. These PSF isolates were applied to the soils after multiplying them in farm yard manure. Seeds were then shown in PSF amended soils which resulted in significant increase in growth, measured in terms of height, leaf number and dry biomass over similar increase in control. Effect of *T. flavus* amended was found to be significantly higher in all the tested crops in comparison to the other *Aspergillus* isolates. Enhancement of growth by these phosphate solubilizing fungal isolates was directly associated with the soil phosphate mobilization. The total residual phosphate in un-inoculated soil was found to be much higher than the soil amended with PSF isolates while root and

leaf phosphate contents significantly increased in plants grown in PSF amended soil comparison to control.

Among the several isolates of PGPR obtained from different regions of Darjeeling hills, seven PGPR isolates, *Bacillus pumilus*, *Enterobacter cloacae*, *Paenibacillus polymyxa*, *B. altitudinis*, *B. methylotrophicus*, *Burkholderia symbiont* and *B. aerophilus* were selected for *in vivo* evaluation of their effects on growth different crop plants. In the first set of field trials, effect of PGPR on growth of *Vigna radiata*, *Cicer arietinum*, *Glycine max*, *Triticum aestivum* in field trials well as four varieties of tea (*Camellia sinensis*) (TV-9, TV-20, TV-25 and TV-26) in nursery conditions was evaluated. Results revealed that Seed bacterization followed by application of the bacterial isolates as soil drench to the natural environment could enhance growth of all the tested crop plants. However, *B. altitudinis* followed by *B. pumilus* could enhance growth of all the tested crops more efficiently. Growth of tea seedlings grown under same environmental and physical conditions was enhanced to a greater extent when both the bacterial isolates were applied jointly. In both the cases the growth promotion was found to be correlated with total phosphate content and phosphatase activities of the soil.

In second level of field studies the selected BCA and PGPR isolates were evaluated for their effect in reducing Sclerotial blight and root rot of different legumes and plantation crops. *T. harzianum*, *T. asperellum* were found to efficiently reduce sclerotial blight incidence of *Vigna radiata* caused by *Sclerotium rolfsii* and root rot disease of *Cicer arietinum* caused by *T. cucumeris* when applied in the soil either singly or in combination with another efficient biocontrol fungus *T. flavus*. Similarly, the PGPR under investigation were also effective in suppressing sclerotial blight of *Glycine max* and *Camellia sinensis* caused by *S. rolfsii* grown in pot and nursery conditions and root rots of *Vigna radiata*, *Lycopersicon esculentum* and *Brassica juncea* caused by *Thanatephorus cucumeris* in pot conditions. The reduction of disease by both BCA and PGPR isolates were found to be correlated with the enhancement of key defence enzymes- chitinase (CHT), β -1, 3-glucanase (GLU), Phenyl alanine

ammonia lyase (PAL) and Peroxidase (POX) which increased significantly specially in the presence of the pathogen.

The overall result of the present study has shown that there is there is a huge microbial diversity in the soils of sub Himalayan regions of Darjeeling Hills. The occurrence of functionally diverse groups of phosphate solubilizers, chitin degraders, biocontrol agents, plant growth promoting rhizobacteria in all the tested soil types suggests presence of abundant Beneficial Microorganisms in the region. RAPD and DGGE based genetic relatedness analysis of these beneficial microorganisms suggested that they were not only functionally diverse but also showed significant variation in their genetic makeup. Bio-priming of the seeds and seedlings prior to sowing and after germination proved to be effective in growth enhancement and to induce resistance against fungal root pathogens. Reduction of root diseases by both BCA and PGPR was associated with all the elements commonly known to be involved in the induced systemic resistance which were found to have been enhanced. Regarding the mechanism of action of the beneficial microorganisms, it seems probable that these organisms act through a combination of methods, it is assumed that on one hand these microorganisms secrete metabolites into the soil which in turn elicit responses in the host which was evident by differential expression of enzymes both in the roots and leaves of treated plants and on the other hand suppress pathogen population by antibiotics, HCN and siderophore secretion.

Preface

Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation, decomposition of organic matter, toxin removal and the cycling of carbon, nitrogen, phosphorus, and sulphur. In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth and changes in vegetation. With the rise in utilizing 'organic products' worldwide, the use of Beneficial Microorganisms in agriculture is considered to be the next alternative against harmful chemicals and pesticides. Considering the role of these microbes, the present study was undertaken to isolate and characterize Agriculturally Important Microorganisms from different soil types of one of the Biodiversity hot spots of the world- Darjeeling Hills and to evaluate their effect in enhancing the growth of plants.

The research work presented in this thesis is a part of work that was carried out in Immuno-Phytopathology laboratory, Department of Botany under the network project "Application of microorganisms in Agriculture and Allied sectors (AMAAS) of National Bureau of Agriculturally Important Microorganisms (NBAIM) funded by Indian Council of Agricultural Research (ICAR).

At this juncture, I am immensely grateful to my mentors Professor B.N. Chakraborty and Professor U. Chakraborty for their valuable encouragement, guidance and support in every sphere of my research whose constant guidance, valuable inputs and wise counseling have helped me to sail through this endeavor.

I am indebted to Dr. S.C. Roy, Head Department of Botany and to all my beloved teachers Prof. A.P. Das, Prof. P.K. Sarkar, Dr. A. Sen, Dr. A. Saha and Mr. P. Mandal for their unconditional help and cooperation.

I would also like to express my sincere gratefulness to the Programme Coordinators, UGC-SAP (DRS-I, DRS-II and DRS-III) for the access to the central instrumentation facility.

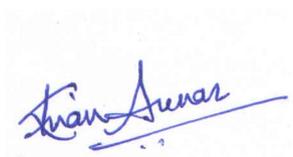
I am grateful to Professor D.K. Arora, former Director and Dr. A.K. Sharma present Director, NBAIM, for providing me the fellowship to carry out this piece of work. I would also like to thank NABIM for allowing me to undergo trainings on 'Microbial community analysis' and 'Chemical taxonomy of microbes' which were immensely helpful to execute this work in a better way. I take this opportunity to express my earnest gratefulness to Prof. P.N. Chowdhry, NCFT, New Delhi, Head Division of Plant Pathology, IARI, New Delhi, Chromus Biotech, Bangalore, and Sophisticated Analytical Instruments Facility- Scanning Electron Microscopy Unit, Bose Institute, Kolkata for identifying, sequencing of rDNA and taking the scanning electron microscopic photographs of the bacterial and fungal cultures.

My journey to this end would not have been fruitful without the support of my valued friends and seniors, I humbly appreciate and acknowledge the help and support that were imparted to me during my early days of research by Dr. Monica Sharma, Dr. Rakhee Das Biswas, Dr. Cyria Tongden, Dr. Mehrab Basnet, Dr. Bijay Moktan and Dr. Kuldeep Rai. I take this opportunity to express my heartfelt thanks to my fellow friends and colleagues Dr. P.L. Dey, Dr. Deepti Pradhan, Mr. Utanka Kr. De, Mr. A.P.

Chakraborty, Ms. Sanjita Allay, Ms. Pushpanjali Ray, Ms. Rohini Lama, Ms. Bhumika Pradhan, Ms. Nishika Jaishee, Ms. Sweata Khati, Ms. Amrita Acharya, Mr. Somnath Roy, Mr. Sibhu Barman, Ms. Basundhara Shrestha as well as all the research scholars of Immuno-Phytopathology Laboratory and Plant Biochemistry Laboratory and other researcher friends of Department of Botany and my beloved friend Late Mr. Tshering Bhutia, who were there in times of need and support. I wish to thank all the field assistants especially Late Mr. Sudarshan Tirky, for lending me their help in field experiments.

I take this opportunity to express my gratitude to my beloved mother Smt. Jasoda Sunar, loving sister Ms. Radhika Sunar and my late father Shri. K.B. Sunar for lending me every support, love and care to sail through every tough and trying times. I am indebted for all the valuable prayer and support of my near and dear ones who were there for me always.

Above all I thank God almighty for giving me strength, knowledge, patience and good health to reach thus far.



(Kiran Sunar)

Immuno-Phytopathology Laboratory

Dept. of Botany, University of North Bengal

Date: 19-08-2013

Contents	Page #
1. Introduction	1
2. Literature Review	9
3. Material and Methods	43
3.1. Girding of study area	43
3.1.1. Darjeeling hill region	45
3.2. Soil sampling strategy	45
3.2.1. Sampling protocol	45
3.2.2. Grid sampling in non uniform ecological zones	45
3.3. Isolation of microorganisms from soil	46
3.3.1. Soil dilution technique	46
3.3.2. Direct soil plating	47
3.3.3. Soil washing technique	47
3.4. Composition of Solid media	47
3.5. Morphological and Microscopical Characterization of isolates	50
3.5.1. Assessment of mycelial growth	50
3.5.1.1. Solid media	50
3.5.1.2. Liquid media	51
3.5.2. Assessment of bacterial growth	51
3.5.3. Microscopical characterization	51
3.5.3.1. Bright field study of fungal spores and mycelia	51
3.5.3.2. SEM studies of fungal isolates	51
3.5.3.3. SEM studies of bacterial isolates	52
3.6. Biochemical characterization of bacterial isolates	52
3.6.1. Gram reaction	52
3.6.2. Catalase	53
3.6.3. Urea digestion	53
3.6.4. H ₂ S production	53
3.7. <i>In vitro</i> characterization of plant growth promoting activities	53
3.7.1. Phosphate solubilizing activity	53
3.7.1.1. Screening for primary phosphate solubilizing activity on PKV medium	53
3.7.1.2. Quantitative measurement of phosphate solubilization	53
3.7.2. IAA production	54
3.7.2.1. Qualitative test for IAA production	54
3.7.2.2. Quantitative test for IAA production	55
3.7.3. Siderophore production	55
3.7.4. HCN production	55
3.7.5. Chitinase production	56
3.7.5.1. Detection in modified Chitinase detection agar	56
3.7.5.2. Quantification of Chitinase activity	56
3.7.6. Protease production	57
3.7.7. Starch hydrolysis	57
3.8. Screening for cellulase production	57
3.8.1. Assay of endocellulase activity	57
3.8.2. Assay of both exo and endocellulase activity	57
3.9. Casein hydrolysis	58
3.10. <i>In vitro</i> testing for antagonism to fungal pathogens	58
3.10.1. Inhibition of mycelial growth in solid medium	58
3.10.2. Sclerotia germination bioassay	58
3.11. Immunological studies	59
3.11.1. Preparation of fungal and bacterial antigen	59

3.11.2. Estimation of protein content	59
3.11.3. SDS-PAGE analysis of soluble proteins	60
3.11.3.1. Preparation of stock solution	60
3.11.3.2. Preparation of gel	61
3.11.3.3. Sample preparation	62
3.11.3.4. Electrophoresis	62
3.11.3.5. Fixing and staining	62
3.11.4. Raising of polyclonal antibodies	62
3.11.4.1. Rabbits and their maintenance	62
3.11.4.2. Immunization	63
3.11.4.3. Bleeding	63
3.11.5. Purification of IgG	63
3.11.5.1. Precipitation	63
3.11.5.2. Column preparation	64
3.11.5.3. Fraction collection	64
3.11.6. Immunodiffusion test	64
3.11.6.1. Preparation of agarose slides	64
3.11.6.2. Diffusion	65
3.11.6.3. Washing, staining and drying of slides	65
3.11.7. Immunoblotting	65
3.11.7.1. Dot immunobinding assay (DIBA)	65
3.11.7.2. Western Blotting	66
3.10.7.2.1. Extraction and estimation of protein	67
3.10.7.2.2. SDS PAGE of protein	67
3.11.7.2.3. Blot transfer process	67
3.11.7.2.4. Immunoprobng	67
3.11.8. Fluorescence antibody staining and microscopy	68
3.11.9. Immunolocalization of Chitinase enzymes by Indirect immunofluorescence staining of leaf, stem and root tissues	68
3.12. <i>In vivo</i> studies for plant growth promotion by PSF and PGPR	69
3.12.1. Mass multiplication and inoculation	69
3.12.1.1. Mass multiplication of PSF	69
3.12.2. Mass multiplication of PGPR isolates	69
3.12.2.1 Application of bacteria	70
3.12.2.2. Soil drench	70
3.12.2.3. Foliar spray	70
3.12.2.4. Seed bacterization	70
3.12.3. Assessment of plant growth promotion by PSF and PGPR	70
3.12.4. Assessment of soil phosphate mobilization by PSF and PGPR Isolates	71
3.12.4.1. Modified Morgan Extraction for Phosphorous from soil	71
3.12.4.2. Estimation of Total phosphate content in soil and plant tissues	71
3.13. <i>In vivo</i> studies of disease suppression by PGPR and BCA	72
3.13.1. Disease assessment with BCA	72
3.13.2. Disease assessment with PGPR	73
3.13.3. Calculation of biocontrol efficacy (BE %) and Disease index (DE %)	73
3.14. Assay of defense enzyme activities enhanced after application of BCA and PGPR	73
3.14.1. β -1, 3-glucanase (β -GLU, EC 3.2.1.38)	74
3.14.2. Chitinase (CHT, EC 3.2.1.14)	74
3.14.3. Phenyl alanine ammonia Lyase (PAL EC 4.3.1.5)	74

3.14.4. Peroxidase (POX, EC1.11.1.7)	75
3.14.5. Acid and Alkaline phosphatase (EC 3.1.3.2 & EC 3.1.3.1)	75
3.14.6. Estimation of phenols contents	76
3.14.6.1. Extraction of phenol contents from leaves and roots	76
3.14.6.2. Estimation of Total phenol	76
3.15. Isolation of genomic DNA	76
3.15.1. Preparation of genomic DNA extraction buffer	77
3.15.2. Extraction of Fungal Genomic DNA	77
3.15.3. Extraction of Bacterial Genomic DNA	78
3.15.4. Purification of genomic DNA	78
3.15.5. Spectrophotometric quantification of Genomic DNA	78
3.15.6. Agarose gel electrophoresis to check DNA quality	79
3.15.7. Preparation of DNA samples for electrophoresis	79
3.15.8. Run gel electrophoresis for DNA fraction	79
3.16. RAPD PCR analysis	79
3.16.1. Amplification conditions for RAPD analysis	79
3.16.2. RAPD- PCR primers	79
3.16.3. Analysis of RAPD band patterns	80
3.16.4. Scoring of individual bands and construction of dendrogram	80
3.17. ITS PCR analysis	80
3.17.1. ITS-PCR primers	80
3.17.2. Amplification conditions	81
3.17.3. Sequencing of rDNA gene	81
3.18. BLAST of Sequence	81
3.19. Submission of rDNA gene to NCBI genbank	81
3.20. Multiple sequence alignment and Phylogenetic analysis	81
3.21. Analysis of rDNA region for DNA molecular weight, nucleotide frequency and ORF	83
3.22. Denaturing Gradient Gel Electrophoresis (DGGE)	83
3.22.1. PCR amplification of genomic DNA of the isolates for DGGE analysis	84
3.22.2. Denature Gradient Gel Electrophoresis of the PCR products	84
3.22.2.1. Reagents and solutions required for DGGE analysis	84
3.22.2.2. Creating the gel sandwich (DCode System BioRad)	84
3.22.2.3. Preparing the gel	85
3.22.2.4. Running a gel	85
3.22.2.5. Staining of gels and photography	85
3.22.2.6. Data analysis.	86
3.22.2.6.1. Scoring of individual bands	86
3.22.2.6.2. UPGMA analysis of the DGGE bands	86
4. Results	87
4.1. Isolation and identification of microorganism from forest soil, riverine soil and agricultural fields	87
4.1.1. Fungal Isolates	99
4.1.1.1. Growth studies in solid medium	99
4.1.1.2. Microscopic observation	99
4.1.1.3. Deposition of fungal isolates to NAIMCC	100
4.1.2. Bacterial isolates	100
4.1.2.1. Biochemical characterization of bacterial isolates	100
4.2. <i>In vitro</i> screening of fungal isolates for plant growth promoting Attributes	130
4.2.1. Phosphate solubilization	130
4.2.1.1. In solid medium	130
4.2.1.2. In liquid medium	130

4.2.2. Exo and Endo Chitinase activities	136
4.2.3. Net Exo-Cellulase activities	136
4.3. Antagonistic activity of the fungal isolates against selected phytopathogens	143
4.3.1. Antagonistic effect of <i>Talaromyces flavus</i> RHS/P-51	143
4.3.1.1. Inhibition in solid medium	143
4.3.1.2. Inhibition by culture filtrate	143
4.3.2. Antagonistic effect of <i>Trichoderma</i> isolates	144
4.3.2.1. Inhibition in solid medium	144
4.3.2.2. Inhibition by culture filtrate	145
4.3.3. Deposition of important microorganism to NAIMCC	148
4.4. <i>In vitro</i> screening of Bacterial isolates for plant growth promoting activities	151
4.4.1. Phosphate solubilization, HCN, Siderophore, IAA and ACC deaminase production	151
4.4.2. Phosphate solubilization, IAA production and ACC deaminase activities in liquid medium	151
4.4.3. Antagonism against fungal pathogens	151
4.5. Morphological and Scanning Electronic Microscopic studies	156
4.5.1. PSF isolates	157
4.5.2. BCA isolates	157
4.5.3. PGPR isolates	158
4.6. Genomic DNA analysis (Agarose gel and Spectrophotometric) for PCR	162
4.6.1. PSF isolates	162
4.6.2. BCA isolates	162
4.6.3. Bacterial isolates	163
4.7. Random Amplified Polymorphic DNA (RAPD) based genetic diversity analysis	167
4.7.1. RAPD based diversity analysis of PSF isolates	167
4.7.1.1. Analysis of polymorphism	168
4.7.1.2. Analysis of Genetic Similarity values	168
4.7.1.3. Dendrogram Construction, PCA analysis, 2D and 3D plot	169
4.7.2. RAPD based diversity analysis of BCA isolates	173
4.7.2.1. Analysis of polymorphism	173
4.7.2.2. Analysis of Genetic Similarity values	173
4.7.2.3. Dendrogram Construction, PCA analysis, 2D and 3D plot	174
4.7.3. RAPD based diversity analysis of Bacterial isolates.	176
4.7.3.1. Analysis of polymorphism of Bacterial isolates	176
4.7.3.2. Analysis of Genetic Similarity values among Bacterial isolates	176
4.7.3.3. Dendrogram Construction, PCA analysis, 2D and 3D plot	178
4.8. Denature Gradient Gel Electrophoresis (DGGE) markers based diversity analysis	184
4.8.1. DGGE based diversity analysis of PSF isolates	184
4.8.1.1. Analysis of polymorphism and genetic similarity values	184
4.8.1.2. Dendrogram Construction, PCA analysis, 2D and 3D plot	185
4.8.2. DGGE based diversity analysis of BCA isolates	185
4.8.2.1. Analysis of polymorphism and genetic similarity value	189
4.8.2.2. Dendrogram Construction, PCA analysis, 2D and 3D plot	189
4.8.3. DGGE based diversity analysis of Bacterial isolates	192

4.8.3.1. Analysis of polymorphism and genetic similarity value	192
4.8.3.2. Dendrogram Construction, PCA analysis, 2D and 3D plot	193
4.8.4. DGGE analysis of BCA isolates with reference to the known identified strains	196
4.8.4.1. Analysis of polymorphism and genetic similarity value	196
4.8.4.2. Dendrogram Construction, PCA analysis, 2D and 3D plot	196
4.9. 18S rDNA sequence and phylogenetic analysis for identification of fungal isolates	200
4.9.1. Pathogen (<i>Thanatephorus cucumeris</i>)	200
4.9.1.1. Multiple sequence alignment and Phylogenetic analysis	200
4.9.1.2. Analysis of Nucleotide frequency, ORF and DNA molecular weight of rDNA sequences	201
4.9.2. Phosphate Solubilizing Fungus-PSF, (<i>Talaromyces flavus</i>)	206
4.9.2.1. Multiple sequence alignment and Phylogenetic analysis	206
4.9.2.2. Analysis of Nucleotide frequency, ORF and DNA molecular weight of rDNA sequences	207
4.9.3. BCA- <i>Trichoderma harzianum</i> , <i>Trichoderma asperellum</i> and <i>Trichoderma erinaceum</i>	214
4.9.3.1. Multiple sequence alignment	215
4.9.3.1.1. <i>Trichoderma harzianum</i> isolates	215
4.9.3.1.2. <i>Trichoderma asperellum</i> isolates	216
4.9.3.1.3. <i>Trichoderma erinaceum</i> isolates	216
4.9.3.2. Phylogenetic analysis of <i>Trichoderma</i> isolates	230
4.9.3.3. Analysis of Nucleotide frequency.	233
4.9.3.3.1. <i>Trichoderma harzianum</i> and <i>T. asperellum</i> isolates	233
4.9.3.3.2. <i>Trichoderma erinaceum</i> isolates	234
4.9.3.4. Analysis of DNA molecular weight of rDNA sequences	235
4.9.3.5. Analysis of Open Reading Frame (ORF) of rDNA gene sequences	235
4.10. 16S rDNA sequence analysis for identification of PGPR isolates	240
4.10.1. 16S rDNA sequencing, Multiple sequence alignment and phylogenetic analysis	240
4.10.1.1. <i>Bacillus</i> isolates	240
4.10.1.2. <i>Enterobacter cloacae</i>	241
4.10.1.3. <i>Paenibacillus polymyxa</i>	241
4.10.1.4. <i>Burkholderia symbionts</i>	241
4.10.2. Phylogenetic analysis of PGPR isolates based on 16S rDNA sequences	255
4.10.3. Analysis of and Nucleotide frequency and DNA molecular weight of 16S rDNA sequences of PGPR isolates	255
4.10.4. Analysis of Open Reading Frame (ORF) of 16S rDNA gene sequences of PGPR isolates	263
4.11. Immunological characterization of <i>T. flavus</i> (PSF) <i>T. harzianum</i> (BCA) and <i>T. cucumeris</i> (Pathogen)	270
4.11.1. Soluble protein	270
4.11.2. Serological Assays of <i>Talaromyces flavus</i> RHS/P-51	270
4.11.3. Serological assay of <i>Trichoderma harzianum</i> RHS/S-559	274
4.11.4. Serological assays of <i>Thanatephorus cucumeris</i> RHS/V-566	276
4.11.5. Indirect immunofluorescence	280
4.12. Effect of selected PSF on plant growth	282
4.12.1. Effect of amendment of soil with PSF isolates on phosphate mobilization	282

4.12.1.1. Soil, root and leaf phosphate contents	282
4.12.1.2. Acid and alkaline phosphatase content	282
4.13. Evaluation of selected PGPR isolates on plant growth promotion	287
4.13.1. Growth promotion of <i>Cicer arietinum</i> , <i>Vigna radiata</i> , <i>Glycine max</i> and <i>Triticum aestivum</i> in field conditions	287
4.13.2. Effect of application of PGPR isolates on phosphate mobilization	287
4.13.2.1. Soil, root and leaf phosphate contents	287
4.13.2.2. Acid and alkaline phosphatase content	288
4.13.3. Growth promotion of Tea seedlings by <i>B. pumilus</i> BRHS/C-and <i>B. altitudinis</i> BRHS/S-73	295
4.13.4. Effects of <i>B. pumilus</i> and <i>B. altitudinis</i> P content	295
4.12.4.1. Soil, root and leaf phosphate contents	295
4.12.4.2. Acid and alkaline phosphatase content	296
4.14. Effect of BCA isolates on inhibiting root diseases	300
4.14.1. Influence of <i>T. flavus</i> and <i>T. harzianum</i> on Sclerotial blight of <i>Vigna radiata</i>	300
4.14.1.1. Disease development	300
4.14.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	300
4.14.1.3. Biochemical changes	302
4.14.2. Influence of <i>T. flavus</i> , <i>T. harzianum</i> and <i>T. asperellum</i> on root rot of <i>Cicer aeritinum</i> caused by <i>Thanatephorus</i> <i>cucumeris</i>	303
4.14.2.1. Disease development	303
4.14.2.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	304
4.14.2.3. Biochemical changes	304
4.15. Effect of PGPR isolates on inhibiting root diseases	308
4.15.1. Root rot disease caused by <i>Thanatephorus cucumeris</i>	308
4.15.1.1. Inhibition of Root rot of <i>Glycine max</i> by <i>Bacillus</i> <i>pumilus</i> , <i>Paenibacillus polymyxa</i> , <i>Enterobacter</i> <i>cloacae</i> and <i>B. altitudinis</i>	308
4.15.1.1.1. Disease development	308
4.15.1.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	310
4.15.1.1.3. Biochemical changes	310
4.15.1.1.4. Tissue and cellular location of chitinase enzyme by FITC labeling	312
4.15.1.2. Inhibition of root rot of <i>Lycopersicon esculentum</i> by <i>Bacillus methylotrophicus</i> , <i>Burkholderia</i> <i>symbionts</i> and <i>Bacillus aerophilus</i>	313
4.15.1.2.1. Disease development	313
4.15.1.2.3. Biochemical changes	315
4.15.1.3. Inhibition of root rot of <i>Brassica juncea</i> by PGPR isolates	319
4.15.1.3.1. Disease development	319
4.15.1.3.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	319
4.15.1.3.3. Biochemical changes	321
4.15.2. Sclerotial blight disease caused by <i>Sclerotium rolfsii</i>	323
4.15.2.1. Inhibition of Sclerotial rot of <i>Glycine max</i> by <i>Bacillus pumilus</i> and <i>B. altitudinis</i>	323
4.15.2.1.1. Disease development	323

4.15.2.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	323
4.15.2.1.3. Biochemical changes	324
4.15.2.1.4. Tissue and cellular location of chitinase enzyme by FITC labeling	327
4.15.2.2. Inhibition of <i>Sclerotial rot</i> of <i>Vigna radiata</i> by <i>PGPR</i> isolates	329
4.15.2.2.1. Disease development	329
4.15.2.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	329
4.15.2.2.3. Biochemical changes	330
4.15.2. 2.4. Tissue and cellular location of chitinase enzyme by FITC labeling	335
4.15.2.3. Inhibition of <i>Sclerotial rot</i> of <i>Camellia sinensis</i> by <i>PGPR</i> isolates	335
4.15.2.3.1. Disease development	335
4.15.2.3.2. Disease incidence (DE %) and biocontrol efficacy (BE %)	337
4.15.2.2.3. Biochemical changes	340
4.15.2.2.4. Tissue and cellular location of chitinase enzyme by FITC labeling	340
4.16. Testing for survivability of pathogens <i>S. rolfsii</i> and <i>T. cucumeris</i> using Immunological formats	344
4.16.1. Testing for survivability of <i>Sclerotium rolfsii</i> in soils treated with Biocontrol fungal isolates and PGPR.	344
4.16.2. Testing for survivability of <i>Thanatephorus cucumeris</i> in soils treated with Biocontrol fungal isolates and PGPR	347
Discussion	348
Conclusion	378
Bibliography	382
Appendices	I-V

List of Tables

Table 1	List of RAPD and ITS universal primers.
Table 2	GIS locations of the sampling sites.
Table 3	Fungal and bacterial population in the forest, rhizosphere and riverine soil samples collected from different regions of Darjeeling District.
Table 4	Morphology and Microscopical Characters of fungal isolates obtained from various sources.
Table 5	NAIMCC accession numbers of common fungal isolates of Darjeeling hills.
Table 6	GIS position and location of the sampling area of source of bacterial isolates.
Table 7	Morphological and biochemical characterization of bacterial isolates.
Table 8	Screening of Phosphate solubilization in solid PVK medium by fungal isolates.
Table 9	<i>In vitro</i> quantification of phosphate solubilization by fungal isolates in modified PVK broth.
Table 10	Chitinase activities of different isolates of <i>Trichoderma</i> obtained from different sources of Darjeeling Hills.
Table 11	Potential PSF isolates deposited to NAIMCC.
Table 12	Evaluation of net exocellulase activity of the fungal isolates.
Table 13	Inhibition of phytopathogenic test fungi by <i>T. flavus</i> RHS/P-51 <i>in vitro</i>
Table 14	Inhibition of phytopathogens by <i>Trichoderma</i> isolates.
Table 15	Inhibition of mycelial growth of <i>S. rolfsii</i> and <i>T. cucumeris</i> by culture filtrate of <i>Trichoderma</i> isolates.
Table 16	Potential BCA isolates deposited to NAIMCC.
Table 17	Quantification of Phosphate solubilizing, IAA production and ACC deaminase activities of bacterial isolates in modified liquid broth medium.
Table 18	<i>In vitro</i> pairing of bacterial isolates with phyto- pathogens for evaluations of antifungal activities.
Table 19	Scanning electron Micrographic characteristics of the bacterial isolates.
Table 20	Spectrophotometrical A_{260}/A_{280} ratio of genomic DNA of PSF and BCA isolates.
Table 21	Spectrophotometrical A_{260}/A_{280} ratio of Bacterial genomic DNA.
Table 22	Total number of polymorphic bands produced by different primers (PSF).
Table 23	Determination of polymorphisms based on the RAPD of PSF isolates.
Table 24	Genetic Similarity matrix obtained as a result of Simqual analysis of RAPD bands of PSF isolates.
Table 25	Total number of polymorphic bands produced by different primers.
Table 26	Genetic similarity matrix, obtained as the result of Simqual analysis of the RAPD bands of BCA isolates.
Table 27	Determination of polymorphisms based on the RAPD.
Table 28	Total number of polymorphic bands produced by different primers.
Table 29	Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of BCA isolates.
Table 30	Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of BCA isolates.
Table 31	Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of PGPR isolates.
Table 32	Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of <i>Trichoderma</i> isolates.
Table 33	Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of <i>T. cucumeris</i> used for analysis with ex-type strain sequence.
Table 34	Nucleotide combinations and frequencies of different combinations of ITS

	sequences of <i>T. cucumeris</i> RHS/V-566.
Table 35	Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of <i>T. flavus</i> .
Table 36	Genbank Accession numbers and geographic location of the Ex- Type strains of <i>Talaromyces flavus</i> .
Table 37	DNA Stats results for 565 residue sequence " <i>Talaromyces flavus</i> strain RHS/P-51- GU324073" starting "TTGTTTTAAC".
Table 38	NCBI accession numbers of Identified <i>Trichoderma</i> isolates.
Table 39	Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of <i>T. harzianum</i> .
Table 40	Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of <i>T. asperellum</i> .
Table 41	Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of <i>T. erinaceum</i> .
Table 42	DNA stats results for 18S rDNA nucleotide frequencies of different isolates of <i>T. harzianum</i> and <i>T. asperellum</i> .
Table 43	DNA stats results for 18S rDNA nucleotide frequencies of different isolates of <i>T. erinaceum</i> .
Table 44	DNA molecular weight of different <i>Trichoderma</i> isolates calculated on the basis of 18S rDNA sequences.
Table 45	NCBI Genbank Accession number of PGPR isolates.
Table 46	Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different Bacilli isolates.
Table 47	Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different <i>Enterobacter</i> isolates.
Table 48	Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different <i>Paenibacillus</i> isolates.
Table 49	Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different isolates of <i>Burkholderis</i> isolates.
Table 50	Nucleotide combinations and frequencies of different combinations of 16S rDNA sequences of PGPR isolates belonging to Bacilli group.
Table 51	Nucleotide combinations and frequencies of different combinations of 16S rDNA sequences of PGPR isolates (Non-Bacilli).
Table 52	DNA molecular weight of different <i>Trichoderma</i> isolates calculated on the basis of 18S rDNA sequences.
Table 53	SDS-PAGE analysis of total soluble proteins of <i>T. flavus</i> , <i>T. harzianum</i> , <i>T. asperellum</i> , <i>T. erinaceum</i> and <i>T. cucumeris</i> .
Table 54	Scoring of dots obtained with DIBA of <i>T. flavus</i> and other heterologous antigens.
Table 55	Analysis of molecular weights of bands obtained in western blot analysis of <i>T. flavus</i> antigen and probed with its homologous antibody
Table 56	Scoring of dots obtained with DIBA of <i>T. harzianum</i> and other heterologous antigens
Table 57	Analysis of molecular weights of bands obtained in western blot analysis of <i>T. harzianum</i> antigen and probed with its homologous antibody.
Table 58	Analysis of molecular weights of bands obtained in western blot analysis of <i>T. harzianum</i> , <i>T. asperellum</i> and <i>T. erinaceum</i> antigens and probed with PAb of <i>T. harzianum</i> (3 rd bleed).
Table 59	Scoring of dots obtained with DIBA of <i>T. cucumeris</i> and other heterogonous antigens.
Table 60	Analysis of molecular weights of bands obtained in western blot analysis of <i>T. cucumeris</i> antigen and probed with its homologous antibody.
Table 61	Alkaline and acid phosphatase content of rhizosphere soil treated with PSF isolates.

Table 62	Effect of PGPR on Alkaline and Acid phosphatase activities of rhizosphere soil.
Table 63	Acidic and alkaline phosphatase activities of the soil following application of the <i>B. pumilus</i> and <i>B. altitudinis</i> .
Table 63a	ANOVA of the data presented in table 60 (Acid Phosphatase activity).
Table 63b	ANOVA of the data presented in table 60 (Acid Phosphatase activity).
Table 64	Sclerotial blight development in the roots of <i>Vigna radiata</i> in presence and absence of <i>T. harzianum</i> and <i>T. flavus</i> in pot conditions.
Table 65	Percent survival of <i>C. aeritinum</i> seedlings in presence and absence of <i>T. harzianum</i> , <i>T. flavus</i> and <i>T. asperellum</i> in pot conditions.
Table 66	Defense enzyme activities in the shoots and roots of <i>Cicer arietinum</i> following inoculation with <i>T. cucumeris</i> and treatment with <i>T. harzianum</i> , <i>T. flavus</i> and <i>T. asperellum</i> .
Table 67	Root rot development in the roots of <i>Glycine max</i> in presence and absence of PGPR isolates in pot conditions.
Table 68	Root rot development in the roots of <i>Lycopersicon esculentum</i> in presence and absence of PGPR isolates in pot conditions.
Table 69	Root rot development in the roots of <i>Brassica juncea</i> in presence and absence of PGPR isolates in pot conditions.
Table 70	Sclerotial blight development in the roots of <i>Glycine max</i> in presence and absence of PGPR isolates in pot conditions.
Table 71	Sclerotial blight development in the roots of <i>Vigna radiata</i> in presence and absence of PGPR isolates in pot conditions.
Table 72	Disease Index of Sclerotial blight incidence of tea seedling following bacterial treatment and pathogen challenge.
Table 73	Changes in the Total Phenol content of the roots and leaves of tea seedlings following PGPR application and pathogen challenge.
Table 73a	ANOVA of the data presented in Table 70, Total Phenol content (Roots).
Table 73b	ANOVA of the data presented in Table 70, Total Phenol content (Leaves).
Table 74	ELISA and DIBA values of rhizosphere soil antigens (<i>S. rolfsii</i>).
Table 75	ELISA and DIBA values of rhizosphere soil antigens (<i>S. rolfsii</i>).
Table 76	ELISA and DIBA values of rhizosphere soil antigenspathogen (<i>T. cucumeris</i>).

List of Figures

- Figure 1 Agricultural crop fields of Darjeeling hills.
Figure 2 Major forests areas of Darjeeling hills.
Figure 3 Major rierrine soil sample collection sites.
Figure 4 Political map of Darjeeling District.
Figure 5 Satellite Image of Darjeeling pointing out the main subdivisions and GIS route.
Figure 6 GIS locations of major forests, agricultural land and river beds of Darjeeling regions.

Figure 7 Fungal and Bcatrial population in three different soil types of Darjeeling Hills.
Figure 8 Radial growth pattern of fungal isolates isolated from forests soil.
Figure 9 Radial growth pattern of fungal isolates isolated from rhizosphere soil.
Figure 10 Radial growth pattern of fungal isolates isolated from riverine soil.
Figure 11 Light Microscopic pictures of the mycelia and spore structures of fungal isolates.
Figure 12 Microscopis characters of different *Trichoderma* isolates.
Figure 13 Growth pattern of different bacterial isolates obtained from rhizosphere soil.
Figure 14 Phosphate solubilizing properties of fungal isolates on Pikovskaya Agar.
Figure 15 Inhibition of root pathogens by *Talaromyces flavus* (RHS/P-51).
Figure 16 Inhibition of *S. rolfsii* and *T. cucumeris* in dual culture assay by *Trichoderma* isolates.

Figure 17 Scaning Electrom Micrograph of fungal hyphae showing interactions.
Figure 18 Evaluation of Phosphate solubilization by bacterial isolates on PKV solid medium.
Figure 19 Characterization of bacterial isolates for PGPR activities *in vitro*.
Figure 20 *In vitro* antifungal activities of PGPR isolates tested against *S. rolfsii* and *T. cucumeris*.

Figure 21 Scaning Electron Micrograph of ascospores of *Talaromyces flavus* (RHS/P-51).
Figure 22 Scanning electron micrograph of conidiophores and conidia of *Trichoderma* isolates.

Figure 23 Scaning Electron Micrograph of bacterial isolates obtained from various sources.
Figure 24 Agarose gel electrophoresis of Genomic DNA of fungal isolates.
Figure 25 Agarose Gel electrophoresis of Bacterial Genomic DNA.
Figure 26 RAPD amplified products of the fungal PSF isolates.
Figure 27 Genetic relatedness analysis among the phosphate solubilizing fungal, RAPD
Figure 28 RAPD analysis of *Trichoderma sp. isolates*.
Figure 29 Genetic relatedness analysis among the *Trichoderma harzianum* and *T. asperellum*.
Figure 30 RAPD analysis of bacterial isolates obtained from various sources (B1-B45).
Figure 31 RAPD analysis of bacterial isolates obtained from various sources (B46-B90).
Figure 32 RAPD analysis of bacterial isolates obtained from various sources (B91-135).
Figure 33 Dendograshowing different bacterial groups in different clades.
Figure 34 Two dimensional (A) and three dimensional plots (B) of bacterial isolates.
Figure 35 Denature gradient gel electrophoresis of the ITS-PCR of PSF isolates.
Figure 36 Analysis of genetic relatedness among the PSF isolates based on DGGE.
Figure 37 Denature gradient gel electrophoresis of the ITS-PCR of BCA isolates.
Figure 38 Analysis of genetic relatedness among the BCA isolates based on DGGE.
Figure 39 Denature gradient gel electrophoresis of the ITS-PCRof PGPR isolates.
Figure 40 Analysis of genetic relatedness among the PGPR isolates based on DGGE.

List of Appendices

APPENDIX A: List of Thesis related publications

APPENDIX B: List of Abbreviations

APPENDIX C: List of Chemicals

- Figure 41 Denature gradient gel electrophoresis of the ITS-PCR of *Trichoderma* isolates.
- Figure 42 Analysis of genetic relatedness among the BCA isolates based on DGGE.
- Figure 43 Chromatogram and sequence deposit of ITS region of *T. cucumeris* RHS/V-566
- Figure 44 Multiple sequence alignment of *T. cucumeris* RHS/V-566.
- Figure 45 The phylogenetic analyses of *T. cucumeris* based on 18S rDNA.
- Figure 46 Chromatogram and sequence deposit of ITS region of *T. flavus* RHS/P-51.
- Figure 47 Multiple sequence alignment of *T. flavus* RHS/P-51.
- Figure 48 Phylogenetic placement of *Talaromyces flavus* (BRHS/P-51) .
- Figure 49 Open Reading Frame (ORF) analysis of "*Talaromyces flavus* strain RHS/P 51"
- Figure 50 Chromatogram and sequence deposit of ITS region of *T. harzianum* RHS/S-559.
- Figure 51 Chromatogram and sequence deposit of ITS region of *T. harzianum* RHS/S-560.
- Figure 52 Multiple sequence alignment of *T. harzianum* isolates with ex-type strain sequences obtained from NCBI genbank database.
- Figure 53 Chromatogram and sequence deposit of ITS region of *T. asperellum* RHS/S-561.
- Figure 54 Multiple sequence alignment of *T. asperellum* isolates.
- Figure 55 Chromatogram and sequence deposit of ITS region of *T. erinaceum* FS/L-20.
- Figure 56 Chromatogram and sequence deposit of ITS region of *T. erinaceum* FS/S-474.
- Figure 57 Chromatogram and sequence deposit of ITS region of *T. erinaceum* FS/S- 475.
- Figure 58 Chromatogram and sequence deposit of ITS region of *T. erinaceum* FS/S- 478.
- Figure 59 Multiple sequence alignment of *T. erinaceum* isolates.
- Figure 60 Neighbourjoinng-Phylogenetic placement of *T. harzianum*, *T. asperellum* and *T. erinaceum* .
- Figure 61 UPGMA-Phylogenetic placement of *T. harzianum*, *T. asperellum* and *T. erinaceum*
- Figure 62 Chromatogram and sequence deposit of ITS region of *B. pumilus* BRHS/C-1.
- Figure 63 Chromatogram and sequence deposit of ITS region of *B.altitudinis* BRHS/P-22.
- Figure 64 Chromatogram and sequence deposit of ITS region of *B. altitudinis* BRHS/S-73.
- Figure 65 Chromatogram and sequence deposit of ITS region of *B. methylotrophicus*.
- Figure 66 Chromatogram and sequence deposit of ITS region of *B. aerophilus* BRHS/B-104.
- Figure 67 Multiple sequence alignment of *Bacillii* isolates.
- Figure 68 Chromatogram and sequence deposit of ITS region of *Enterobacter cloacae*
- Figure 69 Multiple sequence alignment of *Enterobacter cloacae*.
- Figure 70 Chromatogram and sequence deposit of ITS region of *Enterobacter cloacae*
- Figure 71 Multiple sequence alignment of *Paenibacillus polymyxa*..
- Figure 72 Chromatogram and sequence deposit of ITS region of *Burkholderia sp.*
- Figure 73 Multiple sequence alignment of *Burkholderia symbiont*.
- Figure 74 Neighbourjoinng -Phylogenetic placement of PGPR isolates.
- Figure 75 UPGMA-Phylogenetic placement of PGPR isolates.
- Figure 76 SDS-PAGE profiles of fungal proteins.
- Figure 77 Serological assays of *T. flavus*.
- Figure 78 Serological assays of *Trichoderma harzianum* RHS/S-559.
- Figure 79 Serological assays of *Thanatephorus cucumeris* RHS/V-566.
- Figure 80 Indirect immuno-fluorescence *T. cucumeris*.
- Figure 81 Indirect immuno-fluorescence of hypha and phialides of *T. harzianum*.
- Figure 82 Effect of PFS isolates (*A. niger*, *A. melleus*, *A. clavatus* and *T. flavus*) on growth.
- Figure 83 Effect of PSF isolates on growth of different crops in green house condition.
- Figure 84 Effect of PSF on total phosphate contents. Soil (A); Root (B) and Leaves (C).
- Figure 85 Evaluation of growth promotion of *Vigna radiata* by selected PGPR.

- Figure 86 Evaluation of growth promotion of *Cicer arietinum* by selected PGPR.
- Figure 87 Evaluation of growth promotion of *Triticum aestivum* by selected PGPR.
- Figure 88 Evaluation of growth promotion of *Glycine max* by selected PGPR.
- Figure 89 Evaluation of growth promotion of *V. radiata*, *C. arietinum*, *G. max* and *T. aestivum* by PGPR.
- Figure 90 Effect of PGPR application on Total phosphate content of soil, root and leaves.
- Figure 91 Effect of *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73 on growth of tea.
- Figure 92 Evaluation of growth promotion of tea seedlings by *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73.
- Figure 93 Evaluation of Total P-content of soil (A), root (B) and leaves (C) of tea seedlings.
- Figure 94 Sclerotial blight development in *Vigna radiata* in pot conditions.
- Figure 95 Sclerotial blight development in the roots of *Vigna radiata* (disease index)
- Figure 96 Defense enzyme activities in the roots of *Vigna radiata*.
- Figure 97 Seedling survival percentage (A) Disease index (B) of *Cicer arietinum* and Biocontrol efficacy % of BCA isolates following inoculation with *T. cucumeris*.
- Figure 98 Sclerotial blight development in *Cicer arietinum* in presence and absence of *T. harzianum*, *T. flavus* and *T. asperellum* in pot conditions.
- Figure 99 Root rot development in *Glycine max* in presence and absence of PGPR.
- Figure 100 Development of root rot disease of *Glycine max* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index.
- Figure 101 Defense enzyme activities in the roots and leaves of *Glycine max*.
- Figure 102 FITC Labeling of stem, leaf and root tissues of *Glycine max* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73.
- Figure 103 Root rot development in *Lycopersicon esculentum* in presence and absence of PGPR.
- Figure 104 Development of root rot disease of *Lycopersicon esculentum* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation.
- Figure 105 Defense enzyme activities in the roots and leaves of *Lycopersicon esculentum* following treatment with PGPR isolates and pathogen challenge.
- Figure 106 Development of root rot disease of *B. juncea* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation.
- Figure 107 Defense enzyme activities in the roots and leaves of *B. juncea* following treatment with PGPR isolates and pathogen challenge.
- Figure 108 Sclerotial blight development in *Glycine max* in presence and absence of PGPR.
- Figure 109 Development of root rot disease of *G. max* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation.
- Figure 110 Defense enzyme activities in the roots and leaves of *Glycine max* following treatment with PGPR isolates and pathogen challenge.
- Figure 111 FITC Labeling of stem, leaf and root tissues of *Glycine max* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73.
- Figure 112 Sclerotial blight development in *Vigna radiata* in presence and absence of PGPR.
- Figure 113 Development of Sclerotial blight disease of *V. radiata* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation.

- Figure 114 Defense enzyme activities in the roots and leaves of *Vigna radiata* following treatment with PGPR isolates and pathogen challenge GLU and CHT.
- Figure 115 Defense enzyme activities in the roots and leaves of *Vigna radiata* following treatment with PGPR isolates and pathogen challenge PAL and POX.
- Figure 116 FITC Labeling of stem, leaf and root tissues of *Vigna radiata* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73 and pathogen challenge.
- Figure 117 Sclerotial blight development in *Camellia sinensis* in presence and absence of PGPR.
- Figure 118 Development of Sclerotial blight disease of *c. sinensis* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation.
- Figure 119 Defense enzyme activities in the roots and leaves of *C. sinensis* following treatment with PGPR isolates and pathogen challenge β -1,3 Glucanase (A&B), Chitinase (B&C), Phenylalanine ammonial lyase (D&E) and Peroxidase.
- Figure 120 FITC Labeling of leaf tissues of *Camellia sinensis* (TV-20) with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73 and *B. pumilus* and pathogen challenge. TS of leaf treated with *B. pumilus* (A-F), TS of leaf treated with *B. altitudinis* (G-K); TS of leaf of untreated control plant (L).

CHAPTER 1

INTRODUCTION

Soil is known to be a complex microhabitat for two distinctive properties. Firstly, the microbial population in soil is very diverse and secondly soil is a structured, heterogeneous and discontinuous system, generally poor in nutrients and energy sources. The chemical, physical and biological characteristics of these microhabitats differ in both time and space. Diverse microorganisms are essential to a sustainable biosphere and the role of rhizosphere microbial populations for maintenance of root health, nutrient uptake and tolerance of environmental stress is now recognized (Zake *et al.*, 2011). Microorganisms form a vital component of all known ecosystems of earth and represent the richest repertoire of molecular and chemical diversity in nature as they underline basic ecosystem processes. Soil microflora plays the most important role in the soil region of the higher plants. The variable microflora changes the soil fertility conditions to a specific plant and in turn is dependent on the exudates of the roots in the rhizosphere. Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation, decomposition of organic matter, toxin removal and the cycling of carbon, nitrogen, phosphorus, and sulphur. In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth and changes in vegetation (Singh *et al.*, 2011). Soil bacteria and fungi are the key players in various biochemical cycles (BGC) (Trevors, 1998) and are responsible for the cycling of organic compounds. They also influence above-ground ecosystems by contributing to plant nutrition (George *et al.*, 1995) plant health (Smith and Goodman, 1999), soil structure (Wright and Upadhy, 1998) and soil fertility (Karthick *et al.*, 2011).

The study of microbial diversity is also important to solve new and emerging disease problems and to advance biotechnology. New technologies, particularly in nucleic acid analysis, computer science, analytical chemistry, and habitat sampling and characterization place the study of microbial diversity on the cutting edge of science. It is important to study microbial diversity not only for basic scientific

research, but also to understand the link between diversity and community structure and function. Human influences such as pollution, agriculture and chemical applications could adversely affect microbial diversity, and perhaps also above and belowground ecosystem functioning. In addition, a healthy rhizosphere population can help plants deal with biotic and abiotic stresses such as pathogens, drought and soil contamination. The role of rhizospheric organisms in mineral phosphate solubilization was known as early as 1903 and the ability of rhizospheric microorganisms to promote growth by phosphate solubilization is also one of the most studied mechanisms involved in plant growth promotion (Misra *et al.*, 2012). Important genera of mineral phosphate solubilizers include *Bacillus* and *Pseudomonas* (Singh, 2013) while *Aspergillus* and *Penicillium* form the important fungal genera (Nenwani *et al.*, 2010). In soil, phosphate-solubilizing bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population. Generally, the phosphate-solubilizing bacteria outnumber phosphate-solubilizing fungi by 2–150 times (Kucey, 1989). The high proportion of PSM is concentrated in the rhizosphere and is known to be more metabolically active than those isolated from sources other than the rhizosphere. Species of *Aspergillus*, *Penicillium* and yeast have been widely reported solubilizing various forms of inorganic phosphates (Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996).

The use of biological fertilizers in recent times, is receiving attention mainly on account of increased global preference for natural “organic” products. Isolation of microorganisms, screening for desirable characters, selection of efficient strains, production of inoculum and preparation of carrier-based formulation are important steps in the use of this microbe based environment friendly and sustainable technology. When these cultures are introduced into the natural environment, their individual beneficial effects are greatly magnified in a synergistic fashion. A microbial inoculant containing many kinds of naturally occurring beneficial microbes called ‘Effective Microorganisms’ has been used widely in nature and organic farming (Karthick *et al.*, 2011).



Fig. 1. Few of the major forests of Darjeeling hills.

Our understanding of microbial diversity, and concomitantly of species composition, of microbial communities is hampered by the inability to classify microorganisms (Sherriff *et al.*, 2007). Microbes are small and, in general, without conspicuous external characters to classify them morphologically. In addition, classification based on physiological or biochemical features is often not possible because an estimated percentage of 99% of all microorganisms in nature can not be isolated. So, to obtain a better understanding of the role of microbial diversity in ecosystem functioning, other techniques, which complement the traditional microbiological methods are necessary. Pace and co-workers (1986) were the first to realize that this phylogenetic framework of rRNA sequences could be used to design primers and probes. Therefore, approaches detecting the diversity of directly extracted signature molecules of microorganisms, such as fatty acids (Frostegård, *et al.*, 1996) or DNA (Zhou *et al.*, 1997), have been developed. DNA-based characterization techniques have the advantage that specific genes can be amplified from a community mixture or pure culture by PCR and that products of such amplifications can be further characterized, e.g., by subcloning and DNA sequencing. Such data can be directly compared to DNA sequence databases and thus provide information about similarity to already-known genes (Ueda *et al.*, 1995). Genetic fingerprinting techniques provide a pattern or profile of the community diversity based upon the physical separation of unique nucleic acid species (Madhavan *et al.*, 2010). The methods are rapid and relatively easy to perform, but more importantly, they allow the simultaneous analysis of multiple samples, which makes it possible to compare the genetic diversity of microbial communities from different habitats or to study the behaviour of individual communities over time. Application of these in molecular biological techniques allows us to detect and enumerate microorganisms in their natural habitat and so to determine the structure, function and dynamics of selective microbial communities (Jeewon *et al.*, 2013). Genetic fingerprinting techniques to study the diversity and dynamics of microbial communities can be divided into *direct methods*, whereby nucleic acids are extracted and directly analyzed, such as low-molecular-weight (LMW) RNA profiling, or into *indirect methods*, whereby the molecular marker first has to be amplified, which is the case for denaturing gradient gel electrophoresis

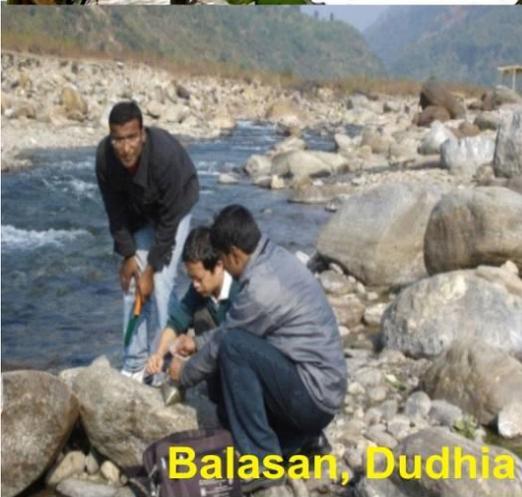


Fig. 2. Major riverine soil sample collection sites of Darjeeling Hills.

(DGGE) or temperature gradient gel electrophoresis (TGGE), singlestranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), bisbenzimidopolyethyleneglycol (Bb- PEG) electrophoresis, restriction fragment length polymorphism (RFLP) or amplified ribosomal DNA restriction analysis (ARDRA), and terminal RFLP (T-RFLP) or fluorescent RFLP (Flu-RFLP). Molecular analysis of genomic DNA of the organism is therefore useful for distinguishing the microbial strains better at intra-species level and these techniques provide valuable information on the magnitude of genetic variability within and between organisms of different species. It has been suggested that molecular fingerprinting techniques are not only helpful in knowing un-cultured communities but also helps to track the populations of known organisms with the help of reference sequences (Sun *et al.*, 2013).

Himalayan region represents a unique combination of plant and soil types that changes drastically with altitude (Kumar *et al.*, 2011). We have till date a very poor record of the Beneficial Microorganisms of the Himalayas especially of the Eastern Himalayan region lying between the latitudes 26° 40' - 29° 30'N and longitudes 88° 5' - 97° 5'E and covering a total area of 93,988 km² comprising Arunachal Pradesh, Sikkim and Darjeeling hills of West Bengal with 83,743, 7,096 and 3,149 km² of area respectively.). In this context the need for survey of efficient microorganisms from the Himalayan belt becomes necessary so as to use them more efficiently as “organic” products in this areas.

India is among the world’s twelve mega-diversity countries and immensely rich in bio-resources. West Bengal represents a good slice of biodiversity of the nation and is commendably bestowed with at least five ecological zones representing not only a variety of ecosystems but also remarkable diversity in its biological resource arena. As such, avenues in tapping upon the state's biodiversity resources and associated knowledge are undoubtedly immense, in order to consequently promote biodiversity-based enterprises in the modern, as well as, traditional sectors, to develop biotechnology industries in the State and also to encourage local level value addition to biodiversity resource. Darjeeling Himalayan hill region is situated on the North-Western side of the state. This region belongs to the Eastern Himalaya range.



Fig. 3. Agricultural crop fields of Darjeeling hills.

The whole of the Darjeeling district except the Siliguri division and a narrow part in the Northern part of Jalpaiguri district constitutes the region. It starts abruptly up from the Terai region. The deep gorge of Teesta has divided this mountainous region into two parts; the Singalila and Darjeeling Ranges run from north to south in the western part. The Singalila range is located along the border of Darjeeling and Nepal; it has four important peaks – Sandakphu, Phalut, Sabargam and Tangu. Isolation of microorganisms useful to improve the plant health from this region of varying altitude and vegetation will be a new attempt to explore the microbial mines of Darjeeling hills.

Keeping this in view, the following major objectives were undertaken to generate the possible information for utilization of microorganisms isolated from the different ecological regions of Darjeeling hills.

- Isolation of microorganisms from rhizosphere soil of Darjeeling hills, their characterization and identification
- Screening of isolates and characterization as phosphate solubilizers, chitin, cellulose and lignin degraders
- Selection of the isolates for their utilization as biocontrol agents against fungal pathogens
- Evaluation of the selected microorganism for plant growth promoting activities
- Molecular diversity analysis of the selected isolates using relevant tools.

CHAPTER 2

LITERATURE REVIEW

Several earlier reviews have outlined the importance of soil microorganisms with respect to Agriculturally Important Microorganisms (AIMs) which are used in a variety of agro-ecosystems both under natural conditions and artificial inoculation for diverse application such as nutrient supply, biocontrol, bioremediation and rehabilitation of degraded lands (Wright and Upadhy, 1998; Smith and Goodman, 1999; Yao *et al.*, 2002; Sharan and Nehra 2011; Bhattacharya and Jha, 2012; Souza *et al.*, 2013). The review presented, has been compiled to focus on the importance of AIMs, their mode of action in promoting plant health as Phosphate solubilizers and Biocontrol Agents, the need of Monitoring these useful agents in the soil following inoculation and the modern day tools to understand their diversity and phylogeny.

Rhizosphere Microflora

Living organisms form three major domains: Bacteria and Archaea, collectively termed prokaryotes, and the Eucarya or eukaryotes. Prokaryotes are distinguished from eukaryotes by the absence of a unit membrane-bound nucleus and, usually, the lack of other cell organelles. Ribosomes in prokaryotes are smaller (70S) than in eukaryotes (80S) and no eukaryote is able to fix atmospheric N₂. The endosymbiotic theory (Margulis, 1993) proposes that the mitochondria and chloroplasts of eukaryotic cells originated as symbiotic prokaryotic cells. The presence of bacterial, circular, covalently closed DNA and 70S ribosomes in mitochondria supports this theory. Despite the apparent, relative simplicity of prokaryotic cells, as a group they have the greater taxonomic and functional diversity. Globally, organic C in prokaryotes is equivalent to that in plants and they contain 10-fold more N. They also possess the most efficient dispersal and survival mechanisms. As a consequence, prokaryotes are of enormous importance in creating, maintaining, and functioning of the soil. Fungi bind soil together, both literally and figuratively, by their filamentous form, their exudates, and their trophic interactions with all other groups of soil organisms.

It is well established that microbial life only occupies a minor volume of soil being localised in hot spots such as the rhizosphere soil (Nannipieri *et al.*, 2003), where microflora has a continuous access to a flow of low and high molecular weight organic substrates derived from roots. This flow, together with specific physical, chemical and biological factors, can markedly affect microbial activity and community structure of the rhizosphere soil (Brimecombe *et al.*, 2001). For many years, soil microbiologists and microbial ecologists differentiated soil microorganisms as 'beneficial' or 'harmful' depending how they affect soil quality, crop growth and yield. Beneficial microorganisms are those that fix atmospheric N, decompose organic wastes and residues, detoxify pesticides, suppress plant diseases and soil-borne pathogens, enhance nutrient cycling and produce bioactive compounds such as vitamins, hormones and enzymes that stimulate plant growth. Soil harbours a phylogenetically diverse community of saprotrophic microorganisms whose physiological activities mediate the biogeochemical cycling of carbon (C) and nitrogen (N) at local, regional and global scales. These communities are structured by the physical environment as well as the availability of growth-limiting resources entering soil (i.e., organic compounds in plant detritus) (Zake *et al.*, 2011).

Agriculturally Important Microorganisms (AIMs)

The rhizosphere harbors an extremely complex microbial community including saprophytes, epiphytes, endophytes, pathogens and beneficial microorganisms. In natural systems, these microbial communities tend to live in relative harmony where all populations generally balance each other out in their quest for food and space (Be' langer and Avis, 2002). In "artificial" systems, *i.e.* agriculture, there is a modification in this natural balance that can drastically alter the microbial community and can lead to loss of beneficial microbes and/or ingress of plant pathogens that may have a devastating effect on plant productivity. In these cases, the integration of beneficial microorganisms into production systems can somewhat shift the balance of the microbial communities toward a population structure more conducive to increased plant health and productivity. Such beneficial rhizosphere organisms are generally termed as Agriculturally Important Microorganisms (AIMs) and are classified into two broad groups based on their primary effects, *i.e.*, their most well known beneficial effect on the plant:

- (i) Microorganisms with direct effects on plant growth promotion [plant growth promoting microorganisms (PGPM)] and
- (ii) Biological control agents (BCA) that indirectly assist with plant productivity through the control of plant pathogens.

In addition to their primary effects on plant productivity and health, respectively, recent work has shown that these beneficial microorganisms possess secondary, *i.e.*, more recently discovered effects that may bestow them increased interest for plant growers (Vassilev *et al.*, 2006; Van-Elsas *et al.*, 2011). More specifically, PGPM have shown activities relating to biocontrol of soilborne pathogens. Conversely, BCA have demonstrated properties that directly promote plant growth (Chakraborty and Chakraborty, 2013). Previous reviews of the role of micro-fauna in the rhizosphere have tended to concentrate on their contribution to gross flows of carbon and nitrogen (Zwart *et al.* 1994) or their role in disease suppression (Curl and Harper 1990). The activity of microorganisms in soil is generally limited by carbon, but not in the rhizosphere where plants steadily supply microorganisms with easily available carbon. Consequently, a specialized microflora typically consisting of fast-growing bacteria results in increased levels of microbial biomass and activity around plant roots (Alphei *et al.* 1996). There is strong top-down control of these bacterial populations by the grazing pressure of microbivorous nematodes and protozoa (Wardle 2002). The release of carbon in the form of root exudates may account for up to 40 percent of the dry matter produced by plants (Lynch and Whipps 1990), even if the C-transfer to exudation was 10–20 percent of total net fixed carbon (Rovira 1991), other microbial symbionts such as mycorrhizae (Smith and Read 1997) or N₂-fixing microorganisms (Ryle *et al.* 1979).

Indirect interactions of microfaunal grazing seem even more important than direct effects due to nutrient release (Bonkowski and Brandt 2002). Protozoa have, for example, been found to increase plant biomass independently of nutrient contents in the plant tissue (Alphei *et al.*, 1996). Thus, in a laboratory experiment with a constant supply of excess nutrients, protozoa increased the biomass of spruce (*Picea abies*) seedlings up to 60 percent (Jentschke *et al.*, 1995). Plants are not simply passive recipients of nutrients, but information from the environment affects their

belowground allocations such as root proliferation (Hodge *et al.*, 1999), formation of symbiotic relationships (e.g. mycorrhizal fungi, Smith and Read 1997; or N₂-fixing bacteria, Ryle *et al.*, 1979), alteration in exudation rates (Wamberg *et al.*, 2003), interactions with free-living bacteria (Mathesius *et al.*, 2003), or production of secondary defence compounds against herbivores (Cipollini *et al.*, 2003).

Phosphate solubilizing microorganisms (PSMs)

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al.*, 2002; Sharan and Nehra, 2011; Hrynkiemicz and Baum, 2011). Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition. Although microbial inoculants are in use for improving soil fertility during the last century, however, a meager work has been reported on P solubilization compared to nitrogen fixation. Soil P dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al³⁺ and Fe³⁺ in acidic, and Ca²⁺ in calcareous or normal soils (Hao *et al.*, 2002). Efficiency of P fertilizer throughout the world is around 10 - 25 % (Isherword, 1998), and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg kg⁻¹ soil (Goldstein, 1994). Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001; Mishra *et al.*, 2012; Pingale and Popat, 2013).

Inorganic forms of P are solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the P ions i.e. PO₄³⁻ directly, releasing P into solution (He *et al.*, 2002).

Evidence of naturally occurring rhizospheric phosphorus solubilizing microorganism (PSM) dates back to 1903 (Khan *et al.*, 2007). Bacteria are more effective in phosphorus solubilization than fungi (Alam *et al.*, 2002). Among the whole microbial population in soil, PSB constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5 % in P solubilization potential (Chen *et al.*, 2006). Number

of PSB among total PSM in north Iranian soil was around 88 % (Fallah, 2006). Microorganisms involved in phosphorus acquisition include mycorrhizal fungi and PSMs (Fankem *et al.*, 2006). Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Igal *et al.*, 2001). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw, 2000). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains (Kucey *et al.*, 1989). A nemato fungus *Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois *et al.*, 2006).

Mechanisms of Phosphorus Solubilization

Some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus, respectively (Khiari and Parent, 2005). Phosphorus solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe *et al.*, 1998). Phosphate solubilization takes place through various microbial processes/mechanisms including organic acid production and proton extrusion (Nahas, 1996, Nenwani *et al.*, 2010).

A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). Phosphorus solubilization is carried out by a large number of saprophytic bacteria and fungi acting on sparingly soluble soil phosphates, mainly by chelation-mediated mechanisms (Whitelaw, 2000). Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, Ca) and decrease the pH in basic soils (Stevenson, 2005). The PSB dissolve the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Deubel *et al.*, 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere is lowered through biotical production of proton / bicarbonate release (anion / cation balance) and gaseous (O₂/CO₂) exchanges. Phosphorus solubilization

ability of PSB has direct correlation with pH of the medium. Release of root exudates such as organic ligands can also alter the concentration of P in the soil solution (Hinsinger, 2001). Organic acids produced by PSB solubilize insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil (Nahas, 1996). Inorganic acids e.g. hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH (Kim *et al.*, 1997, Nenwani *et al.*, 2010; Singh *et al.*, 2013). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar *et al.*, 1999).

Phosphorus mobilization by soil microorganisms

Microorganisms directly affect the ability of plants to acquire P from soil through a number of structural or process-mediated mechanisms. These include (i) an increase in the surface area of roots by either an extension of existing root systems (eg, mycorrhizal associations) or by enhancement of root branching and root hair development (*i.e.* growth stimulation through phytohormones), (ii) by displacement of sorption equilibria that results in increased net transfer of phosphate ions into soil solution or an increase in the mobility of organic forms of P and (iii) through stimulation of metabolic processes that are effective in directly solubilizing and mineralizing P from poorly available forms of inorganic and organic P. These processes include the excretion of hydrogen ions, the release of organic acids, the production of siderophores and the production of phosphatase enzymes that are able to hydrolyse soil organic P (Fig. 3). In particular, organic acids and associated protons are effective in solubilizing precipitated forms of soil P (eg, Fe- and Al-P in acid soils, Ca-P in alkaline soils), chelating metal ions that may be associated with complexed forms of P or may facilitate the release of adsorbed P through ligand exchange reactions (Jones, 1998).

Solubilization of Ca-bound P

Soil phosphates mainly the apatites and metabolites of phosphatic fertilizers are fixed in the form of calcium phosphates under alkaline conditions. Many of the calcium phosphates, including rock phosphate ores (fluoroapatite, francolite), are insoluble in soil with respect to the release of inorganic P (Pi) at rates necessary to support

agronomic levels of plant growth (Goldstein, 2000). Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. Their solubility increases with a decrease of soil pH. Phosphate solubilization is the result of combined effect of pH decrease and organic acids production (Fankem *et al.*, 2006). Microorganisms through secretion of different types of organic acids e.g. carboxylic acid and rhizospheric pH lowering mechanisms dissociate the bound forms of phosphate like $\text{Ca}_3(\text{PO}_4)_2$. Nevertheless, buffering capacity of the medium reduce the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha, 2009). Acidification of the microbial cell surroundings releases P from apatite by proton substitution / excretion of H^+ (accompanying greater absorption of cations than anions) or release of Ca^{2+} (Goldstein, 1994; Illmer and Schinner 1995; Villegas and Fortin 2002). While, the reverse occurs when uptake of anions exceeds that of cations, with excretion of OH^- / HCO_3^- exceeding that of H^+ (Tang and Rengel, 2003). Carboxylic anions produced by PSB, have high affinity to calcium, solubilize more phosphorus than acidification alone (Staunton and Leprince 1996). Complexing of cations is an important mechanism in P solubilization if the organic acid structure favors complexation (Fox *et al.*, 1990). It is controlled by nutritional, physiological and growth conditions of the microbial culture (Reyes *et al.*, 2007), but it is mostly due to the lowering of pH alone by organic acids or production of microbial metabolites (Abd-Alla, 1994). Organic anions and associated protons are effective in solubilizing precipitated forms of soil P (e.g. Fe - and Al - P in acid soils, Ca - P in alkaline soils), chelating metal ions that may be associated with complexed forms of P or may facilitate the release of adsorbed P through ligand exchange reactions (Jones, 1998). Calcium phosphate (Ca-P) release results from the combined effects of pH decrease and carboxylic acids synthesis, but proton release cannot be the single mechanism (Deubel *et al.*, 2000).

Solubilization of Al- / Fe-bound P

Solubilization of Fe and Al occurs via proton release by PSB by decreasing the negative charge of adsorbing surfaces to facilitate the sorption of negatively charged P ions. Proton release can also decrease P sorption upon acidification which increases H_2PO_4^- in relation to HPO_4^{2-} having higher affinity to reactive soil surfaces (Whitelaw, 2000). Carboxylic acids mainly solubilize Al-P and Fe-P (Henri *et al.*,

2008; Khan *et al.*, 2007) through direct dissolution of mineral phosphate as a result of anion exchange of PO_4^{3-} by acid anion, or by chelation of both Fe and Al ions associated with phosphate (Omar, 1998). It is through root colonizing pseudomonads with high-affinity iron uptake system based on the release of Fe^{3+} - chelating molecules i.e. siderophores (Altomare, 1999). Moreover, carboxylic anions replace phosphate from sorption complexes by ligand exchange (Otani *et al.*, 1996; Whitelaw, 2000) and chelate both Fe and Al ions associated with phosphate, releasing phosphate available for plant uptake after transformation. Ability of organic acids to chelate metal cations is greatly influenced by its molecular structure, particularly by the number of carboxyl and hydroxyl groups. Type and position of the ligand in addition to acid strength determine its effectiveness in the solubilization process (Kpombrekou and Tabatabai, 1994). Phosphorus desorption potential of different carboxylic anions lowers with decrease in stability constants of Fe - or Al - organic acid complexes ($\log K_{\text{Al}}$ or $\log K_{\text{Fe}}$) in the order: citrate > oxalate > malonate / malate > tartrate > lactate > gluconate > acetate > formiate (Ryan *et al.* 2001).

Mineralization of organic P

Mineralization of soil organic P (Po) plays an imperative role in phosphorus cycling of a farming system. Organic P may constitute 4-90 % of the total soil P. Almost half of the microorganisms in soil and plant roots possess P mineralization potential under the action of phosphatases (Cosgrove, 1967; Tarafdar *et al.*, 1988). Alkaline and acid phosphatases use organic phosphate as a substrate to convert it into inorganic form. Principal mechanism for mineralization of soil organic P is the production of acid phosphatases (Hilda and Fraga, 2000). Release of organic anions, and production of siderophores and acid phosphatase by plant roots / microbes (Yadaf and Tarafdar, 2001) or alkaline phosphatase (Tarafdar and Claasen, 1988) enzymes hydrolyze the soil organic P or split P from organic residues. The largest portion of extracellular soil phosphatases is derived from the microbial population (Dodor and Tabatabai, 2003). *Enterobacter agglomerans* solubilizes hydroxyapatite and hydrolyze the organic P (Kim *et al.*, 1998). Mixed cultures of PSMs (*Bacillus*, *Streptomyces*, *Pseudomonas* etc.) are most effective in mineralizing organic phosphate (Molla *et al.*, 1984).

Effect of PSMs on Crop Production

Phosphate rock minerals are often too insoluble to provide sufficient P for crop uptake. Use of PSMs can increase crop yields up to 70 percent. Combined inoculation of arbuscular mycorrhiza and PSB give better uptake of both native P from the soil and P coming from the phosphatic rock (Cabello *et al.*, 2005; Pradhan and Shukla, 2005, Singhh *et al.*, 2013, Chakraborty *et al.*, 2013a). Higher crop yields result from solubilization of fixed soil P and applied phosphates by PSB (Zaidi, 1999). Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Ponmurugan and Gopi, 2006). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son *et al.*, 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.*, 2007), while co-inoculation of PSM and PGPR reduced P application by 50 % without affecting corn yield (Yazdani *et al.*, 2009). Inoculation with PSB increased sugarcane yield by 12.6 percent (Sundara *et al.*, 2002). Sole application of bacteria increased the biological yield, while the application of the same bacteria along with mycorrhizae achieved the maximum grain weight (Mehrvarz *et al.*, 2008). Single and dual inoculation along with P fertilizer was 30-40 % better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20 % against sole P fertilization (Afzal and Bano, 2008). Mycorrhiza along with *Pseudomonas putida* increased leaf chlorophyll content in barley. Rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake. Seed yield of green gram was enhanced by 24 % following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* (Zaidi and Khan, 2006). Growth and phosphorus content in two alpine *Carex* species increased by inoculation with *Pseudomonas fortinii* (Bartholdy *et al.*, 2001). Integration of half dose of NP fertilizer with biofertilizer gives crop yield as with full rate of fertilizer; and through reduced use of fertilizers the production cost is minimized and the net return maximized (Jilani *et al.*, 2007).

Soil P precipitated as orthophosphate and adsorbed by Fe and Al oxides is likely to become bio-available by bacteria through their organic acid production and acid

phosphatase secretion. Although, high buffering capacity of soil reduces the effectiveness of PSB in releasing P from bound phosphates; however, enhancing microbial activity through P solubilizing inoculants may contribute considerably in plant P uptake. Phosphorus solubilizing bacteria mainly *Bacillus*, *Pseudomonas* and *Enterobacter* are very effective for increasing the plant available P in soil as well as the growth and yield of crops. So, exploitation of phosphate solubilizing microorganisms through biofertilization has enormous potential for making use of ever increasing fixed P in the soil, and natural reserves of phosphate rocks.

Biological control agents (BCA)

The term biological control and its abbreviated synonym biocontrol have been used in different fields of biology, most notably entomology and plant pathology. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations (Cook, 1993). In both fields, the organism that suppresses the pest or pathogen is referred to as the Biological Control Agent (BCA). More broadly, the term biological control also has been applied to the use of the natural products extracted or fermented from various sources (Cook, 1993). These formulations may be very simple mixtures of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as the target pest or pathogen. While such inputs may mimic the activities of living organisms, non-living inputs should more properly be referred to as biopesticides or biofertilizers, depending on the primary benefit provided to the host plant (Cook, 1993) Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year. **Biological control** of plant diseases including fungal pathogens has been considered a viable alternative method to chemical control. In plant pathology, the term biocontrol applies to the use of microbial antagonists to suppress diseases. Throughout their lifecycle, plants and pathogens interact with a wide variety of organisms. These interactions can significantly affect plant health in various ways (Heydari and Pessarakli, 2010).

Mechanisms of biological control

Direct antagonism

Since biological control is a result of many different types of interactions among microorganisms, scientists have concentrated on characterization of mechanisms occurring in different experimental situations (Audenaert *et al.*, 2002; 1997 Ryu *et al.*, 2004; Inch and Gilbert, 2011). In all cases, pathogens are antagonized by the presence and activities of other microorganisms that they encounter.

Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanism(s) expressed by the biocontrol active microorganisms. In this type of interaction, Hyperparasitism by obligate parasites of a plant pathogen would be considered the most direct type of mechanism because the activities of no other organism would be required to exert a suppressive effect (Harman *et al.*, 2004). In contrast, indirect antagonism is resulted from the activities that do not involve targeting a pathogen by a biocontrol active microorganism. Improvement and stimulation of plant host defense mechanism by non-pathogenic microorganisms is the most indirect form of antagonism (Silva *et al.*, 2004). While many studies have concentrated on the establishment of the importance of specific mechanisms of biocontrol to particular pathosystems, all of the mechanisms described below are likely to be operating to some extent in all natural and managed ecosystems. The most effective biocontrol active microorganisms studied appear to antagonize plant pathogens employing several modes of actions (Cook, 1993).

For example, pseudomonads known to produce the antibiotic 2, 4-diacetylphloroglucinol (DAPG) may also induce host defenses (Kloepper *et al.*, 1980; Lafontaine and Benhamou, 1996; Leeman *et al.*, 1995; Maurhofer *et al.*, 1994; Silva *et al.*, 2004). Additionally, DAPG-producers bacterial antagonists can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of plant through competition for organic nutrients. However, the most important modes of actions of biocontrol active microorganisms are as follows:

Mycoparasitism: In Hyperparasitism, the pathogen is directly attacked by a specific biocontrol agent (BCA) that kills it or its propagules. Four major groups of hyperparasites have generally been identified which include hypoviruses, facultative

parasites, obligate bacterial pathogens and predators. An example of hypoparasites is the virus that infects *Cryphonectria parasitica*, the fungal causal agent of chestnut blight, which causes hypovirulence, a reduction in pathogenicity of the pathogen. This phenomenon has resulted in the control of chestnut blight in many places (Milgroom and Cortesi, 2004). However, the interaction of virus, fungus, tree and environment determines the success or failure of hypovirulence.

In addition to hypoviruses several fungal hypoparasites have also been identified including those that attack sclerotia (e.g., *Coniothyrium minitans*) or others that attack fungal hyphae (e.g. *Pythium oligandrum*). In some cases, a single fungal pathogen can be attacked by multiple hyperparasites. For example, *Acremonium alternatum*, *Acrodontium crateriforme*, *Ampelomyces quisqualis*, *Cladosporium oxysporum* and *Gliocladium virens* are just a few of the fungi that have the capacity to parasitize powdery mildew pathogens (Milgroom and Cortesi, 2004). In contrast to hyperparasitism, microbial predation is more general, non-specific and generally provides less predictable levels of disease control. Some biocontrol agents exhibit predatory behavior under nutrient-limited conditions. Such as *Trichoderma*, a fungal antagonist that produces a range of enzymes that are directed against cell walls of pathogenic fungi (Benhamou and Chet, 1997; McIntyre *et al.*, 2004; Gajera *et al.*, 2013).

Antibiosis: Many microbes produce and secrete one or more compounds with antibiotic activity (Islam *et al.*, 2005). In a general definition antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. It has been shown that some antibiotics produced by microorganisms are particularly effective against plant pathogens and the diseases they cause (Islam *et al.*, 2005). In all cases, the antibiotics have been shown to be particularly effective at suppressing growth of the target pathogen *in vitro* and/or *in situ* conditions. An effective antibiotic must be produced in sufficient quantities (dose) near the pathogen. *In situ* production of antibiotics by several different biocontrol agents has been studied (Thomashow *et al.*, 1990). While several procedures have been developed to ascertain when and where biocontrol agents may produce antibiotics detecting expression in the infection court is difficult because of the heterogenous distribution

of plant-associated microbes and the potential sites of infection (Thomashow *et al.*, 1990).

However, in some cases, the relative importance of antibiotic production by biocontrol bacteria has been demonstrated. For example, mutant strains incapable of producing phenazines (Thomashow and Weller, 1988) or phloroglucinols (Keel *et al.*, 1989) have been shown to be equally capable of colonizing the rhizosphere, but much less capable of suppressing soil borne root diseases than the corresponding wild-type and complemented mutant strains. Many biocontrol strains have been shown to produce multiple antibiotics which can suppress one or more pathogens (Islam *et al.*, 2005). The ability of production of several antibiotics probably results in suppression of diverse microbial competitors and plant pathogens.

Metabolite production: Many biocontrol active microorganisms produce other metabolites that can interfere with pathogen growth and activities. Lytic enzymes are among these metabolites that can break down polymeric compounds, including chitin, proteins, cellulose, hemicelluloses, DNA as well as HCN and Siderophores (Anderson *et al.*, 2004; Martinez-Viveros, 2010; Stals *et al.*, 2010; Hartl *et al.*, 2012). Studies have shown that some of these metabolites can sometimes directly result in the suppression of plant pathogens. For example, control of *Sclerotium rolfsii* by *Serratia marcescens* appeared to be mediated by chitinase expression. It seems more likely that antagonistic activities of these metabolites are indicative of the need to degrade complex polymers in order to obtain carbon nutrition. Microorganisms that show a preference in colonizing and suppression of plant pathogens might be classified as biocontrol agents. For example, *Lysobacter* and *Myxobacteria* that produce lytic enzymes have been shown to be effective against some plant pathogenic fungi (Bull *et al.*, 2002).

Studies have shown that some products of lytic enzyme activity may have indirect efficacy against plant pathogens. For example, oligosaccharides derived from fungal cell walls have been shown to induce plant host defenses. It is believed that the effectiveness of the above compounds against plant pathogens is dependent on the composition and carbon and nitrogen sources of the soil and rhizosphere. For example, in post-harvest disease control, addition of chitosan which is a non-toxic and biodegradable polymer of beta-1, 4-glucosamine produced from chitin by

alkaline deacylation stimulated microbial degradation of pathogens (Benhamou, 2004). Amendment of plant growth substratum with chitosan suppressed the root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato (Lafontaine and Benhamou, 1996).

In addition to the above-mentioned metabolites, other microbial byproducts may also play important roles in plant disease biocontrol (Phillips *et al.*, 2004). For example, Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations (Ramette *et al.*, 2003; Kumar *et al.*, 2012). The production of HCN by certain fluorescent pseudomonads is believed to be effective against plant pathogens. Results of some research studies in this regard have shown that *P. fluorescens* CHA0, an antagonistic bacterium, produces antibiotics including siderophores and HCN, but suppression of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be due primarily to HCN production.

Competition: The nutrient sources in the soil and rhizosphere are frequently not sufficient for microorganisms. For a successful colonization of phytosphere and rhizosphere a microbe must effectively compete for the available nutrients (Loper and Buyer, 1991). On plant surfaces, host-supplied nutrients include exudates, leachates, or senesced tissue. In addition to these, nutrients can also be obtained from waste products of other organisms such as insects and the soil. This is a general believe that competition between pathogens and non-pathogens for nutrient resources is an important issue in biocontrol. It is also believed that competition for nutrients is more critical for soil borne pathogens, including *Fusarium* and *Pythium* species that infect through mycelial contact than foliar pathogens that germinate directly on plant surfaces and infect through appressoria and infection pegs (Loper and Buyer, 1991). It has been shown that non-pathogenic plant-associated microorganisms generally protect the plant by rapid colonization and thereby exhausting the limited available substrates so that none are available for pathogens to grow. For example, effective catabolism of nutrients in the spermosphere has been identified as a mechanism contributing to the suppression of *Pythium ultimum* by *Enterobacter cloacae* (Kageyama and Nelson, 2003). At the same time, these microbes produce metabolites that are effective in suppression of pathogens. These microbes colonize the sites where water and carbon-containing nutrients are most readily available, such as exit

points of secondary roots, damaged epidermal cells and nectaries and utilize the root mucilage.

Competition for rare but essential micronutrients, such as iron, has also been shown to be important in biological disease control. Iron is extremely limited in the rhizosphere, depending on soil pH. In highly oxidized and aerated soil, iron is present in ferric form (Kageyama and Nelson, 2003; Shahraki *et al.*, 2009), which is insoluble in water and the concentration may be extremely low. This very low concentration can not support the growth of microorganisms. To survive in such environment, organisms were found to secrete iron-binding ligands called Siderophores having high ability to obtain iron from the micro-organisms (Shahraki *et al.*, 2009). Almost all microorganisms produce siderophores, of either the catechol type or hydroxamate type (Kageyama and Nelson, 2003).

A direct correlation was established in vitro between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *F. oxysporum* (Elad and Baker, 1985). It was shown that mutants incapable of producing some siderophores, such as pyoverdine, were reduced in their capacity to suppress different plant pathogens (Loper and Buyer, 1991). The increased efficiency in iron uptake of the commensal microorganisms is thought to be a critical factor in their root colonization ability which is a major factor in biocontrol performance of bacterial antagonists.

Induced Systemic Resistance (ISR)

Plants actively respond to a variety of environmental stimulating factors, including gravity, light, temperature, physical stress, water and nutrient availability and chemicals produced by soil and plant associated microorganisms (Moyne *et al.*, 2000; Vallad and Goodman, 2004; Van Loon *et al.*, 1998; Van Peer and Schippers, 1992; Van Wees *et al.*, 1997). Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Induction of host defenses can be local and/or systemic in nature, depending on the type, source and amount of stimulation agents (Audenaert *et al.*, 2002; Vallad and Goodman, 2004; George *et al.*, 2013).

The first pathway called Systemic Acquired Resistance (SAR), is mediated by Salicylic Acid (SA), a chemical compound which is usually produced after pathogen infection and typically leads to the expression of Pathogenesis-related (PR) proteins

(Vallad and Goodman, 2004). These PR proteins include a variety of enzymes some of which may act directly to lyse invading cells, reinforce cell wall boundaries to resist infections, or induce localized cell death (Vallad and Goodman, 2004).

ISR is mediated by Jasmonic Acid (JA) and/or ethylene, which are produced following applications of some nonpathogenic rhizobacteria (Audenaert *et al.*, 2002). Interestingly, the SA- and JA- dependent defense pathways can be mutually antagonistic and some bacterial pathogens take advantage of this to overcome the SAR. For example, pathogenic strains of *Pseudomonas syringae* produce coronatine, which is similar to JA, to overcome the SA-mediated pathway (Vallad and Goodman, 2004). Since the various host-resistance pathways can be activated to variable degrees by different microorganisms and insect feeding, it is therefore possible that multiple stimuli are constantly being received and processed by the plant. Thus, the magnitude and duration of host defense induction will likely vary over time. Only if induction can be controlled, i.e., by overwhelming or synergistically interacting with endogenous signals, will host resistance be increased (Audenaert *et al.*, 2002; De Meyer and Hofte, 1997). Some strains of root-colonizing microorganisms have been identified as potential elicitors of plant host defenses. For example, some biocontrol active strains of *Pseudomonas* sp. and *Trichoderma* sp. are known to strongly induce plant host defenses (Haas and Defago, 2005; Harman *et al.*, 2004). In other instances, inoculation with Plant Growth Promoting Rhizobacteria (PGPR) have been shown to be effective in controlling multiple diseases caused by different fungal pathogens, including anthracnose (*Colletotrichum lagenarium*). A number of chemical elicitors of SAR and ISR such as salicylic acid, siderophore, lipopolysaccharides and 2, 3-butanediol may be produced by the PGPR strains upon inoculation (Ryu *et al.*, 2004).

A substantial number of microbial products have been reported to elicit host defenses, indicating that host defenses are likely stimulated continually during the plant's lifecycle (Ryu *et al.*, 2004). These inducers include lipopolysaccharides and flagellin from Gram-negative bacteria; cold shock proteins of diverse bacteria; transglutaminase, elicitors and α -glucans in Oomycetes; invertase in yeast; chitin and ergosterol in all fungi; and xylanase in *Trichoderma* (Ryu *et al.*, 2004). These findings indicate that plants would detect the composition of their plant-associated microbial communities and respond to changes in the quantity, quality and localization of many different signals. The importance of such interactions is

indicated by the fact that further induction of host resistance pathways, by chemical and microbiological inducers, is not always effective in improving plant health or productivity in the field (Vallad and Goodman, 2004).

Plant immune responses triggered by beneficial microbes

Beneficial soil-borne microorganisms, such as plant growth promoting rhizobacteria and mycorrhizal fungi, can improve plant performance by inducing systemic defense responses that confer broad-spectrum resistance to plant pathogens and even insect herbivores. Different beneficial microbe-associated molecular patterns (MAMPs) are recognized by the plant, which results in a mild, but effective activation of the plant immune responses in systemic tissues. Evidence is accumulating that systemic resistance induced by different beneficials is regulated by similar jasmonate-dependent and ethylene-dependent signaling pathways and is associated with priming for enhanced defense (Van Wees et al 2008). Plant roots become quickly colonized by a diverse microflora of soil-borne bacteria and fungi that may have either beneficial or deleterious effects on the plant. Classical examples of symbiotic microorganisms are mycorrhizal fungi that aid in the uptake of water and minerals, notably phosphate (Harrison, 2005), and Rhizobium bacteria that fix atmospheric nitrogen for the plant (Spaink, 2000). Several other types of beneficial soil-borne microbes, such as plant growth promoting rhizobacteria and fungi, can stimulate plant growth by suppressing plant diseases (Waller *et al.*, 2005) or insect herbivory (Van Oosten *et al.*, 2008). This biological control activity is exerted either directly through antagonism of soil-borne pathogens or indirectly by eliciting a plant-mediated resistance response (Pozo *et al.*, 2007; Liang *et al.*, 2011; George *et al.*, 2013)

Resistance-inducing traits of beneficial microbes

Microbial determinants that contribute to induced resistance as triggered by beneficial microbes are best studied for fluorescent *Pseudomonas* spp. In analogy to the Microbe-Associated Molecular Patterns (MAMPs) flagellin and lipopolysaccharides (LPS) of pathogenic *Pseudomonas* spp. (Nurnberger, 2004), it was found that these cell surface components of beneficial *Pseudomonas* spp. are potent inducers of the host immune response. Purified flagellin and LPS of the nonpathogenic resistance-inducing strains *Pseudomonas fluorescens* WCS417 and

WCS374, and *Pseudomonas putida* WCS358 have differential resistance-inducing activities on *Arabidopsis*, tomato, and bean, suggesting host specificity in the recognition of these beneficial microbe derived MAMPs. Flagellin and LPS mutants of these rhizobacterial strains are nevertheless often as effective as the wild-type strains, suggesting that multiple MAMPs are involved in the activation of the plant's immune response (Bakker, 2007).

Under conditions of low iron availability, most aerobic and facultative anaerobic microorganisms, including fluorescent *Pseudomonas* spp., produce low molecular weight Fe³⁺-specific chelators, so-called siderophores. Competition for iron between fluorescent *Pseudomonas* spp. and plant pathogens is often considered to be the mode of action of these siderophores in disease suppression. However, a role for siderophores in the elicitation of resistance has been reported in several systems as well (Meziane *et al.*, 2005). For instance, in tomato the *P. putida* WCS-358 siderophore pseudobactin-358 triggers systemic resistance, but the pseudobactin358-mutant of this strain does not. In bean, however, this mutant is as effective as the wild-type strain, again indicating that induced systemic resistance (ISR) is activated by multiple MAMPs in this plant–microbe interaction. Interestingly, under low iron conditions several *Pseudomonas* spp. Also produce salicylic acid (SA), a signaling molecule that is known to play an important role in the regulation of pathogen-induced systemic acquired resistance (SAR) (Durrant and Dong 2004). Indeed, SA produced by the siderophore mutant KMPCH of *P. aeruginosa* 7NSK2 was demonstrated to induce disease resistance in tomato. However, in most cases, microbially produced SA does not contribute to enhanced defense, as it is directly channeled into the production of SA-containing siderophores (Mercado-Blanco and Bakker 2007).

Induced defense signaling pathway

It is probable that Microbe-Associated Molecular Patterns (MAMPs) of beneficial microbes and pathogens are recognized in a largely similar manner, ultimately resulting in an enhanced defensive capacity of the plant. However, in plant–beneficial microbe interactions, MAMP-triggered immunity does not ward off the interacting beneficial as it remains accommodated by the plant. This suggests a high

degree of coordination and a continuous molecular dialog between the plant and the beneficial organism. The local and systemic defense responses that are triggered by beneficial and parasitic microorganisms are controlled by a signaling network in which the plant hormones SA, jasmonic acid (JA), and ethylene (ET) play important roles (Glazebrook 2007). There is ample evidence that SA, JA, and ET pathways crosscommunicate, allowing the plant to finely tune its defense response depending on the invader encountered (Koornneef and Pieterse 2008). Well-studied examples of systemically induced resistance are SAR, which is triggered upon infection by necrosis-inducing pathogens and is dependent on SA signaling and ISR, which is triggered by beneficial rhizobacteria, such as *P. fluorescens* WCS417 and requires components of the JA and ET signaling pathway (Pieterse *et al.*,1998). Both pathogen induced SAR and *P. fluorescens* WCS417-triggered ISR are controlled by the transcriptional regulator NPR1 (Pieterse and Van Loon 2004). Because SAR is associated with NPR1-dependent PR gene expression and ISR is not, NPR1 must differentially regulate gene expression, depending on the signaling pathway that is activated upstream of it. Hence, the NPR1 protein is integrating and responding to different hormone-dependent defense pathways. Not only several other rhizobacterial strains but also some beneficial fungi have been shown to induce systemic resistance in a JA-dependent, ET-dependent, and/or NPR1-dependent manner (Ahn *et al.* 2007) while there are also some reports about dependency on SA signaling or requirement of both ISR and SAR components (Conn *et al.*,2008).

Local immune responses triggered by beneficial microbes

Only few plant–beneficial microbe interactions leading to enhanced systemic resistance have been studied for locally induced changes in plant gene expression or metabolism. In most cases only weak, transient, or strictly localized defense-associated responses were elicited, which differs greatly from the massive induction of defense responses triggered during plant–pathogen interactions (Verhagen *et al.*, 2004) Transcriptome analysis of *Arabidopsis* expressing WCS417-ISR revealed a set of 94 genes that were differentially expressed locally in the roots. Knockout mutant analysis of a subset of these WCS417- responsive genes showed that the transcription factor (TF) MYB72 is required in early signaling steps of ISR (Van der Ent *et al.*, 2008). *Arabidopsis* myb72 mutants were incapable of mounting ISR

against both SA-controlled and JA-controlled pathogens, indicating that MYB72 is essential to establish broad-spectrum ISR. Over expression of MYB72 was not sufficient for the expression of ISR. Hence, MYB72 was assumed to act in concert with another signaling component. MYB72 interacted with the EIN3-like TF EIL3 in vitro, making EIL3 a potential candidate in this respect. The interaction with EIL3 links MYB72 function to the ET response pathway involved in ISR, which was previously demonstrated to orchestrate ISR in the roots (Knoester et al 1999). Interestingly, resistance induced in Arabidopsis by the beneficial fungus *Trichoderma asperellum* T34 also appeared to be dependent on MYB72 suggesting that MYB72 functions as a node of convergence in induced defense triggered by soil borne beneficial microorganisms.

Multiple functions of soil microbes

Biochemical mechanisms and metabolites in P-solubilizing microorganisms related to their biocontrol activity Indole-3-acetic acid (IAA) and siderophores, which are among the most frequently studied metabolites with biocontrol functions, are found to be released by microorganisms that express P-solubilizing activity (Sharan and Nehra, 2011). Siderophores are low-molecular-weight, iron-chelating ligands synthesized by microorganisms (Winkelmann 1991). Most bacteria and fungi produce siderophores that differ according to their functional groups. Siderophore production helps a particular microorganism compete effectively against other organisms for available iron. This enhances the growth of the microorganism while limiting iron availability to the competing microorganisms restricts their growth. It is accepted that this mechanism suppresses pathogenic microorganisms (Crowley, 2006). It was also shown that siderophores are beneficial to plants by solubilizing iron formerly unavailable to the plant (Prabhu *et al.* 1996). Similarly, auxins are thought to play a role in host– parasite interactions and particularly the plant-growth regulator IAA is involved in the interaction between a plant pathogen and its host (Hamill, 1993). Various authors have proposed mechanisms of biocontrol action of IAA, which resulted in two main hypotheses: a potential involvement of IAA together with glutathione S-transferases in defense-related plant reactions (Droog, 1997) and an inhibition of spore germination and mycelium growth of different pathogenic fungi (Brown and Hamilton 1993).

Some of the fungi solubilize rock phosphate, presumably by releasing metal-chelating metabolites (Vassilev *et al.*, 2006), we can expect their application as biocontrol microorganisms with simultaneous P-solubilizing activity. P-solubilizing filamentous fungi are also well-known producers of lytic enzymes. Cell-wall-degrading enzymes, such as β -1,3-glucanases, cellulases, proteases, and chitinases are known to be involved in the activity of some microorganisms against phytopathogenic fungi (Chakraborty and Chakraborty, 2013).

One of the most studied approaches in solubilization of insoluble phosphates is the biological treatment of rock phosphates. In recent years, various techniques for rock phosphate solubilization have been proposed, with increasing emphasis on application of P-solubilizing microorganisms. The P-solubilizing activity is determined by the microbial biochemical ability to produce and release metabolites with metal-chelating functions. In a number of studies, it has been shown that agro-industrial wastes can be efficiently used as substrates in solubilization of phosphate rocks. In fermentation conditions, P-solubilizing microorganisms were found to produce various enzymes, siderophores, and plant hormones. Further introduction of the resulting biotechnological products into soil-plant systems resulted in significantly higher plant growth, enhanced soil properties, and biological (including biocontrol) activity. Application of these bio-products in bioremediation of disturbed (heavy metal contaminated and desertified) soils is based on another important part of their multifunctional properties (Vassileva *et al.*, 2010).

Properties of Successful Microbial Inoculum

The majority of soil microorganisms (95–99%) is known to be at least so far nonculturable (Torsvik and Øvreås 2002). However, the basic criterion for subsequent selection and later application of microbial inoculum useful for plant-growth promotion is cultivability and fast multiplication of microorganisms. Information of critical factors influencing plant-microbe-pollutant interactions in soils could lead to an improved selection of microbial inoculum for a microbial-assisted bioremediation. A fundamental condition for subsequent on-site applications of selected microorganisms is their safety for the environment and for humans. Therefore, before field applications, all selected microorganisms have to be precisely identified and toxicologically assessed. Very few different microbial taxa have been

tested so far for their capability to promote plant growth at disturbed and polluted soils and little is known on the microbial spectrum which might be especially relevant to promote plant species in disturbed soils. In general, numerous species of mycorrhizal fungi and soil bacteria which inhabit the rhizosphere can promote plant growth (Compant *et al.*, 2005), e.g., by enzymatic nutrient mobilisation from organic matter (mostly P and N) and production of siderophores (Jing *et al.* 2007) and might be promising also for disturbed or polluted soils. They can contribute essentially to soil aggregation and nutrient availability which is often especially important for disturbed soils. Therefore, enzyme activities can be suitable selection criteria for microbial inoculums for plant growth promotion in disturbed soils. Microbial enzyme activities in the soil were predominantly measured as total potential activities rather than at the level of isolates within a community (e.g., Khan *et al.*, 2007). However, investigations of single strains are necessary for the selection of potential inoculums (Hryniewicz *et al.*, 2010b). High cellulolytic and pectolytic activities of mycorrhizal fungi and rhizosphere bacteria allow the disintegration of living and dead plant tissue and consequently, can enable microorganisms to enter roots. High cellulolytic and pectolytic activities were detected among mycorrhizal fungi (Garbaye, 1994) and their helper bacteria (Duponnois and Plenchette, 2003). Therefore, also cellulolytic and pectolytic activities might be suitable selection criteria. Furthermore, lipolytic activities might be relevant for the selection of microorganisms especially for biodegradation, since they can improve not only the N supply of plants but also promote the biodegradation of organic pollutants (e.g., petroleum-derived wastes) in soils (Hryniewicz *et al.*, 2010a, b). In rhizosphere microbial communities siderophore synthesis might be especially important for successful competition of rhizosphere microorganisms in disturbed soils with extremely low nutrient concentrations. Beside their direct effects on the iron supply of plants, siderophores can contribute additionally to the suppression of pathogens in the rhizosphere through their withhold from iron supply. Furthermore, auxins are recognized as highly active plant growth stimulators, and indole-3-acetic acid (IAA) is a key substance (Woodward and Bartel, 2005). Indole-3-acetic acid (IAA) production is widespread among soil microorganism, mostly ectomycorrhizal fungi (Niemi and Scagel, 2007). Several authors revealed that fungal strains with high

IAA-synthesizing activity induce stronger growth of fine roots and significantly higher numbers of mycorrhizae compared to strains with low activity of IAA.

However, beside these criteria, the selection of suitable combinations of host plants and microbial inoculum is necessary. Specificity of combinations of mycorrhizal fungal and bacterial strains as well as host plants for the remediation of disturbed soils is rarely known. It is still in discussion if a specific fungal selection of particular bacterial strains exists and whether cooperation of these bacterial strains is restricted to given ectomycorrhizal fungi. In several previous published works (Zimmer *et al.*, 2009) it was demonstrated that interactions of mycorrhizal fungi and bacteria can be significantly growth promoting even in situations when the microorganisms used as inoculum does not originate from the same host plant and site. Also several previous studies (Xavier and Germida, 2003) revealed a low specialization of bacterial strains to mycorrhizal fungi and their host plants. This feature of inoculum might assure a broader spectrum for practical applications of microbial inoculum. As a possible mechanism for selection of fungus-associated bacterial strains by ectomycorrhizal fungi, de Boer *et al.* (2005) suggested exudation of soluble fungal storage sugars (usually trehalose), polyols (e.g., mannitol) or organic acids (in particular oxalic acid) which can increase the number of bacteria or exudation of inhibitory chemicals which select antibiotic-resistant bacteria.

Rhizosphere colonization by AIMs

Root exudates released into the soil environment from plants have been traditionally grouped into low- and high-molecular weight compounds. High-molecular weight compounds include polysaccharides, mucilage, and proteins. Plant mucilages are released from the root cap, the primary cell wall between epidermal and sloughed root cap, and epidermal cells (including root hairs). Lysates are released from roots during autolysis. Rhizospheric microorganisms also release microbial mucilages. Collectively, plant and microbial mucilages, microbial cells and their products together with associated organic and mineral matter are referred to as mucigel (Walker *et al.*, 2003). Low-molecular organic compounds released by plant roots include ethylene, sugars, amino acids, vitamins, polysaccharides, and enzymes. The fact the nutritional resources influence population structure and play a role in niche colonization and competition.

Factors Affecting Root Colonization and Efficacy of AIMS

Bacterial root colonization is primarily influenced by the presence of specific bacterial traits required for attachment and subsequent establishment; however, other abiotic and biotic factors play an important role in colonization. When an organism colonizes a root, the process must be confirmed with an array of external parameters including water content, temperature, pH, soil types (texture, organic matter, microaggregate stability, presence of key nutrients such as N, P, K, and Fe), composition of root exudates, and presence of other microorganisms. Plant species is another major determinant of overall microbial diversity (Dakora and Philipps, 2002). The colonization of a fluorescent *Pseudomonas* strain in the potato rhizosphere was reported to be tenfold greater in a sandy loam soil than in clay loam soil. Root colonization of bacteria is negatively affected by predation (protozoa) and parasitism (bacteriophages). Inoculated bacteria must compete with natural inhabitants of the soil for nutrients. The biosynthesis of antagonistic compounds by rhizobacteria such as antibiotics could be affected by nutrient competition.

Antibiotic secretion also plays an important role in the establishment of bacteria in the rhizosphere (De Weert and Bloemberg 2006). In vitro activities exhibited by various PGPR for biocontrol may not provide the identical results under field conditions. The failure of PGPR to produce the desired effects after seed/seedling inoculation is frequently associated with their inability to colonize plant roots. The process of root colonization is complex; several traits associated with survivability, tolerance, competition with indigenous rhizospheric microorganisms, and expression of root colonizing traits are important (Somers and Vanderleyden 2004). In many countries, harsh climatic conditions, population pressures, land constraints, and decline of traditional soil management practices have often reduced soil fertility. Such extreme effects will certainly alter soil's chemical, physical, and biological properties and therefore affect microbial colonization. Biocontrol agents may be affected by indigenous soil microbial communities and they may also influence the community into which they are introduced. Enhancement of introduced PGPR populations leading to enhanced suppression of soil borne pathogens.

A single biocontrol agent is not active against all the pathogens that attack the host plant; a single biocontrol agent is effective against a single pathogen under laboratory

conditions. This may be the reason for the inconsistent performance of biocontrol agents introduced into the field. Naturally occurring biocontrol results from mixtures of agents, rather than from high populations of a single organism. Greater suppression and enhanced consistency against multiple cucumber pathogens were observed using strain mixtures of PGPR. Incompatibility of the co-inoculants may sometimes arise and thus inhibit each other as well as the target pathogens. This is therefore an important prerequisite for successful development of strain mixtures. Even more important is that the inoculant strains may fail to survive and not colonize the root. Patterns of survival and effectiveness with growth phases of plants have not been clearly studied; nor have efforts to distinguish inoculated PGPR from indigenous microbial populations. Thus, various methods are in use to monitor inoculant strains, both genetically modified and non-modified (Ahmed, 2011).

Monitoring of Microbial Inoculants

Substantial range of monitoring methods has been developed for the detection and quantification of microorganisms for various purposes (Morris *et al.*, 2002). Monitoring methods can be divided into three groups: microbiological, direct methods, and molecular methods.

Microbiological Monitoring Methods

These methods are culture-based classical methods and are commonly used to study and monitor soil microbes including those inoculated into the soil system for their survival and colonization on root surfaces as well as in bulk soil. The basic requirement for such methods is the availability of selective media for target organisms to differentiate from native microbes. It is at times difficult to differentiate inoculated organisms from native populations based on morphological characteristics (Lima *et al.*, 2003). Many authors have used the spontaneous mutant of the parent strain resistant to antibiotics such as nalidixic acid and rifampicin in order to differentiate with indigenous bacterial population (Ahmad *et al.* 2006). However, resistance to antibiotics among indigenous populations which can grow on selective media should be first checked before inoculation.

Direct Monitoring

Direct monitoring methods are based on the detection of a specific phenotypic characteristic of the biological agent, for example the emission of fluorescence, to achieve its identification. Bioluminescence is a phenotypic characteristic that can be used to mark biological control/PGPR agents. This technique is based on the introduction of an exogenous reporter gene which encodes for enzymes or proteins responsible for bioluminescence. The most frequently described reporter genes are the *lux* gene from the bacterium *Vibrio fischeri* and *gfp* gene from the jellyfish *Aequorea victoria*. The quantification in direct monitoring is achieved by optical detection methods such as fluorescence microscopy (epifluorescence microscopy), spectrofluorometry, or flow cytometry. Many authors using direct monitoring methods for biological control agents in environmental samples make use of *gfp* markers with flow cytometry (Lowder *et al.*, 2000) and the *gfp/lux* dual marker with flow cytometry and spectrofluorometry to monitor *P. fluorescence* (Unge *et al.*, 1999). Emphasis has been placed on the detection and enumeration of PGPR released in field inoculations as an essential requirement for the assessment of their survival in field conditions. Fluorescent-antibody and selective plating techniques have served as the conventional strategies for detection and isolation of bacteria in environmental samples (Herbert, 1990).

Immunological techniques are useful for both quantification and in situ visualization of bacteria (Mahaffee *et al.*, 1997). They are based on specific antibodies directed against bacterial antigens and can be successfully detected by enzyme-linked immunosorbent assay (ELISA) procedure (Tsuchiya *et al.*, 1995; Chakraborty *et al.*, 2009), the immunofluorescence colony (IFC) staining approach is more informative since it combines quantification (enumeration of colonies marked with antibodies conjugated with fluorescein isothiocyanate) with visualization in planta. Immunomagnetic attraction (specific antibodies linked to iron oxide particles) is also used for quantification (enumeration of bacteria captured with a supermagnet) (Paulitz, 2000). Fluorescence-labeled antibodies have been used with success for detection of root-colonizing *Pseudomonas* strains by immunofluorescence microscopy (Troxler *et al.*, 1997).

Molecular Monitoring Methods

Recent developments in molecular detection techniques have greatly increased the ability to track microorganisms and engineered genetic markers in natural environments (Pickup 1991). Molecular biology techniques that allow the detection of microorganisms in soil include the use of DNA probes, polymerase chain reaction (Ruppel *et al.*, 2006), use of selective markers such as antibiotic resistance genes, and the use of chromogenic markers such as β -galactosidase and β -glucuronidase. None of the techniques mentioned above provides in situ detection in soil, however. DNA hybridization requires extraction of cells and removal of humic material prior to DNA extraction (Ahmad *et al.*, 2011). For monitoring of organisms after introduction into soil, a selective marker that does not interfere with the ability of the strain to survive and, in the case of microorganisms that interacts with plants, to promote plant growth, is needed. A general molecular approach to characterize and detect specific microorganism based on direct DNA isolation and molecular characterization is elaborated in the form of flow chart.

Many workers have used genomic molecular markers to track the biocontrol strain (Broggini *et al.*, 2005). This technique has drawbacks, as the native strain may also have similar molecular markers. To overcome this problem amplified fragment length polymorphism (AFLP), the amplification of repetitive sequence-based PCR (rep PCR), and random amplified polymorphic DNA (RAPD) are recommended. However, these techniques have been used primarily for eukaryotic organisms (Buhariwalla *et al.*, 2005). AFLP, rep PCR, and RAPD have been used for fingerprinting microorganisms. However, when used for the detection of biological control agents they have a significant drawback; in spite of being specific for characterization of a microorganism, they require the isolation of the target strain prior to its detection. An improvement has been made to the above technique by developing sequence characterized amplified regions (SCARs). SCAR markers are obtained by the selection of a unique amplified fragment which differentiates the target strain from others (Chapon *et al.*, 2003).

Microorganisms introduced into the environment undergo a wide variety of processes following their introduction including growth, physiological adaptation, conversion

to nonculturable cells, physical spread, and gene transfer (Van Elsas *et al.*, 1998). Hence, the application of single methods for microbial detection and for evaluation of their activity in the rhizosphere and risk involved is likely to provide only partial information. Both culture-based and culture-independent approaches have their own advantages and limitations. It is suggested that a polyphasic approach would be most practical for monitoring of microbial inoculant in rhizosphere/bulk soil. For robust assessment of the fate and effect of released microbial inoculants/ PGPR, it is therefore necessary to use a combination of techniques as the case may depend upon microbe-to-microbe and microbe-to-plant interactions and other environmental factors. Microscopy, cultivation-based and molecular-based techniques should be developed both for genetically modified and unmodified inoculants released into the rhizosphere or the larger environment. As our understanding of the complex environment of the rhizosphere, of the mechanisms of action of PGPR, and of the practical aspects of inoculant formulation and delivery increase, we can expect to see new PGPR products becoming available. The success of these products will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms (Bowen and Rovira, 1999).

Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculant formulation and delivery. Genetic enhancement of PGPR strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion. The use of multistrain inocula of PGPR with known functions is of interest as these formulations may increase consistency in the field. Alternatively, plant growth-promoting microorganisms with multifarious desirable traits and tolerance to environmental conditions are expected to provide improved results (Imran, 2009). They offer the potential to address multiple modes of action, multiple pathogens, and temporal or spatial variability. The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products with improved effectiveness. However, multiple strain-based inoculants will require more careful monitoring for their survival, colonization, and effectiveness in the root zone.

Diversity analysis of AIMS

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. Since the first estimate of prokaryotic abundance in soil was published, researchers have attempted to assess the abundance and distribution of species and relate this information on community structure to ecosystem function. Present study has investigated the linkage of specific organisms to ecosystem function and an increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The two main drivers of soil microbial community structure, i.e., plant type and soil type, are thought to exert their function in a complex manner. Plant type and soil type both affects the microbial diversity and abundance of soil. It has been reported that statistical analyses of the microbial counts indicated a significant correlation for bacteria ($p < 0.01$) and no significant correlation, for fungi and actinomycetes, however, microbial enumeration indicated that bacteria were most numerous followed by actinomycetes and fungi, respectively (Meliani *et al.*, 2012)

Traditional approaches to the study of microbial diversity have relied on laboratory cultivation of isolates from natural environments and identification by classical techniques, including analysis of morphology, physiological characteristics and biochemical properties. These approaches provide information on fine-scale diversity but suffer from bias, resulting from the media and cultivation conditions employed, and from the inability to grow and isolate significant proportions of natural communities in the laboratory. An alternative approach is the amplification of ribosomal RNA and functional genes from nucleic acids extracted directly from environmental samples, with subsequent analysis by 'fingerprinting' methods or by sequencing and phylogenetic analysis. This approach avoids the need for laboratory cultivation and has provided major insights into species and functional diversity of bacterial and archaeal populations.

An alternative approach, which removes many of the above limitations, is the analysis of genes within environmental samples. These genes may be functional genes, i.e. those coding for proteins performing particular metabolic reactions of

relevance to ecosystem processes. However, most applications have analysed genes encoding the small subunit (SSU) of ribosomal RNA. Analysis of 16S rRNA genes is now widely used for analysis of bacterial populations, and analysis of 18S rRNA genes and internal transcribed spacer (ITS) regions is increasingly being used to analyse fungal populations. Ribosomal rRNA genes are ideal for this purpose in that they possess regions with sequences conserved between all bacteria or fungi, facilitating alignment of sequences when making comparisons, while other regions exhibit different degrees of variation, enabling distinction between different groups. These differences provide the basis for a phylogenetic taxonomy and enable quantification of evolutionary differences between different groups. Discrimination of bacteria, using 16S rRNA gene sequences, is greater than that for fungi, using 18S rRNA sequences, but finer scale information may be obtained by analysis of ITS regions. The presence of regions of rDNA sequence with different degrees of conservation enables the identification of sequences that are common to all bacteria or fungi, or to specific phylogenetic groups, sometimes to the level of species. These sequences may then be used to design primers for the specific amplification, using the polymerase chain reaction (PCR), of rRNA genes belonging to particular groups or to design specific probes for these groups. These primer sequences provide the basis for analysis of species in natural populations. Two approaches may be adopted, the first based on PCR amplification of rRNA genes and the second involving *in situ* detection of rRNA within cells.

Analysis of amplified genes

The first stages in the analysis of rRNA genes in an environmental sample are cell lysis and extraction of DNA, after which DNA is purified to remove material inhibitory to subsequent enzymatic reactions. PCR amplification is then carried out, using primers specific to the microbial groups of interest. Amplification generates a population of rRNA genes, or gene fragments, of equal size, determined by the particular primers used. This population of gene fragments is considered to be representative of the natural microbial population. Most information, and fine scale discrimination between groups, is obtained by cloning the amplified rRNA genes and sequencing members of the clone library. Comparison of sequences with those in databases determines which phylogenetic groups are present and, in many cases,

enables more detailed identification. This approach is particularly useful for studies of bacteria, as 16S rRNA databases are now extensive and comprehensive. They contain sequences of large numbers of laboratory cultures and also of clones obtained from a range of environments, which are not represented in laboratory cultures. Finally, if sufficiently large numbers of clones are sequenced, estimates may be obtained of the relative abundance of different groups. More rapid analysis is achieved using fingerprinting techniques. The most commonly used technique in 16S rRNA studies has been denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1998), which separates products of the same size, but different sequence, by chemical denaturation. Following staining of gels, banding patterns may be used to compare communities, or to compare the same community following perturbations, and band intensities may be used for semi-quantification of relative abundance (McCaig *et al.*, 1999, 2001). In addition, bands may be excised and genes amplified and sequenced for fine scale analysis. A similar approach is adopted in temperature gradient gel electrophoresis (TGGE), where denaturation results from high temperatures (Felske *et al.*, 1998). A number of fingerprinting techniques involve restriction analysis of PCR products, including terminal restriction length polymorphism (tRFLP, Liu *et al.*, 1997) and amplified ribosomal DNA restriction analysis (ARDRA) (Øverås and Torsvik, 1998). In some cases, database information may be used to predict the banding patterns generated using these techniques by particular rRNA gene sequences, providing some information on the identity of organisms present. PCR-based methods, such as competitive PCR (Jansson and Leser, 1996) and real-time PCR (Heid *et al.*, 1996) are also used to quantify gene copies, and hence cell number or biomass. Taking into account the aforementioned intricacies of a typical habitat, Warmink and Van Elsas, (2008).

Controlling the Soil Microflora for Optimum Crop Production and Protection

The idea of controlling and manipulating the soil microflora through the use of inoculants organic amendments and cultural and management practices to create a more favorable soil microbiological environment for optimum crop production and protection is not new. For almost a century, microbiologists have known that organic wastes and residues, including animal manures, crop residues, green manures,

municipal wastes (both raw and composted), contain their own indigenous populations of microorganisms often with broad physiological capabilities.

It is also known that when such organic wastes and residues are applied to soils many of these introduced microorganisms can function as biocontrol agents by controlling or suppressing soil-borne plant pathogens through their competitive and antagonistic activities.

For, many years microbiologists have tried to culture beneficial microorganisms for use as soil inoculants to overcome the harmful effects of phytopathogenic organisms, including bacteria, fungi and nematodes. Such attempts have usually involved single applications of pure cultures of microorganisms which have been largely unsuccessful for several reasons. First, it is necessary to thoroughly understand the individual growth and survival characteristics of each particular beneficial microorganism, including their nutritional and environmental requirements. Second, we must understand their ecological relationships and interactions with other microorganisms, including their ability to coexist in mixed cultures and after application to soils.

There are other problems and constraints that have been major obstacles to controlling the microflora of agricultural soils. First and foremost is the large number of types of microorganisms that are present at any one time, their wide range of physiological capabilities, and the dramatic fluctuations in their populations that can result from man's cultural and management practices applied to a particular farming system. The diversity of the total soil microflora depends on the nature of the soil environment and those factors which affect the growth and activity of each individual organism including temperature, light, aeration, nutrients, organic matter, pH and water. While there are many microorganisms that respond positively to these factors, or a combination thereof, there are many that do not. Microbiologists have actually studied relatively few of the microorganisms that exist in most agricultural soil, mainly because we don't know how to culture them; i.e., we know very little about their growth, nutritional, and ecological requirements.

It is noteworthy that most of the microorganisms encountered in any particular soil are harmless to plants with only a relatively few that function as plant pathogens or potential pathogens. Harmful microorganisms become dominant if conditions develop that are favorable to their growth, activity and reproduction.

Under such conditions, soil-borne pathogens (e.g., fungal pathogens) can rapidly increase their populations with devastating effects on the crop. If these conditions change, the pathogen population declines just as rapidly to its original state. Conventional farming systems that tend toward the consecutive planting of the same crop (i.e., monoculture) necessitate the heavy use of chemical fertilizers and pesticides. This, in turn, generally increases the probability that harmful, disease-producing, plant pathogenic microorganisms will become more dominant in agricultural soils (Higa, 1995; Parr and Hornick, 1994). Chemical-based conventional farming methods are not unlike symptomatic therapy. Examples of this are applying fertilizers when crops show symptoms of nutrient-deficiencies, and applying pesticides whenever crops are attacked by insects and diseases. In efforts to control the soil microflora some scientists feel that the introduction of beneficial microorganisms should follow a symptomatic approach. However, the actual soil conditions that prevail at any point in time may be most unfavorable to the growth and establishment of laboratory-cultured, beneficial microorganisms. To facilitate their establishment, it may require that the farmer make certain changes in his cultural and management practices to induce conditions that will (a) allow the growth and survival of the inoculated microorganisms and (b) suppress the growth and activity of the indigenous plant pathogenic microorganisms. Vegetable cultivars are often selected on their ability to grow and produce over a wide range of temperatures. Under cool, temperate conditions there are generally few pest and disease problems. However, with the onset of hot weather, there is a concomitant increase in the incidence of diseases and insects making it rather difficult to obtain acceptable yields without applying pesticides

New Dimensions for Sustainable Agriculture

Many microbiologists believe that the total number of soil microorganisms can be increased by applying organic amendments to the soil. This is generally true because most soil microorganisms are heterotrophic, i.e., they require complex organic molecules of carbon and nitrogen for metabolism and biosynthesis. Whether the regular addition of organic wastes and residues will greatly increase the number of beneficial soil microorganisms in a short period of time is questionable.

The probability that a particular beneficial microorganism will become predominant, even with organic farming or nature farming methods, will depend on the ecosystem and environmental conditions. It can take several hundred years for various species of higher and lower plants to interact and develop into a definable and stable ecosystem. Even if the population of a specific microorganism is increased through cultural and management practices, whether it will be beneficial to plants is another question. Thus, the likelihood of a beneficial, plant-associated microorganism becoming predominant under conservation-based farming systems is virtually impossible to predict. Moreover, it is very unlikely that the population of useful anaerobic microorganisms, which usually comprise only a small part of the soil microflora, would increase significantly even under natural farming conditions (Chakraborty and Chakraborty, 2013).

This information then emphasizes the need to develop methods for isolating and selecting different microorganisms for their beneficial effects on soils and plants. The ultimate goal is to select microorganisms that are physiologically and ecologically compatible with each other and that can be introduced as mixed cultures into soil where their beneficial effects can be realized.

CHAPTER 3

MATERIALS AND METHOD

3.1. Griding of study area

North Bengal has a total area of 21763.0 sq km stretching from 24°40'28'' N to 27°13' N Latitudes and 87°45'50'' to 89°54'35'' E Longitudes. The entire area comprises of six districts and three important ecological zones.

3.1.1. Darjeeling hill region

Darjeeling district is situated within the state of West Bengal. Kalimpong, Kurseong and Siliguri, are the sub-divisional headquarters of the district. The Hill areas of Darjeeling District are located within the lesser and Sub - Himalayan belts of the Eastern Himalayas. The area is bounded by the Sikkim Himalaya in the north, the Bhutan Himalaya in the east and Nepal Himalaya in the west. The southern foothill belt is demarcated by a highly dissipated platform of terrace deposits extending along the east west axis. The inner belt is defined by a ridgeline stretching from the Darjeeling Hill to the west and Kalimpong Hill to the east, overlooking the southerly flowing Tista valley in between. Prominent rivulets contributing to the Rammam - Rangit basin dissipate the northern slope of Darjeeling Hills. Geographically, the district can be divided into two broad divisions, the hills and the plains. The hilly region of the district are the three hill subdivisions of Darjeeling, Kurseong and Kalimpong. The foothills of Darjeeling Himalayas, which comes under the Siliguri subdivision, is known as the Terai. The major rivers flowing through here are- Teesta River , Mahananda River, the Great Rangit, Mechi, Balason, Lish, Gish, Chel, Ramman, Murti and Jaldhaka river. The Darjeeling hill area is formed of comparatively recent rock structure that has a direct bearing on landslides. Soils of Darjeeling hill areas are extremely varied, depending on elevation, degree of slope, vegetative cover and geolithology. As regards the geology of the land, Darjeeling Hill area represents a unique geo- environmental perception. It is primarily composed of erosional landforms produced by southerly flowing streams, which have exposed a full cross section of different tectonic units. The form units are, however

approximately the same throughout the hill area, having more or less uniform lithology, structure, climate, soil and vegetative covers. The contact between different groups of rocks is represented by thrusts, dipping at high angles towards north. There is various land formations found across the length of the Darjeeling Himalayas. These include Raised terraces, the Siwaliks, the Damuda series, Daling series and Darjeeling Gneiss. The soils of Darjeeling Hill area have developed depending upon the underlying geological structure. But, in general the soils have been developed by both fluvial action and lithological disintegration. The soils that have developed in the Kalimpong area are predominantly reddish in color. Occasional dark soils are found due to extensive existence of phyllitic and schists. Soils in the highlands stretching from the west to the east of the district along most of the interfluvial areas are mainly mixed sandy loam and loamy, while those on the southern slopes of Mirik and Kurseong are mainly clayey loam and reddish in color. Sandy soils are mainly found in the east of the river Tista. The basic soil types are yellow soils, red brown soils and brown forest soils. Red soil and yellow soil have developed on gneiss while brown on schists and shales. Coarse pale yellow to red brown soils are found on the Siwaliks while clayey dark soils are developed on Daling series. On the Darjeeling gneiss, very coarse-grained (50 percent -80 percent) particles are found. All the soils are definitely acidic in nature with the tendency to increase slightly in depth in most cases indicating the absence of bases. Gross cultivated in Darjeeling district is around 63, 786 ha. Important cash crops include Orange, Ginger and Cardamom. Apart from these Tea is one of the most important plantation crop of this region. There are 87 tea gardens spread across the Darjeeling hill which covers roughly 19,000 hectares of the total land area. The major portions of the forests are today found at elevations of 2000 meters and above. The area located in between 1000–2000 meters is cleared either for tea plantation or cultivation. The four major forest types according to altitudinal variation found in Darjeeling Hill Areas are: Tropical moist deciduous forest (300-1000mts);Tropical evergreen lower montane forest (1000-2000mts.) ;Tropical evergreen upper montane forest (2000-3000mts.) ;Temperate forest (3000-3500mts.) ;Sub temperate forest (above 3500mts.).About 30% of the forest covers found in the lower hills are deciduous. Evergreen forest constitutes only about 6% of the total forest coverage. *Shorea robusta* remains the most prominent species of Tropical moist deciduous forest

along with heavy under growth. In the slopes on southern portion of the Tista and the Great Rangit valley and in the Goke forests, this type is found. These species cannot thrive in areas of lower precipitation. Tropical lower montane evergreen forests are found on steep higher slopes, where drainage condition is good; Dhupi (*Cryptomaria Japonica*) is a known variety. The impact of man on this variety is very conspicuous. Tropical upper montane evergreen forests are found in the areas where high humidity along with dense fogs and less sunlight is available. Undergrowth is dense and contains Nettles, Raspberries, Ferns and bamboos. On the steep ridges, Rhododendrons and bamboos are abundant.

3.2. Soil sampling strategy

3.2.1. Sampling protocol

Each ecological zone (Terai and Dooars) of North Bengal have been divided into agriculturally dependent riverine and forest areas according to land use types. One of the most important steps in soil sampling is to collect the sample that represents that area, which means that the sample should be representative. Hence during sampling each zone has been divided into non-uniform random sampling units according to the type of vegetation they represent. While sampling the following points were taken into consideration:

(a) Use of proper sampling tools. (b) Avoiding unusual areas for sampling. (c) Dividing the areas for random sampling. (d) Taking composite sample from each area and (e) Taking proper records of the samples.

3.2.2. Grid sampling in non uniform ecological zones

Many sampling units were not uniform and varied both horizontally and vertically along the landscapes therefore the eco zones were broken into grids with shorter distances between the sampling points. This allows the development of precise sampling maps for further analysis. Since the sampling region falls under varying altitudes while determining the grid, one of the important things that have been considered was the altitude of the sampling unit areas.

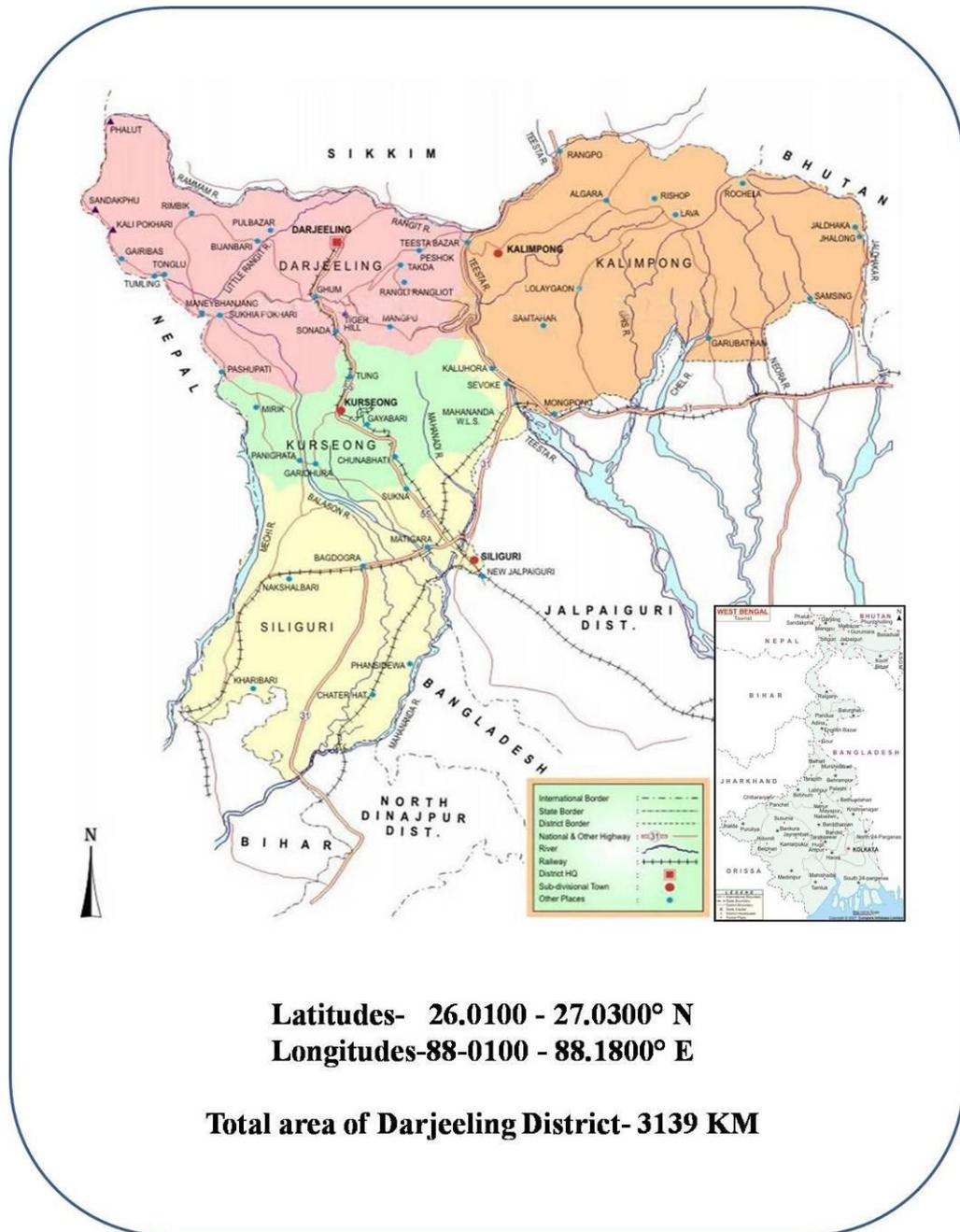


Fig. 4. Map of Darjeeling district showing four main subdivisions

3.3. Isolation of microorganisms from soil

The following plating techniques were adopted for isolation of microorganisms from the collected soil samples

3.3.1. Soil dilution technique

Warcup's soil plate method (1955) for isolating microorganisms from the rhizosphere was followed with a few modifications. This is one of the most popular

methods for isolation and enumeration of soil borne actinomycetes, bacteria and fungi. It favors fungi that sporulates profusely or exist primarily as spores.

3.3.2. Direct soil plating

The process of Thomas and Parkinson (1965) has been adopted with modifications to isolate the fungi. Fungi that don't sporulate and exist as mycelium in soil seldom are isolated by the soil plating method. Soil (5-15 mg) was placed on a sterile culture plate and spread evenly; then 10-15 ml of molten agar medium was added. Finally soil particles were dispersed evenly with swirling motion.

3.3.3. Soil washing technique

Microorganisms not readily isolated from the soil plating technique were obtained from this technique of Watson (1960) where 1 g (air dried equivalent) of soil sample and 200 ml of sterile water were taken in a 500 ml flask, agitated with a blender and allowed to stand for 1-2 minutes, water was poured off and the process was repeated for 30-40 times further agitating by hand. After the final washing step the soil dilution plate method was followed.

3.4. Composition of Solid media

(A) For isolation of PGPR

Nutrient agar medium (NA)

Peptone - 5gm,
Sodium Chloride 5gm,
Yeast extract 1.5 gm,
Beef extract 1.5g,
Agar 20g,
Water 1L

King's B (KB)

Peptone 20.0 g,
K₂HPO₄ 1.5g,
MgSO₄.7H₂O 1.5 g
Glycerol 15 ml
Distilled H₂O- 1L,
pH 7.4 ± 0.2

(B) For isolation of *Trichoderma* species:

Special Nutrient Agar (SNA) (Samuels *et al.* 1998)

KH₂PO₄ 1.0g
MgSO₄.7H₂O 0.5g

KCl	0.2g
KNO ₃	0.5g
Glucose	0.2g
Sucrose	1.0g
Agar	20.0g
Distilled water	1000ml

After sterilization, medium was supplemented with 300µg ml⁻¹L Oxytetracycline.

***Trichoderma* Selective Medium C (TSMC) (Elad *et al.*, 1981)**

MgSO ₄ (7H ₂ O)	0.2g
KH ₂ PO ₄	0.9g
KCl	0.14g
NH ₄ NO ₃	1.0g
Anhydrous Glucose	3.0g
Rose Bengal	0.15g
Agar	20.0g
Distilled water	950 ml

After autoclaving, 50 mL of anti microbial agents (Chloramphenicol 0.25g; Quintozone 0.2g; Captan 0.2g and Metalaxyl 1.6g) was added.

Cellulose Agar Medium (Kuling *et al.*, 2000)

Cellulose powder	30.0g
NaNO ₃	3.0g
(NH ₄)SO ₄	1.0g
KH ₂ PO ₄	1.0g
(NH ₄) ₂ HPO ₄	0.5g
MgSO ₄ .7H ₂ O	0.5g
MnSO ₄ .6H ₂ O	0.02g
Bacto yeast extract	0.3g
FeSO ₄ .7H ₂ O	0.1g
CoCl ₂ .6H ₂ O	0.02g
KCl	0.5g
Agar	18.0g
pH (before autoclaving)	6.5

After sterilization, supplemented with 300µg ml⁻¹L Oxytetracycline.

(C) For identification purpose:

Malt Extract Agar (MEA)

Malt extract	20.0g
Agar	20.0g

Water 1000ml

Oatmeal Agar (OA)

Oatmeal 200g (blended in 600ml water, heated to 40-45° C)

Agar 20.0g (melted in 400ml water)

Both were mixed up, filtered and then autoclaved for 90 min and supplemented with 300µg ml⁻¹L Oxytetracycline.

Cornmeal Dextrose Agar (CMD)

Cornmeal 40.0g

Dextrose 20.0g

Water 1000ml

Filtered before autoclaving for 15min.

Difco cornmeal-dextrose agar 2% (w/v) supplemented with 300µg ml⁻¹L Oxytetracycline.

(D) For maintenance of cultures:

Potato Dextrose Agar (PDA)

Potato 200g

Dextrose 30.0g

Agar 20.0g

Water 1000ml

pH 6.5

After sterilization, PDA was supplemented with 300µg ml⁻¹L Oxytetracycline

(E) Richards agar (RA):

KNO₃ 1.0g

KH₂PO₄ 50g

MgSO₄. 7H₂ O 0.25g

FeCl₃ 0.002g

Sucrose 3.0g

Agar 2.0g

Distilled H₂O 100ml

(F) Carrot juice agar (CJA):

Grated carrot 20.0g

Agar 2.0g

Distilled water 100 ml

(G) Czapek dox agar (CDA):

NaNO₃ 0.20g

KHPO₄ 0.10g

MgSO₄.7H₂O 0.05g

KCl 0.05g

FeSO₄.7H₂O 0.05g

Sucrose 3.0g

Agar 3.0g

Distilled water 100ml

(H) Potato sucrose agar (PSA):

Peeled potato 40.0g

Sucrose 2.0g

Agar 2.0g

Distilled water 100ml

(I) Malt extract peptone agar (MPA):

Malt extract 20.0g

Peptone 1.0g

Dextrose 20.0g

Agar 20.0g

Distilled water 1L

(J) Yeast extract dextrose agar (YDA):

Yeast extract 7.50g

Dextrose 20.0g

Agar 15.0g

Distilled water 1L

(K) Flentze's soil extract agar (FSEA):

Soil extract 1L

Sucrose 1.0g

KH₂PO₄ 0.20g

Dried yeast 0.10g

Agar 25.0g

3.5. Morphological and Microscopical Characterization of isolates

3.5.1. Assessment of mycelial growth.

Mycelial growth of the fungal cultures was assessed in both on solid media and liquid media to know their culture characteristic.

3.5.1.1. Solid media

To assess the growth of fungal culture in solid media, the fungus was first grown on petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 30°C. Agar blocks (6mm diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each Petri dish containing 20ml of different sterilized solid media. The colony diameter was studied at regular interval of time.

3.5.1.2. Liquid media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 30 °C. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Ehrlenmeyer flask (250ml) containing 50 ml of sterilized liquid media Potato dextrose broth (PDB), Richards medium and Nutrient broth (NB) and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60°C for 96 hour, cooled in desiccators and weighed.

3.5.2. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium for 1ml of bacterial suspension was inoculated into the Nutrient broth medium (Peptone - 5.0g, Beef extract - 3.0g, NaCl - 5.0g, Yeast extract - 3.6g, Water - 1000ml, pH – 7.4 ± 0.2) and allowed to grow for 48h. following growth, absorbance was noted in a colorimeter at 600nm. Absorbance was converted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed.

3.5.3. Microscopical characterization

3.5.3.1. Bright field study of fungal spores and mycelia

The isolated fungi were allowed to grow in Petriplates (7cm dia.) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

3.5.3.2. SEM studies of fungal isolates

Selected microorganisms were examined under scanning electron microscopy (SEM). Samples were prepared according to a modification of the method described by King and Brown (1983). Test isolates were grown on PDA plates for 10 days in daylight at room temperature. Small pieces of the agar (less than 1 cm), with aerial sporulating culture attached, were excised from each plate and transferred to the

interior surface of a dry glass Petri dish lid. Efforts were made not to disturb the attached culture. Steps that involved exposing the samples to the atmosphere were performed quickly to minimise air-drying artifacts. The specimen dishes were then placed in vapour diffusion dehydration (VDD) assembly, and a vacuum was drawn. All samples were left in the VDD assembly where a maximum level of dehydration was achieved. The vacuum was released slowly and the specimen dish was removed from the desiccator. Each sample was placed within a separate aluminium “disc cup” (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.5.3.3. SEM studies of bacterial isolates

For scanning electron microscopy of the bacterial cells, 2 days old culture grown in nutrient broth medium were centrifuged at 3000 rpm. The pellet were collected and washed with 0.1 M phosphate buffer saline then the samples were prefixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 6.8 under vacuum followed by dehydrolysis of the sample by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100% and for each ethanol volume incubated for 10 minutes. After stepwise dehydration in graded alcohol, the samples were critical point dried in CO₂ (CPD 030; BAL TEC, Vaduz, Liechtenstein), mounted onto the sample stubs and were coated with 20 nm silver-palladium alloy in a mini sputter coater (SC7620) and examined in a JEOL JSM 5200 Scanning Electron Microscope (Tokyo Japan).

3.6. Biochemical characterization of bacterial isolates

3.6.1. Gram reaction

Gram reaction was carried out as outlined by Buchanan and Gibbson (1974). Smears of test organisms prepared from 24h old culture (on nutrient agar slant) with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, and heat fixed. The smear was then flooded with crystal violet (crystal violet – 2.0g, 95% alcohol- 20ml, ammonium oxalate 1% W/V, aqueous solution – 80ml) stain for 1 min, washed with tap water for 5 sec, flooded with Burke’s iodine

solution (Iodine 1.0g, KI- 2.0g, distilled water 100ml) and allowed to react for 1 min. Slides were washed for 5 sec in 95% ethanol which was poured drop by drop by holding the slides in slanting position till the smears becomes decolorised, rinsed with water and dried. The smears were finally counter stained with safranin (2.5 w/v safranin in 95% ethanol- 10ml, distilled water -100ml) for at least thirty seconds, rinsed with water and dried. The gram character and morphological characters were determined under oil-immersion objectives.

3.6.2. Catalase

Bacterial culture (24 hour old) was flooded with 0.5 ml 10% H₂O₂ solution and gas bubbles production indicated the positive reaction.

3.6.3. Urea digestion

Streaks were made on the slants containing urea medium and incubated at 37° C for 2-7 days. The change in colour of the medium indicates the presence of urease.

3.6.4. H₂S production

Slants containing SIM agar inoculated with the test bacteria and incubated for 48h at 37°C. Darkening along the line of the slants indicated the production of H₂S by the organisms.

3.7. *In vitro* characterization of plant growth promoting activities

3.7.1 Phosphate solubilizing activity

3.7.1.1. Screening for primary phosphate solubilizing activity on PKV medium

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium (Pikovskaya 1948) supplemented with Tricalcium phosphate (TCP) and pH of the medium was adjusted to 7.0 before autoclaving. One gram soil sample was suspended in 9ml sterile distilled water in a tube for serial dilutions, and 1ml aliquots were transferred to PVK medium. The plates were incubated at 28±2°C for 7 days with continuous observation for colony diameter. Transparent (halo) zones of clearing around the colonies of microorganisms indicate phosphate solubilization and each colony was carefully transferred, identified and further used for quantitative determination of phosphate solubilization.

3.7.1.2. Quantitative measurement of phosphate solubilization

Evaluation of phosphate solubilizing activity of both the fungal and bacterial isolates were done by growing the isolates in the two sets of Pikovakaya's liquid medium

amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days at 28⁰C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate is done following ammonium molybdate ascorbic acid method as described by Kundsen and Beegle (1988). Amount of phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovakaya's liquid medium (yeast extract, 0.50 g/L, dextrose, 10.0 g/L, calcium phosphate/rock phosphate, 5.0 g/L, ammonium phosphate, 0.50 g/L, potassium chloride, 0.20 g/L, magnesium sulphate, 0.10 g/L, manganese sulphate, 0.0001g/L, ferrous sulphate, 0.0001 g/L, pH, 6.5) amended with 0.5 % tricalcium phosphate and 0.5 % rock phosphate. Liquid medium (50 ml) was inoculated with 5 % v/v of the spore suspension prepared from the 7 days old culture grown on PDA slants and incubated at room temperature for 4 days with routine shaking at 100 rpm. The initial pH of the medium was recorded with a pH meter fitted with electrode. The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 x g for 5 min. on a table centrifuge.

Phosphate measurement in the medium was estimated by ammonium molybdate-ascorbic acid method where, 2ml of the aliquot of the soil extract was mixed with 8ml of the colorimeter working solution containing 60 g/L ammonium paramolybdate, 1.455 g antimony potassium tartarate, 700ml/L conc. sulfuric acid and 132 ml/L of ascorbic acid and mixed thoroughly and incubated for 20 min. till the colour developed. Percent transmittance of the solution was taken on a colorimeter with the wavelength set at 882 nm.

3.7.2. IAA production

3.7.2.1. Qualitative test for IAA production

10 ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolate and incubated anaerobically at 37° C for 7 days. The culture was centrifuged at 10000 rpm for 15 min and supernatant was taken for analysis.

The supernatant was layered carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the supernatant- reagent interface indicating the production of indole.

3.7.2.2. Quantitative test for IAA production

For detection and quantification of IAA, the selected bacterial cells were grown for 24 h to 48 h in high C/N ratio medium. Tryptophane (0.1 mM) was added in order to enhance acetic acid (IAA) production by the bacteria (Prinsen *et al.* 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.* (1999). For the reaction, 1 ml of reagent, consisting of 12 g FeCl₃ per litre in 7.9 M H₂SO₄ was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm. The amount of IAA produced was calculated with the help of a standard curve.

3.7.3. Siderophore production

The bacterial isolates were characterized for siderophore production following the method of Schwyn and Neiland, (1987) using blue indicator dye, chrome azurol S (CAS). For preparing CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl₃.6H₂O in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates (20 ml per plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

3.7.4. HCN production

Production of hydrocyanic acid was determined using the procedure described by Reddy *et al.* (2008) with slight modification. The selected bacterial isolates were grown at room temperature (37°C) on a rotary shaker in nutrient broth (NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed in side the conical flasks in a hanging position. After incubation at 37°C for 48 hr, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean

test tube containing 10 ml distilled water and the absorbance was measured at 625 nm.

3.7.5. Chitinase production

3.7.5.1. Detection in modified Chitinase detection agar

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 1.0% (w/v) colloidal chitin with 15 g of agar in a medium consisted of (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, yeast extract 0.05g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 30°C for 7-10 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains (Kamil *et al.* 2007).

The colloidal chitin was prepared by following the method described by Mathivanan *et al.* (1997), 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 L of ice cold 95 % ethanol with rapid stirring and kept overnight at 25°C. The precipitation formed was collected by centrifugation at 7000 rpm for 20 min at 4°C and washed with sterile distilled water until the colloidal solution became neutral (pH 7). The prepared colloidal chitin solution (5 %) was stored at 4°C until further use.

3.7.5.2. Quantification of Chitinase activity

Spore suspension (1.0×10^6 spores per mL of culture medium of biocontrol agent were grown in 150 mL flasks containing 20 mL of unbuffered mineral synthetic medium (MSM) supplemented with dried mycelium as the sole carbon source (5 g L^{-1}). The cultures were grown at 30°C for 5 days without shaking. Culture filtrates were centrifuged at 4°C for 10 min at 5000 x g and the clear supernatants were either immediately tested for enzyme activity or stored at -20 °C until assayed.

Chitinase activity was assayed using the colorimetric method described by Molano *et al.* (1977) with minor modifications (Ulhoa, 1992). The assay mixture contained 1 mL of 0.5 % pure chitin (suspended in 50 mM acetate buffer pH=5.2) and 1 mL of enzyme solution. The reaction mixture was incubated for 12 h at 37 °C with shaking and was stopped by centrifugation (5000 g/min) for 10 min and the addition of 1 mL of dinitrosalicylate (DNS) reagent (Miller, 1959).

3.7.6. Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.* (1995).

3.7.7. Starch hydrolysis

Detection of starch hydrolysis was detected by streaking the isolate on sterilized starch agar plate (NA + 0.1% soluble starch) and incubating for five days at 37° C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.8. Screening for cellulase production

Microorganism showing cellulase activities were screened in the medium containing only cellulose as the carbon source. Both exo and endo cellulase activities were determined as the amount of glucose released from the substrate. The amount of glucose released by exocellulase activity of the microorganism during the growth period was measured following the DNS method of Miller (1972). To determine endo and exo β -1, 4 glucanase activity and the amount of glucose released in the medium corresponding to the amount of substrate utilized combined assay was conducted using filter paper assay (FPA) method of Miller (1972).

3.8.1. Assay of endocellulase activity

The amount of glucose released by endocellulase activity of fungal hyphae during their growth using cellulose as C source was measured following the DNS method of Miller (1972) where the culture filtrate was collected from the fermentation media by centrifugation. Culture filtrate (1 ml) was taken in a test tube and equalized with 2ml of distilled water. To the prepared culture filtrate, 3 ml of DNS reagent was added. The contents in the test tubes are heated in a boiling water bath for 5 min. After heating, the contents were allowed to cool at room temperature. At the time of cooling, 1 ml of freshly prepared 40% sodium potassium tartarate solution was added. After cooling, the absorbances were recorded at 510 nm in a U.V. vis spectrophotometer. The amount of reducing sugar was determined using a standard graph.

3.8.2. Assay of both exo and endocellulase activity

A combined assay for endo and exo cellulose activity in culture filtrate is carried out by FPA (Filter Paper Assay). The substrate used is Whatman No. 1 filter paper which was homogenized in 0.2 M sodium acetate buffer, pH 5.5 (5 mg in 20 ml buffer). 0.5

ml of culture filtrate was added to 2ml of substrate. The mixture was incubated at 35°C for one hour, 2 ml of DNS reagent was added and reaction was terminated. Then it was heated in a boiling water bath for 5 min following which 1 ml of potassium sodium tartarate (40%) was added to the warm tubes. The tubes were allowed to cool and the absorbance was recorded at 540 nm in a U.V. vis spectrophotometer.

3.9. Casein hydrolysis

The mild agar was streaked with the bacteria and was incubated at 37° C and observed for the clear zone around the streaks.

3.10. *In vitro* testing for antagonism to fungal pathogens

3.10.1. Inhibition of mycelial growth in solid medium

For *in vitro* evaluation of antagonistic activity of both PGPR and fungal isolates, the following fungal pathogens viz., *Sclerotium rolfsii*, *Thanatophorous cucumeris*, *Rhizoctonia solani* and *Macrophomina phaseolina*. were used. The fungal pathogens were obtained from Immuno-Phytopathology Laboratory, Department of Botany, N.B.U. and were maintained with regular sub culturing in PDA for subsequent tests. Isolated microorganisms were tested for their *in vitro* antifungal activity against plant pathogens by dual inoculation technique. In case of fungal isolates, both the test organisms and the pathogens were grown separately in the petriplates and inocula were cut from the growing region and placed in fresh sterile PDA plates. In each plate, inoculum block of the isolate and of the test pathogen were placed 4 cm apart on the agar medium. The culture plates were seeded with the potential antagonist and the test pathogen at a distance determined by their growth rate (Klingstrom and Johansson, 1973). Interactions were observed at different intervals from 4th day onwards. For each test three replicates were used.

Whereas in case of PGPR isolates, the bacteria were streaked on one side of the Petri plate containing Potato Dextrose Agar (PDA) medium and 5mm fungal pathogen block was placed at the other side of the plate at a distance of 5 cm, incubated for 2-7 days at 28^o±2^oC and inhibition zone towards the fungal colony in individual plate was measured. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolate. For each test three replicate plates were used.

3.10.2. Sclerotia germination bioassay

For assessing the effect of active principle from cell free culture filtrate of BCA and PGPR on sclerotial germination of *Sclerotium rolfsii*, the mature sclerotia were scrapped off from the culture grown on PDA medium. Sclerotia were then soaked in cell free culture filtrate for 1h and placed on sterile black filter paper which were also aseptically soaked in the culture filtrate for at least 30 min. The black filter paper was then placed in a sterile Petri plates. Sclerotia soaked in distilled water and uninoculated sterile PDB served as control. On each soaked filter paper 50 sclerotia were placed with at least three replicates. These were allowed to germinate for 2-4 days after which percent germination was determined.

3.11. Immunological studies

3.11.1. Preparation of fungal and bacterial antigen

Fungalmycelial and Bacterial cell protein were prepared following the method as outlined by Chakarborty and Saha (1994). In case of fungal isolates, mycelial mats were harvested from 5-7 days old culture, washed with 0.2% NaCl and again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM MgCl₂ in ice bath. The homogenate mixture was kept for 2h or overnight at 4°C and then centrifuged at 10,000rpm for 30 min at 4°C to eliminate cell debris. The supernatant was collected and stored in -20°C and used as antigen for the preparation of antiserum.

Prior to injection quantity Total Soluble Protein was measured and these was also analyzed by 12 % SDS-PAGE.

3.11.2. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al.*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO₄ and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% Na₂ CO₃ in 0.1 NaOH) was added. This was incubated for 15 minutes at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 minutes following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.11.3. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for detailed analysis of protein profile following the method of Laemmli (1970).

3.11.3.1. Preparation of stock solution

Following stock solutions were prepared.

A. Acrylamide and N'N' – methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4°C and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris Buffer

- i) 1.5M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for further use.
- ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for use.

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris- Glycine electrophoresis buffer

Tris running buffer consists of 25mM Tris base, 250mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1L distilled water.

F. SDS gel loading buffer

This buffer contains 50 mM Tris –HCl (pH 6.8), 10 mM β- mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M β- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water

3.11.3.2. Preparation of gel

Mini slab gel was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8 cmx10 cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb +1cm). After pouring the resolving gel solution, it was immediately overlaid with isobutanol and kept for polymerization for 1h.

After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and overlaid with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	Resolving gel		5% Stacking gel
	10% (ml)	12%(ml)	(ml)
Distilled water	2.95	2.45	2.10
30% acrylamide	2.50	3.00	0.10
Tris*	1.90	1.90	0.38
10%SDS	0.075	0.070	0.030
10%APS	0.075	0.070	0.030
TEMED**	0.003	0.003	0.003

*For 1.5 M tris pH 8.8 in resolving gel and for 1M Tris pH 6.8 in stacking gel

** N, N, N', N' -Tetramethyl ethylene diamine.

3.11.3.3. Sample preparation

Sample (50µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 3 minutes to denature the proteins. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97, 4000; Biovine Serum Albumin-68,000; Albumin-43,000; Carbolic Anhydrase-29,000; Soybean Trypsin inhibitor-20,000; Lysozyme-14,300) was similarly treated as the other samples and loaded in a separate well.

3.11.3.4. Electrophoresis

Electrophoresis was performed at 18mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.11.3.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved, 45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

The gel was removed from the fixer and stained in this stain solution for 4 hours at 37°C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5:4.5:1) at 37°C with constant shaking until the background become clear.

3.11.4. Raising of polyclonal antibodies

3.11.4.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal antigens in New Zealand white male rabbits approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera Reserves for plant Pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After

each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.11.4.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera, intramuscularly injections of 1ml antigen(protein extracted) mixed with 1ml of Freund's complete adjuvant (Genei) were given into each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Methods of Alba and Devay (1985) and Chakraborty and Saha (1994) were followed for immunization.

3.11.4.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their back on a wooden board fixed at an angle of 60°, and the rabbits were held tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5-10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37°C for 1hr for clotting. After clotting, the clot was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and distributed in 1 ml vials and stored at -20°C as crude antisera. The serum was used for double diffusion analysis, dot immunobinding assay and indirect immunofluorescence study.

3.11.5. Purification of IgG

3.11.5.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2ml) was diluted with two volume of distilled water and an equal volume of ammonium sulphate was taken and adjusted to pH to 6.8, the mixture was stirred for 16h at 20° C in magnetic stirrer. The precipitate thus formed was collected by Centrifugation at 12,000 rpm for 1h at 22°C. Supernatant was discarded and pellet was used for further steps.

3.11.5.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30cm height) and allowed to settle for 2h. After the column material had settled 25ml of buffer (0.02M sodium phosphate, pH 8.0) washing was given to the column material.

3.11.5.3. Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 m to 0,03 M. the initial elution buffer (1) was 0.02 M sodium phosphate (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column buffer (2) was soaked into buffer (1) thereby producing a continuous raise in morality. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV-Vis spectrophotometer (DIGISPEC-200GL).

3.11.6. Immunodiffusion test

3.11.6.1. Preparation of agarose slides

The glass slides (6cm x 6cm) were degreased using ethanol 90%v/v: diethyl ether (1:1v/v) and ether, then dried in hot air oven. After drying the plates were sterilized inside the petriplate each containing one plate. Conical flask with Tris-Barbiturate buffer (pH 8.6) is placed in boiling water bath. Agar/ agarose (0.9%) was boiled over water bath to dissolve the agar at 90 ° C for next 15 min. Then pinch of 0.1% (w/v) sodium azide was added and mixed well. For the preparation of agarose gel, the molten agarose is poured (6 to 10 ml) on the grease free sterilized slide with the help of a sterile pipette in laminar air flow chamber and allow it to solidify, after solidification cut 3-7 wells (6mm diameter) with sterilized cork borer distance of 1.5 to 2cm away from central well and 2.0 to 2.5 cm from well to well.

3.11.6.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1976). Antigen plus undiluted Antisera appropriately diluted were poured into wells with sterile 60-8- micropipette ($50\mu\text{l}/\text{well}^{-1}$) antisera in middle. Slides were kept in moist chamber at 25C for 72h. Precipitations reaction was observed in the agar gel only in cases where common antigen was present.

3.11.6.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45ml methanol, 45ml distilled water and 10ml glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50 ° C

3.11.7. Immunoblotting

3.11.7.1. Dot immunobinding assay (DIBA)

Dot immunobinding assay was performed following the method suggested by Lange *et al.* (1989) with modifications. Following buffers were used for dot immunobinding assay.

- a. Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.
- b. Tris buffer saline (10mM Oh 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.
- c. Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.
- d. Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM MgCl_2

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size $0.45\mu\text{m}$, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), $4\mu\text{l}$, was loaded in each well and allowed to dry for 30 min at room temperature. Load $5\mu\text{l}$ (antigen) test sample on to NCM and allow it to

dry for 30 minute at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker, respective polyclonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4 °C for overnight. The membrane was then washed gently in running tap water for three minutes, thrice followed by washing in TBST (pH 7.4), (Wakemen and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37°C. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.11.7.2. Western Blotting

Western Blotting has been performed following the method of Wakeham and White (1996). The following buffers were used for Western blotting.

Stock solutions

- (i) All the stock solutions and buffers used in SDS-Gel preparation for Western blotting were as mentioned earlier in SDS-Gel electrophoresis.
- (ii) Transfer buffer (Towbin buffer) :
(25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).
Tris-3.03g, Glycine -14.4g, 200 ml Methonal (adjusted to 1L, with dist. Water).
- (iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)
Preparation was as mentioned in ELISA.
- (iv) Blocking solution
Casein hydrolysate -5% in PBS, Sodium azide -0.02%, Tween-20 - 0.02%.
- (v) Washing buffers : Washing buffer -1 : PBS: Washing buffer -2 : (50mM Tris-HCl, 150mM NaCl, pH 7.5). Tris-6.07 gm; NaCl – 8.78gm; made upto 1 lit with distilled water.
- (vi) Alkaline phosphatase buffer : (100mM NaCl, 5mM MgCl₂ , Tris-HCl, pH9.5) Tris-12.14gm; NaCl, 5.84gm; MgCl₂ -1.015gm; made upto 1lit with double distilled water.

- (vii) Substrate: NBT: 5mg NBT in 100µl of 70% N,N-dimethyl formamide
BCIP: 2.5mg BCIP in 50µl of 100% N, N-dimethyl formamide. Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline phosphatase buffer.
- (viii) Enzyme. (Alkaline phosphatase tagged with antirabbit goat IgG)
Alkaline phosphatase buffer; enzyme (1;10,000).
- (ix) Stop solution 0.5M EDTA solution in PBS, pH 8.0) EDTA sodium salt-
0.0372 gm in 200µl distilled water, added in 50ml of PBS.

3.11.7.2.1. Extraction and estimation of protein:

Protein extraction and estimation was done as described earlier.

3.11.7.2.2. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.11.7.2.3. Blot transfer process

After the gel run, the SDS-PAGE gel was transferred to pre-chilled (Towbin) buffer and equilibrated for 1h. The nitrocellulose membrane (BIO-RAD, 0.45 µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves, and soaked in Towbin buffer for 15min. The transfer process was done in Trans-Blot SD Semi-Dry Transfer cell (Bio-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the Semi-dry cell. And the pre-wetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out with a glass rod. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed. The blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1 h. and proceeded for immunological probing.

3.11.7.2.4. Immunoprobng

After drying the NCM for at least 1h, blocking of the unbound sites of NCM was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min. with gentle shaking on a platform shaker at room temperature. Subsequently the membrane was incubated with antibody (IgG) solution (blocking solution : PBS [1:1, v/v + IgG, diluted as 1:100 or as per require ment]. The bag was sealed leaving space

for few air bubbles and incubated at 4°C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250 ml PBS (washing buffer -1). Final washing was done in 200ml washing buffer -2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with anti-rabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed for 3 times in washing buffer-2. Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed upto the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.11.8. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia was done using FITC labeled goat anti-rabbit IgG following the method of Chakraborty and Saha (1994). Fungal mycelia were grown in liquid Richards's medium as described earlier. After four days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and was washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 hour at RT. The mycelia washed thrice with PBS- Tween pH 7.2 as mentioned above and treated with Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC-conjugate) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 minute at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.11.9. Immunolocalization of Chitinase enzymes by Indirect immunofluorescence staining of leaf, stem and root tissues

Localization of Chitinase enzyme expression in the leaf, stem and root tissues of different test crops were conducted following the method of Chakraborty and Saha, 1994. Cross section of healthy, infected and treated roots were cut and immersed in PBS, pH 7.2. These section were treated with primary antibody raised against Chitinase enzyme which was diluted (1:50) in PBS and incubated for 1 hour at RT. After incubation, section were washed thrice with PBS- Tween pH 7.2 for 15 minute

and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescence (FITC). The sections were incubated for 30 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluorescence of the tissue sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.12. *In vivo* studies for plant growth promotion by PSF and PGPR

3.12.1. Mass multiplication and inoculation

3.12.1.1. Mass multiplication of PSF

PSF and BCA isolates were grown separately in the PDA medium for sporulation over a period of 4-5 days after which harvested spore mass (10^6 spores / ml) was suspended in sterile distilled water. For mass multiplication of the PSF, well decomposed FYM heaps were used whereas sand maize meal was used for BCAs. Spore suspension (100 ml) was used to inoculate 5 Kg of FYM. The FYM was first moistened slightly to optimize the PSF growth and kept in polythene bags in shade for 10 days. The mixture was regularly raked every third day during the total of this 10 days period.

Mass culture of fungal pathogens were prepared in sterilized sand maize meal media, in which washed and sterilized sand:water:maize meal ratio of (9:1.5:1; w:w:v) was taken in autoclavable plastic bag and conical flasks (150g) was sterilized at 20 lb for 20 minutes method followed by Biswas and Sen (2000) which was inoculated with mycelial bits of pathogen taken from the margin of actively growing culture and incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 15-20 days. The two weeks old cultures were used for inoculating the soil. The rhizosphere of each of potted plants was inoculated with 100g of pathogen inoculum prepared in sand maize meal media. Regular watering of the plants was done to assure the successful establishment of the pathogen.

3.12.2. Mass multiplication of PGPR isolates

In case of PGPR isolates, the bacterial isolates were cultured in nutrient broth medium with shaking at 30°C at 120 rpm for 48 h. At the end of the log phase, bacterial cultures were centrifuged at 3000 rpm for 5 min and the supernatant was discarded. Pellet was scraped into sterile distilled water. The aqueous suspensions were diluted as necessary to maintain the bacterial concentration at 10^6 cells/ml. This

suspension was utilized for seed bacterization of test crops and for direct application in pot and field condition.

3.12.2.1 Application of bacteria

3.12.2.2. Soil drench

The bacteria were grown in NB for 48 h at 28°C and centrifuged at 2000 rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3×10^6 cfu ml⁻¹.

The bacterial suspension was applied to the pots and rhizosphere of plants in the field conditions. Applications were done @ 0f 100 ml per pot at regular interval.

3.12.2.3. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3×10^6 cfu ml⁻¹ after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the test crop plants. The spraying was done forth nightly till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

3.12.2.4. Seed bacterization

Seeds of test plants were surface sterilized with sodium hypochlorite and rinsed in distilled water after which seeds were dried under sterile air stream. Bacterial suspension containing 3×10^6 cfu ml⁻¹ was taken in 500 ml glass beakers. The seeds were soaked in bacterial suspension using 0.2% sterilized carboxymethyl cellulose as an adhesive. The seeds soaked in sterile distilled water served as control. Bacterized seeds were sown in fields in rows supplemented with 0.05% (wt/ wt) Rock phosphate ((RP-140; P=18.8%)) and allowed to germinate. Growth promotions were evaluated after 15 d in terms of increase in root and shoot length and increase in root and shoot biomass in comparison to control. Each treatment was carried out in three replications with at least 50 plants in each replicate under same physical and environmental conditions.

3.12.3. Assessment of plant growth promotion by PSF and PGPR

The experiment was conducted on different types of crop plants which included; legumes (*Cicer aeritenium*, *Glycine max*, *Vigna radiata*, *Pisum sativum* and *Phaseolous vulgaris*) Cereals (*Triticum aestivum* and *Oryza sativa*) as well as a

plantation crop (*Camelia sinensis*), under greenhouse condition as well as field conditions to assess the efficacy of selected fungal and bacterial isolates to promote plant growth. The growth promotion was assessed in seedling by comparing the increase in height, number of leaves, root length, shoot length, root shoot fresh and dry biomass as well as yield of the treated plants to the untreated control plants under the same environmental and physical condition (temperature 35-30°C; R.H. 60-80%; 16h photoperiod). The experiments consisted of at least five replicates in each treatment incompletely randomized design.

In case of some of the crops whose seed were bacterized or treated, the plant growth promoting activity were also assessed based on the seedling vigour index, seed germination percentage, root and shoot length of individual seedling to work out the vigour index using the formula suggested by Baki and Anderson (1973). Vigour Index = (mean shoot length = root length) x % germination. The experiment was carried out in three replicates with 30 seeds in each plate.

3.12.4. Assessment of soil phosphate mobilization by PSF and PGPR isolates

3.12.4.1. Modified Morgan Extraction for Phosphorous from soil

Approximately 4cm of air dried, sieved soil of 10 g root or leaf tissue oven dried was put into 50ml extraction flasks. For colourless filtrate, 1cm³ of activated carbon (charcoal) was added to each flask. 20 ml of the modified morgan extractant (add 28.74 ml glacial acetic acid as added to a 40L carboxy containing approximately 20L distilled water. And 1825 ml concentrated NH₄OH was added to each flask. Diluted to 40 L with distilled water and mixed well. The pH of the solution was maintained at 4.8± 0.05 by adding concentrated NH₄OH or acetic acid to each flask. It was shaken at 180 oscillations per minute for 15 minutes on reciprocating shaker and finally filtrate was filtered through a medium porosity filter paper. (Ref.....)

3.12.4.2. Estimation of Total phosphate content in soil and plant tissues

Quantitative estimation of phosphate was carried out following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle as described earlier.

3.13. *In vivo* studies of disease suppression by PGPR and BCA

3.13.1. Disease assessment with BCA

Inoculum of Biocontrol agents was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 °C for 10 days. To each pot containing the pathogen (*Thanatophorous cucumeris* and *Sclerotium rolfsii*) infested or control soil (2000 g), 10 g of the wheat bran colonized by the biocontrol agent was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.* (2006).

In order to determine the effects of biocontrol agents (BCA) on disease reduction, four treatments were taken in each case: 1- Untreated control, 2-Inoculated with pathogen, 3- Inoculation with BCA isolates and 4-Inoculation with both BCA isolate and fungal pathogen. Disease assessment was done after 15 days of inoculation.

The rhizosphere of plants pre- treated with the antagonists or without treatment was inoculated with pathogen. In pre- treated plants, pathogen inoculation was done 3 days after application of antagonist. The inoculated plants were examined after 15 days.

Disease intensity was assessed on the basis of above ground and under ground symptoms. (Roots, colour, rotting, leaves withering, shoot tip withering, defoliation etc.). Percentage of disease incidence was calculated by dividing the number of diseased plants by total number of plants and then multiplying by hundred while disease intensity was calculated by using 0-6 scale as adopted by Mathew and Gupta (1996).

0 = No symptoms;

1 = Small roots turn rotten lesion appeared at the collar region;

2 = Middle leaves start wilting and 10-20% of root turn brown;

3 = Leaves wilted and 20-40% roots become dry with browning of shoot;

4 = Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%;

5 = 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%

6 = Whole plants die, since 100% roots were dried.

13.13.2. Disease assessment with PGPR

Ability of PGPR isolates to suppress root diseases of different test crops caused by *Thanatophorous cucumeris* and *Sclerotium rolfsii* were tested in glass house condition in potted plants with 5 plants per pot with three replicates for each treatment. Inoculation of the rhizosphere of test crops with the pathogen and disease assessment was done following the method of Chakraborty *et al.* (2006). For co-inoculations, the PGPR strains to be tested were first applied to the rhizosphere of 15 d old plants as aqueous suspension (10^8 cells/ml) prior to pathogen inoculation and after three days, plants were inoculated with pathogen. The experiment included four treatments: 1- Healthy; 2-Treated with Bacterium but Un-inoculated with the pathogen; 3- Untreated but inoculated with pathogen ; 4- treated inoculated.

Disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation.

Disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms as follows: Root rot index: 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – shoot tips also starts withering; 60–70% roots affected; 5 – whole plants starts withering ; 6–Whole plant die, with upper withered leaves still remaining attached; roots fully rotted.

13.13.3. Calculation of biocontrol efficacy (BE %) and Disease index (DE %)

For calculating biocontrol efficacy (BE) of both the BCAs and PGPR the disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms.

Disease Incidence and Biocontrol efficiency was calculated as described by Xue *et al.*, (2013) using the following formula:

Disease incidence (DI)

$$= \left[\frac{\sum (\text{The number of plants in this index} \times \text{Disease index})}{(\text{Total number of plants investigated} \times \text{highest disease index})} \right] \times 100 \%$$

Biocontrol efficacy (BE)

$$= \left[\frac{(\text{Disease incidence of control} - \text{disease incidence of bacteria treated plants})}{\text{Disease incidence of control}} \right] \times 100 \%$$

Disease incidence of control] x 100 %

3.14. Assay of defense enzyme activities enhanced after application of BCA and PGPR

3.14.1. β -1, 3-glucanase (β -GLU, EC 3.2.1.38)

Estimation of β -1, 3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.* (1991). The crude enzyme extract of 62.5 μ l was added to 62.5 μ l of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 μ l of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as μ g glucose released $\text{min}^{-1} \text{g}^{-1}$ fresh tissue.

3.14.2. Chitinase (CHT, EC 3.2.1.14)

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10 μ l of 1M Na-acetate buffer (pH4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30 μ l of 1M K-PO₄ buffer (pH7.1) and 20 μ l Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70 μ l of 1M Na-borate buffer (pH9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C. The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as μ g GlcNAc released /min/ g fresh wt. tissue.

3.14.3. Phenyl alanine ammonia Lyase (PAL EC 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1gm root and leaf sample was crushed in 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of β mercaptoethanol in ice cold temperature. The slurry was Centrifuge in 15000 rpm for 20 minutes at 4°C. Supernatant was collected and after recording its volume, was immediately used for assay or stored at -20°C.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300 μ M sodium borate (pH 8.8), 0.3 ml of 30 μ M L-phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 °C the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as μ g cinnamic acid produced in 1 min g⁻¹ fresh weight of tissues.

3.14.4. Peroxidase (POX, EC1.11.1.7)

The reaction mixture contained 1 ml of 0.2M Na-phosphate buffer (pH5.4), 1.7 ml dH₂O, 100 μ l crude enzyme, 100 μ l O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H₂O₂. O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H₂O₂ (Chakraborty *et al.*, 1993). Specific activity expressed as the increase in ΔA 465/g tissue/min.

3.14.5. Acid and Alkaline phosphatase (EC 3.1.3.2 & EC 3.1.3.1)

About 2 g portions of each soil sample was used for enzyme extraction and assays. The activities of enzymes were expressed according to method of Tominaga and Takeshi (1974) with modifications. For acid phosphatase assay, soil samples were extracted in 5 ml of 50 mM sodium acetate buffer (pH 5.0) using a chilled mortar and pestle which was then transferred into a tube and solution was shaken well. 1 ml of 5 mM p-nitrophenyl phosphate solution was added to the tube. All the tubes along with control were allowed to incubate at 37°C for 1 h. After incubation, 2 ml of solution was transferred into centrifuge tubes. Centrifugation was performed at 3000 rpm for 2 min at 4°C. Finally the supernatant was transferred into clean cuvettes and the reaction was terminated by addition of 4.0 ml of 100 mM NaOH. The amount of p-nitrophenol liberated was determined from the absorbance at 400 nm. Enzyme activity was expressed as mmol p-nitrophenol liberated/sec/g of soil. The procedure for the assay of alkaline phosphatase was similar to acid phosphatase except that for enzyme extraction and incubation 100 mM sodium bicarbonate buffer (pH 10.0) was used.

3.14.6. Estimation of phenols contents

3.14.6.1. Extraction of phenol contents from leaves and roots

Phenols was extracted from the fresh leaves following the method of Mahadeven and Sridar (1982). 1 g of fresh root/leaf cut into small pieces put in boiling alcohol in a water bath for 5-10 minutes (4ml alcohol /gm tissue). After 15 minutes of boiling it was cooled and crushed in mortar and pestle thoroughly at room temperature. The extract was passed through two layers of cheese cloth and then filtered through Whatmann No.1 filter paper. Final volume was adjusted with 80% ethanol. The whole experiment was done in dark to prevent light induced degradation of phenol.

3.14.6.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteu's reagent, following the method of Mahadevan and Sridhar (1982). To 1ml of test solution with 10^{-2} and 10^{-1} dilution (leaf and root) plus 1ml of 1N folin -ciocalteu reagent (1:1) followed by 2ml of 20% sodium carbonate solution (Na_2CO_3) is taken in test tube, mix well and boil in water bath for exactly 1minute. After cooling, dilute upto 25ml by adding distilled water. Absorbance of the blue colored solution was measured in a systronic photometric colorimeter Modle 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.15. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. For bacteria, the growth was taken for 24 hr. Liquid nitrogen was used for crushing the cell mass for both cases.

3.15.1. Preparation of genomic DNA extraction buffer

The following buffers for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

Lysis Buffer

50 mM Tris, pH 8.0

100 mM EDTA

100mM NaCl

1% SDS

Genomic DNA Buffer

10 mM Tris, pH 8.0

0.1 mM EDTA

20% SDS

CTAB Buffer

2% CTAB
1.5% PVP K 30
1.4 mM NaCl
20 mM EDTA
100mM Tris HCL pH 8.0
0.1% B-mercaptoethanol

3.15.2. Extraction of Fungal Genomic DNA

Isolation of fungal genomic DNA was carried out as outlined by Ma *et al.* 2001. The mycelia (3-4 days old) were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60⁰C followed by centrifugation at 12,000 rpm for 15 min., whereas genomic DNA was extracted from isolates of bacteria and actinomycetes using CTAB buffer. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried. . Finally the pellets were air dried and suspended in 1X TE buffer and stored at 4⁰C until further use.

3.15.3. Extraction of Bacterial Genomic DNA

Isolation of genomic DNA from bacterial DNA was performed by growing the bacterium in 25 ml nutrient broth medium at 30⁰C on a shaker. The broth culture was centrifuged at 6000 rpm for 5 min. Pellets were collected and suspended in 0.4 ml of 1X TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA pH 8.0). The suspension was centrifuged again and the pellet was collected and re-suspended in SET buffer (20mM Tris-HCL, 75mM NaCl, 25mM EDTA, pH-8.0) 10µl of Lysozyme was added and incubated for 30-60 min at 37⁰C. Next after this initial incubation 10% SDS and 10 µl of Proteinase-K was added and incubated at 55⁰C for 60 min. Next 0.3 vol. of 5M NaCl and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and incubated at room temperature with gentle extraction for 30 min. The suspension was then centrifuged at 5000 rpm for 15 min. in a cooling centrifuge, the aqueous phase was removed to a fresh tube. To the clear aqueous phase 0.1 vol of 3M sodium acetate (pH 4.8) was added followed by addition of 1 vol of chilled

absolute ethanol and incubated in room temperature for 30 min with gentle extraction. Next the extraction was centrifuged at 10000 rpm for 15 min in a cooling centrifuge and the supernatant was discarded carefully. The pellet was washed in 70% ethanol and centrifuged at 10000 rpm for 10 min in a cooling centrifuge. Finally the pellets were air dried and suspended in 1X TE buffer and stored at 4°C until further use.

3.15.4. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.15.5. Spectrophotometric quantification of Genomic DNA

The pure sample is (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), can use spec to measure amount of UV irradiation absorbed by the bases. For quantitating DNA or RNA, readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

1 O.D. at 260 nm for RNA molecules = 40 ng/ul of RNA

The reading at 280 nm gives the amount of protein in the sample.

Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.15.6. Agarose gel electrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be

used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.15.7. Preparation of DNA samples for electrophoresis

Preparing an agarose gel involves melting a specified amount (0.8%) of agarose in 1X TBE buffer, cooling the solution, and pouring it into the gel casting tray with ethidium bromide. Gels solidify in 15-20 minutes.

3.15.8. Run gel electrophoresis for DNA fraction

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 minutes. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.16. RAPD PCR analysis

RAPD analysis of the genomic DNA was conducted using 10 bp long decamer primer as described by Caetano-Annoles *et al.*, 1991.

For RAPD, random primers were selected (Table-1). Genomic DNA was randomly amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 μ l, containing 78 μ l deionized water, 10 μ l 10 X Taq pol buffer, 1 μ l of 1 U Taq polymerase enzyme, 6 μ l 2 mM dNTPs, 1.5 μ l of 100 decamer primer and 1 μ l of 50 ng template DNA.

After PCR amplification, PCR product (20 μ l) was mixed with loading buffer (8 μ l) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

3.16.1. Amplification conditions for RAPD analysis

Temperature profile, 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.16.2. RAPD- PCR primers

The following primers are used in the study.

3.16.3. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and then the image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software.

3.16.4. Scoring of individual bands and construction of dendrogram

The RAPD patterns of each isolate was evaluated, assigning character state “1” to indicate the presence of band in the gel and “0” for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. All reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed *in silico* into similarity matrix using NTSYSpc-Numerical Taxonomy System Biostatistics, version 2.11W, (Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's coefficients. The result generated in this analysis was then used to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc. The isolates were then group isolates with identical band patterns for a given primer. A two (2-D) and three dimensional (3-D) principal component analysis was constructed to group the individuals and test the relationship using EIGEN programme (NTSYS-PC).

3.17. ITS PCR analysis

All isolates of BCA, PGPR as well as one of the pathogen *T. cucumeris* were taken up for ITS-PCR amplification and was carried out according to the method of Stafford *et al.*, (2005). Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol

blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.17.1. ITS-PCR primers

3.17.2. Amplification conditions

Temperature profile, 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C (for fungal isolates) 59 °C (for bacterial isolates) and for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.17.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bi-directionally using the ITS primer pairs by Genei Bangalore. A chromatogram was generated which provided the sequence informations.

3.18. BLAST of Sequence

The DNA sequences were analyzed using the alignment software of BLAST algorithm (<http://ingene2.upm.edu.my/Blast>, Altschul *et al.*, 1990) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence (<http://ncbi.nlm.nih.gov/blast>).

3. 19. Submission of rDNA gene to NCBI genbank

The rDNA sequences were deposited to NCBI GenBank through BankIt sequence submission tool and approved as the ITS sequence after complete annotation and given accession numbers.

3.20. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.*, 1994), included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent

Table 1. RAPD and universal ITS primers

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
AA-04	CAGGCCCTTC	10	38.2	70
OPA-4	AATCGGGCTG	10	39.3	60
A-11	AGGGGTCTTG	10	31.8	76
A-5	AGGGGTCTTG	10	36,8	73
OPD6	GGGGTCTTGA	10	32.8	83
OPA1	CAGGCCCTTC	10	38.2	70
ITS-Primers pairs				
<i>Talatomyces flavus</i> and <i>Thanatophorous cucumeris</i>				
T/ITS-1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS-4	TCCTCCGCTTATTGATATGC	20	61.5	45
<i>Trichoderma isolates</i>				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45
Bacterial 16S rDNA Universal primers				
16Srrna	AGAGTRTGATCMTYGCTWA*	19	54.5	42
16S rrna	CGYTAMCTTWTTACGRCT	18	58.5	40

* Degenerate universal primer for 16Sr RNA gene

lengthy or excessive numbers of gaps. The default parameters were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor

equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method (Sneath and Sokal, 1973). Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.*, 2007.

3.21. Analysis of rDNA region for DNA molecular weight, nucleotide frequency and ORF.

Combinations and percentage of occurrence of different nucleotide in the entire sequence was calculated using the bioinformatics algorithm from the website-www.ualberta.ca/~stothard/javascript/dna_stats.html. The DNA Molecular weight of rDNA sequences was calculated with the help of DNA weight calculator (www.ualberta.ca/~stothard/javascript/dna_mw.html). Similarly number of ORFs in the given sequence was calculated with the help of online bioinformatics tool from-www.ualberta.ca/~stothard/javascript/orf_find.html and www.star.mit.edu/orf/runapp_html.html.

3.22. Denaturing Gradient Gel Electrophoresis (DGGE)

3.22.1. PCR amplification of gnomonic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel electrophoresis was performed according to the method of (Zhao *et al.*, 2006). 18S/16S DNA (200 bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end (F352T: 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM MgCl₂ (Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of dNTPs, and 1 U of *Taq* DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5 min, followed by 6 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with “The Decode Universal Mutation Detection System” (Bio-Rad Laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed

several times so as to optimize suitable concentration and finally 20 to 60% denaturant was found optimal for the best result.

3.22.2. Denature Gradient Gel Electrophoresis of the PCR products

3.22.2.1. Reagents and solutions required for DGGE analysis

40% Acrylamide:bisacryl-amide (37.5:1)

50 x DGGE/TAE buffer solution

Trizma-Base:	484.4 grams
Sodium-Acetate:	272.0 grams
trisodium EDTA	37.2 grams
H ₂ O	2 liters

pH 7.40 adjusted with about 230 ml of glacial acetic acid.

Preparation of Denaturants

100% Denaturant:	
Urea	42.0 grams
38.5% Acrylamide (makes a 6.5% gel)	16.9 ml
50x DGGE/TAE	2.0 ml
Formamide	40.0 ml
Filled up to 100 ml with distilled H ₂ O.	
0% Denaturant	
38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

3.21.2.2. Creating the gel sandwich (DCode System BioRad)

Large glass-plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned again with 96% ethanol. Both 1mm spacers were also cleaned with 96% ethanol and placed on the large glass plates. The clamps were screwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass-plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment card slid between the glass plates to align the spacers. The clamps were screwed and the alignment of the glass-plates was checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

3.22.2.3. Preparing the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to following table.

UF solution	[UF](%)	[Acrylamid/Bis] (%)	Volume UF solution (ml)	Volume APS 10% (μl)	Volume TEMED (μl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

APS and TEMED was added to the low and high solutions according to table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closest to the outlet of the gradient mixer and the low concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

3.22.2.4. Running a gel

Fresh 0.5x TAE buffer was added to the buffer tank to the mark "Fill". The DCode™ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel. The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode™ was then switched off and the lid taken off after 1 minute. The sandwich holder was slid into the buffer tank, with the red dot of the cathode at the right side. The DCode™ pump and the stirrer underneath the tank were switched on (300 rpm) until samples were loaded.

3.22.2.5. Staining of gels and photography

Before taking DGGE units out from the tank, the run - evaporated H₂O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50XTAE buffer and the gel was stained for

5 minutes and destained with running buffer. The gel was photographed under UV transilluminator.

3.22.2.6. Data analysis.

3.22.2.6.1. Scoring of individual bands

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme BioProfil 1D and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods, photographs of the gels were scanned into a computer and saved as graphics files.

3.22.2.6.2. UPGMA analysis of the DGGE bands

Variability among the different groups of isolates were detected on the basis of the banding pattern obtained on denature gradient gel. All reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed *in silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W) as in case of RAPD analysis. However, a more complex analysis involved cladistic analysis of data and reconstruction of the phylogenetic tree. A two (2-D) and three dimensional (3-D) principal component analysis was constructed to provide another means and test the relationship among different tested groups using EIGEN programme (NTSYS-PC).

CHAPTER 4

RESULTS

4.1 Isolation and identification of microorganism from forest soil, riverine soil and agricultural fields

Soil samples were collected from the three subdivisions of Darjeeling District *i.e.* Kalimpong, Kurseong and Mirik which includes the area between 24⁰40'28'' N to 27⁰13' N Latitudes and 87⁰45'50'' to 89⁰54'35'' E Longitudes. Source of soil samples include forests, river basins and agricultural fields. Geographically, the district can be divided into two broad divisions, the hills and the plains. The hilly regions of the district are the three hill subdivisions of Darjeeling, Kurseong and Kalimpong. The foothill of Darjeeling Himalayas, which comes under the Siliguri subdivision, is known as the Terai. The major rivers flowing through here are- Teesta River, Mahananda River, the Great Rangit, Mechi, Balason, Reli and Jaldhaka rivers. There are 87 tea gardens spread across the Darjeeling hill which covers roughly 19,000 hectares of the total land area. The major portions of the forests are today found at elevations of 2000 meters and above. The area located in between 1000–2000 meters is cleared either for tea plantation or cultivation. The four major forest types according to altitudinal variation found in Darjeeling Hill Areas are: Tropical moist deciduous forest (300-1000 m); Tropical evergreen lower montane forest (1000-2000 m.); Tropical evergreen upper montane forest (2000-3000 m.); Temperate forest (3000-3500 m.); Sub temperate forest (above 3500 m.). About 30% of the forest covers found in the lower hills are deciduous. Soil samples collected from the different regions of Darjeeling hill were coded accordingly and analyzed for fungal and microbial populations. The samples collected from forest were coded as Sukna forest (FS/S), Lohagarh forest (FS/L), Tindharey (FS/Tn), Ghyabari forest (FS/G), Rongli Rongek (FS/Rr), Maneydara forest (FS/Md), Sandakhpu forest (FS/S), Rimbhik Forest (FS/R), Thorpu Forest (FS/T), Tagdha Forest (FS/Td), Rambi Saal Forest (FS/R), Lamahata (FS/L), Cinchona Forest (FS/C), Mongpong Forest (FS/M), Pedong (FS/P), Rishyup Valley (FS/Ry), Lava (FS/Lv). Similarly soil samples collected from agricultural

fields from all the sub divisions of Darjeeling district were also coded accordingly as Tea (RHS/T), *Cryptomeria japonica* (RHS/C), Orange (RHS/O), Paddy (RHS/P), Bamboo (RHS/B), Soyabean (RHS/Sb), Squash (RHS/S), *Brassica juncea* (RHS/Br), Cardamom(*Amomum subulatum*) (RHS/Cr), *Rhododendron* (RHS/R), Amliso (*Thyasaenolena latifolia*) (RHS/Am) and *Alnus nepalensis* (RHS/A). Soil samples were also collected randomly from the main river basins and coded accordingly. These were Teesta (RS/T), Balasan (RS/B), Rangit (RS/R), Reli (RS/RI) as well as from Mirik lake (RS/M) (Fig.s 1-3).

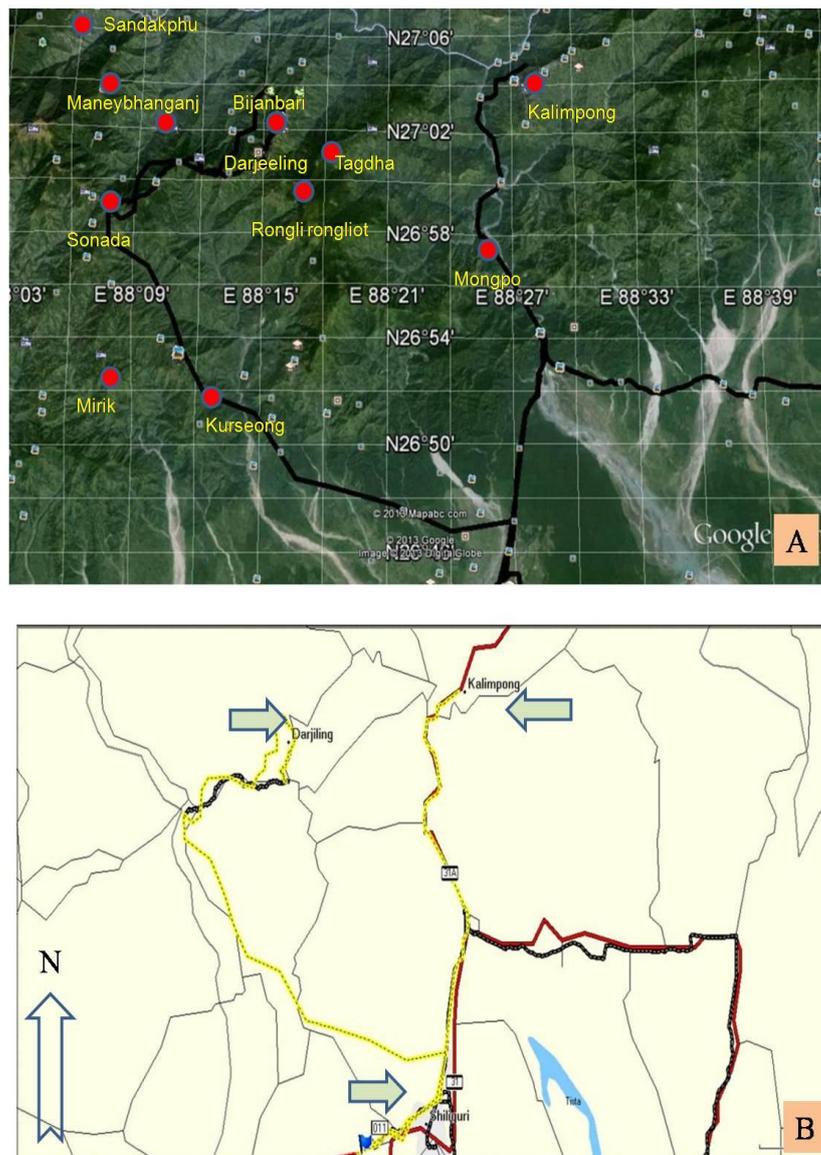


Fig. 5. Satellite Image of Darjeeling district pointing out the main subdivisions (A), GIS route map of the study area obtained with the help of Gramin navigator of GPS tool (B).

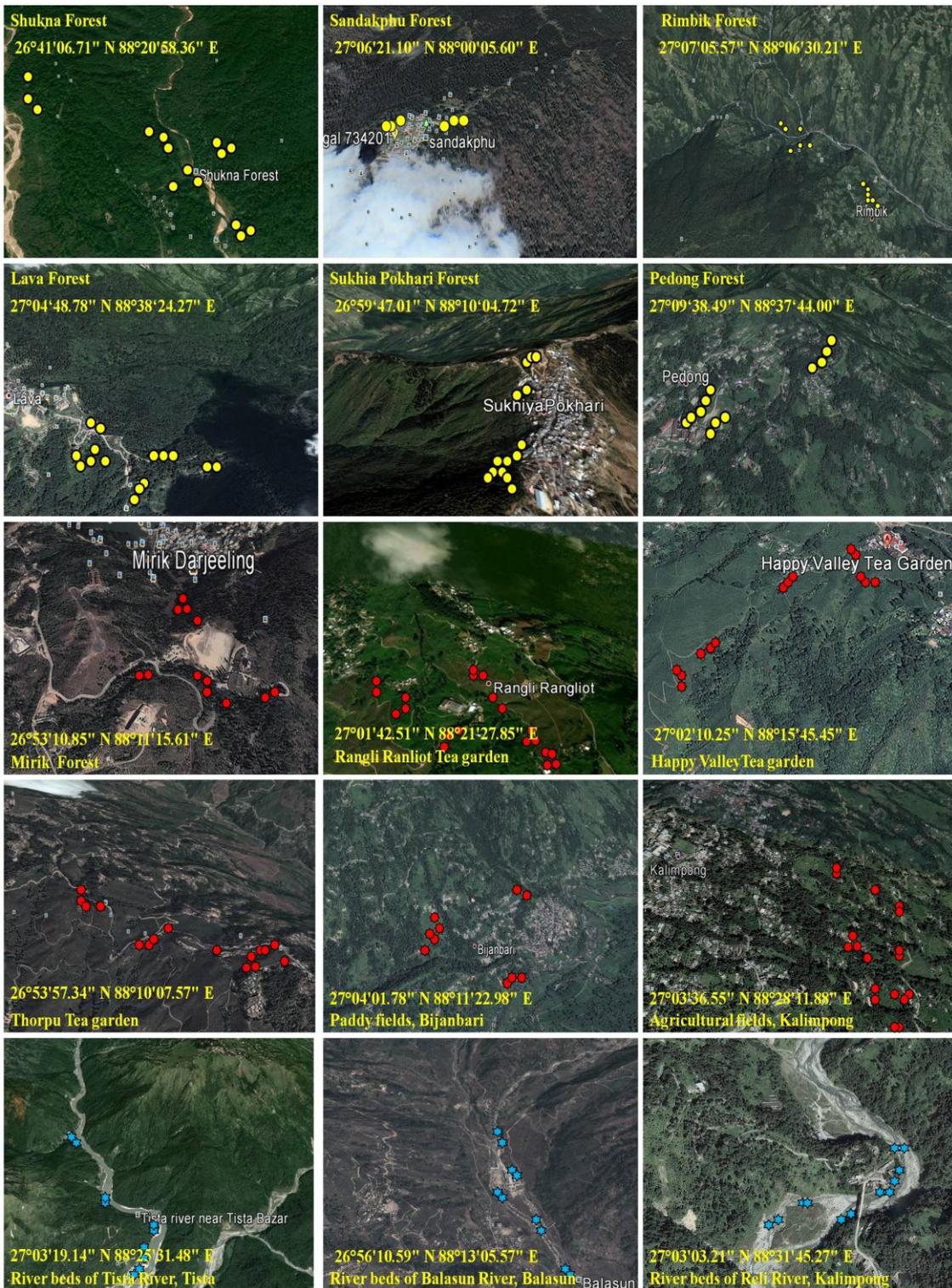


Fig. 6. GIS locations of major forests, agricultural land and river beds of Darjeeling regions.

Table 2. GIS locations of the sampling sites

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Elev (m)	Texture	Soil pH
KURSEONG SUBDIVISION (FOREST SOIL)						
Sukna forest (FS/S)	FS/SI	Sukna	26°41'06.71" N 88°20'58.36" E	126	Clay	4.38
	FS/SII	Sukna	26°41'08.33" N 88°20'54.16" E	126	Clay	4.38
	FS/SIII	Sukna	26°41'11.21" N 88°16'55.36" E	126	Clay	4.38
	FS/SIV	Sukna	26°42'14.72" N 87°22'48.36" E	126	Clay	4.38
	FS/SV	Sukna	26°42'44.56" N 87°22'33.30" E	126	Clay	4.38
Lohagarh Forest (FS/L)	FS/LI	Lohagarh	26°48'19.82" N 88°11'51.07" E	396	Clay	5.15
	FS/LII	Lohagarh	26°48'15.56" N 88°11'12.30" E	396	Clay	5.15
	FS/LIII	Lohagarh	26°48'22.10" N 88°11'10.16" E	396	Clay	5.15
	FS/LIV	Lohagarh	26°48'19.82" N 88°11'51.07" E	396	Clay	5.15
	FS/LV	Lohagarh	26°48'40.60" N 88°11'33.15" E	396	Clay	5.15
Tindharey (FS/Tn)	FS/Tn I	Kurseong	26°41'06.71" N 88°20'58.38" E	126	Clay	4.08
	FS/Tn II	Kurseong	26°41'55.12" N 88°17'48.27" E	126	Clay	4.08
	FS/Tn III	Kurseong	26°42'16.41" N 88°19'50.44" E	126	Clay	4.08
	FS/Tn IV	Kurseong	26°42'04.66" N 88°19'22.73" E	126	Clay	4.08
Ghyabari Forest (FS/G)	FS/G I	Kurseong	26°51'15.27" N 88°46'41.54" E	1300	Clay	4.16
	FS/G II	Kurseong	26°51'20.43" N 88°46'22.76" E	1300	Clay	4.16
	FS/G III	Kurseong	26°51'13.10" N 88°46'52.13" E	1300	Clay	4.16
	FS/G IV	Kurseong	26°51'22.09" N 88°46'15.24" E	1300	Clay	4.16
	FS/G V	Kurseong	26°51'37.18" N 88°46'62.03" E	1300	Clay	4.16
DARJEELING SUBDIVISION (FOREST SOIL)						
Rongli Rongek Forest	FS/Rr I	Ronglirongek	27°01'42.51" N 88°21'27.85" E	1382	Clay	4.75
	FS/Rr II	Ronglirongek	27°01'47.44" N 88°21'21.50" E	1382	Clay	4.75
	FS/Rr III	Ronglirongek	27°01'38.02" N 88°21'13.22" E	1382	Clay	4.75
	FS/Rr IV	Ronglirongek	27°01'42.51" N 88°21'27.85" E	1382	Clay	4.75
	FS/Rr V	Ronglirongek	27°01'76.14" N 88°21'44.09" E	1382	Clay	4.75
Maney Dara Forest	FS/Md I	Rimbik	27°37'15.47" N 88°06'38.27" E	2150	Clay	4.28
	FS/Md II	Rimbik	27°37'20.05" N 88°06'22.14" E	2150	Clay	4.28
	FS/Md III	Rimbik	27°37'26.10" N 88°06'27.20" E	2150	Clay	4.28
	FS/Md IV	Rimbik	27°37'18.07" N 88°06'33.10" E	2150	Clay	4.28
Sandakhpu	FS/S I	Sandakphu	27°06'21.10" N 88°00'05.60" E	3487	Clay	4.55
	FS/S II	Sandakphu	27°06'11.06" N 88°00'11.16" E	3487	Clay	4.55
	FS/S III	Sandakphu	27°06'25.20" N 88°00'17.40" E	3487	Clay	4.55
Rimbhik Forest	FS/R I	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	4.38
	FS/R II	Rimbik	27°07'08.27" N 88°06'32.11" E	1970	Clay	4.38
	FS/R III	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	3.38
	FS/R IV	Rimbik	27°07'11.43" N 88°06'10.18" E	1970	Clay	3.38
	FS/R V	Rimbik	27°07'15.10" N 88°06'22.10" E	1970	Clay	3.38
	FS/R VI	Rimbik	27°07'21.55" N 88°06'33.28" E	1970	Clay	3.38
Thorpu Forest (FS/Th)	FS/T I	Thorpu	26°53'57.34" N 88°10'07.57" E	1527	Clay	4.26
	FS/T II	Thorpu	26°53'57.40" N 88°10'10.25" E	1527	Clay	4.26
	FS/T III	Thorpu	26°53'57.45" N 88°10'13.80" E	1527	Clay	4.55
	FS/T IV	Thorpu	26°53'58.20" N 88°10'22.70" E	1527	Clay	4.55
	FS/T V	Thorpu	26°53'58.18" N 88°10'22.57" E	1527	Clay	4.55
Tagdah Forest (FS/Td)	FS/Td I	Tagdha	27°02'15.25" N 88°21'37.19" E	1476	Clay	4.55
	FS/Td II	Tagdha	27°02'20.04" N 88°21'44.20" E	1476	Clay	4.35
	FS/Td III	Tagdha	27°07'37.43" N 88°22'48.23" E	1476	Clay	4.35
	FS/Td IV	Tagdha	27°07'15.16" N 88°22'48.27" E	1476	Clay	4.35
Rambi Saal Forest (FS/R)	FS/R I	Rambi	26°57'59.42" N 88°25'43.29" E	285	Sandy	5.36
	FS/R II	Rambi	26°57'63.12" N 88°25'46.73" E	285	Sandy	5.36
	FS/R III	Rambi	26°57'77.22" N 88°25'48.05" E	285	Sandy	5.36
	FS/R IV	Rambi	26°57'83.15" N 88°25'63.88" E	285	Sandy	5.36

Contd.....

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Elev (m)	Texture	Soil pH
KALIMPONG SUBDIVISION (FOREST SOIL)						
Lamahata	FS/L I	Kalimpong	27°03'36.55" N 88°28'11.88" E	1046	Clay	4.68
	FS/L II	Kalimpong	27°03'36.44" N 88°28'86.72" E	1046	Clay	4.68
(FS/L)	FS/L III	Kalimpong	27°03'37.20" N 88°28'12.43" E	1046	Clay	4.68
	FS/L IV	Kalimpong	27°03'37.31" N 88°28'12.05" E	1046	Clay	4.68
Cinchona Forest	FS/CI	Mongpong	26°58'25.39" N 88°22'12.19" E	1102	Clay	4.11
(FS/C)	FS/C II	Mongpong	26°58'25.14" N 88°22'12.04" E	1102	Clay	4.10
	FS/C III	Mongpong	26°58'27.30" N 88°22'11.38" E	1102	Clay	4.10
	FS/C IV	Mongpong	26°58'27.44" N 88°22'11.76" E	1102	Clay	4.10
	FS/C V	Mongpong	26°58'24.22" N 88°22'10.72" E	1102	Clay	4.11
Mongpong Forest	FS/M I	Mongpong	26°58'26.95" N 88°22'02.58" E	1127	Clay	4.08
(FS/M)	FS/M II	Mongpong	26°58'21.90" N 88°22'00.55" E	1127	Clay	4.08
	FS/M III	Mongpong	26°58'21.42" N 88°22'00.18" E	1127	Clay	4.08
	FS/M IV	Mongpong	26°58'27.27" N 88°22'00.15" E	1127	Clay	4.08
	FS/M V	Mongpong	26°58'26.95" N 88°22'00.28" E	1127	Clay	4.08
	FS/M VI	Mongpong	26°58'26.56" N 88°22'02.58" E	1127	Clay	4.08
Pedong	FS/P I	Pedong	27°01'11.59" N 88°34'20.96" E	1522	Clay	5.18
(FS/P)	FS/P II	Pedong	27°01'13.77" N 88°34'26.33" E	1522	Clay	5.18
	FS/P III	Pedong	27°01'22.60" N 88°34'25.06" E	1522	Clay	5.18
	FS/P IV	Pedong	27°01'11.42" N 88°34'10.62" E	1522	Clay	5.18
Rishyup Valley	FS/Rv I	Kalimpong	27°06'42.45" N 88°38'28.48" E	1733	Clay	5.42
(FS/Ry)	FS/Rv II	Kalimpong	27°06'42.40" N 88°38'28.12" E	1733	Clay	5.42
	FS/Rv III	Kalimpong	27°06'30.15" N 88°38'25.21" E	1733	Clay	5.42
	FS/Rv IV	Kalimpong	27°06'30.57" N 88°38'25.67" E	1733	Clay	5.42
Lava	FS/Lv I	Kalimpong	27°04'48.78" N 88°38'24.27" E	1680	Clay	5.16
(FS/Lv)	FS/Lv II	Kalimpong	27°04'52.33" N 88°38'45.30" E	1680	Clay	5.16
	FS/Lv III	Kalimpong	27°04'27.15" N 88°38'14.03" E	1680	Clay	5.16
RHIZOSPHERE SOIL SAMPLE						
	RHS/T I	Ghyabari	26°51'15.27" N 88°46'41.54" E	1300	Clay	4.36
	RHS/T II	Ghyabari	26°51'15.22" N 88°46'32.12" E	1300	Clay	4.36
	RHS/T III	Ghyabari	26°51'22.17" N 88°46'41.27" E	1300	Clay	4.36
<i>Camellia sinensis</i>	RHS/T IV	Ghyabari	26°51'22.08" N 88°46'41.43" E	1300	Clay	4.36
(Tea)	RHS/T V	Saureni	26°51'53.61" N 88°11'48.17" E	1363	Clay	4.50
(RHS/T)	RHS/T VI	Saureni	26°51'53.70" N 88°11'48.21" E	1363	Clay	4.50
	RHS/T VII	Saureni	26°51'54.33" N 88°11'41.06" E	1363	Clay	4.50
	RHS/T VIII	Singmari	27°46'11.32" N 88°43'22.17" E	2010	Clay	4.60
	RHS/T IX	Singmari	27°46'11.24" N 88°43'22.02" E	2010	Clay	4.60
	RHS/T X	Singmari	27°46'13.22" N 88°43'10.43" E	2010	Clay	4.60
	RHS/T XI	Singmari	27°46'13.14" N 88°43'20.47" E	2010	Clay	4.60
	RHS/T VIII	Darjeeling	27°02'10.25" N 88°15'45.45" E	2118	Clay	4.18
	RHS/T IX	Darjeeling	27°02'10.63" N 88°15'45.11" E	2118	Clay	4.18
	RHS/T X	Singmari	27°46'11.52" N 88°43'22.43" E	2010	Clay	4.60
	RHS/T XI	Singmari	27°46'11.32" N 88°43'22.17" E	2010	Clay	4.60
	RHS/T VIII	Darjeeling	27°02'10.25" N 88°15'45.45" E	2118	Clay	4.18
	RHS/T IX	Darjeeling	27°02'10.22" N 88°15'45.36" E	2118	Clay	4.18
	RHS/T X	Darjeeling	27°02'17.25" N 88°15'20.10" E	2118	Clay	4.18
	RHS/T XII	Darjeeling	27°02'17.15" N 88°15'20.63" E	2118	Clay	4.18
	RHS/T XIII	Darjeeling	27°02'22.40" N 88°15'77.46" E	2118	Clay	4.18
	RHS/T XI	Thorpu	26°53'57.34" N 88°10'07.57" E	1527	Clay	4.57
	RHS/T XII	Thorpu	26°53'57.14" N 88°10'07.50" E	1527	Clay	4.57
	RHS/T XIII	Thorpu	26°53'57.88" N 88°10'07.37" E	1527	Clay	4.57
	RHS/T XIV	Rongli	27°01'42.51" N 88°21'27.85" E	1382	Clay	4.57
	RHS/T XV	Rongli	27°01'42.66" N 88°21'27.73" E	1382	Clay	4.78
	RHS/T XVI	Rongli	27°01'42.37" N 88°21'27.05" E	1382	Clay	4.78

Contd.....

Type of soil sample	Soil Sample Code	Geographic al Location	GIS location of sampling	Eleva (m)	Texture	Soil pH
<i>Cryptomeria japonica</i>	RHS/C I	Mirik	26°53'10.12" N 88°11'15.11" E	1606	Clay	4.78
(RHS/C)	RHS/C II	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Clay	5.15
	RHS/C III	Mirik	26°53'11.44" N 88°11'14.10" E	1606	Clay	5.15
<i>Citrus reticulata</i>	RHS/O I	Mirik	26°53'11.25" N 88°11'14.76" E	1606	Clay	5.36
(RHS/O)	RHS/O II	Mirik	26°53'14.88" N 88°11'10.18" E	1606	Clay	5.36
	RHS/O III	Mirik	26°53'14.55" N 88°11'10.84" E	1606	Clay	5.36
<i>Oryza sativa</i>	RHS/P I	Bijanbari	27°04'01.78" N 88°11'22.98" E	820	Clay	4.38
(Paddy)	RHS/P II	Bijanbari	27°04'01.40" N 88°11'22.83" E	820	Clay	4.38
(RHS/P)	RHS/P III	Bijanbari	27°04'01.63" N 88°11'22.12" E	820	Clay	4.38
	RHS/P IV	Kalimpong	27°03'21.42" N 88°24'24.01" E	1043	Clay	4.38
	RHS/P V	Kalimpong	27°03'24.44" N 88°24'24.16" E	1043	Clay	4.38
	RHS/P VI	Kalimpong	27°03'21.16" N 88°24'24.83" E	1043	Clay	4.38
<i>Dendrocalamus latiflorus</i>	RHS/B I	Ghyabari	26°51'15.27" N 88°46'41.54" E	1300	Clay	5.15
(Bamboo)	RHS/B II	Ghybaria	26°51'15.35" N 88°46'41.22" E	1300	Clay	5.15
(RHS/B)	RHS/B III	Ghyabari	26°51'22.12" N 88°46'11.36" E	1300	Clay	5.20
	RHS/B IV	Kalimpong	27°03'21.42" N 88°24'24.01" E	1043	Clay	5.22
	RHS/B V	Kalimpong	27°03'21.44" N 88°24'24.18" E	1043	Clay	5.22
	RHS/B VI	Kalimpong	27°03'22.83" N 88°24'23.14" E	1043	Clay	5.22
	RHS/B VII	Saureni	26°51'53.61" N 88°11'48.17" E	1363	Clay	4.50
	RHS/B VIII	Saureni	26°51'53.66" N 88°11'48.10" E	1363	Clay	4.50
	RHS/B IX	Saureni	26°51'68.14" N 88°11'22.20" E	1363	Clay	4.50
<i>Glycine max</i>	RHS/Sb I	Bijanbari	27°04'01.78" N 88°11'22.98" E	820	Clay	5.15
(Soybean)	RHS/Sb II	Bijanbari	27°04'01.27" N 88°11'22.73" E	820	Clay	5.46
(RHS/Sb)	RHS/Sb III	Bijanbari	27°04'01.36" N 88°11'22.18" E	820	Clay	5.46
<i>Sechium edule</i>	RHS/S I	Mirik	26°51'53.61" N 88°11'48.17" E	1363	Clay	4.46
(Squash)	RHS/S II	Mirik	26°51'53.70" N 88°11'48.18" E	1363	Clay	4.46
(RHS/S)	RHS/S III	Mirik	26°51'53.05" N 88°11'48.88" E	1363	Clay	4.46
	RHS/S IV	Pedong	27°01'11.59" N 88°34'20.96" E	1522	Clay	5.38
	RHS/S V	Pedong	27°01'11.47" N 88°34'20.77" E	1522	Clay	5.38
	RHS/S VI	Pedong	27°01'11.13" N 88°34'20.40" E	1522	Clay	5.38
<i>Brassica juncea</i>	RHS/Br I	Kalimpong	27°03'36.55" N 88°28'11.88" E	1012	Clay	5.26
(RHS/Br)	RHS/Br II	Kalimpong	27°03'36.43" N 88°28'11.12" E	1012	Clay	5.26
	RHS/Br III	Kalimpong	27°03'36.20" N 88°28'11.43" E	1012	Clay	5.26
<i>Amomum subulatum</i>	RHS/Cr I	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Clay	4.46
(Cardamom)	RHS/Cr II	Mirik	26°53'10.44" N 88°11'15.43" E	1606	Clay	4.46
(RHS/Cr)	RHS/Cr III	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Clay	4.46
	RHS/Cr IV	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Clay	4.46
	RHS/Cr V	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Clay	4.46
<i>Rhododendron</i>	RHS/R I	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	4.25
(RHS/R)	RHS/R II	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	4.25
	RHS/R III	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	4.25
	RHS/R IV	Rimbik	27°07'05.57" N 88°06'30.21" E	1970	Clay	4.25
	RHS/R V	Maneydara	27°03'25.41" N 88°01'30.23" E	2812	Clay	4.25
	RHS/R VI	Maneydara	27°03'25.41" N 88°01'30.23" E	2812	Clay	4.25
<i>Thysaenolena latifolia</i>	RHS/Th I	Pedong	27°01'11.59" N 88°34'20.96" E	1522	Clay	5.20
	RHS/Th II	Pedong	27°01'11.62" N 88°34'20.18" E	1522	Clay	5.20
	RHS/Th III	Pedong	27°01'11.44" N 88°34'20.23" E	1522	Clay	5.20
<i>Alnus nepalensis</i>	RHS/A I	Lava	27°04'48.78" N 88°38'24.27" E	1680	Clay	4.66
(RHS/A)	RHS/A II	Lava	27°04'48.60" N 88°38'24.37" E	1680	Clay	4.66
	RHS/A III	Lava	27°04'48.55" N 88°38'24.21" E	1680	Clay	4.66
	RHS/A IV	Lava	27°04'48.10" N 88°38'24.86" E	1680	Clay	4.66

Contd.....

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Elev (m)	Texture	Soil pH
Teesta River	RS/T I	Teesta	27°03'19.14" N 88°25'31.48" E	1655	Sandy	5.16
	RS/T II	Teesta	27°03'22.80" N 88°25'11.30" E	1655	Sandy	5.30
	RS/T III	Teesta	27°03'27.11" N 88°25'40.15" E	1655	Sandy	5.22
	RS/T IV	Teesta	27°03'13.38" N 88°25'33.88" E	1655	Sandy	5.16
	RS/T V	Teesta	27°03'17.27" N 88°25'12.20" E	1655	Sandy	4.83
Balasan	RS/B I	Balasan	26°56'10.59" N 88°13'05.57" E	805	Sandy	5.45
	RS/B II	Balasan	26°56'11.88" N 88°13'13.63" E	805	Sandy	5.25
	RS/B III	Balasan	26°56'18.59" N 88°13'08.22" E	805	Sandy	5.15
	RS/B IV	Balasan	26°56'23.17" N 88°13'11.46" E	805	Sandy	5.15
	RS/B V	Balasan	26°56'10.59" N 88°13'05.57" E	805	Sandy	5.45
Rangit	RS/R I	Malli	27°07'40.60" N 88°17'06.63" E	309	Sandy	5.45
	RS/R II	Malli	27°07'40.63" N 88°17'06.12" E	309	Sandy	5.24
	RS/R III	Malli	27°07'40.40" N 88°17'06.57" E	309	Sandy	5.30
	RS/R IV	Malli	27°07'40.12" N 88°17'06.27" E	309	Sandy	5.46
	RS/R V	Malli	27°07'40.18" N 88°17'06.33" E	309	Sandy	5.12
Reli	RS/Re I	Kalompong	27°03'03.21" N 88°31'45.27" E	581	Sandy	4.88
	RS/Re II	Kalimpong	27°03'03.21" N 88°46'45.17" E	581	Sandy	4.25
	RS/Re III	Kalimpong	27°03'03.21" N 88°58'45.44" E	581	Sandy	5.32
	RS/Re IV	Kalimpong	27°03'03.21" N 88°61'45.73" E	581	Sandy	5.12
Mirik Lake	RS/Mr I	Mirik	26°53'10.85" N 88°11'15.61" E	1606	Sandy	4.88
	RS/Mr II	Mirik	26°53'10.44" N 88°11'15.03" E	1606	Sandy	4.50
	RS/Mr III	Mirik	26°53'10.21" N 88°11'15.19" E	1606	Sandy	4.55
	RS/Mr IV	Mirik	26°53'10.30" N 88°11'15.43" E	1606	Sandy	4.16

Soil samples were collected at 10-15 cm depth with the help of proper sampling tools and stored in polythene bags and kept in cold until further analysis. The samplings were divided into ununiform zone and random sampling patterns were followed according to the type of vegetation they represent. Unusual areas for sampling were avoided. Sampling area was properly divided and recorded by GPS tools (Garmin) (Fig. 5 & 6) and proper records like soil pH, texture of the samples were taken (Table 2). The plating techniques were adopted for isolation of microorganisms (soil borne bacteria and fungi) from the collected soil samples using Warcup's soil plating method. Direct soil plating technique was adopted to isolate the fungi that don't sporulate and exist as mycelium in soil seldom, where as soil washing technique was used to obtain microorganisms that not readily isolated from the soil plating technique.

In case of isolation of fungi 10^3 - 10^5 serial dilution were made and 10^4 - 10^7 dilution were used for isolation of bacteria. Different media like Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's Agar (RA), Carrot juice agar (CJA) Czapek-Dox agar (CDA), Flentze's soil agar (FSEA), Malt extract peptone dextrose agar (MPDA), Yeast extract- dextrose agar (YDA), Special Nutrient Agar (SNA), *Trichoderma* Selective Medium C (TSMC), Cellulose Agar Medium, Malt Extract Agar (MEA), Oatmeal Agar (OA), Cornmeal Dextrose Agar (CMD), Nutrient agar (NA). Optimum temperature 28°C for incubation of fungi and 37°C for bacteria were maintained. The number of fungi and bacteria formed colonies on the Fig.s were counted and the microbial populations obtained from different rhizosphere, forest soil and riverine soil were determined. Microbial population determined in soils, ranged between 4×10^3 - 6×10^4 cfu in case of fungi and 5×10^6 cfu- 6×10^6 cfu in case of bacteria. In case of forest soil samples, highest fungal population was recorded in soil samples collected from Sukna forest whereas lowest was in Sandakphu forest. Similarly, highest bacterial population was recorded in case of soil samples obtained from Lohagarh forest, whereas the lowest was from Sandaphu soil sample. In case of Rhizosphere soil samples, highest fungal population was found in the soil obtained from the rhizosphere of *Amomum subulatum* (Cardamom) and the lowest was found in rhizosphere soil sample of *Rhododendron* growing in higher altitude regions of Darjeeling district. Similarly highest bacterial population recorded in Rhizosphere soil sample of *Camellia sinensis* and lowest in *Rhododendron*. In case of microbial populations in soils obtained from the river beds of major rivers of Darjeeling district, highest fungal population was recorded in the soil sample obtained from Reli river Kalimpong and lowest in Rangit river, the highest bacterial population was also found in Reli river soil samples and the lowest in soil samples obtained from Balasan River bed from Mundakothi (Table 3. Fig. 7).

Table 3. Fungal and bacterial population in the forest, rhizosphere and riverine soil samples collected from different regions of Darjeeling District

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10 ⁴ /g)	Mean	Bacterial Population (cfu x 10 ⁶ /g)	Mean
Kurseong Subdivision					
Sukna Forest (FS/S)	FS/SI	6.24	6.628	4.57	4.36
	FS/SII	6.15		4.23	
	FS/SIII	6.33		4.15	
	FS/SIV	7.14		4.43	
	FS/SV	7.28		4.44	
Lohagarh Forest (FS/L)	FS/LI	5.43	5.97	5.33	4.82
	FS/LII	5.68		5.22	
	FS/LIII	6.33		5.12	
	FS/LIV	6.15		4.12	
	FS/LV	6.26		4.33	
Tindharey (FS/Tn)	FS/Tn I	6.17	6.22	5.77	5.40
	FS/Tn II	6.33		5.12	
	FS/Tn III	6.27		5.28	
	FS/Tn IV	6.14		5.46	
Ghyabari Forest (FS/G)	FS/G I	6.15	5.94	5.11	4.80
	FS/G II	6.33		4.33	
	FS/G III	5.88		5.22	
	FS/G IV	5.43		4.15	
	FS/G V	5.72		5.23	
Rongli Rongek Forest (FS/Rr)	FS/Rr I	5.43	5.42	5.33	4.33
	FS/Rr II	5.25		5.13	
	FS/Rr III	5.88		4.76	
	FS/Rr IV	5.12		4.65	
	FS/Rr V	5.34		4.33	
Maney Dara Forest (FS/Md)	FS/Md I	5.27	5.23	5.16	5.37
	FS/Md II	5.20		5.43	
	FS/Md III	5.32		5.76	
	FS/Md IV	5.14		5.15	
Sandakhpu (FS/S)	FS/S I	5.16	4.71	3.22	3.17
	FS/S II	4.22		3.16	
	FS/S III	4.76		3.13	
Rimbhik Forest (FS/R)	FS/R I	5.26	5.23	4.22	4.21
	FS/R II	5.40		4.12	
	FS/R III	5.15		4.65	
	FS/R IV	5.22		4.25	

Contd.....

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10 ⁴ /g)	Mean	Bacterial Population (cfu x 10 ⁶ /g)	Mean
Darjeeling Subdivision					
Thorpu Forest (FS/T)	FS/T I	6.18	6.26	5.15	5.46
	FS/T II	6.17		5.37	
	FS/T III	6.25		5.11	
	FS/T IV	6.44		5.82	
Tagdha Forest (FS/Td)	FS/Td I	5.14	5.21	4.32	3.96
	FS/Td II	5.12		4.36	
	FS/Td III	5.26		3.12	
	FS/Td IV	5.35		3.15	
Rambi Saal Forest (FS/R)	FS/R I	5.43	5.76	4.22	4.15
	FS/R II	5.22		4.23	
	FS/R III	6.23		4.02	
	FS/R IV	6.16		4.15	
Kalimpong Sub Division					
Lamahata (FS/L)	FS/L I	6.10	6.43	5.15	4.83
	FS/L II	6.36		4.73	
	FS/L III	6.44		4.28	
	FS/L IV	6.82		5.16	
Cinchona Forest (FS/C)	FS/CI	5.43	5.90	4.22	4.33
	FS/C II	5.86		4.73	
	FS/C III	5.77		4.15	
	FS/C IV	6.54		4.26	
	FS/C V	6.33		4.33	
Mongpong Forest (FS/M)	FS/M I	6.82	6.40	4.13	4.88
	FS/M II	6.15		4.22	
	FS/M III	6.33		4.65	
	FS/M IV	6.45		4.18	
	FS/M V	6.54		4.88	
	FS/M VI	6.13		4.26	
Pedong (FS/P)	FS/P I	5.73	5.80	4.27	4.29
	FS/P II	6.12		4.11	
	FS/P III	5.87		4.73	
	FS/P IV	5.50		4.08	
Rishyup Valley (FS/Ry)	FS/Rv I	6.73	6.50	4.13	4.38
	FS/Rv II	6.17		4.82	
	FS/Rv III	6.29		4.36	
	FS/Rv IV	6.83		4.22	
Lava (FS/Lv)	FS/Lv I	5.33	5.44	4.73	4.59
	FS/Lv II	5.73		4.18	
	FS/Lv III	5.27		4.88	

Contd.....

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10 ⁴ /g)	Mean	Bacterial Population (cfu x 10 ⁶ /g)	Mean
RHIZOSPHERE SOIL SAMPLES					
<i>Camellia sinensis</i> (Tea) (RHS/T)	RHS/T I	6.32	5.81	4.73	5.53
	RHS/T II	5.45		5.12	
	RHS/T III	6.15		6.15	
	RHS/T IV	6.78		4.27	
	RHS/T V	5.33		4.25	
	RHS/T VI	5.15		5.15	
	RHS/T VII	5.73		5.64	
	RHS/T VIII	6.14		6.27	
	RHS/T IX	4.58		6.34	
	RHS/T X	5.33		5.42	
	RHS/T XI	6.27		5.28	
	RHS/T XII	6.83		6.22	
	RHS/T XIII	6.15		4.18	
	RHS/T XIV	5.28		4.15	
	RHS/T XV	5.76		5.39	
	RHS/T XVI	5.43		5.82	
	RHS/T XVII	6.36		6.18	
	RHS/T XVIII	4.72		5.32	
	RHS/T XIX	4.74		6.37	
	RHS/T XX	6.82		6.73	
	RHS/T XXI	6.17		6.28	
	RHS/T XXII	6.40		6.43	
<i>Cryptomeria japonica</i> (RHS/C)	RHS/C I	6.22	6.21	5.26	4.79
	RHS/C II	6.27		4.74	
	RHS/C III	6.15		4.39	
<i>Citrus reticulata</i> (RHS/O)	RHS/O I	5.76	5.39	4.22	4.23
	RHS/O II	5.15		4.16	
	RHS/O III	5.27		4.33	
<i>Oryza sativa</i> (Paddy) (RHS/P)	RHS/P I	6.17	6.13	4.27	4.47
	RHS/P II	5.18		4.32	
	RHS/P III	6.76		4.33	
	RHS/P IV	6.43		4.72	
	RHS/P V	5.82		4.82	
	RHS/P VI	6.44		4.36	
<i>Dendrocalamus latiflorus</i> (Bamboo) (RHS/B)	RHS/B I	5.83	5.46	4.18	4.17
	RHS/B II	5.18		4.27	
	RHS/B III	5.73		4.03	
	RHS/B IV	5.11		4.24	
	RHS/B V	5.20		4.21	
	RHS/B VI	5.76		4.18	

Contd.....

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10 ⁴ /g)	Mean	Bacterial Population (cfu x 10 ⁶ /g)	Mean
<i>Dendrocalamus latiflorus</i> (Bamboo) (RHS/B)	RHS/B VII	5.27	5.47	4.33	4.44
	RHS/B VIII	5.43		4.74	
	RHS/B IX	5.73		4.27	
<i>Glycine max</i> (Soybean) (RHS/Sb)	RHS/Sb I	6.13	6.51	5.18	5.03
	RHS/Sb II	6.36		4.33	
	RHS/Sb III	6.82		4.88	
<i>Sechium edule</i> (Squash) (RHS/S)	RHS/S I	6.72	6.42	5.33	4.89
	RHS/S II	6.15		5.15	
	RHS/S III	6.44		5.73	
	RHS/S IV	6.13		4.73	
	RHS/S V	6.24		4.12	
	RHS/S VI	6.82		4.33	
<i>Brassica juncea</i> (RHS/Br)	RHS/Br I	5.72	5.43	5.87	5.10
	RHS/Br II	5.43		5.23	
	RHS/Br III	5.15		4.22	
<i>Amomum subulatum</i> (Cardamom) (RHS/Cr)	RHS/Cr I	6.18	6.45	4.78	5.26
	RHS/Cr II	6.88		5.33	
	RHS/Cr III	6.42		5.28	
	RHS/Cr IV	6.45		5.17	
	RHS/Cr V	6.32		5.33	
<i>Rhododendron</i> (RHS/R)	RHS/R I	5.33	4.90	3.22	3.39
	RHS/R II	4.26		3.74	
	RHS/R III	5.15		3.21	
	RHS/R IV	5.22		3.16	
	RHS/R V	4.28		3.42	
	RHS/R VI	5.18		3.62	
<i>Thyasaenolena latifolia</i> (Amliso)	RHS/A I	6.33	6.25	4.23	4.37
	RHS/A II	6.25		4.73	
	RHS/A III	6.18		4.15	
<i>Alnus nepalensis</i> (RHS/A)	RHS/A I	6.28	6.22	4.27	4.34
	RHS/A II	6.82		4.33	
	RHS/A III	5.88		4.65	
	RHS/A IV	5.93		4.13	

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10 ⁴ /g)	Mean	Bacterial Population (cfu x 10 ⁶ /g)	Mean
Teesta River (RS/T)	RS/T I	4.73	4.39	3.24	3.39
	RS/T II	4.13		3.73	
	RS/T III	4.56		3.11	
	RS/T IV	4.21		3.27	
	RS/T V	4.33		3.64	
Balasan (RS/B)	RS/B I	4.27	4.72	3.75	3.34
	RS/B II	4.16		3.16	
	RS/B III	5.13		3.43	
	RS/B IV	5.22		3.22	
	RS/B V	4.83		3.16	
Rangit (RS/R)	RS/R I	4.37	4.46	3.74	3.69
	RS/R II	4.28		3.82	
	RS/R III	4.76		3.68	
	RS/R IV	4.28		3.55	
	RS/R V	4.63		3.68	
Reli (RS/Re)	RS/Re I	5.16	5.25	4.82	4.62
	RS/Re II	5.22		4.74	
	RS/Re III	5.28		4.15	
	RS/Re IV	5.37		4.77	
Mirik Lake (RS/Re)	RS/Mr I	5.18	4.59	3.14	3.37
	RS/Mr II	4.33		3.28	
	RS/Mr III	4.62		3.33	
	RS/Mr IV	4.24		3.74	

4.1.1. Fungal Isolates

4.1.1.1. Growth studies in solid medium

Pure cultures of fungal isolates obtained from various sources were maintained in PDA slants. The morphology and growth pattern on solid medium were studied for 4-6 days at 28⁰C on PDA medium. Nature of mycelial growth, rate of growth and time of sporulation were noted. Unique radial growth pattern were observed each group of fungal isolates which have been categorized as isolates obtained from forest soil (Fig. 8), agricultural soil (Fig. 9) and Riverrine soil (Fig. 10).

4.1.1.2. Microscopic observation

Microscopic observations of spores and mycelia of the fungal isolates were made and photographs were taken with the help of Ocular-attached stereo digital camera. Microscopical observations in terms of spore type, size, attachments, spore ornamentation as well as mycelial type have been presented in detail in Table 4; Fig. 11. Most of the commonly occurring fungal isolates have been

identified on the basis of growth patterns and microscopic observations. It was found that most of the fungal isolates belonged to the genera A total of 637 fungal isolates were obtained from soil samples collected from different regions of Darjeeling district of North Bengal. The dominant fungal isolates belonged to the genera *Absidia*, *Acremonium*, *Alternaria*, *Aspergillus*, *Byasiochlamus*, *Colletotrichum*, *Drechslera*, *Emericella*, *Fusarium*, *Curvularia*, *Gonronella*, *Macrophomina*, *Noesertoria*, *Paecilomyces*, *Penicellium*, *Pseudoeutatum*, *Rhizoctonia*, *Rhizopus*, *Sclerotianum*, *Sporotrichum*, *Syncephalastrum*, *Talaromyces*, *Thanetophorus* and *Trichoderma* . Similarly alarge number of *Trichoderma* islates were also obtained which were initially identified on the basisof their Microscopic characters as *T. harzianum*, *T. asperellum*, *T. erinaceum* and *T. viride* (Fig. 12).

4.1.1.3. Deposition of common fungal isolates to the National Agriculturally Important Culture Collection (NAIMCC)

Commonly occurring fungal isolates were initially identified on the basis of their morphological characters by National Centre of Fungal Taxonomy, New Delhi and respective NCFT identification code have been provided to them. These cultures were finally deposited to the National Agriculturally Important Microbial Culture Collection (NAIMCC) of National Bureau of Agriculturally Important Microorganisms (NBAIM), Maunath Bhnjan, UP, and their accession numbers have been provided in Table 5.

4.1.2. Bacterial isolates

A total of 135 bacterial isolates were obtained from various sources. Among them 39 were obtained from forest soil, 73 from rhizosphere of agricultural crops and 23 from riverine soil. The source and GIS location of the soil samples was recorded and coded according to their respective locations (Table 6).

4.1.2.1 Biochemical characterization of bacterial isolates

All the bacterial isolates obtained from various sources were initially categorized as Gram +ve and Gram –ve isolates. Their morphology (Fig. 13), pigmentation and basic biochemical characterizations like H₂S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, protease production, urase production, cellulase production and indolae production were characterized. The detail results have been presented in Table 7. Result revealed that out of 135 bacterial isolates, 109

were gram positive whereas 26 were gram negative. Most of the isolates showed a positive result for catalase activity. The most common and abundant bacterial species were *Bacillus sp.*, *Micrococcus sp.*, *Coryneform sp.* *Staphylococcus sp.* *Serratiasp.* *Paenibacillus sp.*, *Pseudomonas sp.*, *Enterobacter sp.* well as *Bukholderia sp.*

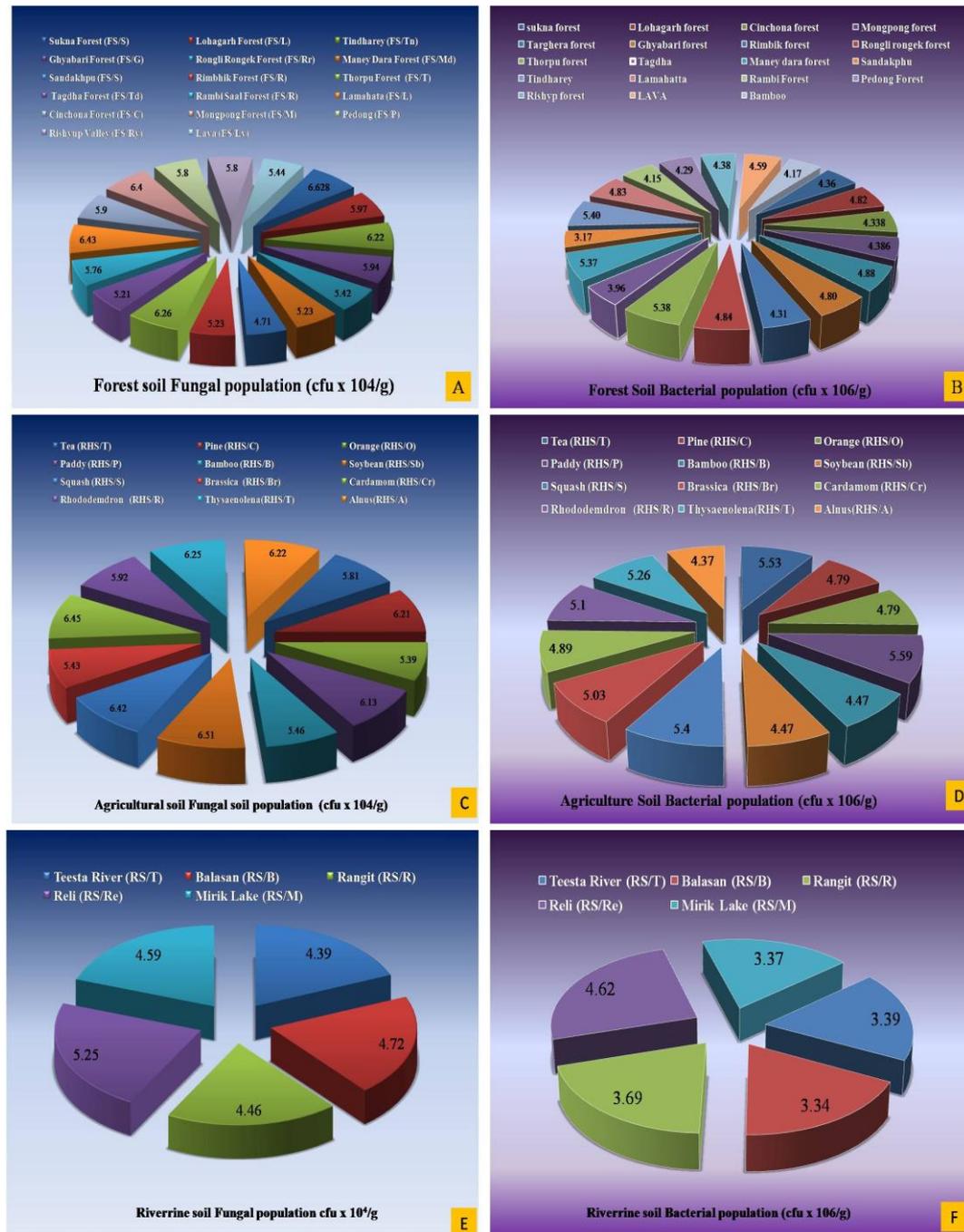


Fig. 7. Fungal and Bacterial population in three different soil types of Darjeeling Hills-Forest soil, Agricultural soil, and Riverine soil. Fungal population (A,C &E), Bacterial population (B,D &F).

Table 4. Morphology and Microscopical Characters of fungal isolates obtained from various sources

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/P-47, RHS/P-48, RHS/P-412	<i>Absidia cylindrospora</i> (3)	<p>Colonies: The rapid growing, flat, woolly to cottony, and olive gray colonies mature within 4 day.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Rhizoid: Rhizoids are rarely observed. When present, the sporangiophores arise on stolons from points between the rhizoids, but not opposite the rhizoids</p> <p>Sporangiophores : are branched and arise in groups of 2-5 at the internodes. They often produce arches. Sporangiophores carry pyriform, relatively small (20-120 µm in diameter) sporangia. A septum is usually present just below the sporangium in the sporangiophore. The sporangiophore widens to produce the funnel-shaped apophysis beneath the sporangium</p>
RHS/T-08, RHS/T-10	<i>Acremonium fusidioides</i> (2)	<p>Colonies: Colonies reaching 8-12 mm diam in ten days at 20°C on PDA, ochraceous-brown, powdery;</p> <p>Mycelia: Hyaline, aseptate, white.</p> <p>Conidia: Conidia catenulate, of two kinds: (a) predominantly slightly pigmented, fusiform with truncate ends, 6.4 x 2.1 µm, (b) globose, hyaline, slightly warty, 3.4-4.7 µm diam.</p>
RHS/T-534	<i>Alternaria alternate</i> (1)	<p>Colonies: Colonies grow rapidly and reaches a diam of 5- 15 cm following incubation at 25°C for 7 days on PDA. The colony is flat, downy to woolly and is covered by grayish, short, aerial hyphae</p> <p>Mycelia: Hyaline, septate, brown hyphae when mature</p> <p>Conidia: The end of the conidium nearest the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia</p> <p>Conidiophore: Conidiophores are also septate and brown in color, occasionally producing a zigzag appearance. They bear simple or branched large conidia (7-10 x 23-34 µm) which have both transverse and longitudinal septations. These conidia may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obclavate, darkly pigmented, muriform, smooth or roughened.</p>

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/L-04, FS/L-40, FS/C-140, FS/C143, FS/Td-165, FS/Td-173, FS/S-177, FS/Rr-140, FS/S-112, FS/S-113, FS/R-262, FS/Tn-57, FS/Tn-58, RHS/P-37, FS/G-101, FS/L-105, FS/L-106, FS/L-107, FS/L -117, FS/L-281, FS/S-282, FS/S-283, FS/Md-284, FS/Md-286, FS/Md-287, FS/Md-365, FS/Md-366, RHS/P-200, RHS/A-82, RHS/T-198, RHS/T- 272, RHS/T-421, RHS/T-422, RHS/T-580, RHS/S-518, RHS/S-530, RHS/M-401, RHS/G-296, RHS/G-297, RHS/S-518, RHS/S-530, RHS/Br-570, RHS/Cd-610, RHS/R-548, RHS/A-30, RHS/A-33, RHS/P-45, RHS/P-48, RHS/D-280, RHS/T-581, RS/B-160, RS/P/14, RS/Md-288, RS/T-137, RS/B-164, RS/Re-231, RS/Re-235	<i>A. niger</i> (57)	<p>Colonies: Colonies growing fast and reaching up to 5-6 cm in 3-4 days on PDA medium and typically black powdery on MEA and PDA at 24-28°C.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia in large radiating heads. Conidia brown, ornamented with warts and ridges, subspherical, 3.5-5.0 µm diam. Conidiophores arising from long, broad, thick walled mostly brownish, sometimes branched foot cells.</p> <p>Conidiophore: Consisting of a dense felt of conidiophores. Conidiophore stipes smooth-walled, hyaline. Vesicles subspherical, 50-100 µm diam.</p>
FS/Rv-330, FS/Rv-323, RHS/P-38, RHS/P -56, RHS/P -54, RHS/P-114, RHS/T-99, RHS/T-190, RHS/T-383, RHS/T-386, RHS/Cd-603, RHS/A-77, RS/B-163	<i>A. clavatus</i> (13)	<p>Colonies: Colonies on Czapek and malt agar usually spreading, occasionally floccose, blue-green, mycelium white, inconspicuous.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia smooth walled, conidial heads clavate, usually splitting into several divergent columns.</p> <p>Conidiophore: Conidiophores very long, 500-900 µm long, smooth-walled, hyaline to slightly brown near vesicle. Vesicle clavate, 15-75 µm diam. Phialide 7-10 x 2-3,5 µm; metulae absent. Conidia smooth-walled, ellipsoidal, 3-4,5 x 2,5-3,5 µm diam.</p>

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/L-42, FS/L-13, FS/L-17, FS/L-18, FS/G-226, FS/S-64, FS/L-41, FS/S-63, FS/S-24, FS/S-278, RHS/T-449, RHS/T-450, RHS/T-274, RHS/T-275, RHS/T-402, RHS/T-331, RHS/T-332, RHS/S-303, RHS/Cd-606, RHS/R-549, RHS/Am-34, RHS/R-12, RHS/P-201, RHS/P-202, RHS/P-205, RS/R-115, RS/T-182, RS/T-183, RS/P-61, RS/P-05, RS/T-236, RS/M-368.	<i>A. melleus</i> (32)	<p>Colonies: Fast growing reaching up to 2.5-4-5 cm diam in 4-5 days on PDA. Creamy buff to pinkish cinnamon at first but later becoming ochraceous due to presence of the sclerotia.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia globose to subglobose, smooth walled or irregularly roughened, 2.8-3.5 μm diam.</p> <p>Conidiophore: Conidiophore usually 0.5-2 μm tall, but rarely 3 μm tall. thickwalled, pigmented and conspicuously roughened. The rodlet pattern of the conidia is observed.</p>
FS/Lv-354, FS/P-214, FS/P-215, FS/S-457, FS/S-459, RHS/T-453, RHS/T-389, RHS/G-299, RHS/Br-579, RHS/A-79, RS/T-59, RS/P-60	<i>A. nidulans</i> (12)	<p>Colonies: Colonies growing rapidly, green, cream-buff or honey-yellow; reverse dark purplish.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia spherical, rugulose, subhyaline, green in mass, 3-4 μm diam. Conidial heads short, columnar, up to 80 μm long</p> <p>Conidiophore: Conidiophore stipes brownish, 60-130 x 2.5-3.0 μm. Vesicles hemispherical, 8-10 μm diam.</p>
RHS/T554, RHS/S-306, RHS/S-307, RHS/P-36, RHS/P-203, RHZ/P-204, RHS/Cd-346	<i>A. parasiticus</i> (7)	<p>Colonies: Colonies on Czapek agar at 25°C attaining a diameter of 2.5-3.5 cm within 7 days, usually consisting of a dense felt of green conidiophores</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia globose, 3.5-5.5 μm in diam, yellow-green, conspicuously rough-walled. Conidial heads green, radiate</p> <p>Conidiophore: Conidiophores mostly 300-700 μm long, hyaline, rough-walled. Vesicles subglobose, 20-35 μm in diam. Phialides usually borne directly on the vesicle, 7-9 x 3-4 μm, hyaline to pale green.</p>

Contd.....

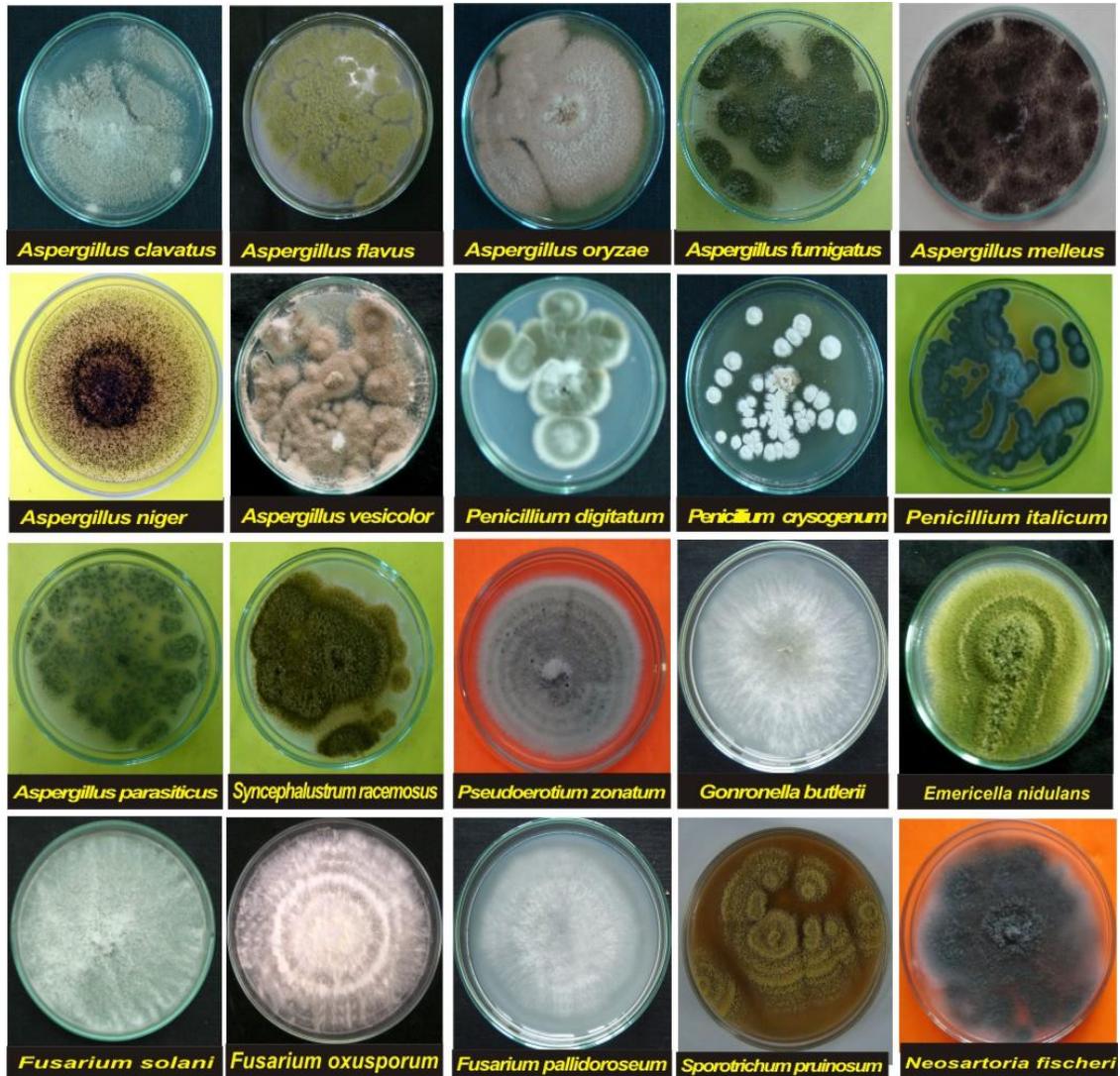


Fig. 8. Radial growth pattern of fungal isolates on PDA medium isolated from different forest soils of Darjeeling hills.

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/P-220, RS/T-190, RS/286, FS/L-104, FS/M-67, FS/P-217, FS/R-522, FS/La-352, 353, FS/L-6, FS/T-62, FS/Td-170, RHS/T-11, RHS/T-427, RHS/T-70, RHS/T-71, RHS/T-431, RHS/T-433RHS/C-316, RHS/P-122, RHS/P-123, RHS/B-224, RHS/B-247, RHS/Cd-21, RHS/Cd-350, RS/T-134, RS/B-15, RS/R-227, RS/R-228, RS/M-377	<i>Aspergillus flavus</i> (29)	Colonies: Colonies on Czapek and PDA usually spreading, yellow green, reverse colourless to dark red brown, occasionally dominated by hard sclerotia, white at first, becoming red brown to almost black with age, 400-700 µm diam. Mycelia: hyaline, aseptate Conidia: Conidial heads typically radiate, splitting into several poorly defined columns, rarely exceeding 500-600 µm diam., mostly 300-400 µm. columnar up to 300-400 µm. Conidiophore: Conidiophores thick-walled, hyaline, coarsely roughened, usually less than 1 µm long, 10-20 µm diam. Vesicles elongated when young, becoming subglobose to globose, 25-45 µm diam.; both metulae and phialides present.
FS/R-263, FS/R-264, RHS/P-209, RHS/B-220, RHS/T-531, RHS/P-43, RHS/T-585, RHS/T-586, RHS/T-588,	<i>A. fumigatus</i> (9)	Colonies: Colonies fast growing reaching up to 1-2.5 cm diam in 4-5 days on PDA at 24-26°C. Dark blue-green, consisting of a dense felt of powdery conidiophores, intermingled with aerial hyphae. Mycelia: hyaline, aseptate Conidia: Conidia verrucose, (sub) spherical, 2.5-3.0 µm diam Conidial heads columnar; conidiogenous cells uniseriate. Conidiophore: Pigmented conidiophores stipes smooth-walled, often green in the upper part. Vesicles subclavate, 20-30 µm wide.
RHS/P-500, RHS/P-507, RHS/P-126, RHS/P-127, RHS/B-248, RHS/Br-635, RHS/T-8RHS/TI-35, RHS/TI-617, RHS/M-449	<i>A. oryzae</i> (11)	Colonies: Colonies growing rapidly, pale greenish-yellow, olive-yellow or with different shades of green, typically with dull brown shades with age. Mycelia: hyaline, aseptate Conidia: Conidial heads radiate to loosely columnar, 150-300 µm diam. Conidia (sub)spherical to ovoidal, 4.5-8.0 (-10.0) x 4.5-7.0 µm, smooth-walled to roughened, greenish to brownish. Conidiophore: Conidiophore stipes hyaline, up to 4-5 mm in length.

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/C-141, FS/M-219, FS/R-318, FS/G-96, FS/G-96, FS/Td-170, FS/Td-171	<i>A. vesicolor</i> (7)	<p>Colonies: Colonies reaching 2-3 cm diam on CzA and 4-5 cm diam on MEA in two weeks at 25°C; variable in colour, light yellowish, pink to flesh-coloured, ochre or orange yellow to yellowish green.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: Conidia globose, echinulate, mostly 2-3 µm diam</p> <p>Conidiophore: Conidiophores colourless or yellowish, smooth-walled, to 500-700 µm long; vesicles elongate with metulae and phialides.</p>
RHS/T-273, RHS/T-593, RHS/T-10	<i>A. ustus</i> (3)	<p>Colonies: Colonies on potato dextrose agar at 25°C are white to yellow to drab gray to brown, but never green. Spreading broadly.</p> <p>Mycelia: Aseptate and hyaline</p> <p>Conidia: Conidial heads are radiate to loosely columnar and biseriate and irregularly elongate.</p> <p>Conidiophore: Conidiophores 30-350 µm, smooth-walled, and brown. Vesicles are globose to subglobose, 7-16 µm in diameter. Metulae and phialides cover the upper portion of the vesicle. Conidia are globose, 3-4.5 µm, with very rough walls.</p>
RHS/P-46, RHS/P- 210	<i>Drechslera</i> <i>sp.</i> (2)	<p>Colonies: Grey, brown or blackish brown, often hairy, sometimes velvety. Reaching to a diam of 4-5 cm in 7 days on PDA.</p> <p>Mycelia: Hyaline, aseptate mycelium mostly immersed. Setae and hyphopodia absent.</p> <p>Conidia: Conidia solitary, also sometimes catenate or forming secondary conidiophores, acropleurogenous, simple, straight or curved, clavate, cylindrical rounded at the ends, ellipsoidal, fusiform or obclavate.</p> <p>Conidiophore: macronematous, mononematous, straight, often geniculate, unbranched or in a few species loosely branched, brown. Conidiogenous cells polytretic, integrated, terminal, frequently becoming intercalary, sympodial, cylindrical, cicatrized.</p>

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/P-107	<i>Byssochlamus nivius</i> (1)	Colonies: Colonies (OA) extremely variable, effuse, whitish to greysh, with pinkish patches; reverse dark brown with vinaceous stains. Generally producing intraepidermal acervuli. With a dense layer of with subulate or cylindrical hyaline phylides. Mycelia: hyaline, aseptate Conidia: Conidia straight, cylindrical, obtuse at the apex, 9-24 x 3.0-4.5 µm.
RHS/T-267, RHS/T-260	<i>Colletotrichum gloeosporioides</i> (2)	Colonies: Colonies (OA) extremely variable, effuse, grey to brown, with pinkish patches; reverse dark brown with vinaceous stains. Generally producing intraepidermal acervuli. With a dense layer of with subulate or cylindrical hyaline phylides. Mycelia: hyaline, aseptate Conidia: Conidia straight, cylindrical, obtuse at the apex, 9-24 x 3.0-4.5 µm. Appressoria: 6-20 x 4-12 µm, clavate or irregular.
RHS/T-582, RHS/T-462	<i>Curvularia lunata</i> (2)	Colonies: Colonies (PDA) expanding, black, hairy Mycelia: Hyaline, aseptate. Conidia: Conidia olive brown Curved ellipsoidal, 3- septate, rounded at the apex slightly acuminate at the base, the middle septum below the centre and the third cell strongly curved, 20-30x 9-15µm. Conidiophore: Conidiophore erect, pigmented, geniculated from sympodial elongations, 3-10 septate.
RHS/M-509, RHS/C-316, RHS/S-425	<i>Emenicella nidulans</i> (3)	Colonies: Growing rapidly, green, cream-buff or honey-yellow; reverse dark purplish. Reaches upto 4-6 cm in dia in 7 days of growth on PDA and MEA Mycelia: Hyaline, aseptate Conidia: Conidial heads short, columnar, up to 80 µm long. Conidiophore: Conidiophore stipes brownish, 60-130 x 2.5-3.0 µm. Vesicles hemispherical, 8-10 µm diam. Conidiogenous cells biseriate, 5.9 x 2-3 µm. Metulae 5.6 x 2.3 µm. Conidia spherical, rugulose, subhyaline, green in mass, 3-4 µm diam.

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/G-28, RHS/Cd-346	<i>Fusarium sp.</i> (2)	<p>Colonies: Growing rapidly, with white to cream-coloured aerial mycelium. Colony reaching up to 4-5 cm diam in 7 days of growth on OMA</p> <p>Mycelia: hyaline, aseptate</p> <p>Conidia: Microconidia usually abundant, produced on elongate, sometimes verticillate conidiophores, 8-16 × 2.0-4.5 μm. Chlamydospores frequent, singly or in pairs, terminal or intercalary, smooth- or rough-walled, 6-10 μm diam</p>
RHS/M-497, RS/M-380	<i>Gonronella butleri</i> (2)	<p>Colonies: Colonies slow growing, stolons and rhizoids poorly differentiated, whitish and sometimes.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Sporangiophores: Erect and mostly branched, Sporangia globose, columella reduced, supported by a conspicuous apophysis.</p> <p>Spore: Oval mostly flattened on one side, 2.2-4.5 x 1.6- 2.5 μm.</p>
RHS/G-253, RHS/Cd-347	<i>Macrophomina phaseolina</i> (2)	<p>Colonies: Pycnidia dark brown, solitary or gregarious on leaves and stems, immersed, becoming erumpent, 100-200 μm diam</p> <p>Mycelia: hyaline, aseptate</p> <p>Conidia: Conidia hyaline, ellipsoid to obovoid, 14-30 x 5-10 μm</p> <p>Conidiophore: Conidiophores (phialides) hyaline, short obpyriform to cylindrical, 5-13 x 4-6 μm.</p>
RHS/S-293	<i>Phialomyces veroiti</i> (1)	<p>Colonies: Colonies effuse, at first yellow, later greyish olive.</p> <p>Mycelia: Mycelium partly superficial, partly immersed. Stroma none</p> <p>Conidia: Conidia catenate, dry, semi-endogenous or acrogenous, simple, broadly ellipsoidal to limoniform, golden brown, verrucose, usually with a small, hyaline papilla or connective at each end, 0-septate</p> <p>Conidiophore: macronematous, mononematous, unbranched or branched at the apex, stipe and branch or branches each bearing terminally a small number of phialides; stipe straight or flexuous, hyaline or pale straw coloured, smooth. Conidiogenous cells monophialidic, discrete, determinate, lageniform.</p>

Contd.....

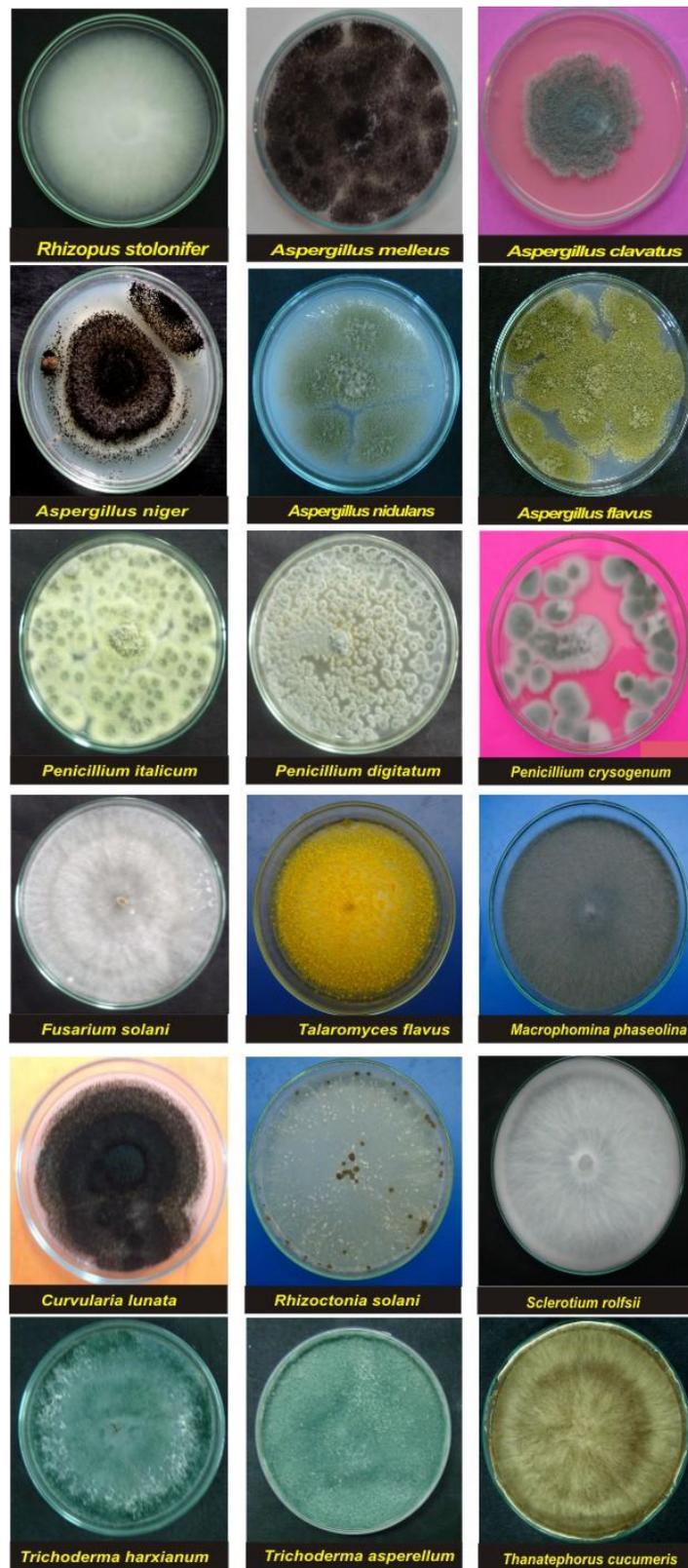


Fig. 9. Radial growth pattern of fungal isolates on PDA medium isolated from Rhizosphere soil of different crops of Darjeeling hills.

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
Fs/s-108	<i>Noesertoria fischeri</i> (1)	<p>Colonies: Colony white to cream coloured cleistothecia, exudate clear texture velutinous</p> <p>Mycelia: Hyaline, aseptate</p> <p>Conidia: conidia sparse and grey green</p> <p>Ascospore: Ascospores with two longitudinal thin Ascospores are spherical, conidial heads spherical to ellipsoidal.</p>
FS/S-109, FS/Rv-317, FS/La-185, RHS/R-555, RHS/T-455, RHS/T-590, RHS/C-338, RS/T-285, FS/Lv-355, FS/S-67, FS/S-459, RHS/R-555, RHS/T-437,	<i>Penicillium digitatum</i> (13)	<p>Colonies: Olive green, slow growing, reaching upto 1-1.5 cm diam in 10 days of growth on PDA at 25°. Mostly greenish brown, reverse uncoloured or dull tan. Mycelia: Hyaline, aseptate</p> <p>Conidia: Large cylindrical conidia divergently branched penicilli with a broadly truncate base and evenly rounded tip, smooth walled, ellipsoidal to cylindrical and 3.5-5.5 x 2.8-6 µm.</p> <p>Conidiophore: Terverticillate, appressed elements, born from subsurface or aerial hyphae.</p>
RHS/T-594, RHS/M-403, RHS/P-414, RHS/R-555	<i>Penicillium italicum</i> (4)	<p>Colonies: Colonies grow restrictedly, reaching 2.0-2.5 cm diam in 10-14 days at 25° on CzA. Grey greenish, reverse pale grey to yellow brown.</p> <p>Mycelia: hyaline, aseptate</p> <p>Conidiophores: Usually loosely synnematus particularly in the colony margins, smooth walled, two-staged branched with appressed penicilli.</p> <p>Phidies cylindrical, slender with a short but distinct neck.</p> <p>Conidia: Smooth-walled, ellipsoidal to cylindrical, 3.5-5 x 2.2-3.5 µm</p>
RHS/P-121, RHS/P-122	<i>Pseudeurotium zonatum</i>	<p>Colonies: Colonies slow growing reaching up to 2.5-3.0 cm diam in 10-14 days at 25° on OMA. First whitish, floccose and becomes grey with the development of ascomata.</p> <p>Ascomata: Globose, glabrous, 100-230 µm diam, wall composed of angular iso-diametric or elongate cell in surface view, mostly 4-8 µm diam.</p> <p>Asci: Globose, pyriform to ellipsoidal, 7-9 x 6.5 µm, becomes olive brown when mature.</p>

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/L-265,FS/G-97, RHS/T-269, RHS/T-271, RHS/C-312, RS/Re-230,	<i>Penicillium chrysogenum</i> (6)	<p>Colonies: Thin spreading colonies reaching 2.0-4 cm diam in 10-14 days at 25° on CzA. Valvety, grass green to bluish green colony, reverse pale to bright yellow.</p> <p>Mycelia: hyaline, aseptate</p> <p>Conidiophores: Two to three staged branched at rather wide angles.</p> <p>Conidia: Smooth walled, ellipsoidal, 3.0-4 x 2.8-3.5 µm diam or globose to sub globose.</p>
FS/L-01, FS/L-02	<i>Rhizopus stolonifer</i>	<p>Colonies: Very fast growing and expanding, up to 1 cm high, whitish to greyish-brown, stolon hyaline to brown.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Sporangiophore: Singly or in tufts, brown, 1-2 mm high, 18 µm wide, mostly unbranched, sometimes with brownish swellings up to 20-25 µm diam. Rhizoids abundantly branched, up to 250 µm long, brownish. Sporangia spherical, 50-250 µm diam, brownish-grey to black; columella comprising 50-70% of sporangium, black, mostly 100-200 µm diam columella sub globose to oval pale brown 70-120 µm diam. Sporangiospores greyish-green, angular, subspherical to ellipsoidal, longitudinally striate, 6-8 x 4.5-5.0 µm</p> <p>Chlamydospore: Absent in stolon and scarce in submerged hyphae. Single or in chains, spherical to ovoidal, 10-35 µm diam, hyaline, smooth-walled. Zygosporangia, dark, spherical, 150-200 µm, Suspensors unequal, spherical and conical sometimes globose. Heterothallic.</p>
RHS/T-308	<i>Syncephalastrum racemosum</i>	<p>Colonies: Colonies fast growing reaching to a diameter of 2-5-4 cm. dark grey in colour. Reverse dark brown to dark grey.</p> <p>Mycelia: hyaline, aseptate</p> <p>Sporangiophores: Erect, ascending and stolon like with adventitious rhizoids, racemosely branched. Septa below the vesicles when mature. The terminal head of the sporingiosphores contain globose merosporangia (10-40 µm diam) containing 3-18 spores.</p>

Contd.....

Isolate	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/G-302	<i>Rhizoctonia solani</i>	<p>Colonies: Colonies fast growing reaching up to 4.5-5.5 cm diam after 10-14 days of growth on MEA at 25°. Fruitbody loosely adnate, hypochnoid to pellicular, usually whitish or cream-coloured.</p> <p>Mycelia: Hyaline, septate</p> <p>Conidia: Basidia short-cylindrical, 20 x 12 µm, normally with four, rather stout and 8-10 µm long sterigmata.</p> <p>Spore: Spores ellipsoid, thin-walled, smooth, hyaline, 8-12 x 5-6 µm but varying in size, adaxial side mostly convex or straight, producing secondary spores although not seen in all specimens, inamyloid, indextrinoid, acyanophilous.</p>
RHS/P-46	<i>Rhizoctonia oryzae</i>	<p>Colonies: Expanding, up to 1 cm high, whitish to greyish-brown on MEA at 30°C.</p> <p>Mycelia: Hyaline, aseptate</p> <p>Sporangiophore: Singly or in tufts, brown, 1-2 mm high, 18 µm wide, mostly unbranched, sometimes with brownish swellings up to 50 µm diam. Rhizoids sparingly branched, up to 250 µm long, brownish. Sporangia spherical, 50-250 µm diam, brownish-grey to black. greyish-green, angular, subspherical to ellipsoidal, longitudinally striate, 6-8 x 4.5-5.0 µm</p> <p>Chlamydospore: Single or in chains, spherical to ovoidal, 10-35 µm diam, hyaline, smooth-walled. Zygosporangia. Zygosporangia red to brown, spherical or laterally flattened, 60-140 µm, with flat projections. Suspensors unequal, spherical and conical. Heterothallic</p>
RHS/V-466	<i>Thanetophorus cucumeris</i>	<p>Colonies: Colonies fast growing, reaching 6-10 cm diam within 3-4 days at 25°C on PDA. At first colourless, rapidly become brown.</p> <p>Mycelia: Sub-hyaline to pale brown, septated, thick walled, cell 100-225 µm long constricted near the septa.</p> <p>Sclerotia: Developing from irregular agglomerations of uniform moniliform hyphae, irregular in outline and about 1 mm diam, confluent to conspicuous to crusts, often in zones near the margin, brown.</p>

Contd.....

Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/P-49	<i>Sporotrichum pruinosum</i>	<p>Colonies: Colonies slow growing, distinct greyish or pinkish blue colour, reverse dark brown to grayish.</p> <p>Mycelia: hyaline, aseptate</p> <p>Conidia: Blastoconidia from unbranched conidiophores ellipsoidal to ovoid pyriform or nearly cylindrical, 5.8 x 3.5 µm. Chlamydospores terminal or intercalary, hyaline, (sub)globose to broadly ellipsoidal or more rarely pyriform, 11- 60 µm diam with granular contents and thick walls (up to 4.5 µm). Arthroconidia hyaline, cylindrical or rather irregular, often with granular contents, thin-walled, but sometimes thick-walled and more ellipsoidal.</p> <p>Conidiophore: Conidiophores simple or typically branched. Branching racemose, each branch forming a terminal blastoconidium. Blastoconidia from branched conidiophores hyaline, subglobose to ellipsoidal or ovoidal, 10 x 8.5 µm.</p>
RHS/P-50, RHS/P-51, RHS/P-54, RHS/P-120	<i>Talaromyces flavus</i>	<p>Colonies: Colonies spreading broadly, reaching 7-8 cm diam in 7-14 days at 25°C on MEA. Ascomata forming a yellowish layer, reverse orange to orange brown.</p> <p>Ascomata: 200-700 µm diam, confluent, ripening within two weeks. The initials consists of long vermiform ascogonia, 100-240 x 3.5-4.5 µm, with slender entwining antheridia.</p> <p>Ascospores: Yellow, broadly ellipsoidal, spiny, 3.5-5.0 x 2.5-3.2 µm.</p> <p>Conidiophores: Rarely formed, sometimes forming grayish green sectors.</p>

Contd.....

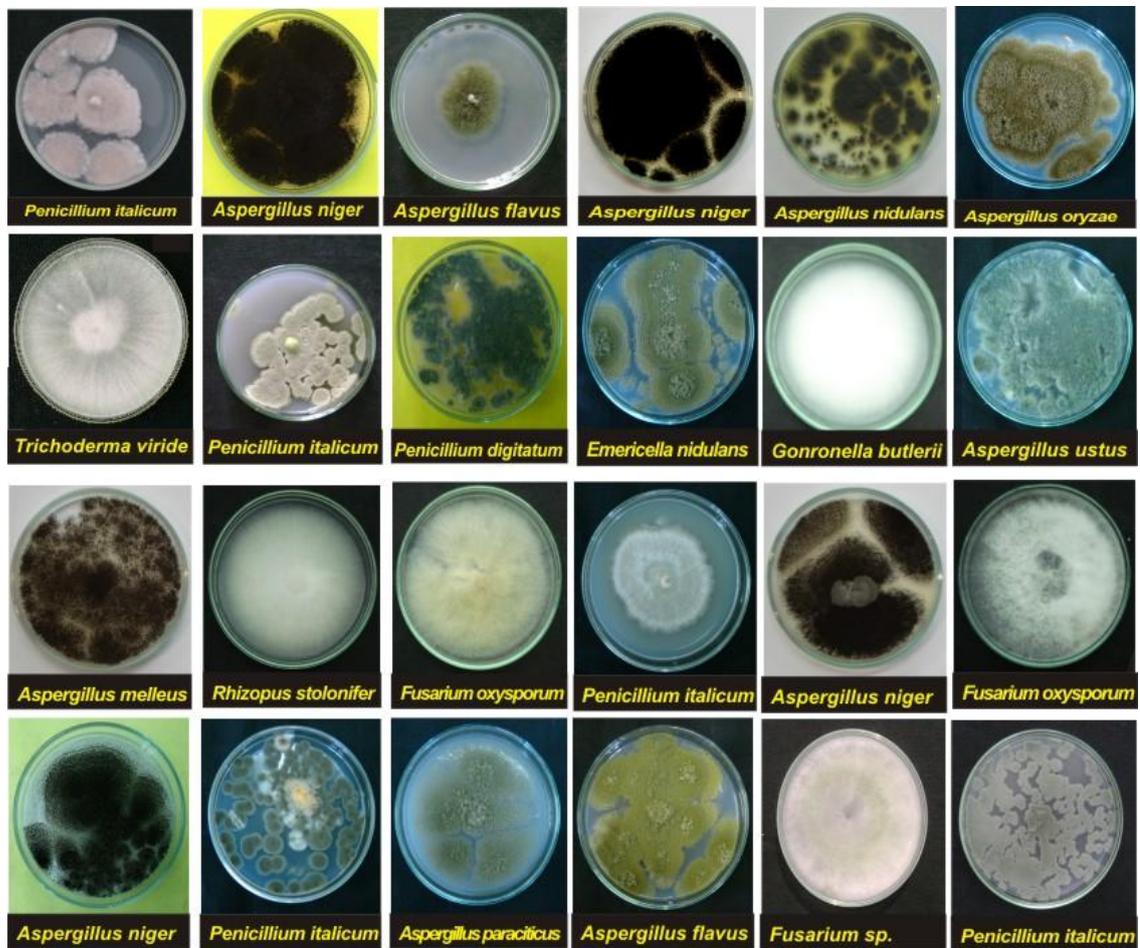


Fig. 10. Radial growth pattern of fungal isolates on PDA medium isolated from different Riverrine soils of Darjeeling hills.

Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/Td-166, FS/Td-168, RHS/T-439, RHS/T-626, RHS/Rd-551	<i>T. erinacium</i>	<p>Colonies: Fast growing, reaching up to 9-12 cm diam in 5-7 days at 25°C on PDA. Colony pale green to olive green in colour.</p> <p>Conidiophore: Conidial structure shows that phialides are regularly branched and shows pyramidal arrangement. Each phialide is broadened at the base and tapers at the tip consisting smooth rounded conidia is cluster. The branching of the conidiophores are irregular and are distantly apart from each other.</p> <p>Phialides Typically arising in whorls of 2-3 at the tips of short branches, cylindrical sometimes with an elongated neck, straight, 5.0-10.5 µm long, 1.5-2.0 µm wide at the base, arising from a cell 2.0-3.0 µm wide.</p> <p>Conidia: Conidia in whorls, ellipsoidal, smooth, 3.5µm X 3µm in diameter.</p>
FS/L-186, FS/R-256, FS/R-426, RHS/T-584, RHS/B-245, RHS/G-251, RHS/Rd-547, RHS/Am-624, FS/L 116,	<i>T. viride</i>	<p>Colonies: Fast growing, reaching up to 8-9 cm diam in 5-7 days at 25°C on PDA. Colony pale green to olive green in colour.</p> <p>Conidiophore: Typically Pyramidally branched comprising a fertile central axis or the central axis 100-150 µm long sometimes lateral branches at widely-spaced intervals when near the tip of the conidiophore and arising at closer intervals when more distant from the tip; phialides arising singly from the main axis or in whorls of 2-3 at the tips of lateral branches or at the tip of the conidiophore. The central axis 2.2-3.2µm wide.</p> <p>Phialides Typically arising singly directly from the main axis or at the tip of a short lateral branch or in whorls of 2-3 at the tips of short branches, cylindrical to somewhat swollen in the middle and sometimes with an elongated neck, straight, hooked or sinuous, 7.0-11.5 µm long, 1.8-2.5 µm wide at the base, arising from a cell 2.2-3.2 µm wide.</p> <p>Conidia: Globose, distinctly roughened, confluent to discrete, 3.5-4.5 µm diam.</p>

Contd.....

Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/C- 149, FS/M- 367, FS/L-358, FS/S-476, FS/G-96, FS/Rr-74, FS/Md-289, FS/R-542, FS/Th-485, FS/R-640, FS/R-641, RHS/T-438, RHS/T-600, RHS/Cd-606, RHS-Am-623, RHS/P-572, RHS/S-559, RHS/S-560, RHS/Br-633	<i>Trichoderma harzianum</i>	<p>Colonies: Fast growing, dark green, reaching upto 9 cm diam. In 5 days in 25° on PDA.</p> <p>Conidiophores: Typically with paired branches forming over 150 µm of the length of terminal branches. Cells supporting the phialides equivalent in width to, or at most only slightly wider than, the base of phialides arising from them.</p> <p>Phialides: 6.5-6.7 µm long, 2.5-3.5µm wide at the widest point 1.6-2.5 µm at the base; supporting cell 2.4-3.6 µm;</p> <p>Terminal phialides in a whorl or solitary, typically cylindrical or at least not conspicuously swollen in the middle and longer than the subterminal phialides.</p> <p>Conidia: subglobose to ovoidal, 3.5 to 4.0 µm long, smooth, green.</p>
FS/L-118, FS/M-363, FS/L-188, FS/Sd-478, FS/R-439, FS/Th-488, RHS/T-341, RHS/S-561, RHS/Br-634, RHS/Cd-601, RHS/Cd-604, FS/Tn-39	<i>T. asperellum</i>	<p>Colonies: Fast growing, light green, reaching upto 9-11 cm diam in 5 days in 25° on PDA.</p> <p>Conidiophores: Conidiophores or phialides are fertile along their length and appear as plumes, regularly branched, with lateral branches being more or less uniformly spaced and paired, the longest branches occurring the farthest from the tip. Phialides form at the tips of branches in verticillate or 'cruciate whorls,' Phialides are straight and tend to be slightly wider in the middle than at the base.</p> <p>Phialides: 5.5-6.6 µm long, 2.0-2.5µm wide at the widest point 1.5-2.0 µm at the base; supporting cell 2.0-2.8 µm;</p> <p>Conidia: Conidia are more ovoidal and round and warty. Warts are slightly more irregular and pyramidal and are unevenly dispersed and parts of individual conidia are smooth 1.5 X 2 µm in diameter</p>

Table 5. NAIMCC accession numbers of common fungal isolates of Darjeeling hills

Name of the Culture	Code	NCFT Id.No.	NAIMCC acc.no.
<i>Absidia cylindrospora</i>	RHS/P 47	NCFT-3782	NAIMCC-F-02913
<i>Alternaria alternata</i>	FS/S 534	NCFT- 4414	NAIMCC-F-02900
<i>Aspergillus flavus</i>	RHS/P 419	NCFT-4005	NAIMCC-F-02889
<i>Aspergillus flavus</i>	FS/L 201	NCFT-3787	NAIMCC-F-02905
<i>Aspergillus fumigatus</i>	FS/R 557	NCFT-4146	NAIMCC-F-02909
<i>Aspergillus fumigatus</i>	RHS/M 498	NCFT-3997	NAIMCC-F-02891
<i>Aspergillus niger</i>	RHS/M 492	NCFT-3994	NAIMCC-F-02890
<i>Aspergillus oryzae</i>	RHS/M 449	NCFT-4001	NAIMCC-F-02914
<i>Aspergillus parasiticus</i>	FS/R 554	NCFT-4147	NAIMCC-F-02910
<i>Aspergillus versicolor</i>	RHS/M 506	NCFT-4002	NAIMCC-F-02911
<i>Byssochlamus nivus</i>	RHS/P107	NCFT-3783	NAIMCC-F-02885
<i>Curvularia lunata</i>	RHS/T 556	NCFT-4149	NAIMCC-F-02904
<i>Emericella nidulans</i>	RHS/M 509	NCFT-4007	NAIMCC-F-02892
<i>Fusarium pallidoroseum</i>	FS/S 538	NCFT-4150	NAIMCC-F-02896
<i>Gonronella butlerii</i>	RHS/M 497	NCFT-3996	NAIMCC-F-02894
<i>Neosartoria fischeri</i>	FS/S 108	NCFT-3791	NAIMCC-F-02895
<i>Paecilomyces varioti</i>	RHS/P 293	NCFT-4145	NAIMCC-F-02887
<i>Penicillium digitatum</i>	RHS/P 555	NCFT-4163	NAIMCC-F-02888
<i>Penicillium chrysogenum</i>	RHS/J 97	NCFT-4153	NAIMCC-F-02906
<i>Penicillium digitatum</i>	RS/D 285	NCFT-4143	NAIMCC-F-02908
<i>Penicillium italicum</i>	FS/M 536	NCFT-4151	NAIMCC-F-02907
<i>Pseudoerotium zonatum</i>	RHS/P 120	NCFT-3785	NAIMCC-F-02886
<i>Sporotricum pruinosum</i>	RHS/M 496	NCFT- 3784	NAIMCC-F-02893
<i>Syncephalustrum racemosus</i>	RHS/B 301	NCFT-4148	NAIMCC-F-02912
<i>Thanatophorus cucumeris</i>	RHS/V566	NCFT-4005	NAIMCC-F-02903

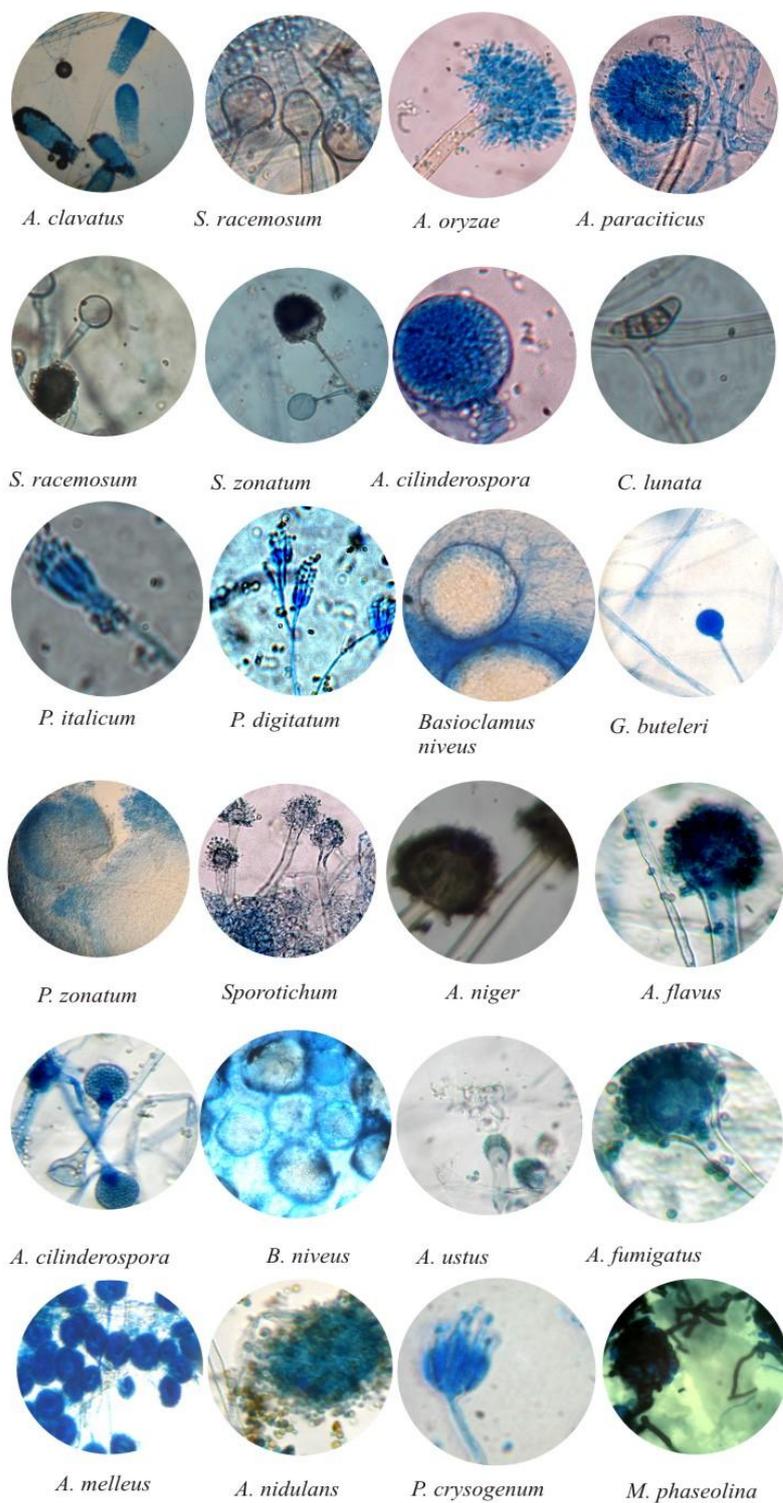


Fig. 11. Light Microscopic characters of the mycelia and spore structures of the most common fungal isolates obtained from forest, agricultural and rivrine soils of Darjeeling district.

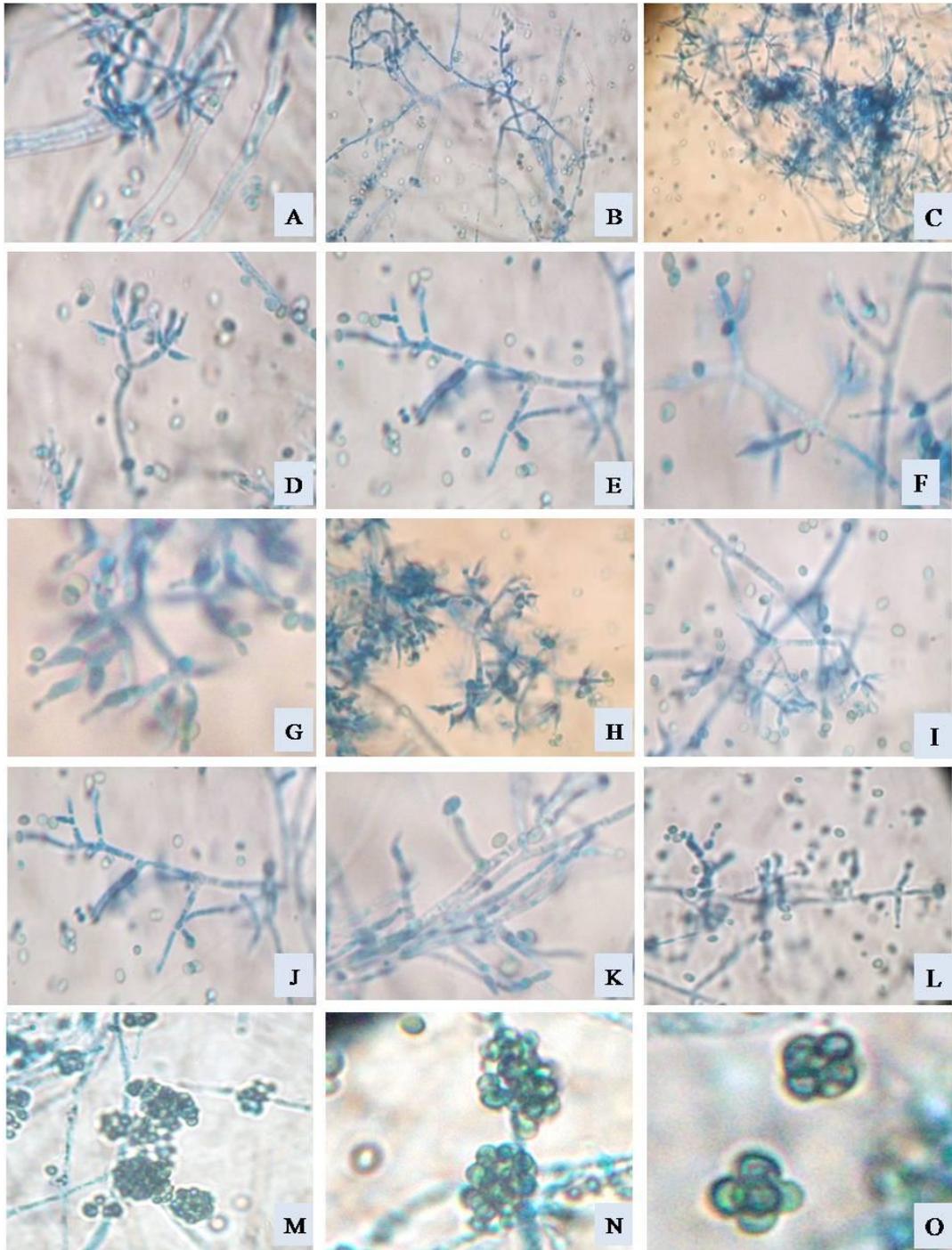


Fig. 12. Microscopic characters of different *Trichoderma* isolates; *T. asperellum*-RHS/S-561 (A-F); *T. erinaceum*-FS/L-20 (G-I); *T. viride* (J-K); *T. harzianum*-RHS/S-559 (M-O).

Table 6. GIS position and location of the sampling area of source of bacterial isolates

Type of soil sample	Bacterial code	Geographical Location	GIS location of sampling	Elevation (m)
Sukna forest (BFS/S)	BFS/S I	Sukna	26°41'06.71" N 88°20'58.36" E	126
	BFS/S II	Sukna	26°41'08.33" N 88°20'54.16" E	126
	BFS/S III	Sukna	26°41'11.21" N 88°16'55.36" E	126
	BFS/S IV	Sukna	26°42'14.72" N 87°22'48.36" E	126
	BFS/S V	Sukna	26°42'44.56" N 87°22'33.30" E	126
Lohagarh Forest (BFS/L)	BFS/L I	Lohagarh	26°48'19.82" N 88°11'51.07" E	396
	BFS/L II	Lohagarh	26°48'15.56" N 88°11'12.30" E	396
	BFS/L III	Lohagarh	26°48'22.10" N 88°11'10.16" E	396
	BFS/L IV	Lohagarh	26°48'19.82" N 88°11'51.07" E	396
	BFS/L V	Lohagarh	26°48'40.60" N 88°11'33.15" E	396
Tindharey (BFS/Tn)	BFS I	Kurseong	26°41'06.71" N 88°20'58.38" E	126
	BFS II	Kurseong	26°41'55.12" N 88°17'48.27" E	126
	BFS III	Kurseong	26°42'16.41" N 88°19'50.44" E	126
	BFS IV	Kurseong	26°42'04.66" N 88°19'22.73" E	126
Ghyabari Forest (BFS/G)	BFS/G I	Kurseong	26°51'15.27" N 88°46'41.54" E	1300
	BFS/G II	Kurseong	26°51'20.43" N 88°46'22.76" E	1300
	BFS/G III	Kurseong	26°51'13.10" N 88°46'52.13" E	1300
	BFS/G IV	Kurseong	26°51'22.09" N 88°46'15.24" E	1300
	BFS/G V	Kurseong	26°51'37.18" N 88°46'62.03" E	1300
Rongli Rongek Forest (BFS/Rr)	BFS/Rr I	Ronglirongek	27°01'42.51" N 88°21'27.85" E	1382
	BFS/Rr II	Ronglirongek	27°01'47.44" N 88°21'21.50" E	1382
	BFS/Rr III	Ronglirongek	27°01'38.02" N 88°21'13.22" E	1382
	BFS/Rr IV	Ronglirongek	27°01'42.51" N 88°21'27.85" E	1382
	BFS/Rr V	Ronglirongek	27°01'76.14" N 88°21'44.09" E	1382
Maney Dara Forest (BFS/Md)	BFS/Md I	Rimbik	27°37'15.47" N 88°06'38.27" E	2150
	BFS/Md II	Rimbik	27°37'20.05" N 88°06'22.14" E	2150
	BFS/Md III	Rimbik	27°37'26.10" N 88°06'27.20" E	2150
	BFS/Md IV	Rimbik	27°37'18.07" N 88°06'33.10" E	2150
Sandakhpu (BFS/Sd)	BFS/Sd I	Sandakphu	27°06'21.10" N 88°00'05.60" E	3487
	BFS/Sd II	Sandakphu	27°06'11.06" N 88°00'11.16" E	3487
	BFS/Sd III	Sandakphu	27°06'25.20" N 88°00'17.40" E	3487
Rimbhik Forest (BFS/R)	BFS/R I	Rimbik	27°07'05.57" N 88°06'30.21" E	1970
	BFS/R II	Rimbik	27°07'08.27" N 88°06'32.11" E	1970
	BFS/R III	Rimbik	27°07'05.57" N 88°06'30.21" E	1970
	BFS/R IV	Rimbik	27°07'11.43" N 88°06'10.18" E	1970
	BFS/R V	Rimbik	27°07'15.10" N 88°06'22.10" E	1970
	BFS/R VI	Rimbik	27°07'21.55" N 88°06'33.28" E	1970

Contd.....

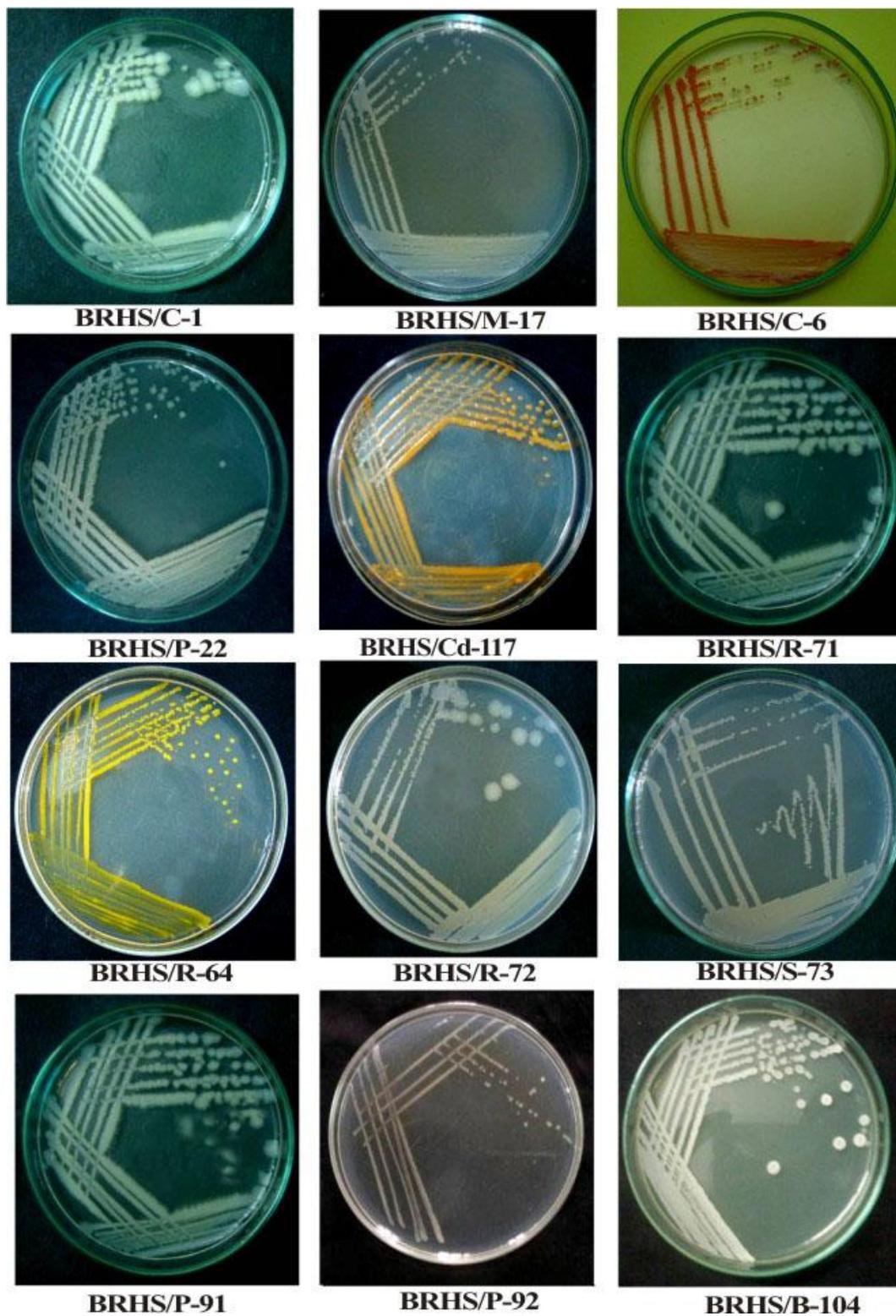


Fig. 13. Growth pattern of different bacterial isolates on NA medium obtained from rhizosphere soil of different hosts from Darjeeling hills.

Type of soil sample	Bacterial isolate code	Geographical Location	GIS location of sampling
Thorpu Forest (BFS/T)	BFS/T I	Thorpu	26°53'57.34" N 88°10'07.57" E
	BFS/T II	Thorpu	26°53'57.40" N 88°10'10.25" E
	BFS/T III	Thorpu	26°53'57.45" N 88°10'13.80" E
	BFS/T IV	Thorpu	26°53'58.20" N 88°10'22.70" E
	BFS/T V	Thorpu	26°53'58.18" N 88°10'22.57" E
Tagdha Forest (BFS/Td)	BFS/Td I	Tagdha	27°02'15.25" N 88°21'37.19" E
	BFS/Td II	Tagdha	27°02'20.04" N 88°21'44.20" E
	BFS/Td III	Tagdha	27°07'37.43" N 88°22'48.23" E
	BFS/Td IV	Tagdha	27°07'15.16" N 88°22'48.27" E
Rambi Saal Forest (BFS/R)	BFS/R I	Rambi	26°57'59.42" N 88°25'43.29" E
	BFS/R II	Rambi	26°57'63.12" N 88°25'46.73" E
	BFS/R III	Rambi	26°57'77.22" N 88°25'48.05" E
	BFS/R IV	Rambi	26°57'83.15" N 88°25'63.88" E
Lamahata (BFS/L)	BFS/L I	Kalimpong	27°03'36.55" N 88°28'11.88" E
	BFS/L II	Kalimpong	27°03'36.44" N 88°28'86.72" E
	BFS/L III	Kalimpong	27°03'37.20" N 88°28'12.43" E
	BFS/L IV	Kalimpong	27°03'37.31" N 88°28'12.05" E
Cinchona Forest (BFS/C)	BFS/C I	Mongpong	26°58'25.39" N 88°22'12.19" E
	BFS/C II	Mongpong	26°58'25.14" N 88°22'12.04" E
	BFS/C III	Mongpong	26°58'27.30" N 88°22'11.38" E
	BFS/C IV	Mongpong	26°58'27.44" N 88°22'11.76" E
	BFS/C V	Mongpong	26°58'24.22" N 88°22'10.72" E
Mongpong Forest (BFS/M)	BFS/M I	Mongpong	26°58'26.95" N 88°22'02.58" E
	BFS/M II	Mongpong	26°58'21.90" N 88°22'00.55" E
	BFS/M III	Mongpong	26°58'21.42" N 88°22'00.18" E
	BFS/M IV	Mongpong	26°58'27.27" N 88°22'00.15" E
	BFS/M V	Mongpong	26°58'26.95" N 88°22'00.28" E
	BFS/M VI	Mongpong	26°58'26.56" N 88°22'02.58" E
Pedong (BFS/P)	BFS/P I	Pedong	27°01'11.59" N 88°34'20.96" E
	BFS/P II	Pedong	27°01'13.77" N 88°34'26.33" E
	BFS/P III	Pedong	27°01'22.60" N 88°34'25.06" E
	BFS/P IV	Pedong	27°01'11.42" N 88°34'10.62" E
Rishyup Valley (BFS/Ry)	BFS/Ry I	Kalimpong	27°06'42.45" N 88°38'28.48" E
	BFS/Ry II	Kalimpong	27°06'42.40" N 88°38'28.12" E
	BFS/Ry III	Kalimpong	27°06'30.15" N 88°38'25.21" E
	BFS/Ry IV	Kalimpong	27°06'30.57" N 88°38'25.67" E
Lava (BFS/Lv)	BFS/Lv I	Kalimpong	27°04'48.78" N 88°38'24.27" E
	BFS/Lv II	Kalimpong	27°04'52.33" N 88°38'45.30" E
	BFS/Lv III	Kalimpong	27°04'27.15" N 88°38'14.03" E

Contd.....

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling
<i>Camellia sinensis</i> (Tea) (BRHS/T)	BRHS/T I	Ghyabari	26°51'15.27" N 88°46'41.54" E
	BRHS/T II	Ghyabari	26°51'15.22" N 88°46'32.12" E
	BRHS/T III	Ghyabari	26°51'22.17" N 88°46'41.27" E
	BRHS/T IV	Ghyabari	26°51'22.08" N 88°46'41.43" E
	BRHS/T V	Saureni	26°51'53.61" N 88°11'48.17" E
	BRHS/T VI	Saureni	26°51'53.70" N 88°11'48.21" E
	BRHS/T VII	Saureni	26°51'54.33" N 88°11'41.06" E
	BRHS/T VIII	Singmari	27°46'11.32" N 88°43'22.17" E
	BRHS/T IX	Singmari	27°46'11.24" N 88°43'22.02" E
	BRHS/T X	Singmari	27°46'13.22" N 88°43'10.43" E
	BRHS/T XI	Singmari	27°46'13.14" N 88°43'20.47" E
	BRHS/T XII	Darjeeling	27°02'10.25" N 88°15'45.45" E
	BRHS/T XIII	Darjeeling	27°02'10.63" N 88°15'45.11" E
	BRHS/T XIV	Singmari	27°46'11.52" N 88°43'22.43" E
	BRHS/T XV	Singmari	27°46'11.32" N 88°43'22.17" E
	BRHS/T XVI	Darjeeling	27°02'10.25" N 88°15'45.45" E
	BRHS/T XVII	Darjeeling	27°02'10.22" N 88°15'45.36" E
	BRHS/T XVIII	Darjeeling	27°02'17.25" N 88°15'20.10" E
	BRHS/T XIX	Darjeeling	27°02'17.15" N 88°15'20.63" E
	BRHS/T XX	Darjeeling	27°02'22.40" N 88°15'77.46" E
	BRHS/T XXI	Thorpu	26°53'57.34" N 88°10'07.57" E
	BRHS/T XXII	Thorpu	26°53'57.14" N 88°10'07.50" E
	BRHS/T XXIII	Thorpu	26°53'57.88" N 88°10'07.37" E
	BRHS/T XXIV	Rongli	27°01'42.51" N 88°21'27.85" E
	BRHS/T XXV	Rongli	27°01'42.66" N 88°21'27.73" E
	BRHS/T XXVI	Rongli	27°01'42.37" N 88°21'27.05" E
<i>Cryptomeria japonica</i> (BRHS/C)	BRHS/C I	Mirik	26°53'10.12" N 88°11'15.11" E
	BRHS/C II	Mirik	26°53'10.85" N 88°11'15.61" E
	BRHS/C III	Mirik	26°53'11.44" N 88°11'14.10" E
<i>Citrus reticulate</i> (BRHS/O)	BRHS/O I	Mirik	26°53'11.25" N 88°11'14.76" E
	BRHS/O II	Mirik	26°53'14.88" N 88°11'10.18" E
	BRHS/O III	Mirik	26°53'14.55" N 88°11'10.84" E
<i>Oryza sativa</i> (Paddy) (BRHS/P)	BRHS/P I	Bijanbari	27°04'01.78" N 88°11'22.98" E
	BRHS/P II	Bijanbari	27°04'01.40" N 88°11'22.83" E
	BRHS/P III	Bijanbari	27°04'01.63" N 88°11'22.12" E
	BRHS/P IV	Kalimpong	27°03'21.42" N 88°24'24.01" E
	BRHS/P V	Kalimpong	27°03'24.44" N 88°24'24.16" E
	BRHS/P VI	Kalimpong	27°03'21.16" N 88°24'24.83" E

Contd.....

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling
<i>Dendrocalamus latiflorus</i> (Bamboo) (BRHS/B)	BRHS/B I	Ghyabari	26°51'15.27" N 88°46'41.54" E
	BRHS/B II	Ghyabaria	26°51'15.33" N 88°46'41.22" E
	BRHS/B III	Ghyabari	26°51'22.12" N 88°46'11.36" E
	BRHS/B IV	Kalimpong	27°03'21.42" N 88°24'24.01" E
	BRHS/B V	Kalimpong	27°03'21.44" N 88°24'24.18" E
	BRHS/B VI	Kalimpong	27°03'22.83" N 88°24'23.14" E
	BRHS/B VII	Saureni	26°51'53.61" N 88°11'48.17" E
	BRHS/B VIII	Saureni	26°51'53.66" N 88°11'48.10" E
	BRHS/B IX	Saureni	26°51'68.14" N 88°11'22.20" E
<i>Glycine max</i> (Soybean) (BRHS/Sb)	BRHS/Sb I	Bijanbari	27°04'01.78" N 88°11'22.98" E
	BRHS/Sb II	Bijanbari	27°04'01.27" N 88°11'22.73" E
	BRHS/Sb III	Bijanbari	27°04'01.36" N 88°11'22.18" E
<i>Sechium edule</i> (Squash) (BRHS/S)	BRHS/S I	Mirik	26°51'53.61" N 88°11'48.17" E
	BRHS/S II	Mirik	26°51'53.70" N 88°11'48.18" E
	BRHS/S III	Mirik	26°51'53.05" N 88°11'48.88" E
	BRHS/S IV	Pedong	27°01'11.59" N 88°34'20.96" E
	BRHS/S V	Pedong	27°01'11.47" N 88°34'20.77" E
<i>Brassica juncea</i> (BRHS/Br)	BRHS/S VIII	Pedong	27°01'11.13" N 88°34'20.40" E
	BRHS/Br I	Kalimpong	27°03'36.55" N 88°28'11.88" E
	BRHS/Br II	Kalimpong	27°03'36.43" N 88°28'11.12" E
<i>Amomum subulatum</i> (Cardamom) (BRHS/Cr)	BRHS/Br III	Kalimpong	27°03'36.20" N 88°28'11.43" E
	BRHS/Cr I	Mirik	26°53'10.85" N 88°11'15.61" E
	BRHS/Cr II	Mirik	26°53'10.44" N 88°11'15.43" E
	BRHS/Cr III	Mirik	26°53'10.85" N 88°11'15.61" E
	BRHS/Cr IV	Mirik	26°53'10.85" N 88°11'15.61" E
<i>Rhododendron</i> (BRHS/R)	BRHS/Cr V	Mirik	26°53'10.85" N 88°11'15.61" E
	BRHS/R I	Rimbik	27°07'05.57" N 88°06'30.21" E
	BRHS/R II	Rimbik	27°07'05.57" N 88°06'30.21" E
	BRHS/R III	Rimbik	27°07'05.57" N 88°06'30.21" E
	BRHS/R IV	Rimbik	27°07'05.57" N 88°06'30.21" E
	BRHS/R V	Maneydara	27°03'25.41" N 88°01'30.23" E
Amliso (<i>Thyasaenolena latifolia</i>) (BRHS/Tl)	BRHS/R VI	Maneydara	27°03'25.41" N 88°01'30.23" E
	BRHS/Tl I	Pedong	27°01'11.59" N 88°34'20.96" E
	BRHS/Tl II	Pedong	27°01'11.62" N 88°34'20.18" E
<i>Alnus nepalensis</i> (BRHS/A)	BRHS/Tl III	Pedong	27°01'11.44" N 88°34'20.23" E
	BRHS/A I	Lava	27°04'48.78" N 88°38'24.27" E
	BRHS/A II	Lava	27°04'48.60" N 88°38'24.37" E
	BRHS/A III	Lava	27°04'48.55" N 88°38'24.21" E
	BRHS/A IV	Lava	27°04'48.10" N 88°38'24.86" E

Contd.....

Type of soil sample	Bacterial isolate Code	Geographical Location	GIS location of sampling
Teesta River (BRS/T)	BRS/T I	Teesta	27°03'19.14" N 88°25'31.48" E
	BRS/T II	Teesta	27°03'22.80" N 88°25'11.30" E
	BRS/T III	Teesta	27°03'27.11" N 88°25'40.15" E
	BRS/T IV	Teesta	27°03'13.38" N 88°25'33.88" E
	BRS/T V	Teesta	27°03'17.27" N 88°25'12.20" E
Balasan (BRS/B)	BRS/B I	Balasan	26°56'10.59" N 88°13'05.57" E
	BRS/B II	Balasan	26°56'11.88" N 88°13'13.63" E
	BRS/B III	Balasan	26°56'18.59" N 88°13'08.22" E
	BRS/B IV	Balasan	26°56'23.17" N 88°13'11.46" E
	BRS/B V	Balasan	26°56'10.59" N 88°13'05.57" E
Rangit (BRS/R)	BRS/R I	Malli	27°07'40.60" N 88°17'06.63" E
	BRS/R II	Malli	27°07'40.63" N 88°17'06.12" E
	BRS/R III	Malli	27°07'40.40" N 88°17'06.57" E
	BRS/R IV	Malli	27°07'40.12" N 88°17'06.27" E
	BRS/R V	Malli	27°07'40.18" N 88°17'06.33" E
Reli (BRS/Re)	BRS/Re I	Kalompong	27°03'03.21" N 88°31'45.27" E
	BRS/Re II	Kalimpong	27°03'03.21" N 88°46'45.17" E
	BRS/Re III	Kalimpong	27°03'03.21" N 88°58'45.44" E
	BRS/Re IV	Kalimpong	27°03'03.21" N 88°61'45.73" E
Mirik Lake (BRS/M)	BRS/M I	Mirik	26°53'10.85" N 88°11'15.61" E
	BRS/M II	Mirik	26°53'10.44" N 88°11'15.03" E
	BRS/M III	Mirik	26°53'10.21" N 88°11'15.19" E
	BRS/M IV	Mirik	26°53'10.30" N 88°11'15.43" E

Table 7. Morphological and biochemical characterization of bacterial isolates

Code	Shape	Pigment	Spore	Gram reaction	H ₂ S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
BRHS/C-1	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus pumilus</i>
BRHS/C -2	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus pumilus.</i>
BRHS/C -3	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BRHS/C -4	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/C -5	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/C -6	Sp	R	-	-	-	+	-	-	+	+	+	+	+	+	-	<i>Staphylococcus sp.</i>
BRHS/C -7	Sp	Y	-	+	-	-	+	-	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/M-8	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/M -9	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/M -10	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/M -11	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/M -12	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/M -13	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/M -14	Sp	R	-	+	-	-	-	-	+	+	-	-	+	+	-	<i>Staphylococcus sp.</i>
BRHS/M -15	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/M -16	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/M -17	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/M -18	Rd	R	-	-	-	+	-	+	+	+	+	+	-	-	-	<i>Serratia sp.</i>
BRHS/M -19	Rd	R	-	-	-	+	-	+	+	+	+	+	-	-	-	<i>Serratia sp.</i>
BRHS/M -20	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/M -21	Rd	W	+	+	-	+	+	+	+	+	+	+	-	+	-	<i>Bacillus altitudinis</i>
BRHS/P-22	Rd	W	+	+	-	+	+	+	+	+	+	+	-	+	-	<i>Bacillus altitudinis</i>
BRHS/P -23	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/P -24	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/P -25	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/M-26	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/M-27	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/M-28	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/M-29	Sp	W	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/M-30	Sp	R	-	+	-	+	-	-	+	+	-	-	+	+	-	<i>Staphylococcus sp.</i>
BFS/S- 31	Rd	W	+	+	-	-	+	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/S-32	Rd	W	+	+	-	-	+	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/S-33	Rd	W	+	+	-	-	+	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/S-34	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp</i>
BFS/S-35	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BFS/S-36	Sp	R	-	+	-	-	-	-	+	+	-	-	+	+	-	<i>Staphylococcus sp.</i>
BRS/Mr-37	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRS/Mr-38	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRS/Mr-39	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/Mr-40	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/Mr-41	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRS/Mr-42	Sp	R	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Serratia sp.</i>
BRS/Mr-43	Sp	R	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Serratia sp.</i>
BRS/Mr-44	Rd	W	+	-	-	-	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/Mr-45	Sp	R	-	+	-	-	-	-	+	+	-	-	+	+	-	<i>Staphylococcus sp.</i>

R=Red; Y=Yellow; W=White; Rd=Rod; Sp=Spherical; += Activity present; -=Activity absent

Contd.....

Code	Shape	Pigment	Spore	Gram reaction	H ₂ S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
BFS/Md-46	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-47	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-48	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-49	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BFS/Md-50	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BFS/Md-51	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BFS/Md-52	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/Md-53	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/Md-54	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-55	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-56	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/Md-57	Rd	W	+	-	-	-	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/T-58	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/T-59	Rd	W	+	-	-	-	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRS/T-60	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRS/T-61	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRS/T-62	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BRHS/R-63	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/R-64	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/R-65	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/R-66	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/R-67	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/R-68	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/R-69	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/R-70	Rd	W	+	-	-	-	+	+	+	+	+	+	-	+	-	<i>Pseudomonas</i>
BRHS/R-71	Rd	W	-	-	-	+	+	+	+	-	-	+	-	-	-	<i>Enterobacter cloacae</i>
BRHS/R-72	Rd	W	+	+	-	+	+	+	+	+	+	+	+	+	-	<i>Paenibacillus polymyxa</i>
BRHS/R-73	Rd	W	+	+	-	+	+	+	+	+	+	+	-	+	-	<i>Bacillus altitudinis</i>
BRHS/S-74	Rd	W	-	-	-	+	+	+	+	-	-	+	-	-	-	<i>Enterobacter sp.</i>
BRHS/S-75	Rd	W	-	-	-	+	+	+	+	-	-	+	-	-	-	<i>Enterobacter sp.</i>
BRHS/S-76	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/S-77	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/S-78	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/S-79	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRHS/S-80	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRHS/S-81	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Pseudomonas sp.</i>
BRHS/S-82	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/S-83	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/S-84	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRHS/S-85	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRHS/S-86	Sp	Y	-	+	-	+	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BRHS/S-87	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/S-88	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/S-89	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BRHS/P-90	Sp	R	-	+	-	+	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>

R=Red; Y=Yellow; W=White; Rd=Rod; Sp=Spherical; += Activity present; -=Activity absent

Contd.....

Code	Shape	Pigment	Spore	Gram reaction	H ₂ S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
BRHS/P-91	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>B. methylotrophicus</i>
BRHS/P-92	Rd					+										<i>Bukholdaria symbiont</i>
FS/T-93	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
FS/T-93	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
FS/T-94	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
FS/T-95	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
FS/T-96	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
FS/T-97	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRHS/B-98	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BRHS/B-99	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BRHS/B-100	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRHS/B-101	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Coryneform sp.</i>
BRHS/B-102	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/B-103	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/B-104	Rd	W	+	+	-	+	-	+	+	+	+	+	-	+	-	<i>Bacillus aerophilus</i>
BRHS/B-105	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/B-106	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/B-107	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRHS/Cd-108	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BRHS/Cd-109	Sp	R	-	+	-	-	-	-	+	-	+	-	+	-	-	<i>Staphylococcus sp.</i>
BRHS/Cd-110	Rd	R	-	-	-	-	-	+	+	+	+	+	-	-	-	<i>Serratia sp.</i>
BRHS/Cd-111	Rd	R	-	-	-	-	-	+	+	+	+	+	-	-	-	<i>Serratia sp.</i>
BRHS/Cd-112	Rd	W	-	-	-	-	+	+	+	-	-	+	-	-	-	<i>Enterobacter sp.</i>
BRHS/Cd-113	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/Cd-114	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/Cd-115	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/Cd-116	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BRHS/Cd-117	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRS/R-118	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Aerococcus sp.</i>
BRS/R-119	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BRS/R-120	Rd	W	+	-	-	-	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BRS/R-121	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BRS/R-122	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRS/R-123	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRS/R-124	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus subtilis</i>
BRS/R-125	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/C-126	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/C-127	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/C-128	Sp	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	<i>Micrococcus sp.</i>
BFS/C-129	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/C-130	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/C-131	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/C-132	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus sp.</i>
BFS/C-133	Rd	W	+	-	-	+	+	+	+	+	+	+	-	+	-	<i>Psudomonas sp.</i>
BFS/C-134	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>
BFS/C-135	Rd	W	+	+	-	-	-	+	+	+	+	+	-	+	-	<i>Bacillus cereus</i>

R=Red; Y=Yellow; W=White; Rd=Rod; Sp=Spherical; += Activity present; -=Activity absent

4.2. *In vitro* screening of fungal isolates

4.2.1. Phosphate solubilization

4.2.1.1. In solid medium

A total of 637 fungal isolates were obtained from the major forest, agricultural fields and river basins of Darjeeling hills. Out of the total collection, 205 isolates were obtained from forest, 373 from agricultural and 59 isolates were obtained from river basins. All the fungal isolates were initially screened for their ability to solubilize insoluble phosphate *in vitro* in Pikovskaya's (PVK) agar medium. Formation of halo zones around the fungal colony indicated a positive result for phosphate solubilization. After this initial screening in solid medium, a total of 150 fungal isolates showed phosphate solubilizing activity. The efficacy of each phosphate solubilizing isolate has been recorded in terms of the diameter of halo zone formed around the fungal colony after 4 and 7 days of growth at $25\pm 2^{\circ}\text{C}$ (Table 8).

4.2.1.2. In liquid medium

Once a positive result was obtained on PVK solid medium, the phosphate solubilizing fungal isolates (PSF) were quantified for their ability to solubilize two insoluble phosphates (Tricalcium phosphate and Rock phosphate) in modified PVK broth medium. The initial total P- content of both the TCP and RP were 920 and 592 mg/L. The amount of Total phosphate solubilized by individual isolates has been presented in Table 9, The initial pH of the culture medium was 7. A drop in the pH of the culture filtrate was also recorded after 7 days of incubation. Among the total collection of phosphate solubilizers, four isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) were found to solubilize rock phosphate and tricalcium phosphate more efficiently than rest of the others. *T. flavus* (RHS/P-51) was found to be most efficient phosphate where it could solubilize maximum of 870 mg/L of Tricalcium phosphate and 392 mg/L of Rock phosphate *in vitro*. Potential PSF isolates were deposited to the National Agriculturally Important Microbial Culture Collection (NAIMCC) of National Bureau of Agriculturally Important Microorganisms (NBAIM), Maunath Bhnjan, UP, and their accession numbers have been provided in Table 10.

Table 8. Screening of Phosphate solubilization in solid PVK medium by fungal isolates

	Isolate	Diameter of the halo zone (cm)			Isolate	Diameter of the halo zone (cm)		
		4 d	7 d			4 d	7 d	
<i>A. niger</i>	FS/L-04	0.41±0.05	0.76±0.06	<i>A. niger</i>	RHS/M-401	0.31±0.02	0.67±0.04	
	FS/L-40	0.41±0.04	0.75±0.05		RHS/G-296	0.34±0.02	0.65±0.06	
	FS/C-140	0.40±0.03	0.72±0.05		RHS/G-297	0.27±0.02	0.54±0.08	
	FS/C143	0.25±0.02	0.58±0.03		RHS/S-518	0.33±0.08	0.62±0.07	
	FS/Td-165	0.42±0.05	0.70±0.06		RHS/S-531	0.34±0.07	0.65±0.02	
	FS/Td-173	0.30±0.06	0.62±0.03		RHS/Br-570	0.37±0.03	0.68±0.03	
	FS/S-177	0.33±0.04	0.64±0.04		RHS/Cd-610	0.43±0.08	0.73±0.06	
	FS/R-344	0.33±0.04	0.62±0.02		RHS/R-548	0.40±0.07	0.72±0.08	
	FS/S-112	0.37±0.03	0.67±0.07		RHS/A-30	0.32±0.08	0.68±0.01	
	FS/S-113	0.35±0.05	0.67±0.03		RHS/A-33	0.41±0.03	0.70±0.08	
	FS/R-262	0.30±0.04	0.64±0.04		RHS/P-45	0.38±0.06	0.68±0.03	
	FS/Tn-57	0.32±0.02	0.63±0.07		RHS/P-48	0.37±0.02	0.65±0.06	
	FS/Tn-58	0.33±0.05	0.62±0.03		RHS/D-280	0.37±0.04	0.66±0.05	
	RHS/P-37	0.37±0.04	0.68±0.05		RHS/T-581	0.35±0.05	0.65±0.08	
	FS/G-101	0.36±0.04	0.66±0.05		RS/B-160	0.36±0.08	0.67±0.02	
	FS/L-105	0.37±0.05	0.70±0.02		RS/P/14	0.40±0.04	0.74±0.03	
	FS/L-106	0.42±0.05	0.62±0.06		RS/Md-288	0.40±0.01	0.71±0.05	
	FS/L-103	0.40±0.05	0.65±0.04		RS/T-137	0.33±0.03	0.60±0.06	
	FS/L -117	0.43±0.04	0.63±0.05		RS/B-164	0.42±0.05	0.72±0.03	
	FS/L-281	0.30±0.03	0.60±0.06		RS/Re-231	0.28±0.06	0.58±0.08	
	FS/S-282	0.32±0.03	0.60±0.03		RS/Re-235	0.28±0.01	0.54±0.00	
	FS/S-283	0.37±0.05	0.61±0.02		<i>A. melleus</i>	FS/L-42	0.22±0.014	0.57±0.02
	FS/Md-284	0.42±0.05	0.63±0.06			FS/L-13	0.30±0.015	0.58±0.05
	FS/Md-286	0.44±0.05	0.62±0.02			FS/L-17	0.30±0.026	0.57±0.02
	FS/Md-287	0.38±0.05	0.64±0.03			FS/L-18	0.32±0.024	0.53±0.04
FS/Md-365	0.27±0.05	0.62±0.02	FS/G-226	0.37±0.022		0.55±0.03		
FS/Md-366	0.23±0.05	0.63±0.07	FS/S-64	0.32±0.025		0.52±0.03		
RHS/P-200	0.36±0.05	0.60±0.02	FS/L-41	0.21±0.028		0.55±0.03		
RHS/A-82	0.33±0.05	0.68±0.03	FS/S -63	0.25±0.015		0.51±0.02		
RHS/T-198	0.37±0.05	0.63±0.07	FS/S-24	0.25±0.014		0.54±0.03		
RHS/T-272	0.43±0.05	0.65±0.05	FS/S-278	0.24±0.024		0.56±0.03		
RHS/T-421	0.43±0.05	0.58±0.02	RHS/T-449	0.27±0.015		0.53±0.02		
RHS/T-422	0.40±0.05	0.68±0.06	RHS/T-450	0.30±0.022		0.52±0.03		
RHS/T-580	0.38±0.05	0.64±0.08	RHS/T-274	0.33±0.028		0.55±0.04		
RHS/S-518	0.43±0.05	0.63±0.07	RHS/T-275	0.37±0.017	0.58±0.04			
RHS/S-492	0.32±0.05	0.64±0.02	RHS/T-402	0.37±0.014	0.56±0.04			

FS=Forest soil;RHS=Rhizosphere of soil; RS= Riverrine soil; Values mean of three replicate sets; ±= SE

Contd.....

	Isolate	Diameter of the halo zone (cm)			Isolate	Diameter of the halo zone (cm)			
		4 d	7 d			4 d	7 d		
<i>A. melleus</i>	RHS/T-331	0.28±0.05	0.55±0.02	<i>A. nidulans</i>	FS/Lv-354	0.12±0.07	0.36±0.010		
	RHS/T-332	0.33±0.02	0.53±0.03		FS/P-214	0.22±0.02	0.42±0.015		
	RHS/S-303	0.27±0.03	0.42±0.01		FS/P-215	0.18±0.04	0.38±0.011		
	RHS/Cd-606	0.30±0.02	0.46±0.02		FS/S-457	0.24±0.04	0.53±0.022		
	RHS/R-549	0.33±0.02	0.63±0.04		FS/S-459	0.22±0.06	0.54±0.020		
	RHS/Am-34	0.27±0.02	0.52±0.03		RHS/T-453	0.27±0.03	0.56±0.018		
	RHS/R-12	0.30±0.08	0.58±0.02		RHS/T-389	0.25±0.07	0.43±0.014		
	RHS/P-201	0.20±0.01	0.53±0.01		RHS/G-299	0.33±0.08	0.47±0.023		
	RHS/P-202	0.21±0.01	0.50±0.01		RHS/Br-579	0.25±0.03	0.44±0.018		
	RHS/P-205	0.27±0.01	0.54±0.02		RHS/A-79	0.22±0.06	0.53±0.022		
	RS/R-115	0.25±0.01	0.53±0.01		RS/T-59	0.18±0.04	0.48±0.016		
	RS/T-182	0.22±0.01	0.40±0.02		RS/P-60	0.17±0.02	0.43±0.018		
	RS/T-183	0.30±0.01	0.55±0.02		<i>P. digitatum</i>	FS/S-109	0.35±0.02	0.56±0.023	
	RS/P-61	0.27±0.01	0.41±0.02			FS/Rv-317	0.28±0.07	0.60±0.020	
RS/P-05	0.22±0.01	0.42±0.01	FS/La-185	0.32±0.04		0.58±0.024			
RS/T-236	0.20±0.01	0.40±0.01	RHS/R-515	0.36±0.07		0.68±0.026			
RS/M-368	0.23±0.02	0.43±0.02	RHS/T-455	0.34±0.01		0.57±0.028			
<i>A. fumigatus</i>	FS/R-263	0.22±0.01	0.44±0.01	RHS/T-590		0.25±0.08	0.63±0.022		
	FS/R-264	0.23±0.01	0.51±0.01	RHS/C-338		0.32±0.02	0.66±0.018		
	RHS/P-209	0.27±0.01	0.50±0.02	RS/T-285		0.30±0.01	0.61±0.017		
	RHS/B-220	0.22±0.01	0.43±0.01	FS/Lv-355		0.27±0.06	0.58±0.023		
	RHS/T-531	0.27±0.01	0.41±0.02	FS/S-67		0.33±0.07	0.62±0.026		
	RHS/P-43	0.27±0.01	0.47±0.02	FS/S-459		0.38±0.08	0.64±0.021		
	RHS/T-585	0.24±0.02	0.52±0.03	RHS/R-505		0.35±0.06	0.63±0.025		
	RHS/T-586	0.18±0.01	0.34±0.01	RHS/T-437		0.27±0.05	0.47±0.018		
	RHS/T-588	0.20±0.01	0.22±0.01	<i>P. italicum</i>		RHS/T-594	0.32±0.05	0.54±0.026	
	<i>A. clavatus</i>	FS/Rv-323	0.20±0.01		0.61±0.02	RHS/M-403	0.26±0.01	0.56±0.024	
		FS/Rv-330	0.23±0.01		0.63±0.02	RHS/P-414	0.22±0.03	0.43±0.028	
		RHS/P-38	0.20±0.01		0.69±0.02	RHS/R-555	0.29±0.08	0.56±0.026	
		RHS/P-56	0.26±0.01		0.52±0.02	<i>P. crysogenum</i>	FS/L-265	0.33±0.04	0.57±0.02
		RHS/P-54	0.20±0.01		0.62±0.01		FS/G-97	0.34±0.01	0.65±0.04
RHS/P-114		0.25±0.01	0.53±0.01		RHS/T-269		0.32±0.06	0.62±0.02	
RHS/T-99		0.30±0.01	0.62±0.02		RHS/T-271		0.23±0.03	0.60±0.05	
RHS/T-190		0.22±0.02	0.64±0.02		RHS/C-312		0.31±0.01	0.64±0.02	
RHS/T-383		0.23±0.01	0.54±0.02		RS/Re-230		0.26±0.08	0.63±0.02	
RHS/T-386		0.22±0.01	0.60±0.01		<i>T. flavus</i>		RHS/P-50	0.45±0.04	0.75±0.02
RHS/Cd-603		0.25±0.01	0.55±0.01				RHS/P-51	0.46±0.02	0.82±0.02
RHS/A-77		0.24±0.01	0.47±0.01				RHS/P-54	0.38±0.08	0.80±0.02
RS/B-163		0.27±0.01	0.45±0.02				RHS/P-120	0.40±0.02	0.75±0.02

FS=Forest soil;RHS=Rhizosphere of soil; RS= Riverrine soil; Values mean of three replicate sets; ±= SE

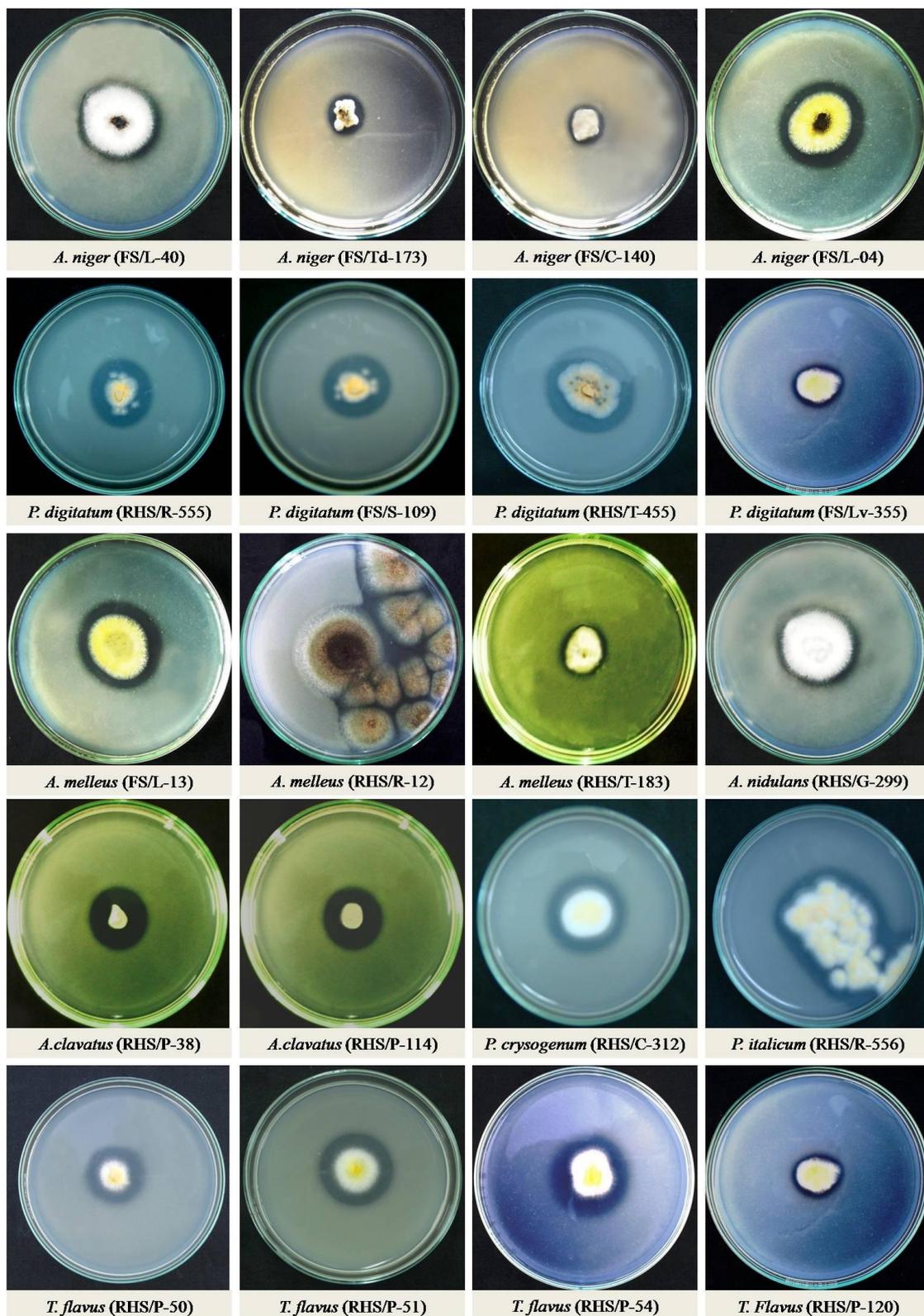


Fig. 14. Phosphate solubilizing properties of fungal isolates on Pikovskaya Agar showing characteristic halo zone formation around the colonies.

Table 9. *In vitro* quantification of phosphate solubilization by fungal isolates in modified PVK broth

	Isolate	Phosphate solubilized ($\mu\text{g/ml}$)			Isolate	Phosphate solubilized ($\mu\text{g/ml}$)		
		TCP*	RP**			TCP*	RP**	
<i>A. niger</i>	FS/L-04	856 \pm 10.58	366 \pm 8.76	<i>A. niger</i>	RHS/M-401	812 \pm 12.15	312 \pm 5.43	
	FS/L-40	847 \pm 11.41	370 \pm 8.22		RHS/G-296	823 \pm 14.22	334 \pm 6.07	
	FS/C-140	824 \pm 09.06	344 \pm 7.25		RHS/G-297	816 \pm 12.56	362 \pm 6.13	
	FS/C143	711 \pm 08.03	345 \pm 9.15		RHS/S-518	804 \pm 13.52	365 \pm 7.44	
	FS/Td-165	734 \pm 08.86	346 \pm 8.92		RHS/S-531	822 \pm 17.12	321 \pm 3.21	
	FS/Td-173	830 \pm 10.75	352 \pm 6.76		RHS/Br-570	736 \pm 09.24	316 \pm 3.27	
	FS/S-177	802 \pm 11.91	343 \pm 6.85		RHS/Cd-610	788 \pm 08.33	288 \pm 6.42	
	FS/R-344	843 \pm 11.03	341 \pm 7.31		RHS/R-548	831 \pm 11.63	286 \pm 5.32	
	FS/S-112	842 \pm 09.92	367 \pm 8.38		RHS/A-30	846 \pm 10.43	376 \pm 5.16	
	FS/S-113	842 \pm 12.76	354 \pm 5.09		RHS/A-33	812 \pm 11.23	338 \pm 4.73	
	FS/R-262	848 \pm 12.85	363 \pm 6.92		RHS/P-45	811 \pm 11.78	318 \pm 6.42	
	FS/Tn-57	795 \pm 11.31	350 \pm 6.30		RHS/P-48	727 \pm 08.78	327 \pm 6.33	
	FS/Tn-58	852 \pm 10.99	360 \pm 7.38		RHS/D-280	835 \pm 09.43	315 \pm 7.23	
	RHS/P-37	830 \pm 13.57	350 \pm 7.09		RHS/T-581	836 \pm 09.22	363 \pm 6.21	
	FS/G-101	809 \pm 10.38	352 \pm 8.92		RS/B-160	815 \pm 11.45	342 \pm 4.82	
	FS/L-105	802 \pm 10.09	354 \pm 8.30		RS/P/14	852 \pm 12.67	360 \pm 5.34	
	FS/L-106	807 \pm 13.15	345 \pm 8.92		RS/Md-288	782 \pm 10.43	319 \pm 6.33	
	FS/L-103	849 \pm 12.92	374 \pm 9.76		RS/T-137	746 \pm 07.54	311 \pm 6.15	
	FS/L -117	807 \pm 09.76	349 \pm 9.85		RS/B-164	733 \pm 07.62	313 \pm 5.82	
	FS/L-281	813 \pm 13.85	344 \pm 8.31		RS/Re-231	822 \pm 08.12	327 \pm 6.31	
	FS/S-282	807 \pm 11.31	355 \pm 1.99		RS/Re-235	846 \pm 10.44	362 \pm 7.18	
	FS/S-283	842 \pm 08.99	287 \pm 7.57		<i>A. melleus</i>	FS/L-42	730 \pm 11.98	260 \pm 8.91
	FS/Md-284	841 \pm 11.57	342 \pm 7.38			FS/L-13	717 \pm 11.82	281 \pm 8.03
	FS/Md-286	837 \pm 10.38	360 \pm 8.09			FS/L-17	720 \pm 11.82	279 \pm 9.55
	FS/Md-287	806 \pm 08.09	336 \pm 7.92			FS/L-18	724 \pm 13.07	276 \pm 6.55
	FS/Md-365	807 \pm 08.92	362 \pm 8.55			FS/G-226	710 \pm 11.70	252 \pm 7.92
FS/Md-366	817 \pm 10.30	355 \pm 6.55	FS/S-64	712 \pm 11.86		211 \pm 6.76		
RHS/P-200	838 \pm 09.54	345 \pm 5.57	FS/L-41	733 \pm 12.36		214 \pm 5.85		
RHS/A-82	838 \pm 08.92	350 \pm 9.38	FS/S -63	729 \pm 11.54		232 \pm 5.31		
RHS/T-198	841 \pm 12.38	346 \pm 9.92	FS/S-24	720 \pm 12.51		238 \pm 5.42		
RHS/T-272	816 \pm 10.38	334 \pm 6.92	FS/S-278	709 \pm 13.17		239 \pm 5.57		
RHS/T-421	840 \pm 10.09	375 \pm 6.76	RHS/T-449	716 \pm 10.43		225 \pm 3.21		
RHS/T-422	804 \pm 11.09	336 \pm 8.31	RHS/T-450	701 \pm 11.25		272 \pm 4.76		
RHS/T-580	807 \pm 11.32	340 \pm 7.99	RHS/T-274	704 \pm 12.64		274 \pm 4.22		
RHS/S-518	713 \pm 10.05	210 \pm 6.43	RHS/T-275	733 \pm 07.43		213 \pm 3.16		
RHS/S-492	764 \pm 12.47	312 \pm 5.21	RHS/T-402	725 \pm 11.33	234 \pm 3.81			

FS=Forest soil; RHS=Rhizosphere of soil; RS= Riverrine soil; *Tricalcium phosphate; ** Rock Phosphate; Values are mean of three replicate experiments. \pm = Standard Error.

Contd.....

	Isolate	Phosphate solubilized ($\mu\text{g/ml}$)			Isolate	Phosphate solubilized ($\mu\text{g/ml}$)			
		TCP*	RP**			TCP*	RP**		
A. <i>melleus</i>	RHS/T-331	713 \pm 07.13	212 \pm 4.32	A. <i>nidulans</i>	FS/Lv-354	413 \pm 06.44	213 \pm 6.73		
	RHS/T-332	733 \pm 06.44	265 \pm 6.12		FS/P-214	426 \pm 07.23	176 \pm 6.22		
	RHS/S-303	710 \pm 11.09	285 \pm 5.76		FS/P-215	433 \pm 05.33	138 \pm 6.43		
	RHS/Cd-606	716 \pm 10.92	242 \pm 5.85		FS/S-457	421 \pm 07.16	214 \pm 5.87		
	RHS/R-549	729 \pm 11.30	250 \pm 5.31		FS/S-459	427 \pm 07.37	225 \pm 4.32		
	RHS/Am-34	712 \pm 10.92	240 \pm 6.38		RHS/T-453	473 \pm 05.38	275 \pm 5.54		
	RHS/R-12	736 \pm 14.24	238 \pm 7.38		RHS/T-389	512 \pm 07.32	282 \pm 4.33		
	RHS/P-201	810 \pm 09.25	309 \pm 6.91		RHS/G-299	412 \pm 06.44	274 \pm 4.86		
	RHS/P-202	750 \pm 09.91	317 \pm 6.03		RHS/Br-579	422 \pm 05.32	289 \pm 4.17		
	RHS/P-205	727 \pm 11.27	243 \pm 6.20		RHS/A-79	465 \pm 07.16	321 \pm 5.27		
	RS/R-115	724 \pm 08.25	270 \pm 6.55		RS/T-59	430 \pm 08.86	320 \pm 5.92		
	RS/T-182	712 \pm 08.13	217 \pm 4.76		RS/P-60	410 \pm 10.36	343 \pm 5.76		
	RS/T-183	718 \pm 11.24	237 \pm 3.21		P. <i>digitatum</i>	FS/S-109	808 \pm 08.55	310 \pm 5.19	
	RS/P-61	688 \pm 11.07	265 \pm 4.83			FS/Rv-317	802 \pm 08.55	255 \pm 5.57	
	RS/P-05	683 \pm 12.36	248 \pm 5.11			FS/La-185	715 \pm 08.17	326 \pm 4.34	
	RS/T-236	714 \pm 09.14	261 \pm 4.32			RHS/R-515	816 \pm 09.44	312 \pm 5.22	
	RS/M-368	726 \pm 07.36	217 \pm 4.89			RHS/T-455	815 \pm 10.43	317 \pm 4.32	
	A. <i>fumigatus</i>	FS/R-263	687 \pm 12.51			251 \pm 4.85	RHS/T-590	788 \pm 10.86	308 \pm 4.76
		FS/R-264	537 \pm 11.54			214 \pm 5.31	RHS/C-338	765 \pm 11.77	314 \pm 5.19
RHS/P-209		538 \pm 10.17	235 \pm 5.90	RS/T-285		739 \pm 11.92	310 \pm 6.85		
RHS/B-220		519 \pm 10.14	288 \pm 5.57	FS/Lv-355	678 \pm 10.67	275 \pm 4.53			
RHS/T-531		543 \pm 04.27	278 \pm 4.33	FS/S-67	632 \pm 09.38	271 \pm 5.97			
RHS/P-43		542 \pm 05.73	213 \pm 4.86	FS/S-459	674 \pm 09.10	311 \pm 4.18			
RHS/T-585		582 \pm 07.88	226 \pm 4.73	RHS/R-505	716 \pm 09.44	212 \pm 5.22			
RHS/T-586		514 \pm 07.15	224 \pm 5.12	RHS/T-437	633 \pm 10.43	316 \pm 4.15			
RHS/T-588		533 \pm 06.43	211 \pm 6.23	P. <i>italicum</i>	RHS/T-594	712 \pm 10.2	327 \pm 5.78		
A. <i>clavatus</i>	FS/Rv-323	799 \pm 08.24	288 \pm 4.23		RHS/M-403	746 \pm 11.74	289 \pm 5.91		
	FS/Rv-330	783 \pm 06.75	252 \pm 4.81		RHS/P-414	728 \pm 09.54	315 \pm 4.73		
	RHS/P-38	714 \pm 10.32	247 \pm 5.76	RHS/R-555	711 \pm 09.32	313 \pm 4.14			
	RHS/P-56	832 \pm 13.91	341 \pm 5.99	P. <i>crysogenum</i>	FS/L-265	642 \pm 08.32	275 \pm 4.87		
	RHS/P-54	825 \pm 13.03	270 \pm 6.57		FS/G-97	615 \pm 10.28	243 \pm 3.22		
	RHS/P-114	727 \pm 11.55	351 \pm 5.38		RHS/T-269	622 \pm 11.43	214 \pm 3.77		
	RHS/T-99	804 \pm 11.55	312 \pm 6.09		RHS/T-271	627 \pm 10.23	261 \pm 3.18		
	RHS/T-190	829 \pm 12.12	340 \pm 5.92		RHS/C-312	583 \pm 10.46	228 \pm 4.55		
	RHS/T-383	812 \pm 12.76	350 \pm 6.55	RS/Re-230	574 \pm 09.34	263 \pm 4.14			
RHS/T-386	762 \pm 10.32	312 \pm 5.50	T. <i>flavus</i>	RHS/P-50	862 \pm 12.85	368 \pm 5.50			
RHS/Cd-603	783 \pm 12.46	283 \pm 5.21		RHS/P-51	870 \pm 11.55	392 \pm 6.09			
RHS/A-77	811 \pm 11.44	316 \pm 6.83	RHS/P-54	859 \pm 12.12	348 \pm 6.92				
RS/B-163	803 \pm 13.72	327 \pm 6.09	RHS/P-120	862 \pm 12.76	350 \pm 5.55				

FS=Forest soil;RHS=Rhizosphere of soil; RS= Riverrine soil; *Tricalcium phosphate; ** Rock Phosphate; Values are mean of three replicate experiments. \pm = Standard Error.

4.2.2. Exo and Endo Chitinase activities

A total of 55 *Trichoderma* isolates were obtained from different sources both on PDA and *Trichoderma* Selective Medium (TSM) and were initially identified on the basis of their morphological and microscopical characters. On the basis of microscopical characters they were identified as *Trichoderma harzianum*, *T. viride*, *T. asperellum* and *T. erinaceum*. A total of 26 isolates of *T. harzianum* 10 isolates of *T. viride*, 13 isolates of *T. asperellum* and 6 isolates of *T. erinaceum* obtained from various sources were tested for their ability to produce Chitinase *in vitro*. The net exo and endo chitinase activities of the isolates were determined spectrophotometrically and expressed as μg N-Acetyl glucosamine released/ ml culture filtrate/h (Endo) and μg N-Acetyl glucosamine released/ g mycelium /h (Exo). The detail results of the analysis presented in Table 11, reveals that all the isolates showed a significant amount of both Exo and Endo Chitinase activities. Among the *T. harzianum* isolates, two isolates designated as RHS/S-559 and RHS/S-560 obtained from the rhizosphere of *Secchium edule* showed maximum amount of both endo and exo Chitinase activities. Whereas among the *T. viride* isolates, isolate RHS/G 251 showed maximum activities. Similarly among the *T. asperellum* isolates, one isolate designated as RHS/S-561 showed maximum activities and among the *T. erinaceum* isolates, isolate RHS/Rd-551 showed maximum endo and exo chitinase activities.

4.2.3. Net Exo-Cellulase activities

All the fungal isolates which showed positive results for phosphate solubilization and chitinase production were tested exo and endo-cellulase activities. The amount of glucose released by endocellulase activity of fungal hyphae during their growth using cellulose as C source was measured and expressed as net exo-cellulase activity of the isolate ($\mu\text{g}/\text{ml}/\text{hr}$). Net exo-cellulase activities of all the tested isolated hae been presented in Table. 12 Among the tested fungal isolates, isolates of *A. niger* (FS/L-04, FS/L-40, FS/C-140, RS/P/14, FS/Td-173 and RHS/T-198), *A. melleus* (FS/L-13, FS/L-17, FS/L-18, RHS/R-12 and RS/P-05), *A. fumigates* (FS/R-263), *A. clavatus* (RHS/P-38, RHS/T-99, and RHS/P-114), *P. digitatum* (RHS/T-455 and RHS/C-338), *P. italicum* (RHS/M-403 and RHS/P-414), *P. crysoenum* (RHS/T-269), *T. flavus* (RHS/P-54, RHS/P-51, RHS/P-50 and RHS/P-120), *T. harzianum* (RHS/S-559 and RHS/S-560), *T. viride* (RHS/B-245 and RHS/G-251 *T. asperellum* (RHS/S-561, RHS/Cd-601 and FS/L-188) and *T. erinacium* (RHS/T-626 and FS/Td-166) showed comparatively higher exo and endo cellulose activities.

Table 10. Chitinase activities of different isolates of *Trichoderma* obtained from different sources of Darjeeling Hills

Isolate	Chitinase activity		Isolate	Chitinase activity	
	Exo	Endo		Exo	Endo
<i>T. harzianum</i>			<i>T. viride</i>		
FS/C- 149	4.33±0.16	21.27±0.86	RHS/B-245	3.04±0.18	16.55±0.72
FS/M- 367	4.26±0.28	23.41±0.66	RHS/G-251	3.63±0.16	17.82±0.68
FS/L-358	3.27±0.22	18.37±0.63	RHS/Rd-547	2.82±0.24	16.80±0.83
FS/S-476	4.18±0.23	20.40±0.68	RHS/Am624	2.75±0.20	18.58±0.65
FS/G-96	4.33±0.17	19.44±0.65	FS/L 116	2.44±0.25	22.73±0.80
FS/Rr-74	4.16±0.21	18.16±0.74	FS/L-118	3.15±0.22	21.68±0.75
FS/Md-289	3.73±0.22	22.43±0.66	<i>T. asperellum</i>		
FS/R-542	3.61±0.18	24.76±0.72	FS/M- 363	3.74±0.17	18.44±0.74
FS/Th-485	3.82±0.23	23.81±0.73	FS/L-188	3.16±0.26	22.72±0.83
FS/R-640	3.27±0.18	23.11±0.63	FS/Sd-478	3.77±0.23	20.43±0.74
FS/R-641	4.31±0.16	24.52±0.67	FS/R-439	4.06±0.17	19.88±0.84
RHS/T-438	4.25±0.24	18.38±0.74	FS/Th-488	3.11±0.15	22.33±0.76
RHS/T-600	4.47±0.22	25.41±0.77	RHS/T-341	3.22±0.27	23.76±0.77
RHS/Cd-606	4.29±0.26	24.14±0.62	RHS/S-561	4.18±0.16	24.63±0.83
RHS-Am-623	4.18±0.16	21.73±0.80	RHS/Br-634	3.22±0.20	20.11±0.68
RHS/P-572	4.24±0.23	25.88±0.82	RHS/Cd-601	3.73±0.26	21.12±0.72
RHS/S-559	4.76±0.16	25.63±0.71	RHS/Cd-604	2.76±0.17	24.10±0.78
RHS/S-560	4.83±0.25	26.77±0.65	FS/Tn-39	3.76±0.24	23.12±0.68
RHS/Br-633	4.12±0.22	22.42±0.69	<i>T. erinacium</i>		
<i>T. viride</i>			FS/Td-166	3.14±0.21	21.33±0.73
FS/L-186	3.17±0.13	17.24±0.83	FS/Td-168	3.27±0.20	20.36±0.66
FS/R-256	3.22±0.17	17.87±0.81	RHS/T-439	4.08±0.16	22.83±0.76
FS/R-426	3.50±0.18	16.55±0.76	RHS/T-626	4.11±0.25	23.28±0.83
RHS/T-584	3.16±0.22	18.20±0.84	RHS/Rd-551	4.15±0.22	25.43±0.63

* Chitinase activity expressed as µg N-Acetyl glucosamine released/ ml culture filtrate/h (Endo) and µg N-Acetyl glucosamine released/ g mycelium /h (Exo) FS=Forest soil;RHS=Rhizosphere of soil; RS= Riverrine soil; Values are mean of three replicate experiments.±= Standard Error.

Table 11. Potential PSF isolates deposited to NAIMCC

Phosphate solubilizing Fungi	Code	NAIMCC acc.no.
<i>Aspergillus clavatus</i>	RHS/P 38	NAIMCC-F-01947
<i>Aspergillus melleus</i>	RS/P 05	NAIMCC-F-01942
<i>Aspergillus melleus</i>	FS/L-13	NAIMCC-F-01943
<i>Aspergillus melleus</i>	FS/L-17	NAIMCC-F-01944
<i>Aspergillus melleus</i>	FS/L-18	NAIMCC-F-01945
<i>Aspergillus niger</i>	FS/L-04	NAIMCC-F-01939
<i>Aspergillus niger</i>	FS/P-14	NAIMCC-F-01941
<i>Aspergillus niger</i>	FS/L040	NAIMCC-F-01946
<i>Talaromyces flavus</i>	RHS/P 51	NAIMCC-F-01948

Table 12. Evaluation of net exocellulase activity of the fungal isolates

Organism	Isolate	Exo and endo cellulase activity (μg reducing sugar produced/ml/h)	Amount reducing sugar due to Endocellulase activity ($\mu\text{g/ml}$)	Net exocellulase activity ($\mu\text{g/ml/h}$)
<i>T. harzianum</i>	FS/C- 149	16.33 \pm 0.21	9.66 \pm 0.12	6.67 \pm 0.23
	FS/M- 367	14.26 \pm 0.16	8.28 \pm 0.18	5.98 \pm 0.25
	FS/L-358	15.11 \pm 0.22	7.33 \pm 0.18	7.78 \pm 0.21
	FS/S-476	14.13 \pm 0.31	7.15 \pm 0.11	6.98 \pm 0.19
	FS/G-96	14.53 \pm 0.36	6.34 \pm 0.13	8.19 \pm 0.15
	FS/Rr-74	16.20 \pm 0.28	8.22 \pm 0.18	7.98 \pm 0.24
	FS/Md-289	18.43 \pm 0.27	9.76 \pm 0.17	8.67 \pm 0.26
	FS/R-542	16.83 \pm 0.19	8.41 \pm 0.11	8.42 \pm 0.16
	FS/Th-485	14.20 \pm 0.22	8.33 \pm 0.19	5.87 \pm 0.19
	FS/R-640	15.34 \pm 0.39	7.44 \pm 0.16	7.90 \pm 0.22
	FS/R-641	17.42 \pm 0.34	9.12 \pm 0.12	8.30 \pm 0.20
	RHS/T-438	17.10 \pm 0.27	8.73 \pm 0.13	8.37 \pm 0.13
	RHS/T-600	16.18 \pm 0.25	7.86 \pm 0.18	8.32 \pm 0.19
	RHS/Cd-606	16.65 \pm 0.21	7.13 \pm 0.23	9.52 \pm 0.14
	RHS-Am-623	15.82 \pm 0.36	8.11 \pm 0.22	7.71 \pm 0.21
	RHS/P-572	15.43 \pm 0.32	7.21 \pm 0.12	8.22 \pm 0.25
	RHS/S-559	16.73 \pm 0.26	9.13 \pm 0.11	7.60 \pm 0.22
	RHS/S-560	14.58 \pm 0.22	9.44 \pm 0.19	5.14 \pm 0.14
	RHS/Br-633	17.42 \pm 0.20	9.76 \pm 0.13	7.66 \pm 0.23
	<i>T. viride</i>	FS/L-186	16.32 \pm 0.18	7.45 \pm 0.15
FS/R-256		16.73 \pm 0.25	9.16 \pm 0.21	7.57 \pm 0.25
FS/R-426		14.36 \pm 0.36	7.33 \pm 0.22	7.03 \pm 0.21
RHS/T-584		14.56 \pm 0.24	7.18 \pm 0.16	7.38 \pm 0.24
RHS/B-245		17.42 \pm 0.17	8.10 \pm 0.24	9.32 \pm 0.18
RHS/G-251		17.36 \pm 0.35	8.44 \pm 0.15	8.92 \pm 0.14
RHS/Rd-547		14.88 \pm 0.30	9.16 \pm 0.18	5.72 \pm 0.22
RHS/Am-624		13.22 \pm 0.27	6.54 \pm 0.23	6.68 \pm 0.24
FS/L 116		13.68 \pm 0.27	6.21 \pm 0.21	7.47 \pm 0.18
<i>T. asperellum</i>		FS/L-118	14.73 \pm 0.36	8.16 \pm 0.12
	FS/M- 363	15.44 \pm 0.24	7.21 \pm 0.16	8.23 \pm 0.14
	FS/L-188	16.33 \pm 0.19	7.83 \pm 0.19	8.50 \pm 0.23
	FS/Sd-478	15.32 \pm 0.19	9.54 \pm 0.20	5.78 \pm 0.12
	FS/R-439	15.81 \pm 0.26	9.21 \pm 0.24	6.60 \pm 0.11
	FS/Th-488	15.43 \pm 0.33	7.16 \pm 0.11	8.27 \pm 0.25
	RHS/T-341	13.20 \pm 0.37	6.30 \pm 0.16	6.90 \pm 0.20
	RHS/S-561	16.77 \pm 0.20	7.56 \pm 0.13	9.21 \pm 0.15
	RHS/Br-634	16.21 \pm 0.24	7.83 \pm 0.23	8.38 \pm 0.18
	RHS/Cd-601	14.55 \pm 0.26	6.43 \pm 0.22	8.12 \pm 0.16
	RHS/Cd-604	16.57 \pm 0.32	8.32 \pm 0.15	8.25 \pm 0.11
	FS/Tn-39	16.54 \pm 0.33	8.84 \pm 0.18	7.70 \pm 0.18

Contd.....

Organism	Isolate	Exo and endo cellulase activity (µg reducing sugar produced/ml/h)	Amount reducing sugar due to Endocellulase activity (µg/ml)	Net exo-cellulase activity (µg/ml/h)
<i>T. erinacium</i>	FS/Td-166	12.34±0.55	7.88±0.17	4.46±0.13
	FS/Td-168	11.44±0.43	6.32±0.22	5.12±0.15
	RHS/T-439	12.62±0.41	7.18±0.26	5.44±0.11
	RHS/T-626	13.55±0.38	7.43±0.15	6.12±0.20
	RHS/Rd-551	11.83±0.35	6.32±0.18	5.51±0.22
<i>A. niger</i>	FS/L04	28.45±0.63	9.56±0.27	18.89±0.58
	FS/L-40	26.13±0.74	8.73±0.11	17.40±0.33
	FS/C-140	27.40±0.60	9.22±0.16	18.18±0.26
	FS/C143	23.76±0.57	6.16±0.25	17.60±0.38
	FS/Td-165	25.33±0.33	7.22±0.22	18.11±0.41
	FS/Td-173	27.43±0.61	9.16±0.16	18.27±0.46
	FS/S-177	22.34±0.69	6.15±0.18	16.19±0.21
	FS/Rr-140	21.73±0.78	8.12±0.20	13.61±0.52
	FS/S-112	18.42±0.39	6.63±0.13	09.79±0.33
	FS/S-113	18.36±0.43	7.10±0.16	11.26±0.36
	FS/R-262	25.84±0.61	7.36±0.15	18.48±0.28
	FS/Tn-57	17.86±0.55	6.48±0.28	11.38±0.43
	FS/Tn-58	26.44±0.72	6.15±0.24	20.29±0.52
	RHS/P-37	23.62±0.60	9.32±0.16	14.30±0.39
	FS/G-101	16.75±0.75	7.14±0.13	09.61±0.27
	FS/L-105	18.33±0.68	6.13±0.11	12.20±0.40
	FS/L-106	22.45±0.65	6.92±0.21	15.53±0.59
	FS/L-107	21.83±0.50	7.16±0.25	14.67±0.51
	FS/L -117	26.55±0.52	8.12±0.20	18.43±0.44
	FS/L-281	18.16±0.73	6.41±0.16	11.75±0.42
	FS/S-282	17.84±0.66	6.28±0.25	11.56±0.30
	FS/S-283	20.55±0.71	7.10±0.21	13.45±0.52
	FS/Md-284	27.30±0.43	8.12±0.15	19.18±0.55
	FS/Md-286	21.33±0.48	7.46±0.19	13.87±0.36
	FS/Md-287	20.16±0.41	6.33±0.26	11.96±0.35
	FS/Md-365	18.27±0.77	7.15±0.24	11.12±0.24
	FS/Md-366	18.40±0.43	6.16±0.20	12.24±0.46
	RHS/P-200	22.36±0.73	9.82±0.16	12.54±0.58
	RHS/A-82	20.93±0.66	8.20±0.18	12.73±0.53
	RHS/T-198	26.44±0.62	7.55±0.23	18.89±0.49
	RHS/T- 272	24.29±0.59	7.16±0.24	17.13±0.36
	RHS/T-421	18.33±0.70	6.47±0.15	11.86±0.31
	RHS/T-422	20.76±0.68	6.17±0.13	14.59±0.43
RHS/T-580	18.37±0.53	7.15±0.20	11.22±0.50	

Contd.....

Organism	Isolate	Exo and endo cellulase activity (µg reducing sugar produced/ml/h)	Amount reducing sugar due to Endocellulase activity (µg/ml)	Net exo-cellulase activity (µg/ml/h)
<i>A. niger</i>	RHS/S-518	23.44±0.78	7.83±0.26	15.61±0.43
	RHS/S-530	24.32±0.63	9.11±0.33	15.21±0.47
	RHS/M-401	18.75±0.77	6.76±0.24	11.99±0.56
	RHS/G-296	23.17±0.62	8.23±0.26	14.94±0.55
	RHS/G-297	24.43±0.83	8.19±0.32	16.24±0.42
	RHS/S-518	16.73±0.66	5.33±0.38	11.40±0.36
	RHS/S-530	22.15±0.72	7.61±0.42	14.54±0.35
	RHS/Br-570	21.37±0.59	6.18±0.36	15.19±0.40
	RHS/Cd-610	17.40±0.54	7.33±0.30	10.07±0.48
	RHS/R-548	21.43±0.55	6.28±0.20	15.15±0.55
	RHS/A-30	21.82±0.63	8.25±0.25	13.57±0.43
	RHS/A-33	20.77±0.68	8.36±0.37	12.41±0.42
	RHS/P-45	24.63±0.77	8.12±0.34	16.51±0.50
	RHS/P-48	25.36±0.58	9.15±0.47	16.21±0.33
	RHS/D-280	18.54±0.53	5.32±0.43	13.22±0.36
	RHS/T-581	20.29±0.64	6.36±0.40	13.93±0.42
	RS/B-160	22.18±0.60	7.10±0.38	15.08±0.30
	RS/P/14	17.45±0.51	6.22±0.39	11.23±0.38
	RS/Md-288	24.71±0.74	8.28±0.22	16.43±0.41
	RS/T-137	25.44±0.70	9.15±0.26	16.29±0.28
	RS/B-164	20.18±0.48	5.77±0.36	14.41±0.51
	RS/Re-231	21.46±0.69	6.11±0.28	15.35±0.43
	RS/Re-235	20.74±0.55	7.76±0.44	12.98±0.37
<i>A. melleus</i>	FS/L-42	15.43±0.52	5.48±0.29	09.95±0.42
	FS/L-13	16.77±0.70	6.10±0.33	10.67±0.48
	FS/L-17	24.35±0.63	7.38±0.39	16.97±0.52
	FS/L-18	23.73±0.62	7.62±0.30	16.11±0.46
	FS/G-226	17.88±0.46	6.91±0.34	10.97±0.35
	FS/S-64	15.60±0.50	5.83±0.26	09.77±0.36
	FS/L-41	21.76±0.63	6.22±0.34	15.54±0.44
	FS/S -63	15.82±0.66	5.13±0.26	10.69±0.38
	FS/S-24	18.16±0.71	5.44±0.37	12.72±0.33
	FS/S-278	17.16±0.52	6.25±0.32	10.91±0.50
	RHS/T-449	17.50±0.43	6.13±0.28	11.37±0.47
	RHS/T-450	16.32±0.46	5.13±0.20	11.19±0.38
	RHS/T-274	25.18±0.58	8.80±0.38	16.38±0.36
	RHS/T-275	22.37±0.52	9.81±0.33	12.56±0.41
	RHS/T-402	20.59±0.63	7.16±0.35	13.43±0.30

Contd.....

Organism	Isolate	Exo and endo cellulase activity (µg reducing sugar produced/ml/h)	Amount reducing sugar due to Endocellulase activity (µg/ml)	Net exo-cellulase activity (µg/ml/h)
<i>A. melleus</i>	RHS/T-331	17.36±0.53	5.43±0.23	11.93±0.53
	RHS/T-332	15.42±0.43	7.22±0.20	08.20±0.44
	RHS/S-303	20.44±0.55	8.36±0.34	12.08±0.58
	RHS/Cd606	14.32±0.43	5.66±0.25	08.66±0.43
	RHS/R-549	15.78±0.64	5.42±0.32	10.36±0.56
	RHS/Am-34	16.73±0.42	5.16±0.21	11.57±0.48
	RHS/R-12	26.78±0.52	8.31±0.27	18.47±0.41
	RHS/P-201	20.14±0.44	7.16±0.32	12.98±0.56
	RHS/P-202	18.33±0.63	6.38±0.33	11.95±0.55
	RHS/P-205	16.54±0.56	5.63±0.21	10.91±0.30
	RS/R-115	16.11±0.62	5.32±0.26	10.79±0.41
	RS/T-182	17.44±0.66	7.15±0.22	10.29±0.33
	RS/T-183	21.27±0.54	8.73±0.32	12.54±0.47
	RS/P -61	18.39±0.44	7.32±0.28	11.07±0.43
	RS/P-05	25.44±0.42	7.57±0.44	17.78±0.68
	RS/T-236	16.73±0.52	5.41±0.27	11.32±0.54
	RS/M-368	15.82±0.32	5.88±0.22	09.94±0.43
<i>A. fumigatus</i>	FS/R-263	17.45±0.33	5.23±0.43	12.22±0.37
	FS/R-264	13.46±0.36	5.16±0.36	08.30±0.33
	RHS/P-209	15.78±0.42	7.43±0.24	08.35±0.27
	RHS/B-220	14.28±0.37	6.82±0.42	07.46±0.22
	RHS/P-43	13.91±0.55	5.67±0.26	08.24±0.34
	RHS/T-585	16.25±0.42	6.17±0.28	10.08±0.44
	RHS/T-586	16.10±0.38	7.91±0.21	08.19±0.47
	RHS/T-588	14.33±0.45	5.21±0.25	09.12±0.27
	RHS/T-531	17.81±0.40	6.33±0.28	11.48±0.44
<i>A. clavatus</i>	RHS/P-114	13.27±0.51	5.11±0.33	08.16±0.28
	FS/Rv-323	21.37±0.33	7.21±0.33	14.16±0.36
	FS/Rv-330	23.41±0.73	6.56±0.35	16.85±0.57
	RHS/T-99	18.72±0.30	6.10±0.26	12.62±0.54
	RHS/T-190	17.38±0.43	6.33±0.27	11.05±0.36
	RHS/P -56	18.52±0.63	7.32±0.38	11.20±0.47
	RHS/P -54	23.56±0.88	7.18±0.43	16.38±0.36
	RHS/P-38	26.32±0.63	8.54±0.33	17.78±0.47
	RHS/T-383	21.22±0.74	6.43±0.27	14.79±0.50
	RHS/T-386	22.57±0.65	5.80±0.20	16.77±0.54
	RHS/Cd-601	18.36±0.62	5.16±0.36	13.20±0.44
	RHS/A-77	20.11±0.60	7.83±0.30	14.28±0.58
	RS/B-163	21.59±0.58	8.32±0.28	13.27±0.42

Contd.....

Organism	Isolate	Exo and endo cellulase activity (μg reducing sugar produced/ml/h)	Amount reducing sugar due to Endocellulase activity ($\mu\text{g/ml}$)	Net exo-cellulase activity ($\mu\text{g/ml/h}$)
<i>A. nidulans</i>	FS/Lv-354	12.46 \pm 0.36	5.33 \pm 0.12	7.13 \pm 0.33
	FS/P-214	14.21 \pm 0.32	6.24 \pm 0.18	7.97 \pm 0.26
	FS/P-215	10.56 \pm 0.37	4.33 \pm 0.22	6.23 \pm 0.22
	FS/S-457	12.16 \pm 0.44	5.12 \pm 0.12	7.04 \pm 0.37
	FS/S-459	12.38 \pm 0.45	6.13 \pm 0.24	6.25 \pm 0.31
	RHS/T-453	14.53 \pm 0.30	5.21 \pm 0.26	9.32 \pm 0.38
	RHS/T-389	14.27 \pm 0.27	5.18 \pm 0.21	9.09 \pm 0.20
	RHS/G-299	11.34 \pm 0.23	4.33 \pm 0.35	7.01 \pm 0.24
	RHS/Br-579	10.26 \pm 0.21	4.73 \pm 0.27	5.53 \pm 0.31
	RHS/A-79	10.93 \pm 0.26	5.44 \pm 0.25	5.49 \pm 0.35
	RS/T-59	14.66 \pm 0.37	7.16 \pm 0.18	7.50 \pm 0.26
	RS/P-60	11.32 \pm 0.31	4.72 \pm 0.15	6.60 \pm 0.35
<i>P. digitatum</i>	FS/S-109	15.27 \pm 0.36	7.16 \pm 0.22	8.11 \pm 0.26
	FS/Rv-317	16.43 \pm 0.22	8.54 \pm 0.28	7.89 \pm 0.37
	FS/La-185	16.73 \pm 0.27	7.11 \pm 0.35	9.62 \pm 0.25
	RHS/R-515	13.28 \pm 0.51	5.73 \pm 0.33	7.55 \pm 0.26
	RHS/T-455	17.14 \pm 0.57	8.21 \pm 0.28	8.93 \pm 0.18
	RHS/T-590	15.20 \pm 0.30	6.37 \pm 0.17	8.83 \pm 0.17
	RHS/C-338	17.44 \pm 0.46	8.10 \pm 0.25	9.34 \pm 0.33
	RS/T-285	13.26 \pm 0.53	4.18 \pm 0.20	9.08 \pm 0.34
	FS/Lv-355	12.44 \pm 0.48	4.73 \pm 0.22	7.71 \pm 0.26
	FS/S-67	16.73 \pm 0.33	6.11 \pm 0.35	10.62 \pm 0.46
	FS/S-459	14.56 \pm 0.42	5.26 \pm 0.30	09.30 \pm 0.36
	RHS/R-505	15.63 \pm 0.47	5.18 \pm 0.21	10.45 \pm 0.32
	RHS/T-437	14.30 \pm 0.37	3.16 \pm 0.18	11.14 \pm 0.36
	<i>P. italicum</i>	RHS/T-594	16.50 \pm 0.47	5.11 \pm 0.32
RHS/M-403		18.32 \pm 0.52	7.12 \pm 0.27	11.20 \pm 0.36
RHS/P-414		18.16 \pm 0.55	6.80 \pm 0.20	11.36 \pm 0.22
RHS/R-555		15.33 \pm 0.41	5.42 \pm 0.26	09.91 \pm 0.18
<i>P. crysogenum</i>	FS/L-265	17.21 \pm 0.46	5.43 \pm 0.37	11.78 \pm 0.26
	FS/G-97	18.37 \pm 0.32	6.32 \pm 0.32	12.02 \pm 0.20
	RHS/T-269	19.43 \pm 0.37	7.32 \pm 0.25	12.11 \pm 0.26
	RHS/T-271	17.48 \pm 0.42	6.71 \pm 0.26	10.77 \pm 0.22
	RHS/C-312	15.13 \pm 0.48	4.28 \pm 0.22	10.85 \pm 0.18
	RS/Re-230	16.44 \pm 0.50	5.43 \pm 0.31	11.01 \pm 0.24
<i>T. flavus</i>	RHS/P-50	24.73 \pm 0.56	8.36 \pm 0.36	16.37 \pm 0.33
	RHS/P-51	28.21 \pm 0.53	7.12 \pm 0.20	21.09 \pm 0.38
	RHS/P-54	27.28 \pm 0.65	8.44 \pm 0.26	18.84 \pm 0.30
	RHS/P-120	22.46 \pm 0.44	6.02 \pm 0.21	16.44 \pm 0.28

FS=Forest soil;RHS=Rhizosphere of soil; RS= Riverrine soil; *Tricalcium phosphate; ** Rock Phosphate; Values are mean of three replicate experiments. \pm = Standard Error.

4.3. Antagonistic activity of the fungal isolates against selected phytopathogens

4.3.1. Antagonistic effect of *Talaromyces flavus* RHS/P-51

4.3.1.1. Inhibition in solid medium

One isolate designated as RHS/P-51 obtained from Paddy rhizosphere which showed phosphate solubilizing activity *in vitro* was tested for its efficiency in inhibiting a number of fungal pathogens. The interactions in the inhibition in percentage were recorded and enlisted in Results revealed that *T.flavus RHS/P-51* could successfully inhibit the pathogens like *S. rolfsii*, *T.cucumeris*, *R. solani* and *M. phaseolina* where the inhibition percentage ranged from 79.86 to 86.86 % (Table 13; Fig. 15 A-F).

4.3.1.2. Inhibition by culture filtrate

Effect of *T. flavus* RHS/P-51 in solid medium was successfully tested in dual Fig. culture. The effect of culture filtrate in inhibiting one of the most notorious root pathogen *S. rolfsii* in terms of inhibition of germination of sclerotia was tested. Sclerotial germination of *S. rolfsii* with cell free culture filtrates of *T. flavus* showed 90-95 % inhibition in comparison to control (Fig. 15 G-I).

Table 13. Inhibition of phytopathogenic test fungi by *T. flavus* RHS/P-51 *in vitro*

Interacting Microorganisms	Diameter of fungal colony after 7 days growth(cm)	% of Inhibition
<i>Sclerotium rolfsii</i>	8.73	-
<i>S. rolfsii</i> + <i>T. flavus</i> RHS/P-51	1.15	86.82
<i>Thanatephorus cucumeris</i>	8.76	-
<i>T. cucumeris</i> + <i>T. flavus</i> RHS/P-51	1.38	84.24
<i>Rhizoctonia solani</i>	7.45	-
<i>R. solani</i> + <i>T. flavus</i> RHS/P-51	1.50	79.86
<i>Macrophomina phaseolina</i>	8.26	-
<i>M. phaseolina</i> + <i>T. flavus</i> RHS/P-51	1.65	80.00

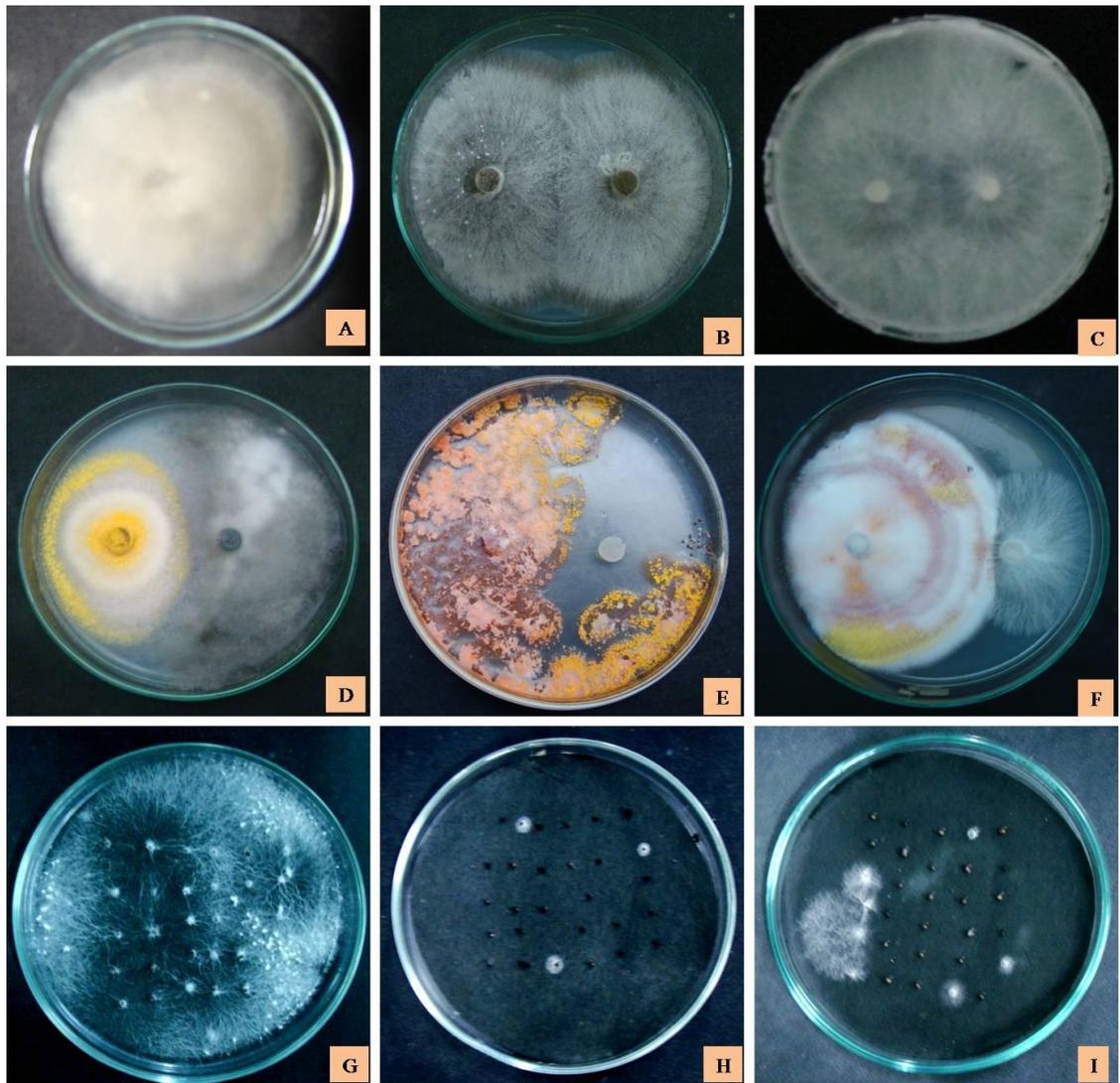


Fig. 15. Inhibition of root pathogens *Talaromyces flavus* (RHS/P-51), *Macrophomina phaseolina* (A), *Thanetophorus cucumeris* (B) and *Sclerotium rolfsii* (C); Inhibition of *M. phaseolina*, *T. cucumeris* and *S. rolfsii* in dual plate culture assay (D-F) and inhibition of sclerotia of *S. rolfsii* by culture filtrate of *T. flavus* in Sclerotia germination bioassay (G-I); Control (G) Inhibition by culture filtrate (Crude) (H) and diluted (1:1) (I).

4.3.2. Antagonistic effect of *Trichoderma* isolates

4.3.2.1. Inhibition in solid medium

A total of twenty six *T. harzianum* isolates, ten *T. viride* isolates, thirteen *T. asperellum* isolates and five *T. erinacium* isolates were obtained from different sources of Darjeeling hills. All these isolates were initially tested for their antagonistic effects against the fungal plant pathogens (*S. rolfsii* and *T. cucumeris*) *in vitro* (Fig. 16). For each of the antagonistic test fungal isolates, 5 mm agar disc taken from 5 days old culture and placed at the periphery of the 90 mm culture Fig.s. Then

same size of another agar disc of selected phytopathogens was similarly placed at the periphery but on the opposing end of the same Petri dish. The percent inhibition in the radial colony growth was calculated by the following formula:

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where, C = Radial growth in control set; T = Radial growth in treated set.

Their interactions in the inhibition in percentage were recorded and enlisted in Table 14; Fig. 16. The overall interactions showed that the isolates of *T. harzianum* were more efficient in inhibiting the tested fungal pathogens *in vitro*. However, among the *T. harzianum* isolates, isolate RHS/S-559 and RHS/S-560 showed maximum inhibitory activities similarly among the *T. asperellum* isolates, RHS/S-561 showed maximum inhibitory effects. Among the *T. erinaceium* isolates the maximum inhibitory effect was recorded in case of RHS/T-439 and among the *T. viride* isolates, FS/L-186 showed maximum inhibitory activities.

Interactions of the antagonists and fungal pathogens were also studied with the help of scanning electron microscopy. The SEM micrographs revealed that the Trichoderma mycelium profusely parasitizes the pathogen mycelium and inhibits its growth. On the later stage of growth the pathogen is completely overgrown by the antagonists (Fig. 17).

4.3.2.2. Inhibition by culture filtrate

Growth of test pathogens in culture filtrate of antagonists was determined with the help of culture filtrate assay. The mycelial weight of fungal pathogens grown in culture filtrate of antagonists was compared with that of control sets and percent inhibition was determined. Results presented in **Table 15** shows that the mycelial growth of *S. rolfsii* was reduced by 75 % when grown in culture filtrate of *T. harzianum* RHS/S-560 whereas the mycelial growth of *T. cucumeris* was reduced by 72% by *T. harzianum* RHS/S-559. Similarly mycelial growth of *S. rolfsii* was reduced by 69 % and the mycelial weight of *T. cucumeris* was reduced by 66 % when grown in the culture filtrate of *T. asperellum* RHS/S-561. Mycelial weight of *S. rolfsii* and *T. cucumeris* was also reduced by 67% when grown in the culture filtrate of *T. viride* FS/L-116 and RHS/G-251. The mycelial weight of both the pathogens was also reduced by 67% when grown in culture filtrate of *T. erinaceium* RHS/T-626.

Table 14. Inhibition of phtytopathogens by *Trichoderma* isolates

Isolate	Colony Diam. (mm)			Colony Diam. (mm)		
	<i>T. sp</i>	<i>Sr</i>	Inhibition %	<i>T. sp</i>	<i>Tc</i>	Inhibition %
<i>Trichoderma harzianum</i>						
FS/C- 147	64	21	67.18	62	27	56.45
FS/C- 149	62	18	70.96	64	26	59.37
FS/M- 367	65	15	76.92	60	26	56.66
FS/L-358	65	17	73.84	58	23	60.34
FS/S-476	61	22	63.93	55	18	67.27
FS/S-477	60	24	60.00	54	20	61.81
FS/G-94	63	15	76.19	53	22	58.49
FS/G-96	60	20	66.66	61	24	60.65
FS/Rr-74	66	23	65.15	63	18	71.42
FS/Md-289	58	23	60.34	56	20	64.28
FS/R-542	61	17	72.13	55	22	60.00
FS/Th-485	62	25	59.67	58	22	65.45
FS/Th-490	60	23	61.66	60	25	58.33
FS/R-640	66	18	72.72	61	17	72.13
FS/R-641	63	16	74.60	63	27	57.14
RHS/T-438	60	18	70.00	60	22	63.33
RHS/T-600	64	23	64.06	62	24	61.29
RHS/Cd-606	63	20	68.25	55	20	63.63
RHS/T-424	62	17	72.58	57	23	59.64
RHS-Am623	61	20	67.21	63	27	57.14
RHS/P-572	60	26	56.66	57	20	64.91
RHS/B-243	63	24	61.90	64	21	67.18
RHS/G-249	57	18	68.42	61	22	63.93
RHS/S-559	68	13	80.88	67	15	77.61
RHS/S-560	65	12	81.53	64	13	79.68
RHS/Br-633	62	21	66.12	65	24	63.07
<i>Trichoderma asperellum</i>						
FS/L-118	53	18	66.03	62	24	61.29
FS/M- 363	55	22	60.00	65	18	72.30
FS/L-188	57	24	57.89	68	25	63.23
FS/Sd-478	52	25	51.92	58	20	51.72
FS/R-439	56	17	69.64	62	23	62.90
FS/Th-488	55	18	67.27	63	25	60.31
RHS/T-341	58	17	70.68	64	20	68.75
RHS/S-561	63	13	79.36	61	25	59.01
RHS/Br-634	58	18	68.96	58	26	55.17
RHS/Cd-601	62	14	77.41	63	25	60.31
RHS/Cd-604	60	23	61.66	58	20	65.51
FS/Tn-39	58	22	62.06	55	23	58.18

T.sp=*Trichoderma* spp; *Sr*= *Sclerotium rolfsii*; *Tc*=*Thanatephorus cucumeris*

Contd.....

Isolate	Colony Diam. (mm)			Colony Diam. (mm)		
	<i>T.sp</i>	<i>Sr</i>	Inhibition %	<i>T.sp</i>	<i>Tc</i>	Inhibition %
<i>Trichoderma viride</i>						
FS/L-186	55	22	66.03	57	17	70.17
FS/R-256	58	21	63.79	62	18	70.96
FS/R-426	53	22	58.49	58	22	62.06
RHS/T-584	57	25	56.14	60	24	60.00
RHS/B-245	55	26	52.72	56	18	67.85
RHS/G-251	54	22	59.25	61	16	73.77
RHS/Rd-547	51	21	58.82	56	21	62.50
RHS/Am624	52	18	65.38	58	18	68.96
FS/L 116	55	20	63.63	63	20	68.25
<i>Trichoderma erinacium</i>						
FS/Td-166	53	18	66.03	58	18	68.96
FS/Td-168	56	22	60.71	62	25	59.67
RHS/T-439	54	17	68.51	60	24	60.00
RHS/T-626	50	21	58.00	56	20	64.28
RHS/Rd-551	55	18	67.27	61	24	60.65

T. sp=*Trichoderma spp.*; *Sr*=*Sclerotium rolfisii*; *Tc*=*Thanatephorus cucumeris*

Table 15. Inhibition of mycelial growth of *S. rolfisii* and *T. cucumeris* by culture filtrate of *Trichoderma* isolates

Isolate	<i>Sclerotium rolfisii</i>		<i>Thanatephorus cucumeris</i>	
	Mycelial dry weight (g)	Inhibition %	Mycelial dry weight (g)	Inhibition %
<i>Trichoderma harzianum</i>				
Control	1.23	-	1.43	-
FS/C- 147	0.44	64.22	0.52	63.63
FS/C- 149	0.45	63.41	0.66	53.84
FS/M- 367	0.38	69.10	0.61	57.34
FS/L-358	0.54	56.09	0.63	55.94
FS/S-476	0.46	62.60	0.67	53.14
FS/S-477	0.51	57.77	0.55	61.53
FS/G-94	0.52	57.72	0.51	64.33
FS/G-96	0.55	55.28	0.64	55.24
FS/Rr-74	0.48	60.97	0.65	54.54
FS/Md-289	0.43	65.04	0.63	55.94
FS/R-542	0.42	65.85	0.56	60.83
FS/Th-485	0.57	53.65	0.45	68.53
FS/Th-490	0.46	62.60	0.48	66.43
FS/R-640	0.44	64.22	0.66	53.84
FS/R-641	0.50	59.34	0.63	55.94

Cont.....

Isolate	<i>Sclerotium rolfsii</i>		<i>Thanetophorus cucumeris</i>	
	Mycelial dry weight (g)	Inhibition %	Mycelial dry weight (g)	Inhibition %
RHS-Am-623	0.47	61.78	0.48	66.43
RHS/P-572	0.56	54.47	0.63	55.94
RHS/B-243	0.54	56.09	0.66	53.84
RHS/G-249	0.41	66.66	0.67	53.14
RHS/S-559	0.33	73.17	0.40	72.02
RHS/S-560	0.30	75.60	0.42	70.62
RHS/Br-633	0.40	67.47	0.63	55.94
<i>Trichoderma asperellum</i>				
Control	1.26	-	1.50	-
FS/L-118	0.62	50.79	0.62	58.66
FS/M-363	0.60	52.38	0.68	54.66
FS/L-188	0.58	53.96	0.60	60.00
FS/Sd-478	0.55	56.34	0.66	56.00
FS/R-439	0.50	60.31	0.57	62.00
FS/Th-488	0.57	54.76	0.55	63.33
RHS/T-341	0.53	57.93	0.63	58.00
RHS/S-561	0.38	69.84	0.51	66.00
RHS/Br-634	0.62	50.79	0.68	54.66
RHS/Cd-601	0.42	66.66	0.60	60.00
RHS/Cd-604	0.44	65.07	0.59	60.66
FS/Tn-39	0.50	60.31	0.65	56.66

4.3.3. Deposition of potential biocontrol agents to the National Agriculturally Important Culture Collection (NAIMCC)

The most potential *Trichoderma* isolates which were initially identified on the basis of their morphological and microscopical character were deposited to the National Agriculturally Important Microbial Culture Collection (NAIMCC) of National Bureau of Agriculturally Important Microorganisms (NBAIM), Maunath Bhnjan, UP, and their accession numbers have been provided in Table 16.

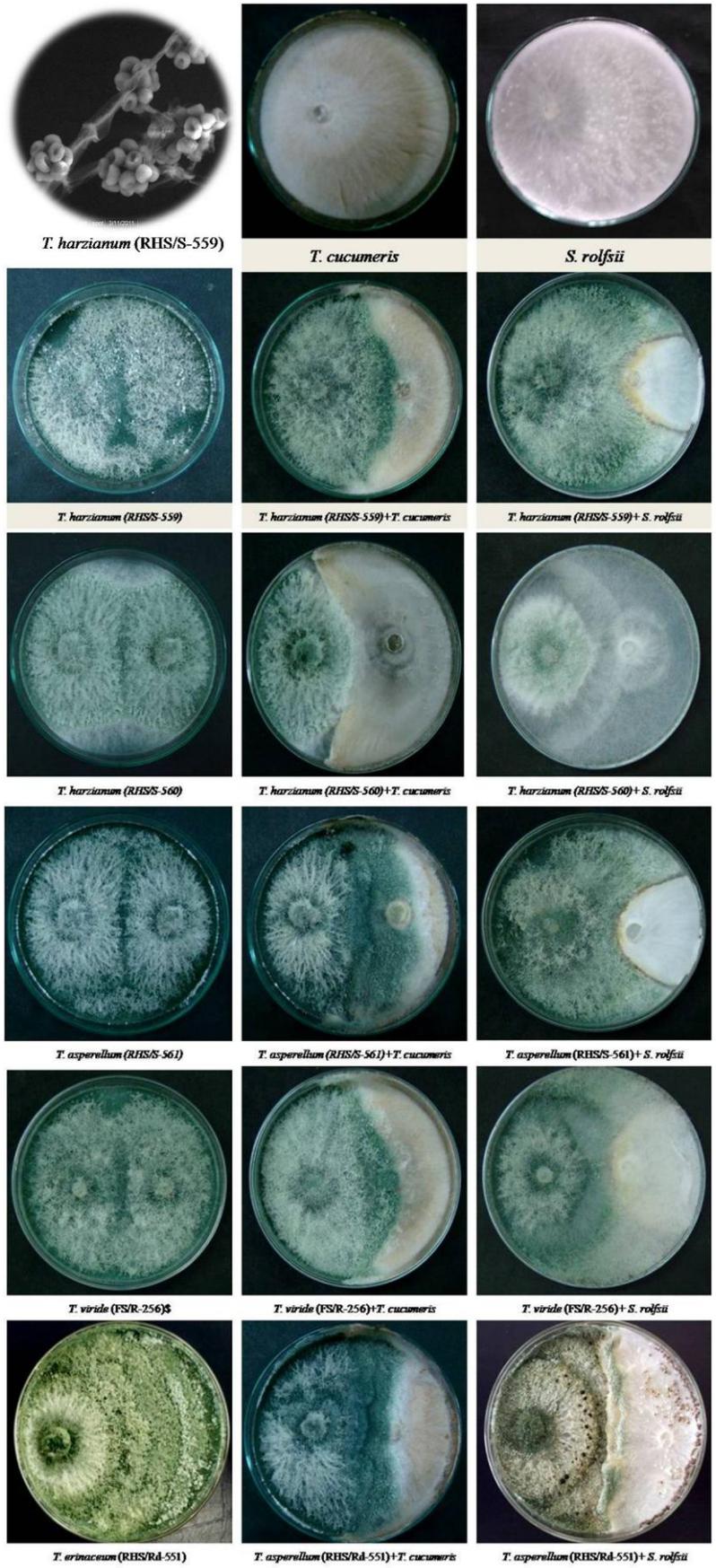


Fig. 16. Inhibition of *S. rolfsii* and *T. cucumeris* in dual plate culture assay by *Trichoderma* isolates.

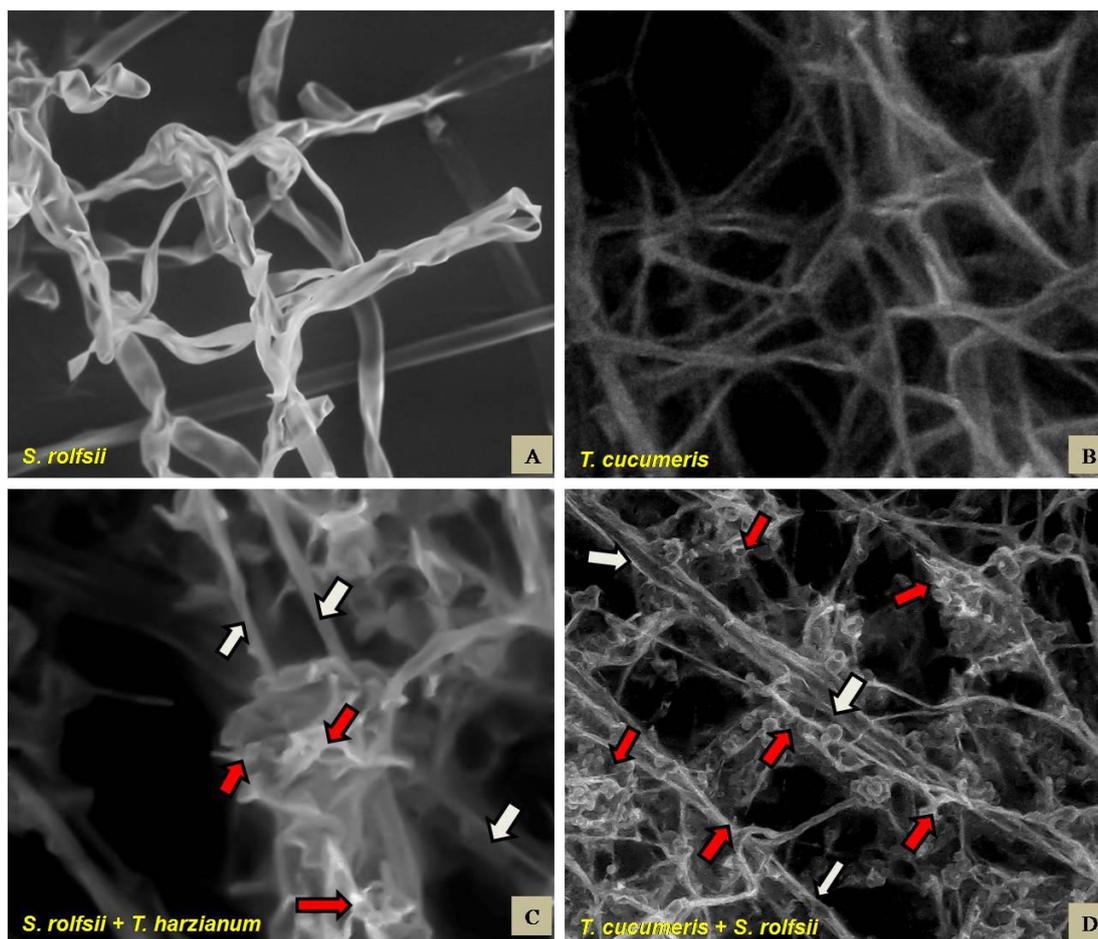


Fig. 17. Scanning Electron Micrograph of fungal hyphae showing interactions between antagonists and fungal pathogens. Control *S. rolfsii* (A); *S. rolfsii* and *T. harzianum* (C); Control *T. cucumeris* (B); *T. cucumeris* and *T. harzianum* (D). Red arrows indicate antagonists mycelium supercoiling around the mycelium of fungal pathogens and white arrows indicate pathogen mycelium.

Table 16. Potential BCA isolates deposited to NAIMCC

Isolates	Code	NAIMCC acc.no.
<i>Trichoderma erinaceum</i>	FS/L-20	NAIMCC-F-01949
<i>Trichoderma erinaceum</i>	FS/S-474	NAIMCC-F-01960
<i>Trichoderma erinaceum</i>	FS/S-475	NAIMCC-F-01953
<i>Trichoderma erinaceum</i>	FS/S-478	NAIMCC-F-01954
<i>Trichoderma harzianum</i>	RHS/S 560	NAIMCC-F-01966
<i>Trichoderma asperellum</i>	RHS/S 561	NAIMCC-F-01967
<i>Trichoderma asperellum</i>	RHS/S 559	NAIMCC-F-01968

4.4. In vitro screening of Bacterial isolates for plant growth promoting activities

4.4.1. Phosphate solubilization, HCN, Siderophore, IAA and ACC deaminase production

A total of 135 bacterial isolates were obtained from various sources of Darjeeling hills. All these isolates were primarily screened for phosphate solubilizing activity in PVK solid medium where the formation of halo zone around the bacterial colony indicated their activity (Fig. 18). Similarly all the phosphate solubilizing isolates were screened for Siderophore and HCN production. The results of this preliminary screening presented in Table 17 shows that 48 isolates solubilized phosphate, 27 isolates showed positive tests for Siderophore production and nine isolates produced HCN. Finally eight bacterial isolates (BRHS/C-1; BRHS/P-22; BRHS/R-71; BRHS/R-72; BRHS/S-7; BRHS/P-91; BRHS/P-92 and BRHS/B-104) which showed positive results for the most important PGPR characters like phosphate solubilization, Siderophore and HCN production were selected for further evaluation (Fig. 19).

4.4.2. Phosphate solubilization, IAA production and ACC deaminase activities in liquid medium

A total of 48 bacterial isolates were found to solubilize phosphate when screened on solid medium . All these isolates were then subjected to three main PGPR tests conducted in liquid broth medium. For quantification of phosphate solubilization in liquid medium, all the isolates were grown in modified PKV broth medium supplemented with Rock phosphate and Tricalcium Phosphate. The results revealed that isolate BRHS/S-73 could solubilize maximum amount of rock and tricalcium phosphate followed by BRHS/C-1, BRHS/R-71, BRHS/R-72, BRHS/P-91, BRHS/P-92 and BRHS/B-104 (Fig. 20). All these isolates were also found to produce IAA and ACC deaminase (Table 17).

4.4.3. Antagonism against fungal pathogens

All the seven bacterial isolates that showed positive result for all the tested PGPR characteristics were tested for their antifungal activities against the fungal pathogens *Sclerotium rolfsii*, *Thanatophorous cucumeris*, *Rhizoctonia solani* and *Macrophomina phaseolina*. Their interactions and percent inhibition has been presented in Table 18; Fig. 21.

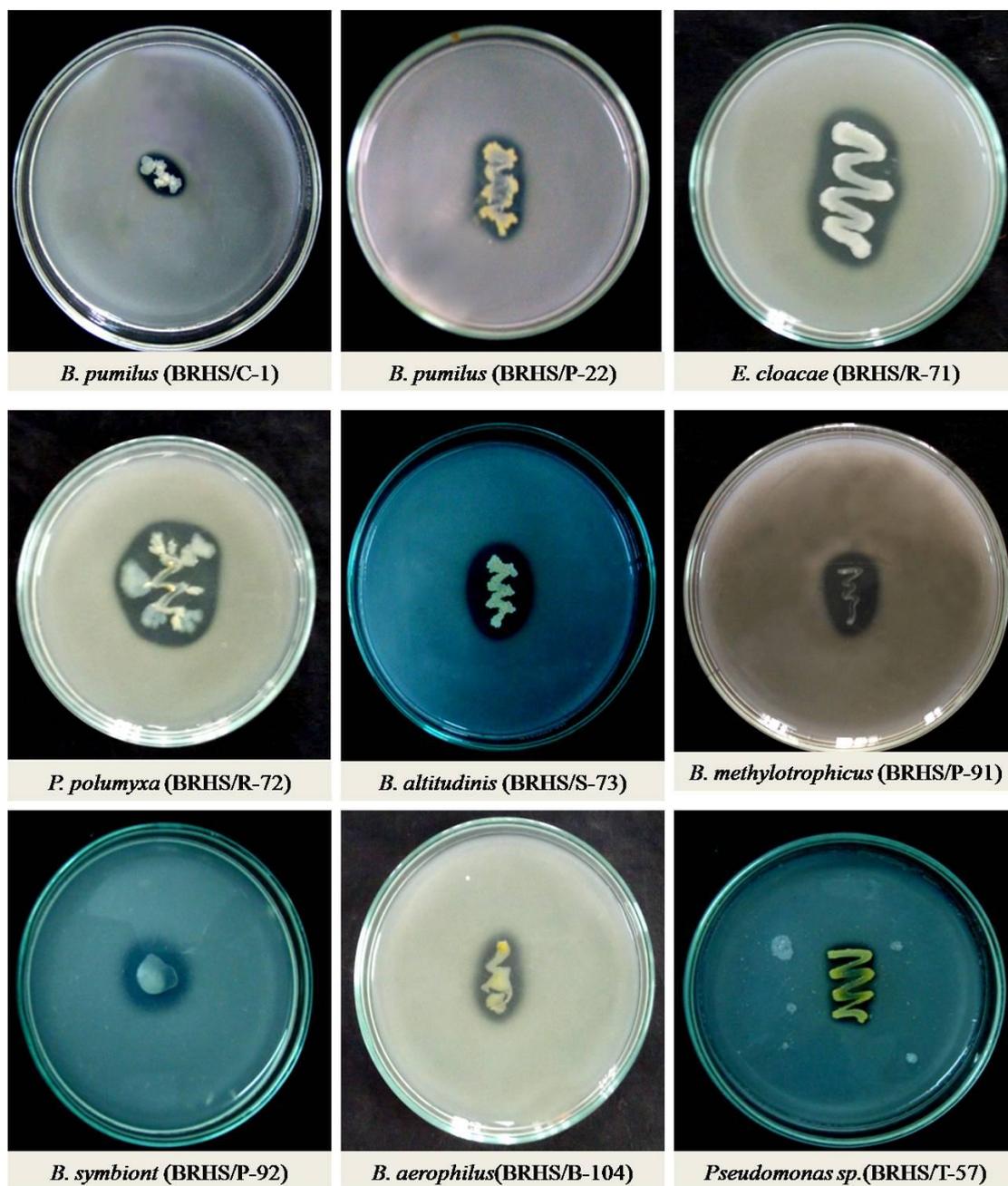


Fig. 18. Evaluation of Phosphate solubilization by bacterial isolates on PKV solid medium.

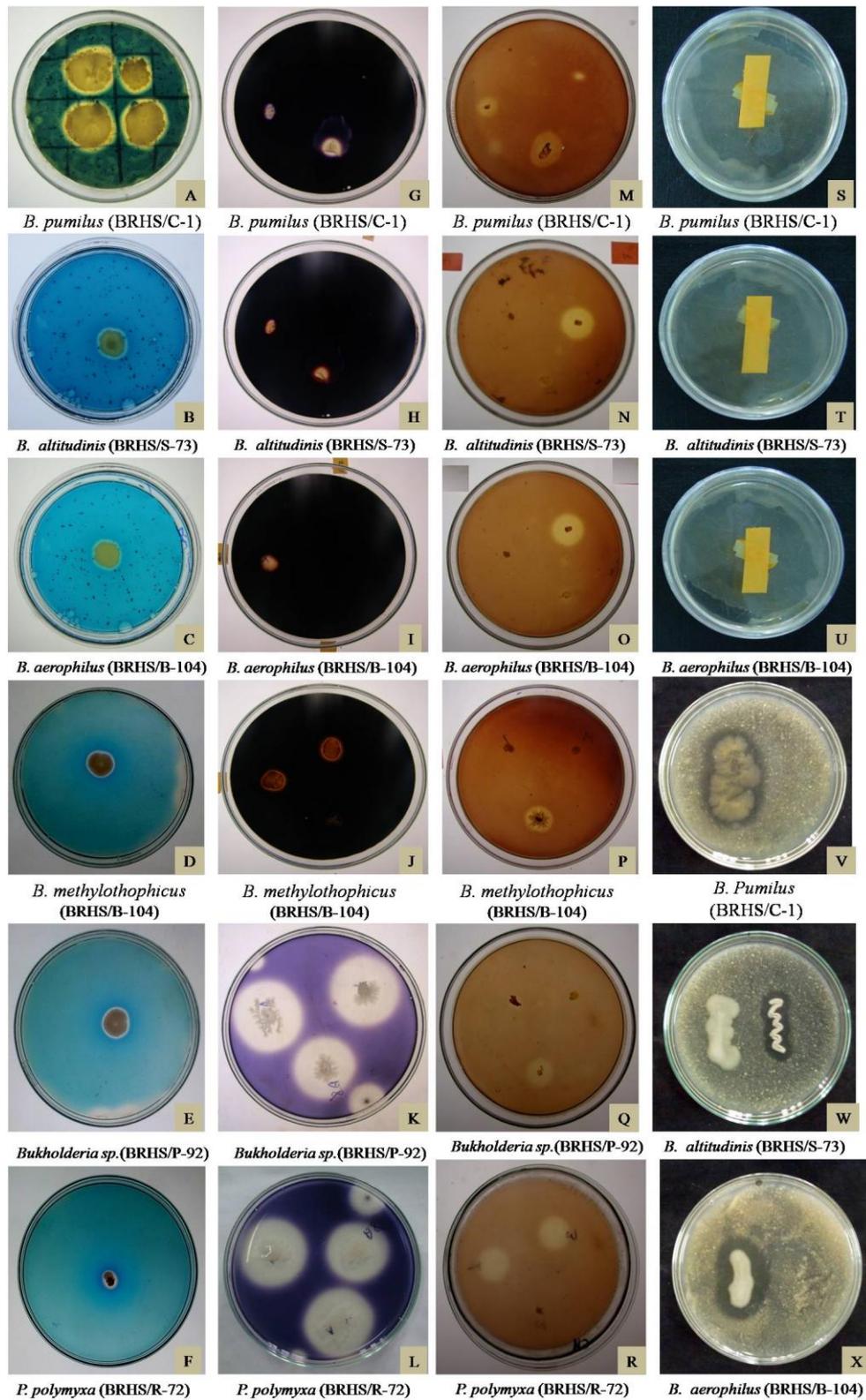


Fig. 19. Characterization of bacterial isolates for PGPR activities *in vitro*. Siderophore production (A-F); Starch hydrolysis (G-L); Chitinase production (M-R); HCN production (S-U); Protease production (V-X).

Table 17. Quantification of Phosphate solubilizing, IAA production and ACC deaminase production of bacterial isolates in modified liquid broth medium

Isolate	ACC Deaminase*	IAA (mg/L)	Phosphate solubilized (µg/ml)		Isolate	ACC Deaminase*	IAA (mg/L)	Phosphate solubilized (µg/ml)	
			TCP**	RP***				TCP**	RP***
			BRHS/C-1	20.60				24.55±2.4	765.66±11.5
BRHS/C-2	-	16.22±1.4	612.44±09.4	212.37±07.4	BRS/T-60	-	-	375.25±12.7	298.55±14.4
BRHS/C-5	-	-	429.66±10.8	317.33±10.4	BRHS/R-71	12.22	10.42±1.1	355.12±12.8	287.36±11.2
BRHS/C-6	-	-	404.66±11.0	278.33±08.4	BRHS/R-72	16.42	8.43±1.7	370.55±11.3	278.55±11.3
BRHS/M-10	-	-	416.44±12.4	308.42±07.4	BRHS/S-73	40.63	31.47±3.7	837.33±9.4	411.67±4.7
BRHS/M-17	-	18.43±1.3	630.33±12.6	383.33±12.5	BRHS/S-74	-	-	601.46±7.8	328.33±8.5
BRHS/M-18	-	12.66±1.1	655.66±13.8	354.66±12.6	BRHS/S-75	-	-	421.32±6.5	213.53±9.9
BRHS/M-19	-	-	428.22±11.6	310.44±12.5	BRHS/S-79	-	-	518.25±4.6	283.16±5.3
BRHS/M -21	-	-	513.88±10.5	203.44±10.4	BRHS/S-80	-	-	602.18±4.3	349.24±9.0
BRHS/P-22	20.20	24.22±1.5	506.29±11.4	316.98±10.8	BRHS/S-81	-	16.3±1.3	657.68±5.9	308.32±7.5
BRHS/P-25	-	16.43±1.5	605.66±12.9	426.33±13.3	BRHS/S-82	-	-	322.72±8.4	121.81±06.3
BFS/M-26	-	18.22±1.2	488.55±13.0	297.66±12.4	BRHS/S-83	-	-	259.33±6.8	125.47±04.1
BFS/M-28	-	11.43±1.1	346.33±12.1	290.33±12.8	BRHS/S-84	-	-	513.44±5.2	172.74±08.6
BFS/M-29	-	-	422.33±17.5	279.66±11.6	BRHS/S-86	-	18.27±3.2	518.25±4.7	283.16±05.3
BFS/S-34	-	-	362.66±17.8	272.33±10.7	BRHS/S-89	-	9.27±0.3	563.12±6.8	218.18±05.8
BFS/S-35	-	-	474.33±14.9	269.66±11.2	BRHS/S-90	-	14.35±1.4	606.66±5.2	324.27±07.4
BRS/Mr-37	-	-	440.58±15.3	280.33±13.4	BRHS/P-91	15.83	13.44±1.5	702.36±11.8	384.56±07.4
BRS/Mr-38	-	-	363.66±11.4	298.33±14.8	BRHS/P-92	18.62	-	714.66±12.4	345.75±08.9
BRS/Mr-39	-	-	518.22±11.3	281.55±13.7	BRHS/B-98	-	-	511.28±11.6	212.44±06.
BRS/Mr-40	-	-	445.78±10.4	235.88±11.5	BRHS/B-99	-	-	487.0±10.1	215.43±08.6
BRS/Mr-42	-	7.82±1.2	368.67±11.5	272.45±14.3	BRHS/B-104	17.14	23.44±1.5	782.33±11.4	387.65±07.6
BFS/Md-46	-	12.44±1.6	345.23±10.7	248.55±14.8	BRS/R-119	-	10.46±1.7	564.20±10.5	213.45±08.3
BFS/Md-55	-	-	312.85±13.8	326.22±13.3	BRS/R-121	-	-	463.22±10.4	255.15±06.5
BFS/Md-56	-	-	452.77±11.5	348.55±12.7	BFS/C-133	-	-	212.43±10.1	352.18±08.2

*ACC deaminase activity expressed as $-\alpha$ -ketobutyrate/ mg/h, **TCP=Tricalcium Phosphate (Total P-920 mg/L) ***RP=Rock Phosphate (Total P-592 mg/L); -= Activity not detected; Data average of three replicate experiments \pm SE

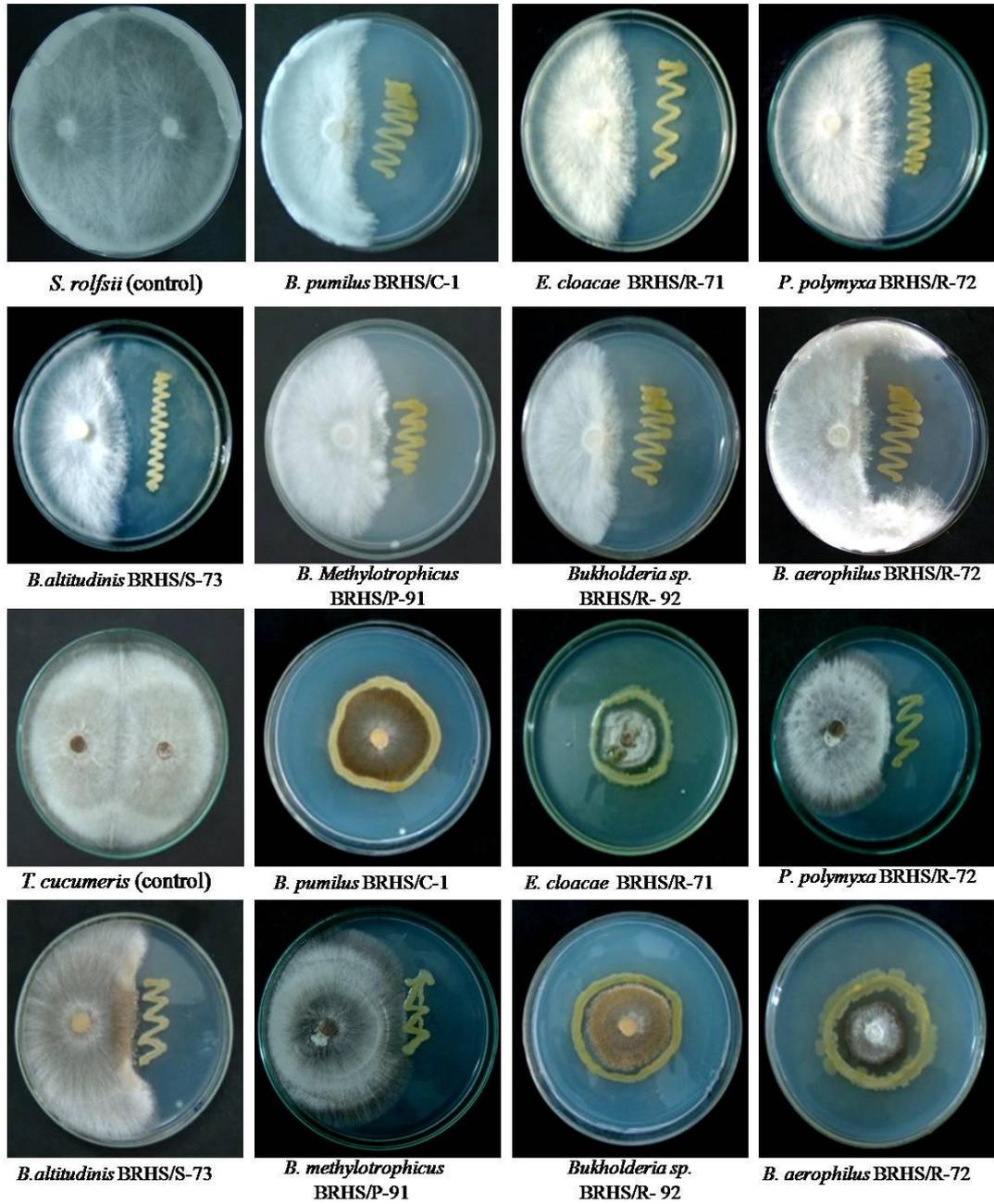


Fig. 20. *In vitro* antifungal activities of PGPR isolates tested against *S. rolfsii* and *T. cucumeris*.

Table 18. *In vitro* pairing of bacterial isolates with phyto- pathogens for evaluations of antifungal activities

Interacting microorganisms	Diameter of fungal colony after 7 days growth(cm)	% of Inhibition
<i>Sclerotium rolfii</i>	8.40±0.14	-
<i>S. rolfii</i> + <i>B. pumilus</i> (BRHS/C-1)	1.35±0.13	83.92±1.82
<i>S. rolfii</i> + <i>E. cloacae</i> (BRHS/R-71)	2.43±0.25	71.07±1.44
<i>S. rolfii</i> + <i>P. ploymyxa</i> (BRHS/R-72)	2.46±0.22	70.71±1.27
<i>S. rolfii</i> + <i>B. altitudinis</i> (BRHS/S-73)	1.80±0.12	78.50±1.40
<i>S. rolfii</i> + <i>B. methylothophilus</i> (BRHS/P-91)	2.25±0.11	73.21±1.18
<i>S. rolfii</i> + <i>Bukholderia sp.</i> (BRHS/P-92)	2.10±0.14	75.00±1.62
<i>S. rolfii</i> + <i>B. aerophilus</i> (BRHS/B-104)	2.00±0.12	76.19±1.14
<i>Thanatephorus cucumeris</i>	8.60±0.12	-
<i>T. cucumeris</i> + <i>B. pumilus</i> (BRHS/C-1)	1.90±0.15	77.90±1.16
<i>T. cucumeris</i> + <i>E. cloacae</i> (BRHS/R-71)	2.56±0.09	70.23±1.24
<i>T. cucumeris</i> + <i>P. ploymyxa</i> (BRHS/R-72)	2.33±0.11	72.90±1.22
<i>T. cucumeris</i> + <i>B. altitudinis</i> (BRHS/S-73)	2.10±0.08	75.50±1.40
<i>T. cucumeris</i> + <i>B. methylothophilus</i> (BRHS/P-91)	2.77±0.15	67.79±1.63
<i>T. cucumeris</i> + <i>Bukholderia sp.</i> (BRHS/P-92)	2.25±0.18	73.83±1.44
<i>T. cucumeris</i> + <i>B. aerophilus</i> (BRHS/B-104)	2.50±0.15	70.93±1.26
<i>Rhizoctonia solani</i>	8.50±0.11	-
<i>R. solani</i> + <i>B. pumilus</i> (BRHS/C-1)	1.73±0.13	79.64±1.33
<i>R. solani</i> + <i>E. cloacae</i> (BRHS/R-71)	3.4±0.22	60.00±1.43
<i>R. solani</i> + <i>P. ploymyxa</i> (BRHS/R-72)	3.12±0.18	63.29±1.16
<i>R. solani</i> + <i>B. altitudinis</i> (BRHS/S-73)	2.80±0.13	67.05±1.40
<i>R. solani</i> + <i>B. methylothophilus</i> (BRHS/P-91)	3.10±0.20	63.52±1.25
<i>R. solani</i> + <i>Bukholderia sp.</i> (BRHS/P-92)	2.95±0.16	65.29±1.30
<i>R. solani</i> + <i>B. aerophilus</i> (BRHS/B-104)	3.16±0.17	62.82±1.28
<i>Macrophomina phaseolina</i>	8.6±0.08	-
<i>M. phaseolina</i> + <i>B. pumilus</i> (BRHS/C-1)	1.83±0.15	78.72±1.88
<i>M. phaseolina</i> + <i>E. cloacae</i> (BRHS/R-71)	2.44±0.17	71.62±1.82
<i>M. phaseolina</i> + <i>P. ploymyxa</i> (BRHS/R-72)	2.50±0.14	70.93±1.45
<i>M. phaseolina</i> + <i>B. altitudinis</i> (BRHS/S-73)	2.20±0.08	74.40±1.30
<i>M. phaseolina</i> + <i>B. methylothophilus</i> (BRHS/P-91)	2.62±0.18	69.53±1.38
<i>M. phaseolina</i> + <i>Bukholderia sp.</i> (BRHS/P-92)	2.33±0.13	72.90±1.74
<i>M. phaseolina</i> + <i>B. aerophilus</i> (BRHS/B-104)	2.25±0.11	73.83±1.84

Values are mean of three replicate experiments.±= Standard Error.

4.5. Morphological and Scanning Electronic Microscopic studies

For morphological and scanning electron microscopic studies of the fungal isolates 7 -10 days sporulated cultures were used to study the spore type, size and ornamentation. The scanning microscopic studies of one of the most potential phosphate solubilizing and biocontrol isolate, *Talaromyces flavus* (RHS/P-51) was conducted. The SEM studies of dominant biocontrol agents like *Trichoderma*

harzianum, *T. asperellum*, *T. viride* and *T. erinaceum* were conducted apart from this, SEM studies of some potential PGPR like *Bacillus pumilus*, *B. altitudinis*, *Paenibacillus polymyxa* and *Enterobacter cloacae* were conducted to study the morphological variation among the PGPR isolates.

4.5.1. PSF isolates

Among the most phosphate solubilizing fungal isolate obtained from different sources, one isolate *T. flavus* RHS/P-51 was selected as potential PGPF. In order to identify this isolate on the basis of morphological characters, scanning electron microscopic studies were conducted. Scanning electron microscopic observation of the ascospores of *T. flavus* revealed that they are ellipsoid, 4-5 µm in length and spinulose. Spines were irregularly disposed on the surface which gives the ascospores a warty appearance (Fig. 21). Spiny wall character of ascospores is reported to be one of the identifying characters for identification for *Talaromyces flavus*.

4.5.2. BCA isolates

Scanning electron microscopic studies of the most dominant Trichoderma isolates were conducted to study the structural variations among them. The most dominant Trichoderma isolates includes, *T. harzianum*, *T. asperellum*, *T. erinaceum* and *T. viride*.

The Scanning Electron Micrographic description of the isolates are as follows:

***Trichoderma harzianum*:** Scanning Electron Microscopic examination of the conidial structure of *T. harzianum* (RHS/S-561) revealed that the conidia were borne on branched phialides. Phialides are broad at the base and slightly tapers at the apical portion bearing conidia in cluster. 6.5-6.7 µm long, 2.5-3.5µm wide at the widest point 1.6-2.5 µm at the base; supporting cell 2.4-3.6 µm. Conidia globose to sub globose in shape with a diameter 3.5 X 4.0 µm in diameter. Conidial surface smooth (Fig. 22 A & B).

***Trichoderma asperellum*:** Scanning Electron Microscopic examination of the conidial structure of *T. asperellum* (RHS/S-559) revealed that Conidiophores or phialides are fertile along their length and appear as plumes, regularly branched, with lateral branches being more or less uniformly spaced and paired, the longest branches occurring the farthest from the tip. Phialides form at the tips of branches in verticillate or 'cruciate whorls,' Phialides are straight and tend to be slightly wider in the middle than at the base. Conidia are more ovoidal and round and warty. Warts are

slightly more irregular and pyramidal and are unevenly dispersed and parts of individual conidia are smooth 1.5 X 2 µm in diameter (Fig. 22C&D).

Trichoderma erinaceum: Scanning Electron Microscopic examination of the conidial structure of *T. erinaceum* (RHS/ 475) revealed that the phialides are regularly branched and shows pyramidal arrangement. Each phialide is broadened at the base and tapers at the tip consisting smooth rounded conidia is cluster. The branching of the phialides are irregular and are distantly apart from each other. Conidia in whorls, ellipsoidal, smooth, 34.5µm X 3µm in diameter (Fig. 22 E & F).

Trichoderma viride: Scanning Electron Microscopic examination of the conidial structure of *T. viride* (RHS/ 478) revealed that the phialides that protrude from the main hyphae tend to be fertile only at the tip and are only sparingly produced along the main axis, branch irregularly, with lateral branches formed at regular intervals along the main axis or infrequently paired. Phialides form singly or in cruciate whorls. Phialides are straight, markedly sinuous or hooked and are almost cylindrical. Conidia roughly ornamented, oval, broadly rounded warts which are evenly distributed. The conidia average 4.1 x 3.4 µm in diameter (Fig. 22G&H).

4.5.3. PGPR isolates

Scanning electron microscopic characters of the most common bacterial isolates have been presented in Table 19, Fig. 23. Bacilli isolates (*B. pumilus* and *B. altitudinis*, *B. cereus*) were characteristically rod shaped whereas *E. cloacae* was rod shaped but but were smaller in length than the Bacilli isolates. Another isolate (BRHS/C-6) was round shaped and quite larger in shape.

Table 19: Scanning electron Micrographic characteristics of the bacterial isolates

Isolate	Cell shape	Cell-Wall	Cell length X Breadth/ diameter
<i>Bacillus pumilus</i> (BRHS/C-1)	Rod	Smooth	2.50 X 1.10 µm
<i>Serratia spp.</i> (BRHS/C-6)	Oval	Smooth	4.50 X 1.12 µm
<i>Bacillus cereus</i> (BRHS/M-17)	Rod	Smooth	2.20 X 1.50 µm
<i>Enterobacter cloacae</i> (BRHS/R-71)	Rod	Smooth	1.50 X 0.75 µm
<i>Paenibacillus polymyxa</i> (BRHS/R-72)	Rod	Smooth	2.20 X 1.15 µm
<i>Bacillus altitudinis</i> (BRHS/S-73)	Rod	Smooth	2.75 X 1.10 µm

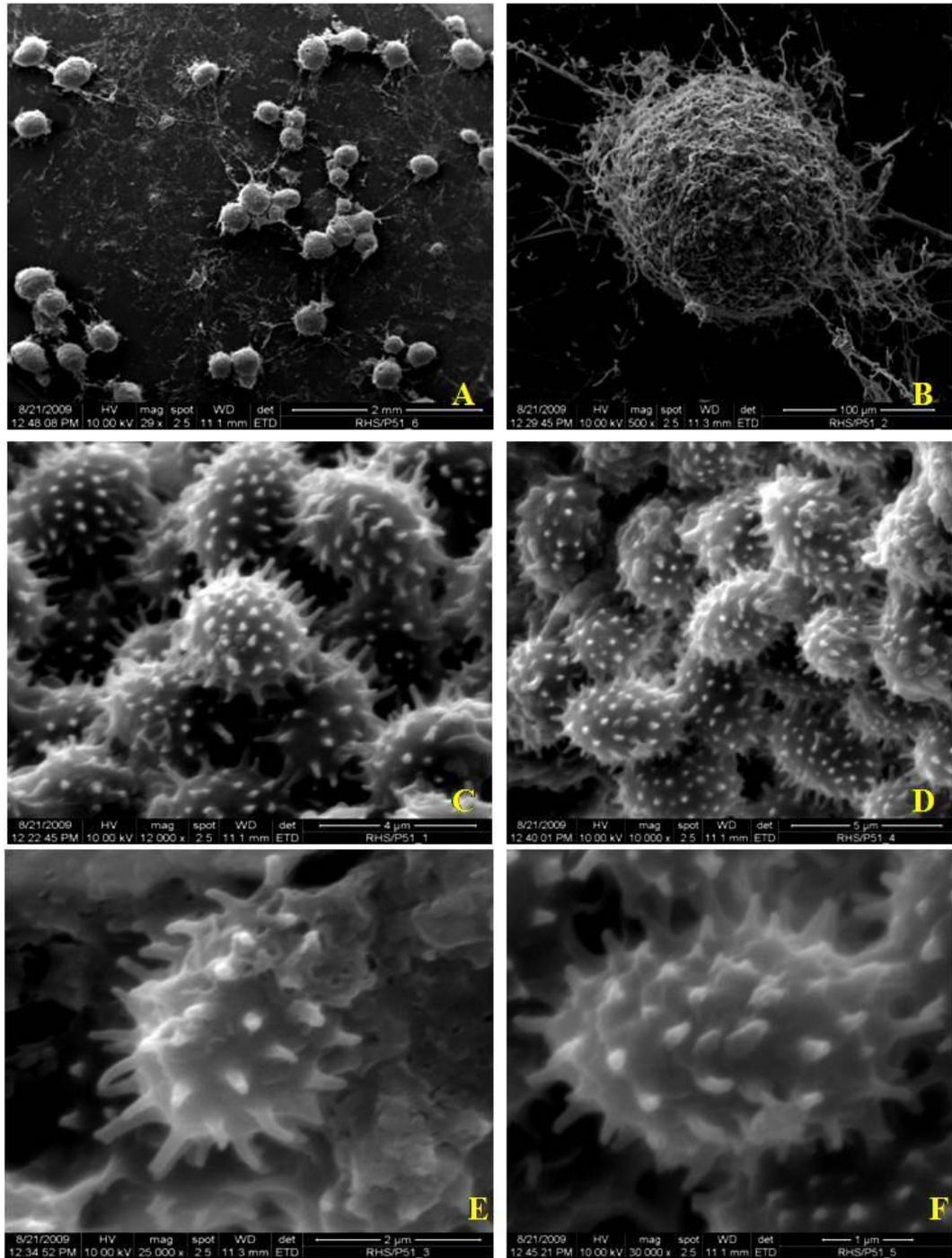


Fig.. 21. Scanning Electron Micrograph of ascospores of *Talaromyces flavus* (RHS/P-51). Oval Ascus [29 X] (A); Magnified view of the ascus showing thick warty outer wall [x 500 X] (B); Ascospores in cluster showing spiny ornamentation on the outer wall [10000-12000 X] (C&D); Single ascospores showing spiny outgrowths on the surface 2-5 μ m in size (E&F).

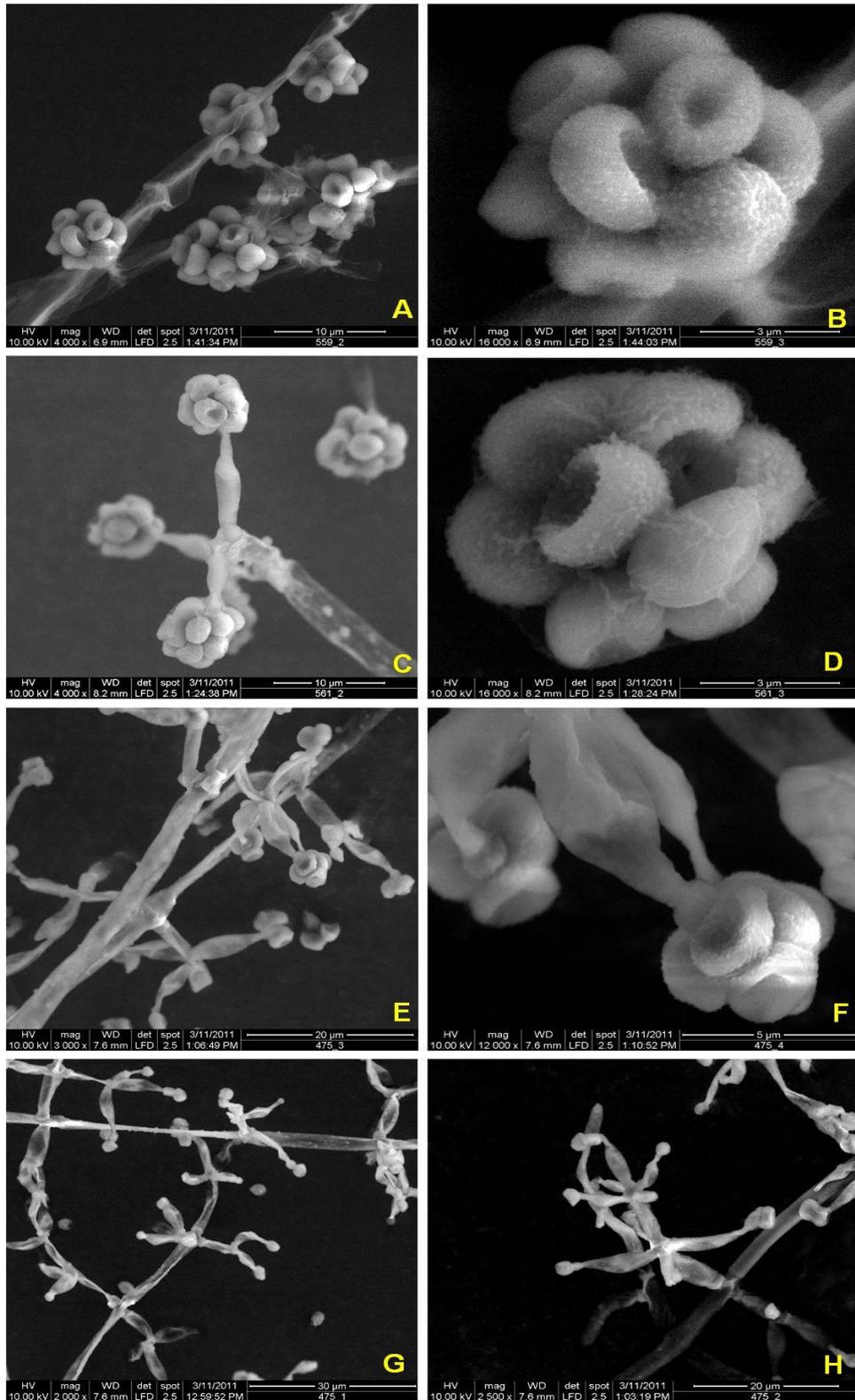


Fig. 22. Scanning electron micrograph of conidiophores and conidia of different *Trichoderma* isolates. *T. harzianum* (RHS/S-559) (A&B); *T. asperellum* (RHS/S-561) (C&D); *T. erinaceum* (RHS/475) (E&F); *T. viride* (RHS/478) (G&H).

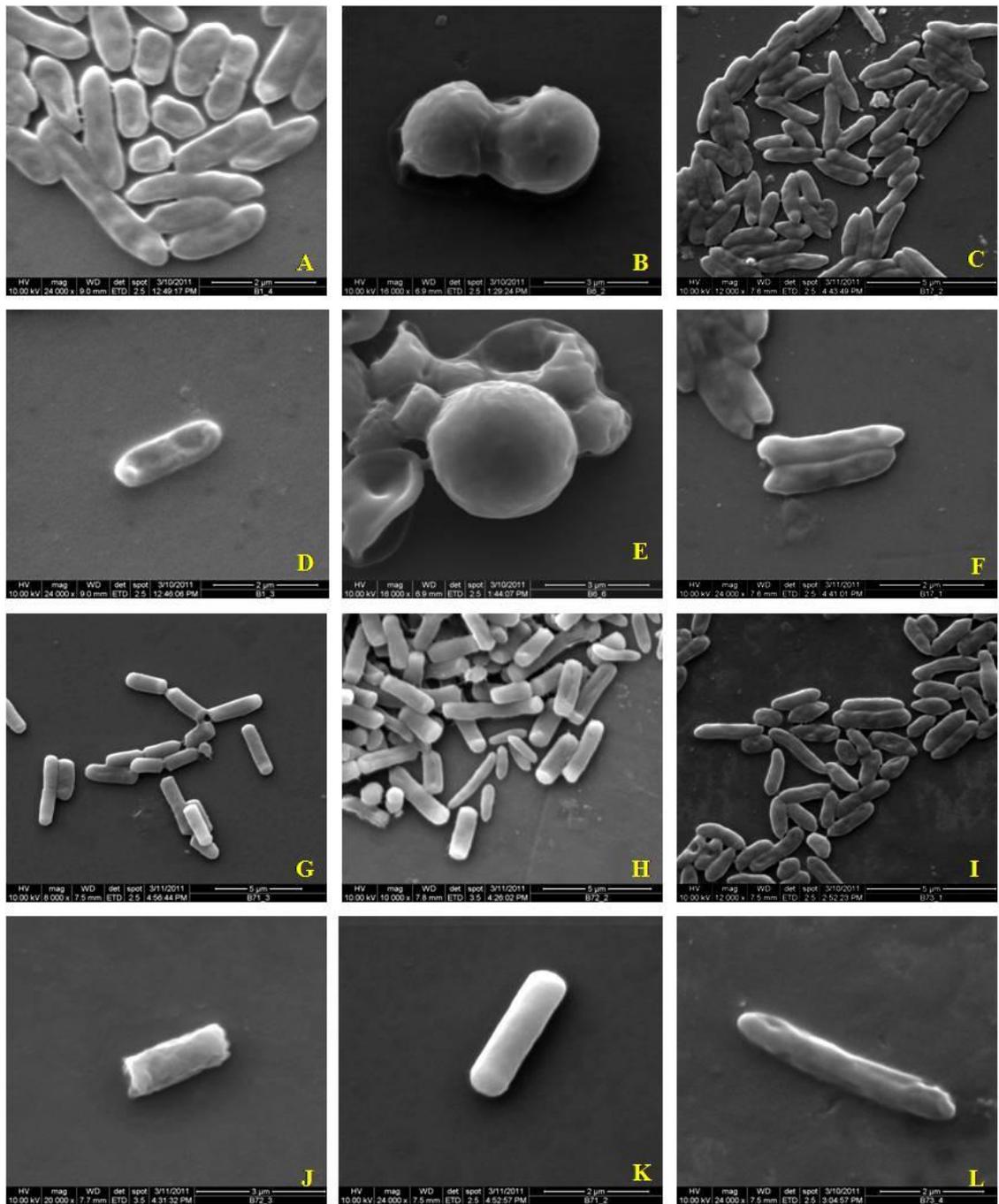


Fig. 23. Scanning Electron Micrograph of bacterial isolates obtained from various sources. *Bacillus pumilus* (BRHS/C-1) (A&D); *Staphylococcus* sp. (BRHS/C-6) (B&E); *B. cereus* (BRHS/M-17) (C&F); *Enterobacter cloacae* (BRHS/R-71) (G&J); *Paenibacillus polymyxa* (BRHS/R-72) (H&K); *B. altitudinis* (BRHS/S-73) (I&L).

4.6. Genomic DNA analysis (Agarose gel and Spectrophotometric) for PCR

4.6.1. PSF isolates

Among the total collection of phosphate solubilizers, four isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) were found to solubilize rock phosphate and tricalcium phosphate more efficiently than rest of the others. Genetic diversity among these phosphate solubilizers were assessed using RAPD markers for which genomic DNA from them were isolated and quantified both with the help of agarose gel electrophoresis as well as spectrophotometric analysis.

When DNA quality of PSF isolates were evaluated by 0.8% agarose gel electrophoresis it was visualized as a clear sharp band. The size of DNA of each isolates were ranging from 1.5-1.8 Kb when compared with the wide range molecular weight marker loaded parallel in the adjoining lane. (Fig. 24, A).

The purity of genomic DNA samples of all fifteen PSF isolates was determined by the ratio of absorbance at A_{260} and A_{280} . The results of the spectrophotometric analysis have been presented in in Table 20 .

4.6.2. BCA isolates

Among the total collection of Biocontrol isolates obtained from Darjeeling hill region, *T. harzianum* (RHS/S 560, RHS/S 561, RHS/M 501, RHS/M 511,) *T. asperellum* (RHS/S 559, RHS/M 512, RHS/M 517) showed highest inhibition against the tehese isolatsted fungal pathogens. When the DNA quality of these *Trichoderma* isolates were evaluated by 0.8% agarose gel electrophoresis it was visualized as a clear sharp band. The size of DNA of each isolates were ranging from 1.5-1.8 Kb when compared with the wide range molecular weight marker loaded parallel in the adjoining lane. (Fig. 24, B). The purity of genomic DNA samples of all fifteen PSF isolates was determined by the ratio of absorbance at A_{260} and A_{280} . The results of the spectrophotometric analysis have been presented in in Table 20.

Table 20. Spectrophotometrical A_{260}/A_{280} ratio of genomic DNA of PSF and BCA isolates

Organism	Isolate nos.	A_{260}	A_{280}	A_{260}/A_{280}
<i>Aspergillus niger</i>	FS/L-04	0.227	0.134	1.694
	RS/P-14	0.216	0.112	1.928
	FS/L-40	0.195	0.098	1.989
	FS/C-140	0.213	0.114	1.868
<i>Aspergillus melleus</i>	RHS/R 12	0.183	0.105	1.742
	FS/L 13	0.231	0.116	1.991
	FS/L 17	0.216	0.097	2.226
	FS/L 18	0.173	0.093	1.860
<i>A. clavatus</i>	RHS/P 38	0.240	0.124	1.935
	RHS/P-114	0.216	0.118	1.830
	RHS/T-99	0.195	0.106	1.839
<i>Talaromuces flavus</i>	RHS/P 50	0.216	0.110	1.963
	RHS/P 51	0.285	0.152	1.875
	RHS/P 54	0.244	0.126	1.936
	RHS/P 120	0.253	0.127	1.992
<i>Trichoderma harzianum</i>	RHS/S 559	0.185	0.097	1.907
	RHS/S 560	0.228	0.125	1.824
	RHS/M 501	0.249	0.152	1.638
<i>Trichoderma asperellum</i>	RHS/M 511	0.188	0.102	1.843
	RHS/S 561	0.217	0.116	1.870
	RHS/M 512	0.184	0.098	1.877
	RHS/M-517	0.192	0.114	1.684

4.6.3. Bacterial isolates

Genomic DNA of all the 135 bacterial isolates were isolated for analysis. The Genomic DNA was then purified and re-suspended in 1X TAE buffer until further use. Agarose gel electrophoresis of the Genomic DNA revealed that they were free of RNA and ranged between 1.2-1.8 Kb when compared with the wide range molecular weight marker loaded parallel in the adjoining lane. (Fig. 25). The purity of genomic DNA samples of all fifteen PSF isolates was determined by the ratio of absorbance at A_{260} and A_{280} . The results of the spectrophotometric analysis have been presented in in Table 21.

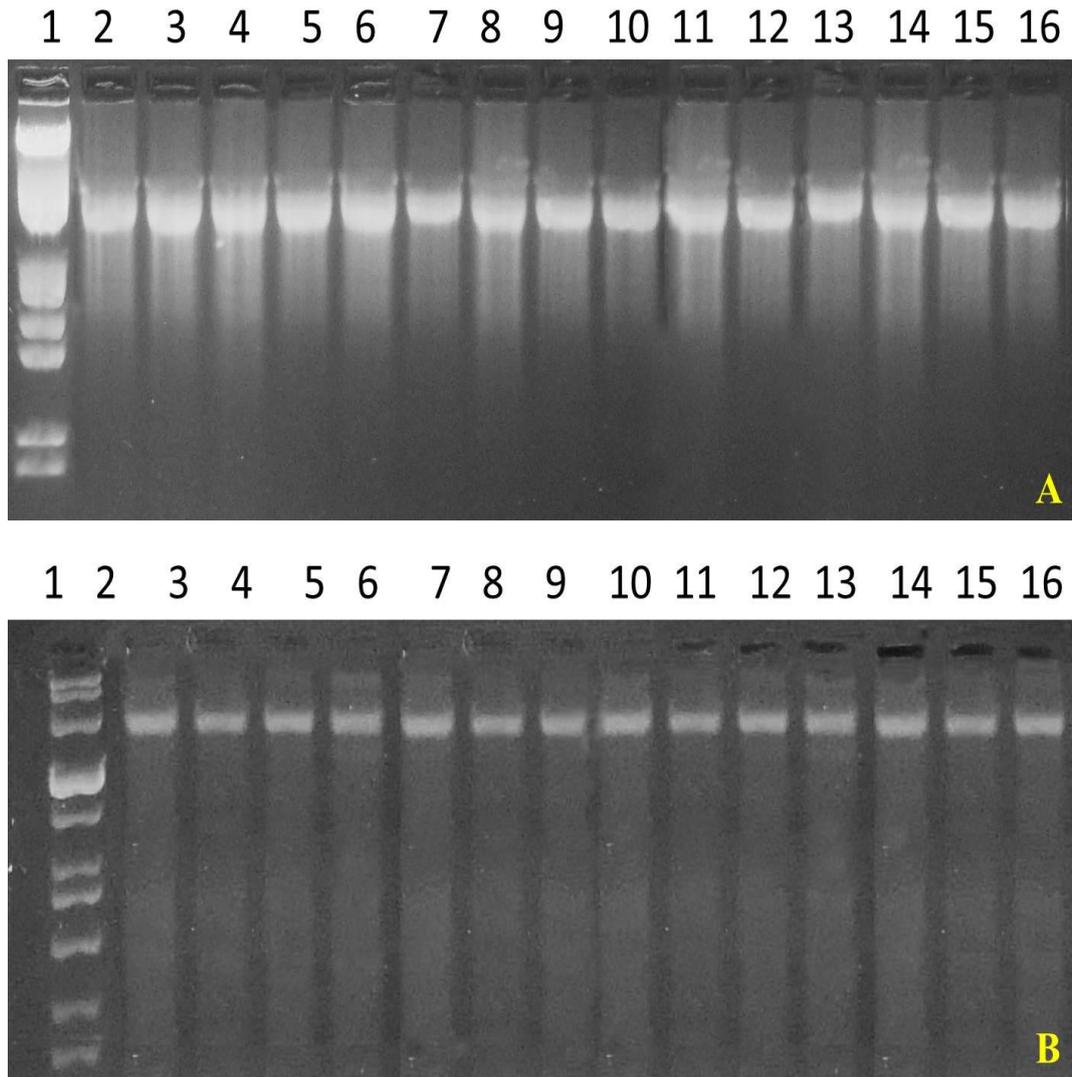


Fig. 24. Agarose gel electrophoresis of Genomic DNA of fungal isolates. (A) PSF isolates lane 1. Wide range Molecular weight marker. 2- *Aspergillus niger* FS/L-04 3- *A. niger* RS/P14, 4- *A. niger* FS/L-40, 5- *A. niger* FS/C-140; 6- *A. melleus* RHS/R 12, 7- *A. melleus* FS/L 13, 8- *A.melleus* FS/L 17, 9-*A. melleus* FS/L 18; 10-*A. clavatus* RHS/P 38,11-*A. clavatus* RHS/P-114, 12-*A. clavatus* RHS/T-99; 13-*Talaromyces flavus* RHS/P 50,14-*T. flavus* RHS/P 51, 15-*T. flavus* RHS/P 54 and 16-*T. flavus* RHS/P 120. (B) *Trichoderma* isolates. Lane 1-wide range DNA marker, Lane 2&3: *T. harzianum* (RHS/S 560) , 4&5- *T. harzianum* (RHS/S 561) ,6&7- *T. asperellum* (RHS/S 559) , 8&9-*T. harzianum* (RHS/M 501) , 10&11-- *T. harzianum* (RHS/M 511), 13&13- *T. asperellum* (RHS/M 512) , 14&15- *T. asperellum* (RHS/M 517).

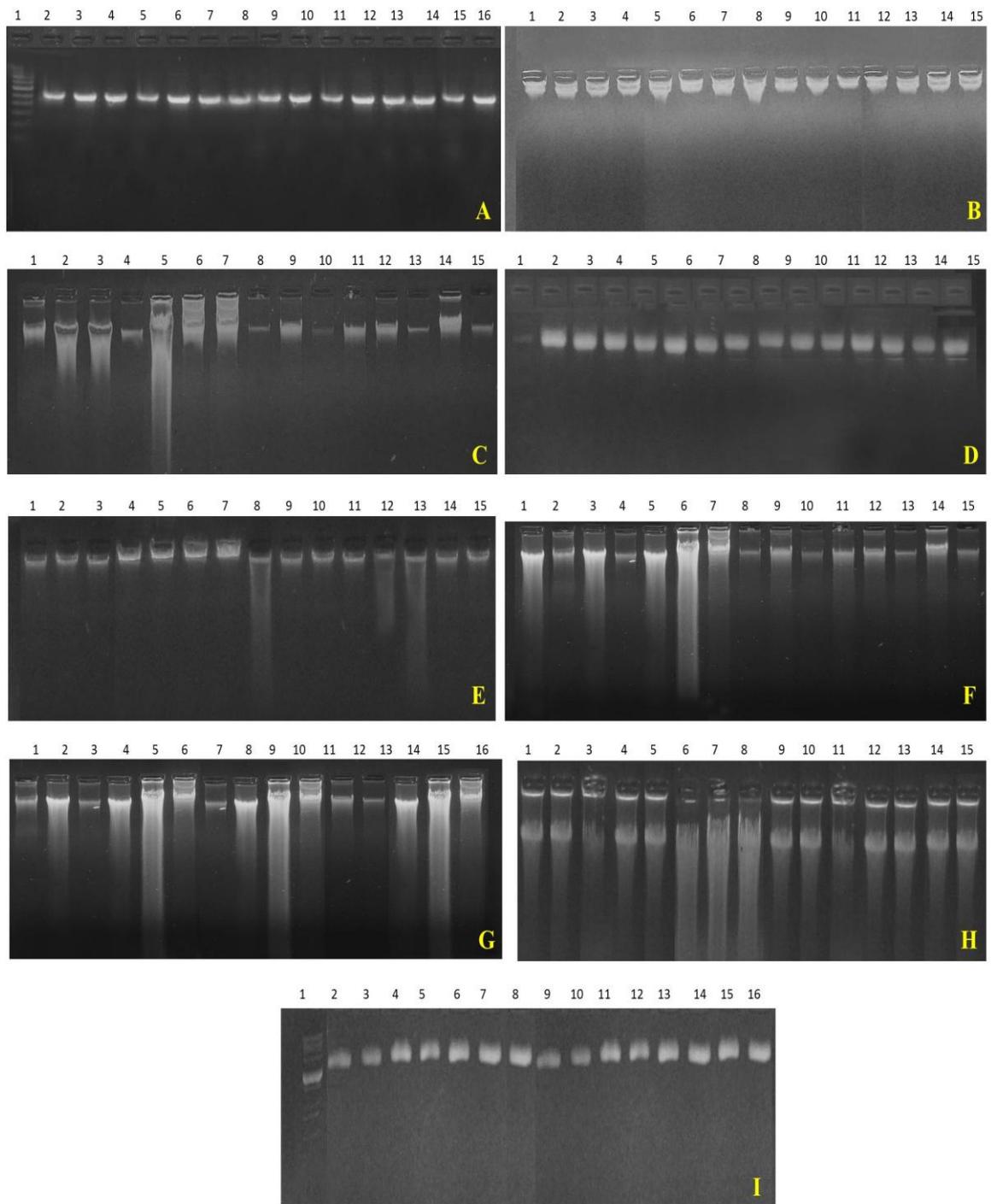


Fig. 25. Agarose Gel electrophoresis Genomic DNA of all the 135 Bacterial isolates obtained from various sources.

Table 21. Spectrophotometrical A₂₆₀/A₂₈₀ ratio of Bacterial genomic DNA

Isolate	A260	A280	A260/280	Isolate	A260	A280	A260/280
BRHS/C-1	0.224	0.115	1.947	BFS/Md-46	0.194	0.103	1.883
BRHS/C -2	0.212	0.119	1.781	BFS/Md-47	0.223	0.116	1.922
BRHS/C -3	0.183	0.098	1.867	BFS/Md-48	0.214	0.121	1.768
BRHS/C -4	0.192	0.095	2.021	BFS/Md-49	0.244	0.128	1.906
BRHS/C -5	0.197	0.112	1.758	BFS/Md-50	0.188	0.122	1.540
BRHS/C -6	0.188	0.103	1.825	BFS/Md-51	0.194	0.133	1.458
BRHS/C -7	0.212	0.114	1.859	BFS/Md-52	0.197	0.108	1.824
BRHS/M-8	0.227	0.125	1.816	BFS/Md-53	0.221	0.114	1.938
BRHS/M -9	0.216	0.116	1.836	BFS/Md-54	0.243	0.124	1.959
BRHS/M -10	0.256	0.138	1.855	BFS/Md-55	0.254	0.132	1.924
BRHS/M -11	0.224	0.134	1.671	BFS/Md-56	0.193	0.102	1.892
BRHS/M -12	0.198	0.105	1.885	BFS/Md-57	0.182	0.095	1.915
BRHS/M -13	0.195	0.102	1.911	BRS/T-58	0.204	0.11	1.854
BRHS/M -14	0.187	0.123	1.52	BRS/T-59	0.265	0.115	2.304
BRHS/M -15	0.225	0.116	1.939	BRS/T-60	0.272	0.138	1.971
BRHS/M -16	0.216	0.115	1.878	BRS/T-61	0.263	0.143	1.839
BRHS/M -17	0.189	0.095	1.989	BRS/T-62	0.232	0.134	1.731
BRHS/M -18	0.244	0.143	1.706	BRHS/R-63	0.197	0.114	1.728
BRHS/M -19	0.256	0.125	2.048	BRHS/R-64	0.198	0.114	1.736
BRHS/M -20	0.21	0.124	1.693	BRHS/R-65	0.231	0.133	1.736
BRHS/M -21	0.204	0.107	1.906	BRHS/R-66	0.268	0.138	1.942
BRHS/P-22	0.188	0.095	1.978	BRHS/R-67	0.21	0.113	1.858
BRHS/P -23	0.195	0.114	1.710	BRHS/R-68	0.253	0.134	1.888
BRHS/P -24	0.226	0.125	1.808	BRHS/R-69	0.194	0.117	1.658
BRHS/P -25	0.224	0.113	1.982	BRHS/R-70	0.188	0.11	1.709
BFS/M-26	0.218	0.124	1.758	BRHS/R-71	0.219	0.133	1.646
BFS/M-27	0.232	0.117	1.982	BRHS/R-72	0.224	0.124	1.806
BFS/M-28	0.199	0.103	1.932	BRHS/S-73	0.243	0.125	1.944
BFS/M-29	0.194	0.11	1.763	BRHS/S-74	0.211	0.126	1.674
BFS/M-30	0.198	0.112	1.767	BRHS/S-75	0.238	0.122	1.950
BFS/S- 31	0.195	0.108	1.805	BRHS/S-76	0.183	0.114	1.605
BFS/S-32	0.262	0.137	1.912	BRHS/S-77	0.199	0.116	1.715
BFS/S-33	0.264	0.141	1.872	BRHS/S-78	0.232	0.121	1.917
BFS/S-34	0.241	0.142	1.697	BRHS/S-79	0.211	0.128	1.648
BFS/S-35	0.195	0.113	1.725	BRHS/S-80	0.239	0.124	1.927
BFS/S-36	0.193	0.11	1.754	BRHS/S-81	0.198	0.103	1.922
BRS/Mr-37	0.274	0.138	1.985	BRHS/S-82	0.197	0.104	1.894
BRS/Mr-38	0.21	0.132	1.590	BRHS/S-83	0.216	0.155	1.393
BRS/Mr-39	0.207	0.124	1.669	BRHS/S-84	0.254	0.136	1.867
BRS/Mr-40	0.185	0.097	1.907	BRHS/S-85	0.211	0.128	1.648
BRS/Mr-41	0.18	0.095	1.894	BRHS/S-86	0.234	0.129	1.813
BRS/Mr-42	0.227	0.115	1.973	BRHS/S-87	0.217	0.152	1.427
BRS/Mr-43	0.265	0.143	1.853	BRHS/S-88	0.264	0.118	2.237
BRS/Mr-44	0.231	0.135	1.711	BRHS/S-89	0.199	0.107	1.859
BRS/Mr-45	0.209	0.118	1.771	BRHS/P-90	0.194	0.098	1.979

Contd.....

Isolate	A260	A280	A260/280	Isolate	A260	A280	A260/280
BRHS/P-91	0.198	0.104	1.903	BRHS/Cd-114	0.193	0.104	1.855
BRHS/P-92	0.211	0.114	1.850	BRHS/Cd-115	0.197	0.11	1.790
FS/T-93	0.243	0.125	1.944	BRHS/Cd-116	0.215	0.117	1.837
FS/T-94	0.187	0.094	1.989	BRHS/Cd-117	0.223	0.122	1.827
FS/T-95	0.216	0.118	1.830	BRS/R-118	0.227	0.126	1.801
FS/T-96	0.224	0.125	1.792	BRS/R-119	0.238	0.131	1.816
FS/T-97	0.265	0.139	1.906	BRS/R-120	0.183	0.095	1.926
BRHS/B-98	0.218	0.132	1.651	BRS/R-121	0.218	0.116	1.879
BRHS/B-99	0.193	0.114	1.692	BRS/R-122	0.197	0.114	1.728
BRHS/B-100	0.182	0.094	1.936	BRS/R-123	0.207	0.118	1.754
BRHS/B-101	0.194	0.094	2.063	BRS/R-124	0.241	0.142	1.697
BRHS/B-102	0.215	0.127	1.692	BRS/R-125	0.182	0.111	1.639
BRHS/B-103	0.263	0.138	1.905	BFS/C-126	0.194	0.114	1.701
BRHS/B-104	0.197	0.114	1.728	BFS/C-127	0.263	0.148	1.777
BRHS/B-105	0.208	0.119	1.747	BFS/C-128	0.245	0.135	1.814
BRHS/B-106	0.193	0.105	1.838	BFS/C-129	0.233	0.136	1.713
BRHS/B-107	0.237	0.143	1.657	BFS/C-130	0.217	0.116	1.870
BRHS/Cd-108	0.262	0.137	1.912	BFS/C-131	0.192	0.097	1.979
BRHS/Cd-109	0.195	0.163	1.196	BFS/C-132	0.187	0.104	1.798
BRHS/Cd-110	0.188	0.112	1.678	BFS/C-133	0.183	0.117	1.564
BRHS/Cd-111	0.265	0.156	1.698	BFS/C-134	0.267	0.145	1.841
BRHS/Cd-112	0.255	0.133	1.917	BFS/C-135	0.254	0.133	1.909
BRHS/Cd-113	0.243	0.142	1.711				

4.7. Random Amplified Polymorphic DNA (RAPD) based genetic diversity analysis

4.7.1. RAPD based diversity analysis of PSF isolates

Genetic relatedness among the phosphate solubilizing isolates *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) was assessed using random deca mer primers. Initially for optimization of DNA concentrations, 5 ng, 10 ng, 15 ng and 20 ng per 25 µl reaction mixture were used. Among these tested concentrations, 20 ng /25 µl reaction mixtures gave the best amplification. Lower or higher concentrations either reduced amplification of produced smearing, respectively. Therefore, in all the subsequent experiments 20 ng temFig. DNA concentration was constantly used.

Altogether 6 random decamer (10 bp) oligonucleotide sequences (primers) were initially screened for determining polymorphism among the PSF isolates to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns. RAPD profiles showed that primer OPD- 5 scored highest bands which ranged between 100bp to 2000bp (Table 22, Fig. 26).

Table 22. Total number of polymorphic bands produced by different primers (PSF)

Primer	No. of RAPD products (bands)		
	Total bands	Polymorphic bands	% polymorphism
OPA-4	8	2	25.00
AA-11	7	3	42.85
AA-05	9	4	44.45
OPD-06	5	2	40.00
OPD-02	8	4	50.00
OPD-05	8	5	62.50

4.7.1.1. Analysis of polymorphism

Out of the 30 loci scored only 12 (40 %) were polymorphic. The size of the bands varied from 200 to 3000 bp. However, the primers differed in their capacity to detect polymorphism. Highest level of polymorphism was recorded in primer OPD-5 (75.00 %) followed by OPB-2 (62.50 %), OPD-6 (40.00 %) and AA-5 (26%) (Table 23).

4.7.1.2. Analysis of Genetic Similarity values

The analysis of Similarity co-efficient reveals that few of the isolates showed highest degree of similarity. The highest degree of similarity between *A. niger* isolates (FS/L-04, RS/P-14 and FS/L-40) was found to be as high as 75 % and that between *T. flavus* isolates (RHS/P-50 and RHS/P-51) was also found to be as high as 75 %. (*i.e.*, moderately low degree of dissimilarity value). On the other hand, *A. niger* and *A. melleus* isolates showed low similarity value of 09.90%, (*i.e.*, moderately high degree of dissimilarity value). The degree of similarity between *T. flavus* and *Aspergillus* isolates ranged from 14.00 % to 22 % (Moderate dissimilar values). The overall result of the data computed in 1/0 matrix showed that the similarity coefficient among the *A. niger* groups ranged from 0.75-1, between *A. niger* group and *A. melleus* group it ranges from 0.09-1, between *A. niger* group and *A. clavatus* group it ranged from 0.30 to 1 and between *A. niger* group and *T. flavus* group it ranged between 0.16-1. (Table 24).

4.7.1.3. Dendrogram Construction, PCA analysis, 2D and 3D plot

A dendrogram was constructed on the basis of shared fragments which shows broadly four major clusters. Cluster-I was linked to Cluster-II with low similarity

coefficient of about 39%. Cluster-II was linked to Cluster- III with a similarity coefficient of about 22% and cluster-III was linked with Cluster- IV with a similarity coefficient of about 19 %. Cluster-I contains *A. niger* isolates Cluster- II contains *A. clavatus* isolates, Cluster- III contains *A. melleus* isolates and Cluster IV contains *T. flaus* isolates. Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software which further showed the degree of similarities among different clusters and the representative organisms in each cluster (Fig. 27, A-C).

Table 23. Determination of polymorphisms based on the RAPD banding patterns

	Primers				Total	% Polymorphism
	AA-5	OPD-5	OPD-6	OPB-2		
Total No. of RAPD products	9	8	5	8	30	-
Total No. of polymorphic bands	2 (25.00)	6 (75.00)	2 (40.00)	5 (62.50)	12	40.00
<i>A. niger</i>	AA-5	OPD-5	OPD-6	OPB-2	Total	% Polymorphism
FS/L-04	2	2	1	2	7	23
RS/P-14	2	2	1	2	7	23
FS/L-40	1	2	1	2	6	20
FS/C-140	1	1	1	2	5	16
<i>A. melleus</i>						
RHS/R-12	2	2	2	2	8	26
FS/L-13	2	2	2	3	9	30
FS/L-17	2	3	2	3	10	33
FS/L-18	4	3	2	3	12	40
<i>A. clavatus</i>						
RHS/P-38	2	1	1	2	6	20
RHS/P-114	2	1	1	2	6	20
RHS/T-99	3	1	1	2	7	23
<i>T. flaus</i>						
RHS/P- 50	1	3	2	1	7	23
RHS/P- 51	1	3	2	1	7	23
RHS/P- 54	1	2	2	1	6	20
RHS/P-120	1	2	2	1	6	20

Table 24. Genetic similarity matrix, obtained as the result of Simqual analysis of the RAPD bands

	FS/L-04	RS/P-14	FS/L-40	FS/C-140	RHS/R-12	FS/L-13	FS/L-17	FS/L-18	RHS/P-38	RHS/P-99	RHS/T-114	RHS/P-50	RHS/P-51	RHS/P-54	RHS/P-120
FS/L-04	1.0000000														
RS/P-14	1.0000000	1.0000000													
FS/L-40	0.7500000	0.7500000	1.0000000												
FS/C-140	0.7500000	0.7500000	1.0000000	1.0000000											
RHS/R-12	0.1000000	0.1000000	0.0909091	0.0909091	1.0000000										
FS/L-13	0.1000000	0.1000000	0.0909091	0.0909091	1.0000000	1.0000000									
FS/L-17	0.1000000	0.1000000	0.0909091	0.0909091	1.0000000	1.0000000	1.0000000								
FS/L-18	0.1000000	0.1000000	0.0909091	0.0909091	1.0000000	1.0000000	1.0000000	1.0000000							
RHS/P-38	0.3333333	0.3333333	0.5000000	0.5000000	0.3000000	0.3000000	0.3000000	0.3000000	1.0000000						
RHS/P-99	0.3333333	0.3333333	0.5000000	0.5000000	0.3000000	0.3000000	0.3000000	0.3000000	1.0000000	1.0000000					
RHS/T-114	0.3333333	0.3333333	0.5000000	0.5000000	0.3000000	0.3000000	0.3000000	0.3000000	1.0000000	1.0000000	1.0000000				
RHS/P-50	0.1666667	0.1666667	0.3333333	0.3333333	0.2000000	0.2000000	0.2000000	0.2000000	0.2857143	0.2857143	0.2857143	1.0000000			
RHS/P-51	0.1666667	0.1666667	0.3333333	0.3333333	0.2000000	0.2000000	0.2000000	0.2000000	0.2857143	0.2857143	0.2857143	1.0000000	1.0000000		
RHS/P-54	0.2000000	0.2000000	0.1666667	0.1666667	0.2222222	0.2222222	0.2222222	0.2222222	0.1428571	0.1428571	0.1428571	0.7500000	0.7500000	1.0000000	
RHS/P-120	0.2000000	0.2000000	0.1666667	0.1666667	0.2222222	0.2222222	0.2222222	0.2222222	0.1428571	0.1428571	0.1428571	0.7500000	0.7500000	1.0000000	1.0000000

" SIMQUAL; coeff=J; " by Cols, += 1.00000, -= 0.00000: 3 15L 15 0

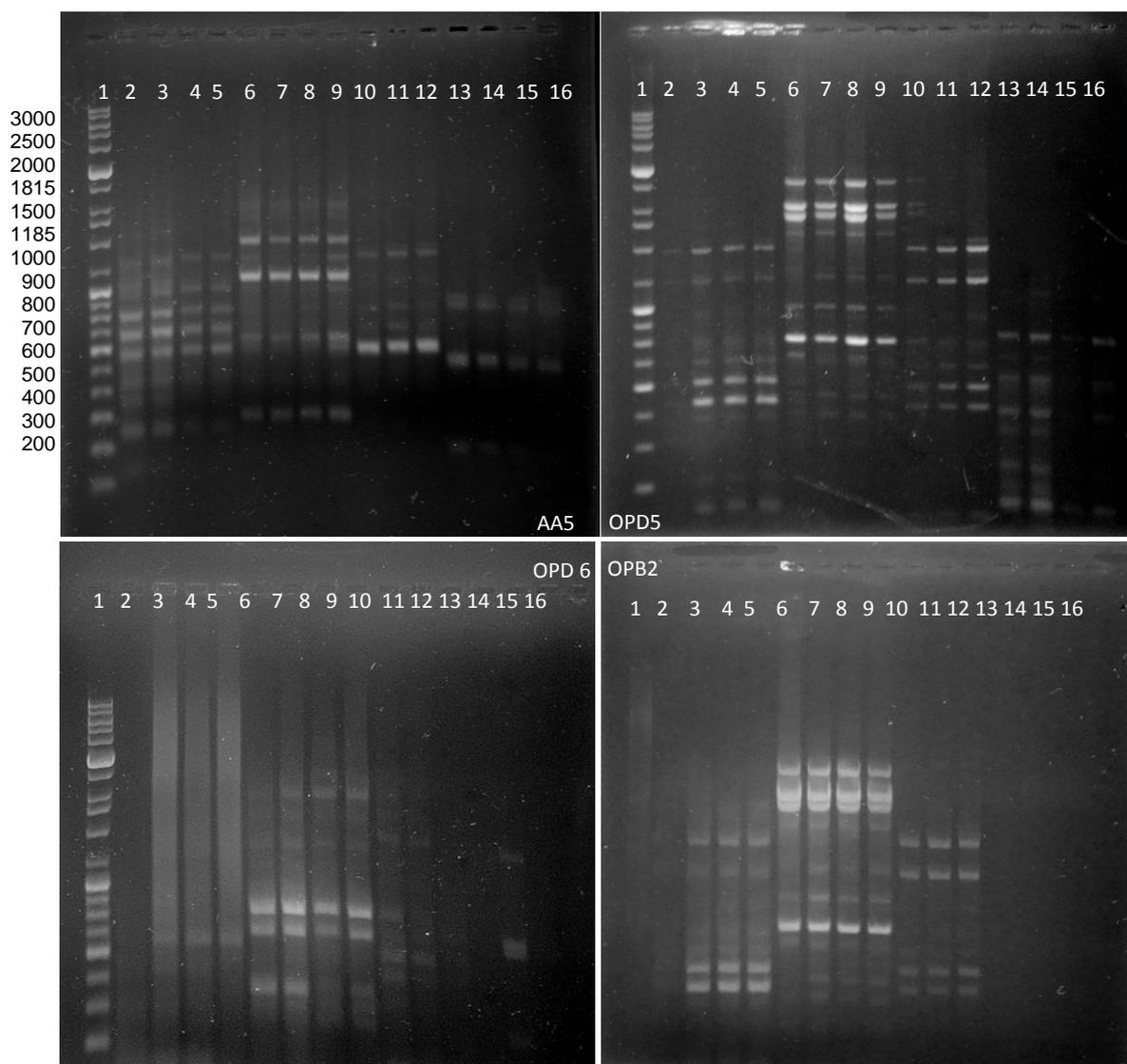


Fig. 26. RAPD amplified products of the fungal isolates. Lane-1, DNA wide range molecular marker.2 *Aspergillus niger* (FS/L 04).3, *Aspergillus niger* (RS/P 14),4, *Aspergillus niger* (FS/LA0).5, *Aspergillus niger* (FS/C-140).6, *Aspergillus melleus* (RHS/R 12).7, *Aspergillus melleus* (FS/L 13),8, *Aspergillus melleus* (FS/L 17).9, *Aspergillus melleus* (FS/L 18), 10, *Aspergillus clavatus* (RHS/P 38), 11, *Aspergillus clavatus* (RHS/P-114). 12, *Aspergillus clavatus* (RHS/T-99).13, *Talaromyces flavus* (RHS/P 50).14 *Talaromyces flavus* (RHS/P 51).15, *Talaromyces flavus* (RHS/P 54).and16, *Talaromyces flavus* (RHS/P 120).

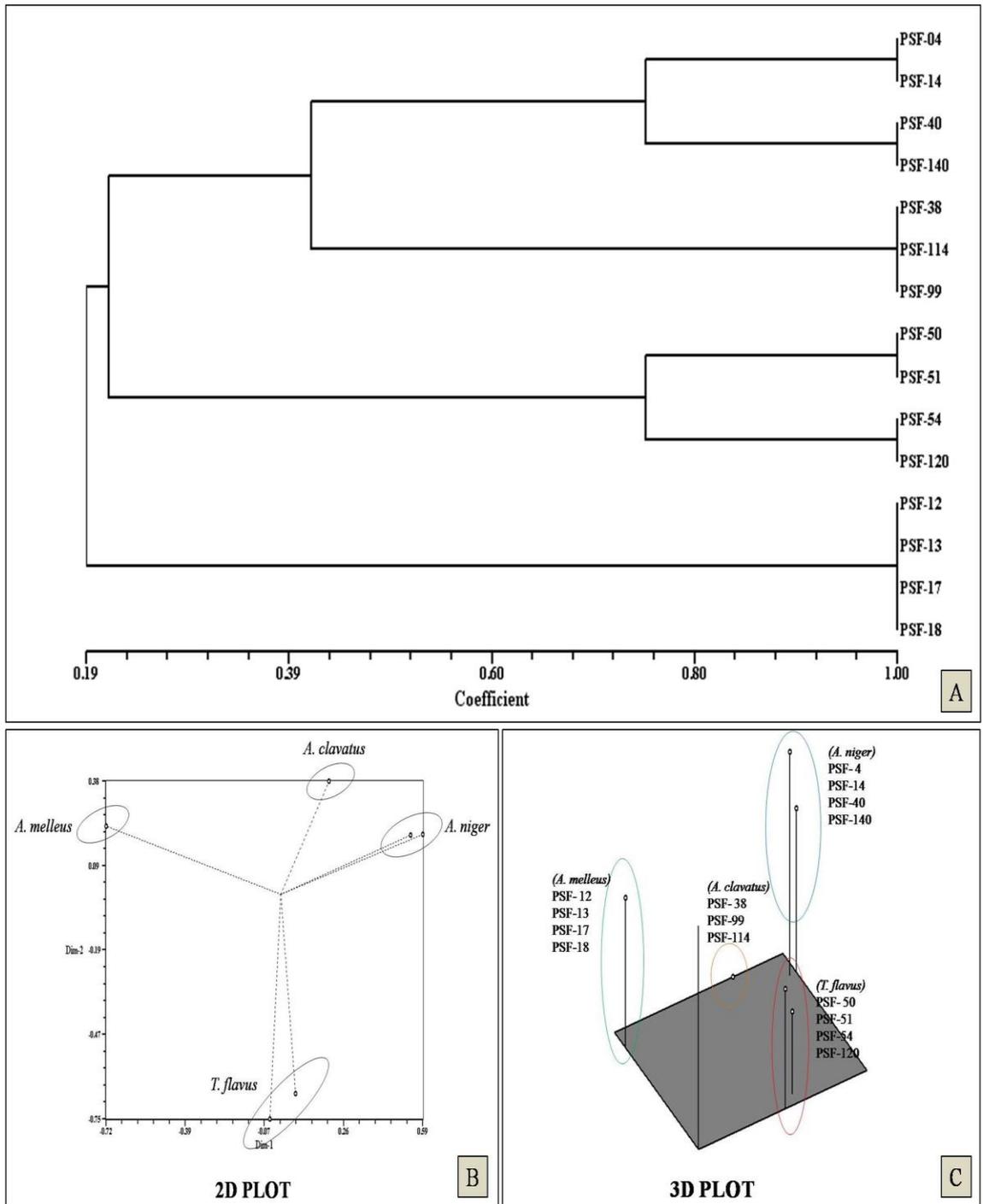


Fig. 27. (A-C). Genetic relatedness analysis among the phosphate solubilizing fungal isoaltes obtained on the basis of RAPD banding patterns. A- Dendrogram showing different fungal groups in different clades (A); Two dimensional and three dimensional plots of the similarity coefficient calculated on the basis of presence or absence of bands using NTSYS- PC software. (B&C).

4.7.2. RAPD based diversity analysis of BCA isolates

Among the Biocontrol agents, isolates of *Trichoderma* were screened for their antagonistic effects against the fungal pathogens, among them *T. harzianum* (RHS/S-559, RHS/S 560, RHS/M 501, RHS/M 511) and *T. asperellum* (RHS/S 561, RHS/M 512, RHS/M 517) isolates showed maximum inhibitory effects against fungal pathogens. DNA concentrations were optimized in the similar manner as in the case of PSF isolates where in all the subsequent experiments 20 ng template DNA concentration was constantly used. Altogether 6 random decamer (10 bp) oligonucleotide sequences (primers) were initially screened for determining polymorphism among the BCA isolates to generate reproducible polymorphisms. However, only two primers AA-5 and AA-11 showed maximum polymorphisms which ranged between 300bp to 3000bp (Table 25, Fig. 28).

4.7.2.1. Analysis of polymorphism

Out of the 17 loci scored only 10 (58.82 %) were polymorphic. The size of the bands varied from 300 to 3000 bp. However, the primers differed in their capacity to detect polymorphism. Highest level of polymorphism was recorded in primer AA-05 (62.50 %) followed by AA-11 (55.55 %) (Table 26).

Table 25. Total number of polymorphic bands produced by different primers

Primer	No. of RAPD products (bands)		
	Total bands	Polymorphic bands	% polymorphism
OPA-4	7	2	28.57
AA-11	9	5	55.55
AA-05	8	5	62.50
OPD-06	7	2	28.57
OPD-02	7	2	28.57
OPD-05	7	2	28.57

4.7.2.2. Analysis of Genetic Similarity values

The Similarity co-efficient reveals that few of the isolates belonging to the same genera and species showed highest degree of similarity. The highest degree of

similarity between *T. harzianum* isolates (RHS/S- 559, RHS/S 560, RHS/M 501) was found to be as high as 85 % and that between *T. asperellum* (RHS/S 561, RHS/M 512, RHS/M 517) was also found to be as high as 75 %. (*i.e.*, moderately low degree of dissimilarity value). On the other hand, the similarity coefficient between *T. harzianum* and *T. asperellum* isolates was 71 %. The degree of similarity between *T. harzianum* and *T. asperellum* isolates ranged from 28.00 % to 71.00 % (Moderate dissimilar values) (Table 27).

4.7.2.3. Dendrogram Construction, PCA analysis, 2D and 3D plot

A dendrogram was constructed on the basis of shared fragments which show broadly two major clusters. Cluster-I was linked to Cluster-II with low similarity coefficient of about 29 %. The Dendrogram also shows that the clustering matches exactly with respective group of organisms. Cluster-I contains *T. harzianum* isolates while Cluster- II contains *T. asperellum* isolates. Two dimensional and three dimensional plots of similarity coefficient within the same groups showed that among the *T. harzianum* isolates isolate RHS/S-559 and RHS/S-560 have the highest similarity coefficient values of 45.33 % (Highest dissimilarity values) and that within the *T. asperellum* isolates, isolate RHS/M 512 and RHS/M-517 have maximum similarity coefficient values of 73 % (Maximum dissimilarity values) (Fig. 29 , A-C).

4.7.3. RAPD based diversity analysis of Bacterial isolates.

Genetic relatedness of all the 135 bacterial isolates obtained from various geographical regions of Darjeeling hills were studied with the help of RAPD formats. Bacterial DNA concentrations were also optimized in the similar manner as in the case of PSF and BCA isolates where in all the subsequent experiments 20 ng template DNA concentration was constantly used. Altogether 6 random decamer (10 bp) oligonucleotide sequences (primers) were initially screened for determining polymorphism among the bacterial isolates to generate reproducible polymorphisms, only one primer OPD-05 showed maximum polymorphisms (63.63 %) among the bacterial isolates, the reproducible RAPD products were electrophoresed and documented, the bands size which ranged between 300bp to 3000bp (Table 25, Fig. 30, 31 & 32).

Table 26. Genetic similarity matrix, obtained as the result of Simqual analysis of the RAPD bands

	RHS/S-559	RHS/S-560	RHS/S-561	RHS/M-501	RHS/M- 511	RHS/M-512	RHS/M-517
RHS/S-559	1.0000000						
RHS/S-560	0.7142857	1.0000000					
RHS/S-561	0.2857143	0.2857143	1.0000000				
RHS/M-501	0.8571429	0.8571429	0.4285714	1.0000000			
RHS/M- 511	0.7142857	0.7142857	0.2857143	0.8571429	1.0000000		
RHS/M-512	0.5000000	0.2857143	0.5000000	0.4285714	0.2857143	1.0000000	
RHS/M-517	0.4285714	0.4285714	0.7500000	0.5714286	0.4285714	0.7500000	1.0000000

" SIMQUAL; coeff=J; " by Cols, += 1.00000, -= 0.00000: 3 15L 15

Table 27. Determination of polymorphisms based on the RAPD banding patterns obtained with the help of two decamer primers

Primers	Total No. of RAPD products	Total No. of polymorphic bands	No. of polymorphic PCR products						
			<i>Trichoderma harzianum</i>				<i>Trichoderma asperellum</i>		
			RHS/S-559	RHS/S-560	RHS/M-501	RHS/M-511	RHS/S-561	RHS/M-512	RHS/M-517
AA-5	8	5 (62.50)	3	3	3	4	3	3	4
AA-11	9	5 (55.55)	2	2	2	2	2	2	3
Total	17	10	5	5	5	6	5	5	7
% Polymorphism	17	58.82	29.41	29.41	29.41	35.29	29.41	29.41	41.17

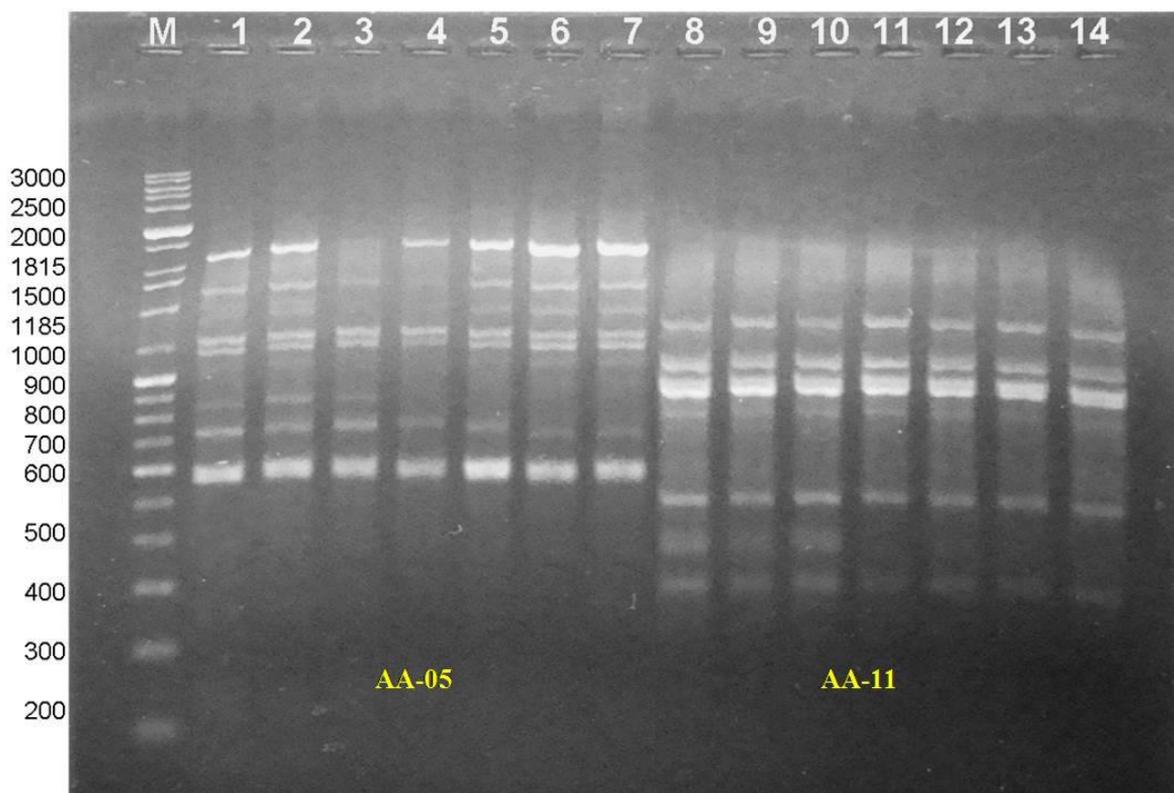


Fig. 28. RAPD analysis of *Trichoderma* sp. isolates. 1 Lane M : Low range DNA marker, Lane 1&8: *T.harzianum* (RHS/S 559) , Lane 2&9: *T. harzianum* (RHS/S 560) , Lane 3&10: *T. asperellum* (RHS/S 561) , Lane 4&11: *T. harzianum* (RHS/M 501) , Lane 5&12: *T. harzianum* (RHS/M 511) , Lane 6&13: *T. asperellum* (RHS/M 512) , Lane 7&14: *T. asperellum* (RHS/M 517) with RAPD primer AA-11 and AA -05 respectively.

4.7.3.1. Analysis of polymorphism of Bacterial isolates

The average number of polymorphic bands produced by the primer OPD-05 was 7 and the highest degree of polymorphism recorded was 63.63 % followed by OPD-02 (57.10 %), AA-11 (40.00%), OPD-06 (37.50 %), AA-05 (33.33%), OPA-04 (28.57 %) (Table 28).

4.7.3.2. Analysis of Genetic Similarity values among Bacterial isolates

The genetic similarity, analysis of Similarity co-efficient reveals that most of the bacterial isolates belonging to the same genera and species showed highest degree of similarity. The similarity co-efficient was maximum in case of isolates obtained from the same geographic locations.

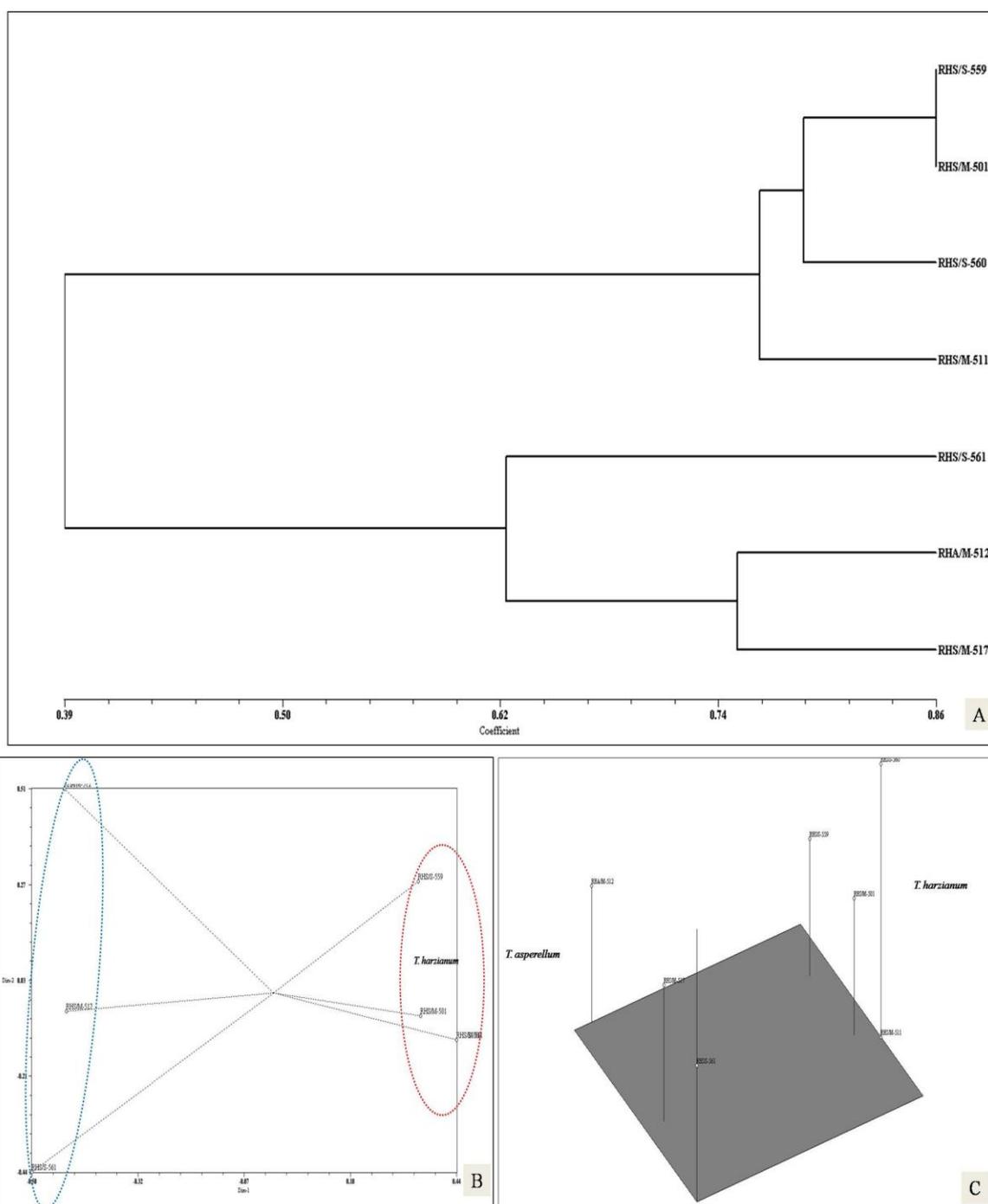


Fig. 29. (A-C). Genetic relatedness analysis among the *Trichoderma harzianum* and *T. asperellum* isoaltes obtained on the basis of RAPD banding patterns. Dendrogram showing different fungal groups in different clades (A); Two dimensional and three dimensional plots of the similarity coefficient calculated on the basis of presence or absence of bands using NTSYS- PC software (B&C).

Table 28. Total number of polymorphic bands produced by different primers

Primer	No. of RAPD products (bands)		
	Total bands	Polymorphic bands	% polymorphism
OPA-4	7	2	28.57
AA-11	5	2	40.00
AA-05	6	2	33.33
OPD-06	8	3	37.50
OPD-02	7	4	57.10
OPD-05	11	7	63.63

4.7.3.3. Dendrogram Construction, PCA analysis, 2D and 3D plot

A dendrogram was constructed on the basis of the RAPD banding patterns obtained with the Primer OPD-05. The Phenogram thus obtained broadly shows four major clusters. Each cluster contained the isolates which showed highest similarity coefficient among themselves. Cluster-I was linked to Cluster-II with low similarity coefficient of about 25%. Cluster-II was linked to Cluster- III with a similarity coefficient of about 15% and cluster-III was linked with Cluster- IV with a similarity coefficient of about 27%. The Dendrogram also shows that the clustering matches exactly with respective group of organisms (Fig. 33). Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software which further showed the degree of similarities among different clusters and the representative organisms in each cluster. However, on the basis of the data obtained on the basis of principal component analysis (2D and 3D plots) it was clear the all the bacterial isolates exhibited a wide degree of genetic diversity which has been represented with a number of dispersed points through the plot area (Fig. 34).

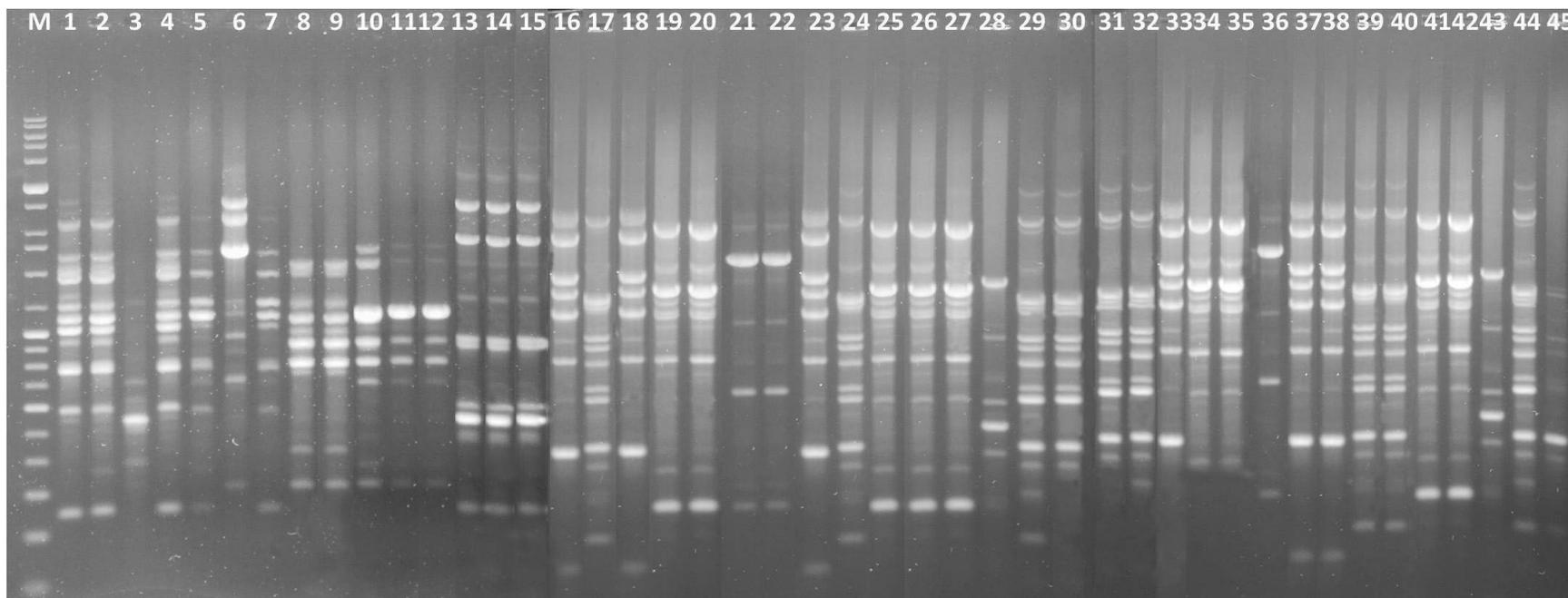


Fig. 30. RAPD analysis of bacterial isolates obtained from various sources. Lanes (M- Wide range molecular weight marker; 1-45- bacterial isolates BRHS/C-1, BRHS/C -2, BRHS/C -3, BRHS/C -4, BRHS/C -5, BRHS/C -6, BRHS/C -7, BRHS/M-8, BRHS/M -9, BRHS/M -10, BRHS/M -11, BRHS/M -12, BRHS/M -13, BRHS/M -14, BRHS/M -15, BRHS/M -16, BRHS/M -17, BRHS/M -18, BRHS/M -19, BRHS/M -20, BRHS/M -21, BRHS/P-22, BRHS/P-23, BRHS/P -24, BRHS/P -25, BFS/M-26, BFS/M-27, BFS/M-28, BFS/M-29, BFS/M-30, BFS/S-31, BFS/S-32, BFS/S-33, BFS/S-34, BFS/S-35, BFS/S-36, BRS/Mr37, BRS/Mr-38, BRS/Mr-39, BRS/Mr-40, BRS/Mr-41, BRS/Mr-42, BRS/Mr-43, BRS/Mr-44 and BRS/Mr-45 respectively.

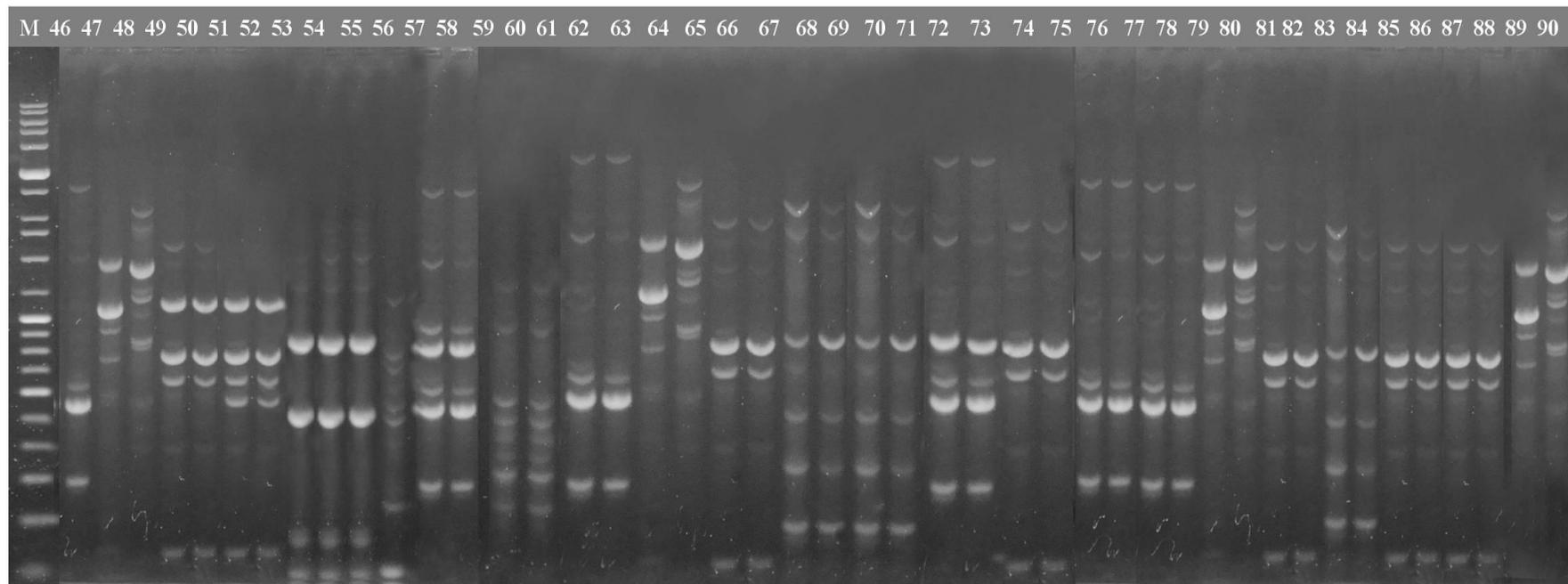
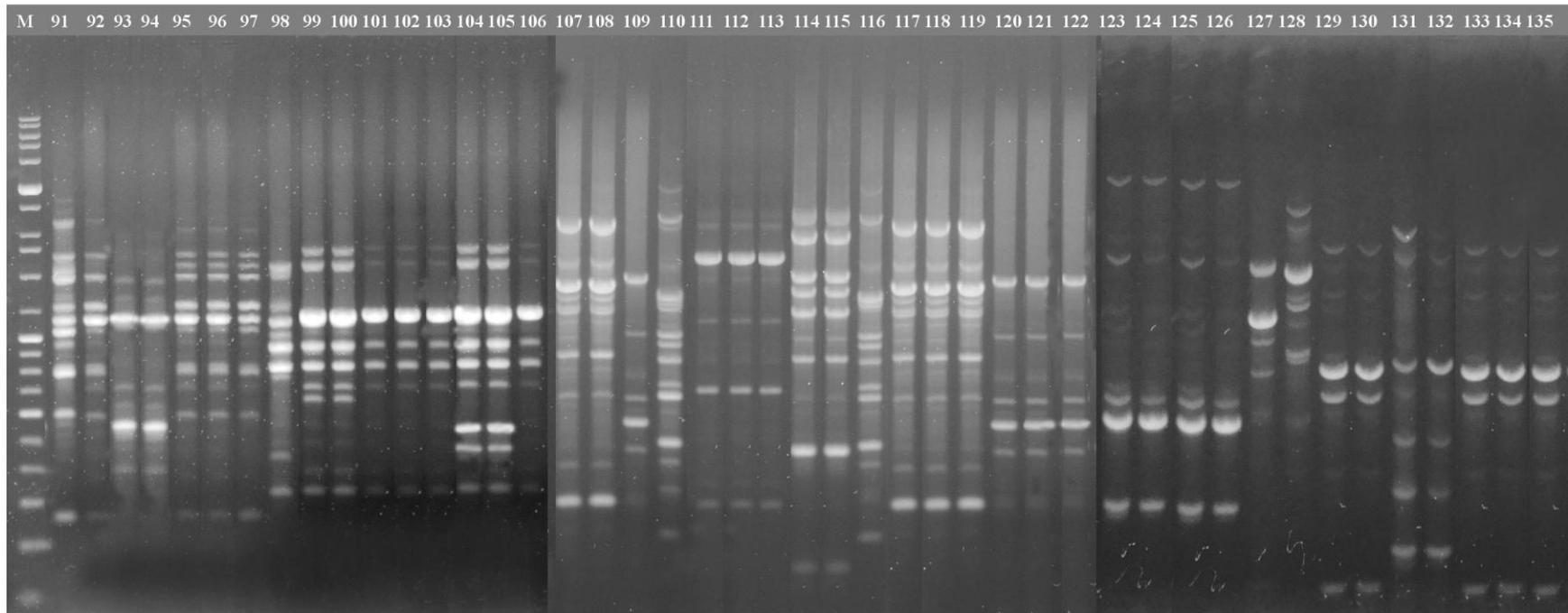


Fig. 31. RAPD analysis of bacterial isolates obtained from various sources. Lanes (M- Wide range molecular weight marker; 46-90- bacterial isolates BFS/Md-46,BFS/Md-47,BFS/Md-48,BFS/Md-49,BFS/Md-50,BFS/Md-51,BFS/Md-52,BFS/Md-53,BFS/Md-54,BFS/Md-55,BFS/Md-56,BFS/Md-57,BRS/T-58,BRS/T-59,BRS/T-60,BRS/T-61,BRS/T-62,BRHS/R-63,BRHS/R-64,BRHS/R-65,BRHS/R-66,BRHS/R-67,BRHS/R-68,BRHS/R 69,BRHS/R-70,BRHS/R-71,BRHS/R-72,BRHS/S-73,BRHS/S-74, BRHS/S-75,BRHS/S-76,BRHS/S-77,BRHS/S-78,BRHS/S-79,BRHS/S-80,BRHS/S-81,BRHS/S-82,BRHS/S-83,BRHS/S-84,BRHS/S-85,BRHS/S-86,BRHS/S-87,BRHS/S-88,BRHS/S-89,BRHS/P-90) respectively.

**Fig.
32.**



RAPD analysis of bacterial isolates obtained from various sources Lanes (M- Wide range molecular weight marker; 96—135- bacterial isolates BRHS/P-91, BRHS/P-92, FS/T-93, FS/T-93, FS/T-94, FS/T-95, FS/T-96, FS/T-97, BRHS/B-98, BRHS/B- 99, BRHS/B-100, BRHS/B-101, BRHS/B-102, BRHS/B-103, BRHS/B-104, BRHS/B-105, BRHS/B-106, BRHS/B-107, BRHS/Cd108, BRHS/Cd-109, BRHS/Cd-110, BRHS/Cd-111, BRHS/Cd-112, BRHS/Cd-113, BRHS/Cd-114, BRHS/Cd-115, BRHS/Cd116, BRHS/Cd-117, BRS/R-118, BRS/R-119, BRS/R-120, BRS/R-121, BRS/R-122, BRS/R-123, BRS/R-124, BRS/R-125, BFS/C-126, BFS/C-127, BFS/C-128, BFS/C-129, BFS/C-130, BFS/C-131, BFS/C-132, BFS/C-133, BFS/C-134, BFS/C-135 respectively.

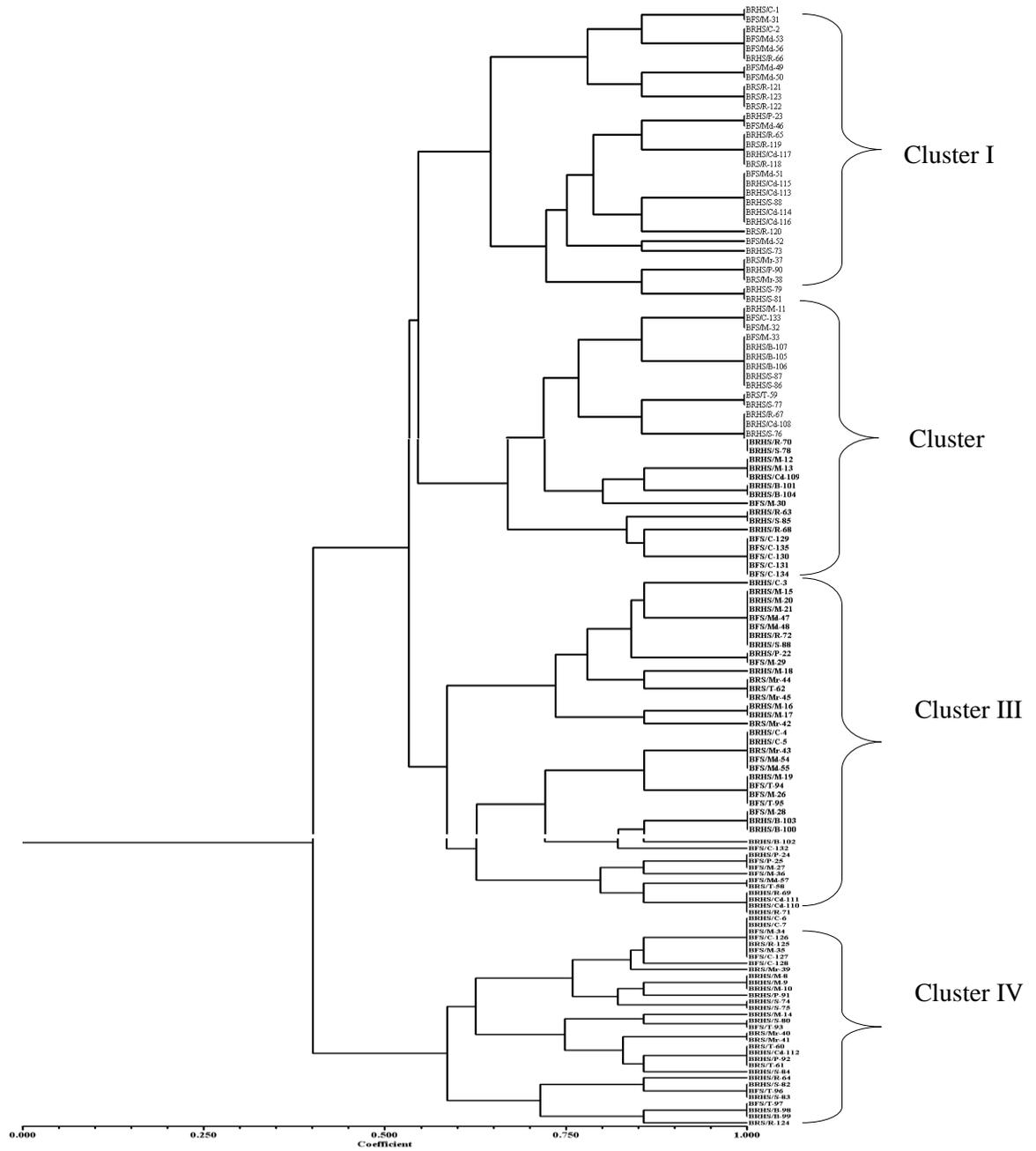


Fig. 33. Dendrograshowing different bacterial groups in different clades obtained while analyzing genetic relatedness analysis among the bacterial isoaltes based on RAPD markers.

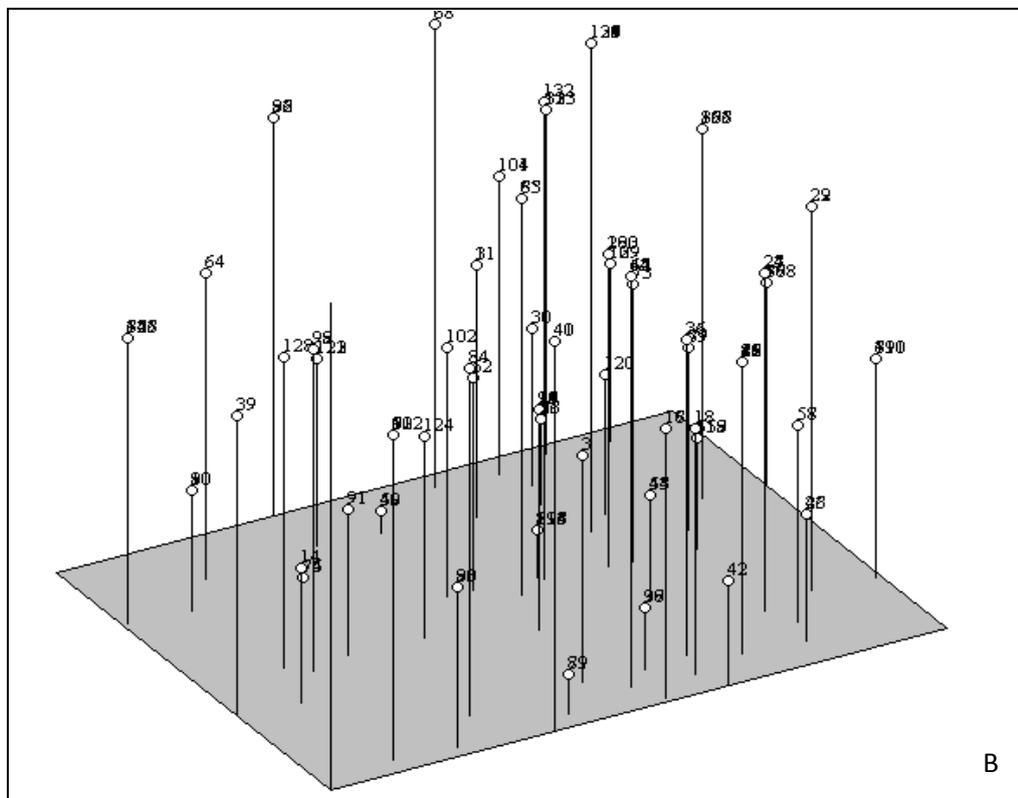
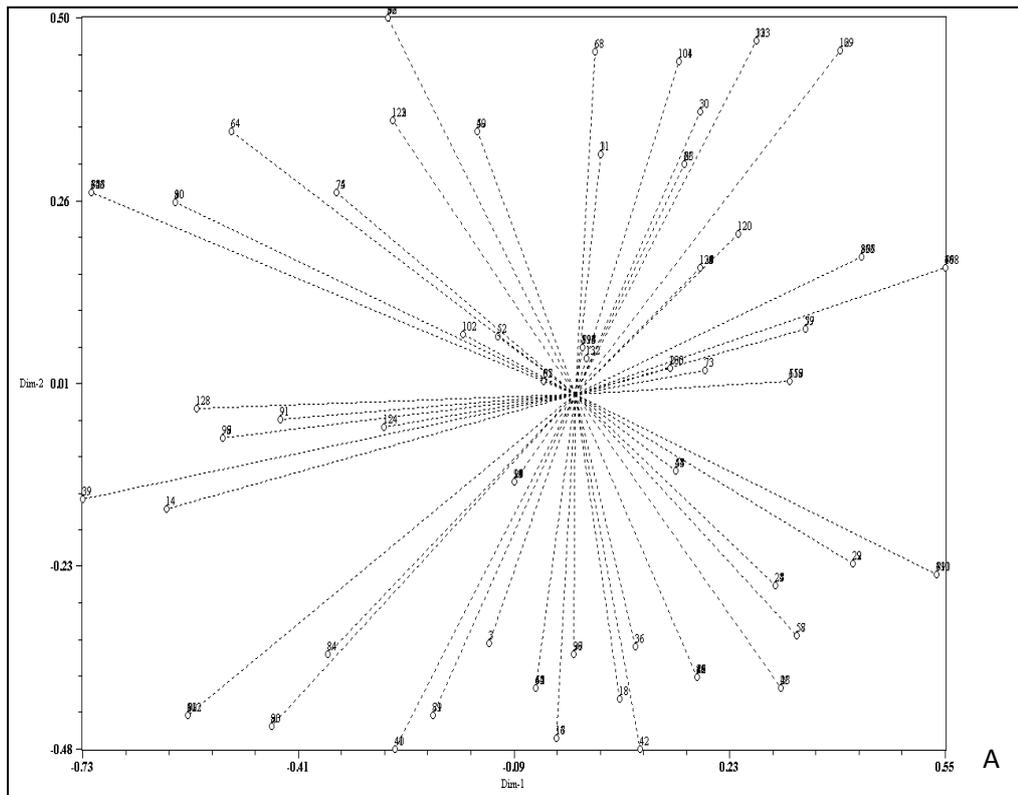


Fig. 34. Two dimensional (A) and three dimensional plots (B) of the similarity coefficient calculated on the basis of presence or absence of bands using NTSYS- PC software.

4.8. Denature Gradient Gel Electrophoresis (DGGE) markers based diversity analysis

Analysis of genetic diversity among different groups of closely related organisms was evaluated on the basis of the sequence difference between their conserved sequence for this the ITS (18S rRNA gene sequences of fungal genome and 16S rDNA gene sequence of bacterial genome) were amplified using a special set of primers, forward primer containing GC clamp at 5' end (F352T: 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3'. The amplified products were then electrophoresed in a perpendicular DGGE performed with "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). A series of gradient ranging from 0-100 %; 0-50 %; 10-80 %; 20-60% was utilized for optimizing a suitable concentration for analyzing the amplicons.

4.8.1. DGGE based diversity analysis of PSF isolates

Denature Gradient Gel electrophoretic analysis of phosphate solubilizing isolates *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) was assessed using GC-fung primers as mentioned above. The uniform PCR products of size 300 bp was obtained. The DGGE electrophoresis yielded a unique and uniform banding pattern of each group of organisms. In case of 0-100 % denaturant, no major difference among the PFS isolates were obtained, however variations in the banding patterns were observed when the denaturing gradient was 20-60%, run time 8 hours in 100 V (**Fig. 35**). All the bands in obtained were scored individually in the form of 0/1 matrix and analysed in NTSYS-PC software.

4.8.1.1. Analysis of polymorphism and genetic similarity values

The Similarity co-efficient conducted using NTSYS-PC software reveals that isolates belonging to the same genera and species showed highest degree of similarity.

The highest degree of similarity among *A. niger* isolates (FS/L-04, RS/P-14 and FS/L-40) was found to be as high 75 % and that among *T. flavus* isolates (RHS/P-50 and RHS/P-51) was also found to be as high as 75 %. (*i.e.*, moderately low degree of dissimilarity value). On the other hand, *A. niger* and *A. melleus* isolates showed low similarity value of 33%, (*i.e.*, moderately high degree of dissimilarity value). The

degree of similarity between *T. flavus* and *Aspergillus* isolates ranged from 16% - 60% (Moderate dissimilar values). The DGGE analysis of the PSF isolates also revealed that there is a significant difference between the isolates obtained from different geographical regions. The degree of similarity between *A. niger* isolates obtained from forest soil (FS/L-04, FS/L-40, FS/C-140) and from riverine soil (RS/P-14) was 16 % (high dissimilarity values) similarly among the *A. melleus* isolates since they were obtained from similar soil samples i.e. forest soil, the degree of similarity between them was found to be as high as 75 %. Whereas the degree of similarity among the *A. clavatus* isolates and *T. flavus* isolates was found to be 75 % and 100 % respectively. However among different group the degree of similarity was found to be much lesser (Table 29).

4.8.1.2. Dendrogram Construction, PCA analysis, 2D and 3D plot

The Dendrogram was constructed by the UPGMA analysis based on the presence and absence of the bands in DGGE which shows four major clusters. Cluster I represented *A. niger*, cluster II represented *T. flavus* group, cluster III represented *A. melleus* group and cluster IV represented *A. clavatus* group. The similarity coefficient ranged from 0.22 to 1. Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software. The 2 dimensional and three dimensional plots showed that there is a significant variation between the groups though the similarity coefficient is not less than 0.22 (Fig. 36).

4.8.2. DGGE based diversity analysis of BCA isolates

Denature Gradient Gel electrophoretic analysis of biocontrol isolates *Trichoderma harzianum* (RHS/S-559, RHS/S-560, RHS/M-501 and RHS/M-511), *T. asperellum* (RHS/S-561, RHS/M-512, RHS/M-517) was assessed using GC-fung primers as mentioned above. The uniform PCR products of size 250 bp was obtained. The DGGE electrophoresis yielded a unique and uniform banding pattern of each group of organisms. In case of 0-100 % denaturant, no major difference among the BCA isolates were obtained, however variations in the banding patterns were observed when the denaturing gradient was 0-50%, run time 8 hours in 100 V (Fig. 37). All the bands in obtained were scored individually in the form of 0/1 matrix and analysed in NTSYS-PC software.

Table 29. Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands

	FS/L-04	RS/P-14	FS/L-40	FS/C-140	RHS/R-12	FS/L-13	FS/L-17	FS/L-18	RHS/P-38	RHS/P-114	RHS/T-99	RHS/P-50	RHS/P-51	RHS/P-51	RHS/P-120
FS/L-04	1.0000000														
RS/P-14	1.0000000	1.0000000													
FS/L-40	0.7500000	0.7500000	1.0000000												
FS/C-140	1.0000000	1.0000000	0.7500000	1.0000000											
RHS/R-12	0.1666667	0.1666667	0.0000000	0.1666667	1.0000000										
FS/L-13	0.1428571	0.1428571	0.0000000	0.1428571	0.7500000	1.0000000									
FS/L-17	0.0000000	0.0000000	0.0000000	0.0000000	0.5000000	0.7500000	1.0000000								
FS/L-18	0.1428571	0.1428571	0.0000000	0.1428571	0.7500000	1.0000000	0.7500000	1.0000000							
RHS/P-38	0.3333333	0.3333333	0.1666667	0.3333333	0.4000000	0.6000000	0.4000000	0.6000000	1.0000000						
RHS/P-114	0.3333333	0.3333333	0.1666667	0.3333333	0.4000000	0.6000000	0.4000000	0.6000000	1.0000000	1.0000000					
RHS/T-99	0.1666667	0.1666667	0.2000000	0.1666667	0.2000000	0.4000000	0.5000000	0.4000000	0.7500000	0.7500000	1.0000000				
RHS/P-50	0.4000000	0.4000000	0.2000000	0.4000000	0.2000000	0.4000000	0.2000000	0.4000000	0.4000000	0.4000000	0.2000000	1.0000000			
RHS/P-51	0.4000000	0.4000000	0.2000000	0.4000000	0.2000000	0.4000000	0.2000000	0.4000000	0.4000000	0.4000000	0.2000000	1.0000000	1.0000000		
RHS/P-51	0.6000000	0.6000000	0.4000000	0.6000000	0.1666667	0.3333333	0.1666667	0.3333333	0.3333333	0.3333333	0.1666667	0.7500000	0.7500000	1.0000000	
RHS/P-120	0.6000000	0.6000000	0.4000000	0.6000000	0.1666667	0.3333333	0.1666667	0.3333333	0.3333333	0.3333333	0.1666667	0.7500000	0.7500000	1.0000000	1.0000000

" SIMQUAL: input=D:\DGGE Analysis\PSF-DGGE\PSF-DGGE-1.NTS, coeff=J " by Cols, += 1.00000, -= 0.00000

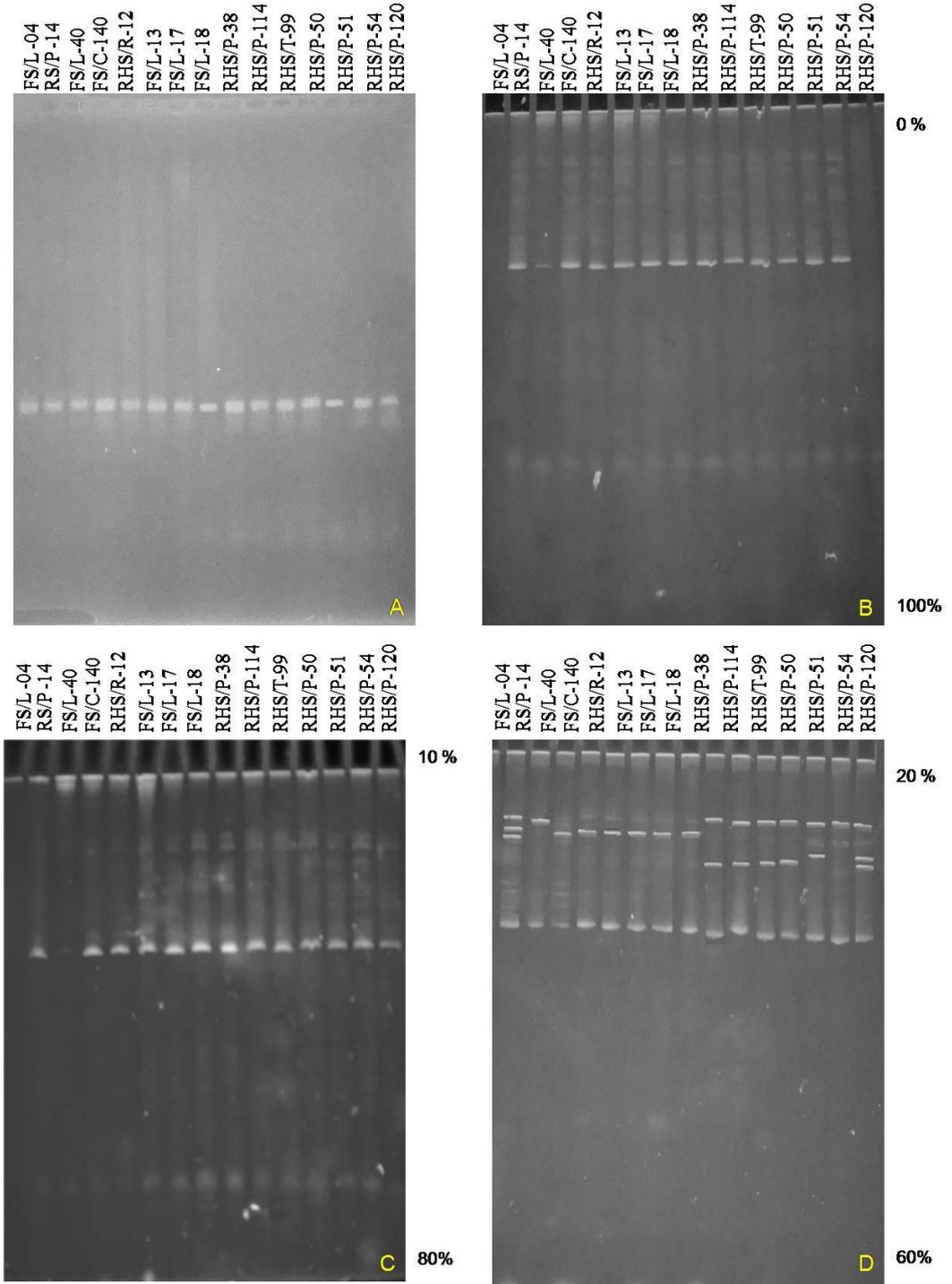


Fig. 35. Denature gradient gel electrophoresis of the ITS-PCR amplified products of PSF isolates *A. niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and *T. flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120). ITS-PCR products -300 bp (A); DGGE analysis- gradient 0-100 %, 12 hrs run, 100 V (B); Gradient 10-80%, 8 hrs run, 100 V; (C); (D) gradient 20-60%, 8 hrs run, 100V (C).

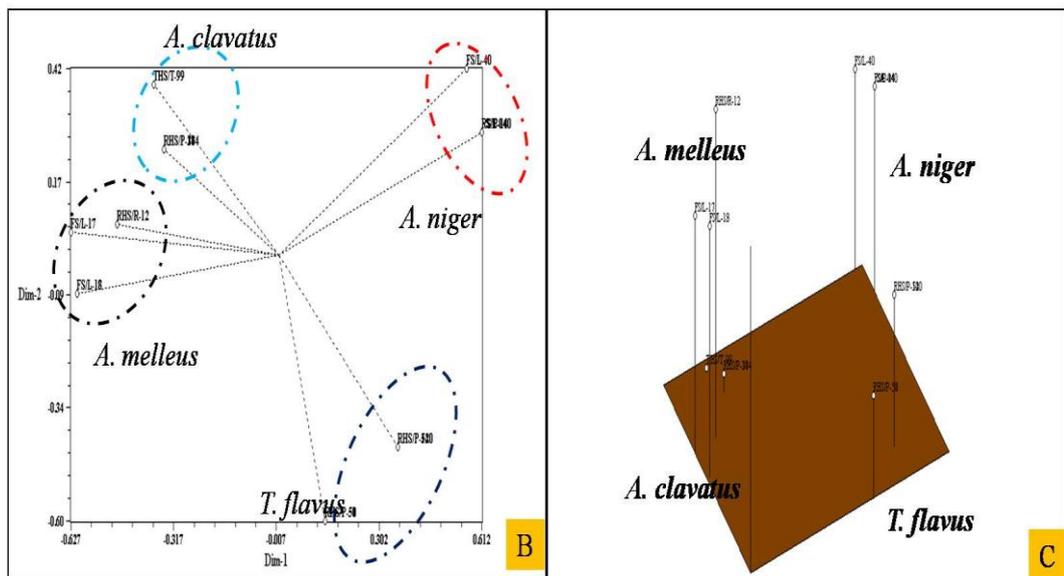
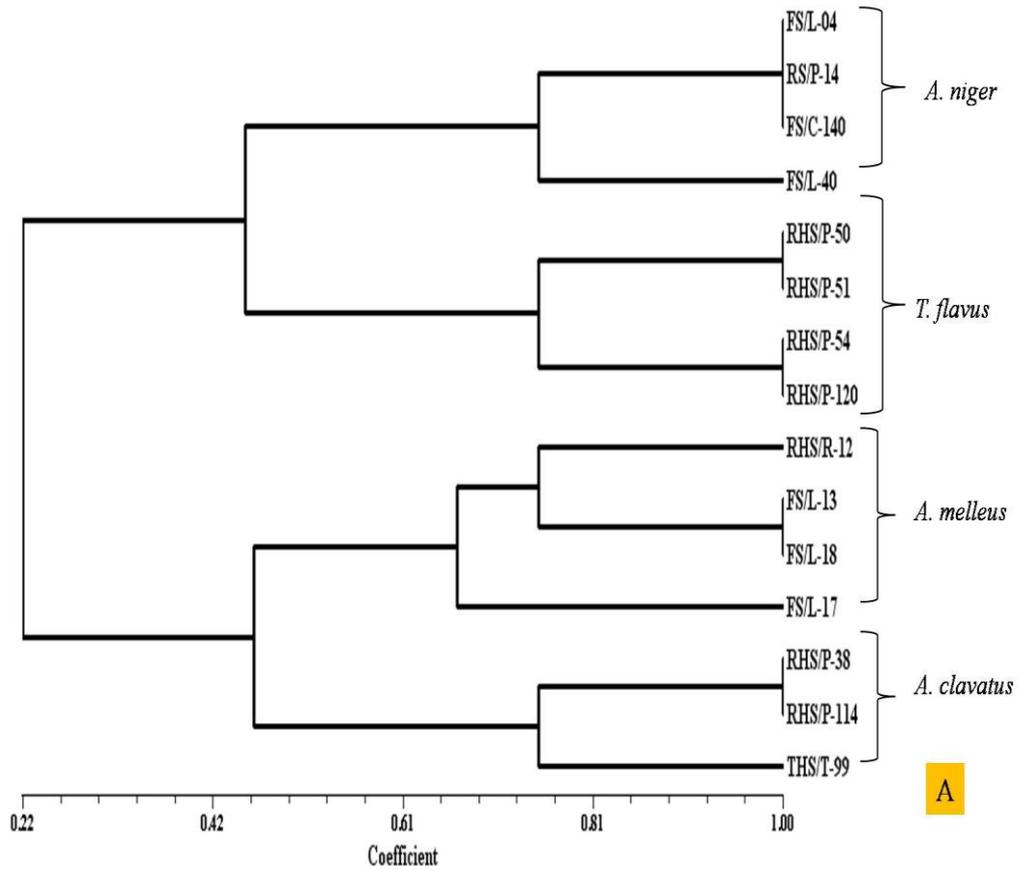


Fig. 36. Analysis of genetic relatedness among the PSF isolates based on DGGE banding pattern. Dendrogram obtained based on the basis of UPGMA analysis showing four major clusters, coefficient ranges from 0.22-1 (A); two and three dimensional plots of the UGMA analysis showing four distinct groups of organisms placed in their respective clades (B & C).

4.8.2.1. Analysis of polymorphism and genetic similarity value

The Similarity co-efficient conducted using NTSYS-PC software reveals that isolates belonging to the same genera and species showed highest degree of similarity.

The highest degree of similarity among the *T. harzianum* (RHS/S-559, RHS/S-560, RHS/M-501 and RHS/M-51) was found to be as high 80 % and that among the *T. asperellum* isolates (RHS/S-561, RHS/M-512, RHS/M-517) it was 66% (*i.e.*, moderately low degree of dissimilarity value). On the other hand the degree of similarity between *T. harzianum* and *T. asperellum* isolates was 66 %. The DGGE analysis of the BCA isolates also revealed that there is a significant difference between the isolates obtained from different geographical regions. The degree of similarity between *T. harzianum* (RHS/S-559, RHS/S-560) and *T. harzianaum* (RHS/M-501, RHS/M-511) was 28 % (moderate dissimilarity values) (Table 30).

Table 30. Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of BCA isolates

	RHS/S-559	RHS/S-560	RHS/M-501	RHS/M-511	RHS/M-561	RHS/M-512	RHS/M-517
RHS/S-559	0.8000000						
RHS/S-560	0.6000000	1.0000000					
RHS/M-501	0.8000000	0.6000000	1.0000000				
RHS/M-511	0.4285714	0.2857143	0.5000000	1.0000000			
RHS/M-561	0.5000000	0.4285714	0.5000000	0.2857143	1.0000000		
RHS/M-512	0.5000000	0.5000000	0.5000000	0.6666667	0.6666667	1.0000000	
RHS/M-517	0.6666667	0.5000000	0.5000000	0.5000000	0.6666667	0.6666667	1.0000000

"SIMQUAL: input=D:\DGGE Analysis\BCA-DGGE\Bacteria DGGE-1.NTS, coeff=J

4.8.2.2. Dendrogram Construction, PCA analysis, 2D and 3D plot

The Dendrogram was constructed by the UPGMA analysis based on the presence and absence of the bands in DGGE which shows two major clusters. Cluster I represented *T. harzianum*, cluster II represented *T. asperellum* group, the similarity co-efficient ranged from 0.45 to 1. Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software. The 2 dimensional and three dimensional plots showed that there is a significant variation between the groups though the similarity co efficient is not less than 0.45 (Fig. 38).

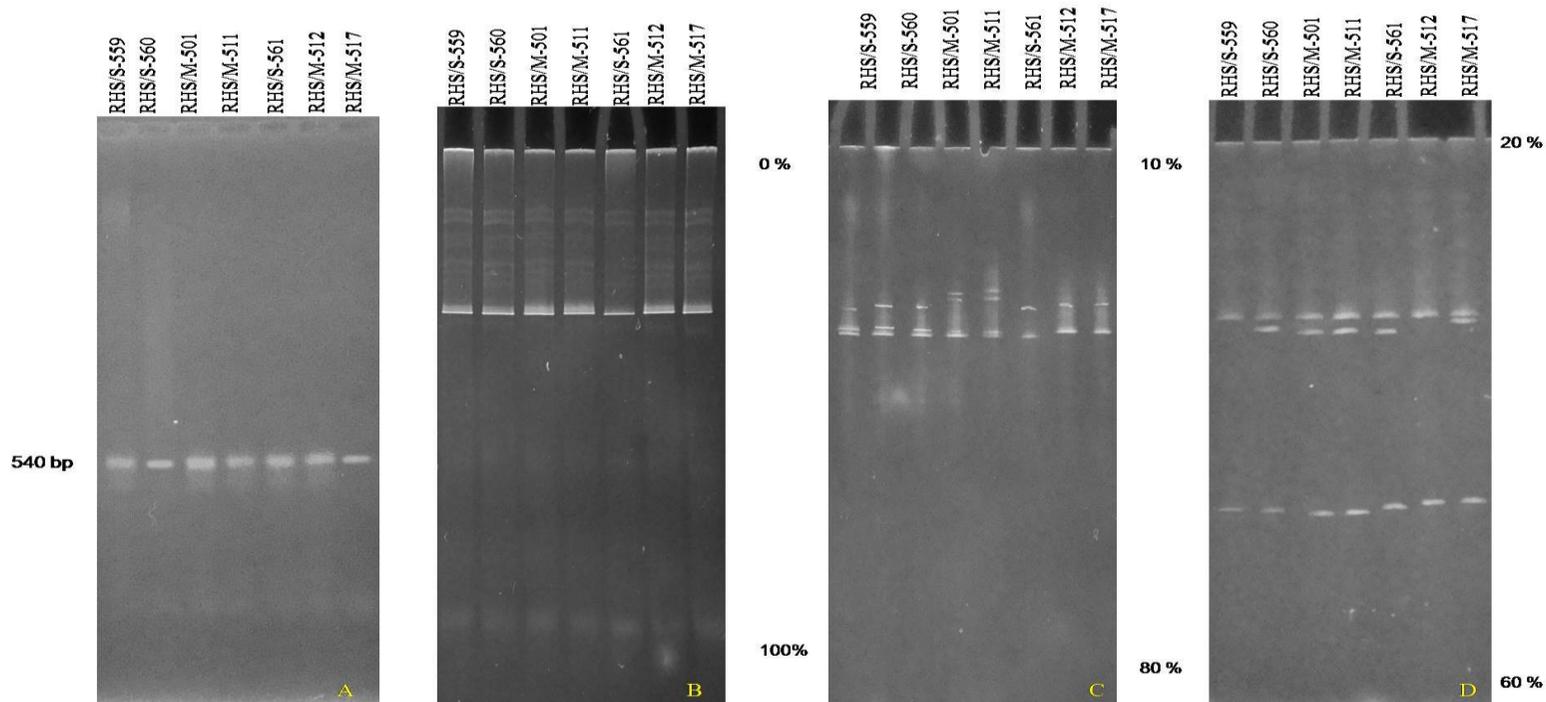


Fig. 37. Denature gradient gel electrophoresis of the ITS-PCR amplified products of BCA isolates *T. harzianum* (RHS/S-559, RHS/S-560, RHS/M-501 and RHS/M-511) and *T. asperellum* (RHS/S-561, RHS/M-512, RHS/M-517). ITS-PCR products -300 bp (A); DGGE analysis-gradient 0-100 %, 12 hrs run, 100 V (B); Gradient 10-80%, 8 hrs run, 100 V; (D) gradient 20-60%, 8 hrs run, 100V (B).

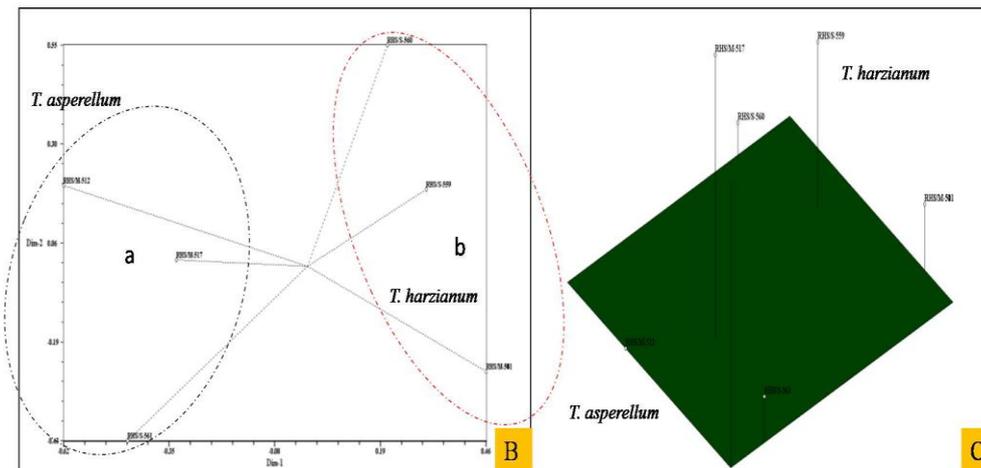
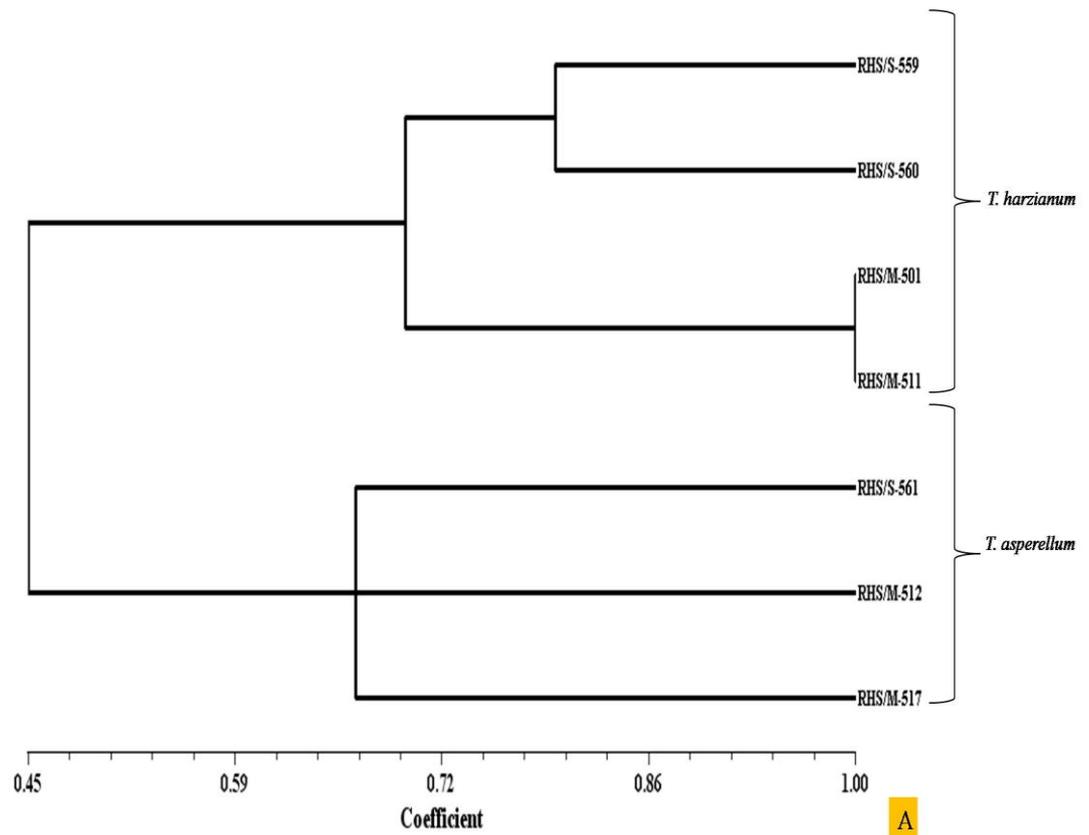


Fig. 38. Analysis of genetic relatedness among the BCA isolates based on DGGE banding pattern. Dendrogram obtained based on the basis of UPGMA analysis showing four major clusters, coefficient ranges from 0.45-1 (A); Two and three dimensional plots of the UPGMA analysis showing four distinct groups of organisms placed in their respective clades (B&C).

4.8.3. DGGE based diversity analysis of Bacterial isolates

A total of 15 bacterial isolates were obtained from different soil samples obtained from Darjeeling Hill regions. On the basis of *in vitro* test for plant growth promoting activities, seven potential isolates were chosen for further evaluation of their beneficial characters. These seven isolates were morphologically dissimilar and were obtained from different geographical locations. They were initially identified on the basis of 16S DNA gene sequence analysis. these isolates were *Bacillus pumilus* (BRHS/C-1), *B. altitudinis* (BRHS/P-22), *Enterobacter cloacae* (BRHS/R-71), *Paenibacillus polymyxa* (BRHS/R-72), *B. altitudinis* (BRHS/S-73), *B. methylotrophicus* (BRHS/P-9), *Bukholderia symbiont* (BRHS/P-92) and *B. aerophilus* (BRHS/B-104). Analyses of genetic relatedness among these PGPR isolates were conducted on the basis of the differences in their conserved sequences. For this 16S rDNA sequences were amplified using specific primers (F352T: 5'-CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519 r: 5'-ACC GCG GCT GCT GGC AC- 3'). The DGGE electrophoresis yielded a unique and uniform banding pattern of each bacterial isolate. In case of 0-100 % denaturant, no major difference among the bacterial isolates was noticed, however variations in the banding patterns were observed when the denaturing gradient was 20-60%, run time 8 hours in 100 V (Fig. 39). All the bands in obtained were scored individually in the form of 0/1 matrix and analysed in NTSYS-PC software.

4.8.3.1. Analysis of polymorphism and genetic similarity value

The Similarity co-efficient conducted using NTSYS-PC software reveals that isolates belonging to the same genera and species showed highest degree of similarity. The analysis of similarity co-efficient values revealed that the individual bacterial isolates were unique in their conserved sequences. The degree of similarity among the *Bacillus* isolates (*Bacillus pumilus* BRHS/C-1, *B. altitudinis* BRHS/P-22, *B. methylotrophicus* BRHS/P-9, *B. aerophilus* BRHS/B-104) is 60%. The degree of similarity between *Bacillus* isolates and *Enterobacter cloacae* (BRHS/R-71), is 42 %, *Paenibacillus polymyxa* (BRHS/R-72) is 33 %, and *Bukholderia symbiont* (BRHS/P-92) is 20%. The DGGE analysis of the PGPR isolates also revealed that there is a significant difference between the isolates obtained from different geographical regions (Table 31).

Table 31. Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of PGPR isolates

	BRHS/C-1	BRHS/P-22	BRHS/R-71	BRHS/R-72	BRHS/S-73	BRHS/P-91	BRHS/P-92
BRHS/B-104							
BRHS/C-1	1.0000000						
BRHS/P-22	0.6000000	1.0000000					
BRHS/R-71	0.4285714	0.4285714	1.0000000				
BRHS/R-72	0.4000000	0.4000000	0.5000000	1.0000000			
BRHS/S-73	0.6000000	1.0000000	0.4285714	0.4000000	1.0000000		
BRHS/P-91	0.6000000	0.3333333	0.4285714	0.1666667	0.3333333	1.0000000	
BRHS/P-92	0.2000000	0.2000000	0.3333333	0.2500000	0.2000000	0.2000000	1.0000000
BRHS/B-104	0.4000000	0.4000000	0.2857143	0.0000000	0.4000000	0.7500000	0.2500000

"SIMQUAL: input=D:\DGGE Analysis\Bacteria DGGE\Bacteria DGGE-1.NTS, coeff=J

4.8.3.2. Dendrogram Construction, PCA analysis, 2D and 3D plot

The Dendrogram was constructed by the UPGMA analysis based on the presence and absence of the bands in DGGE. The Dendrogram clearly shows that each individual isolates were separated however showed similarity with each other. The bacilli isolates were grouped in one single cluster and showed a similarity co-efficient of 0.62 among themselves. The overall similarity co-efficient ranged from 0.23 to 1. Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software. The 2 dimensional and three dimensional plots showed that there is a significant variation between the groups though the similarity co efficient is not less than 0.19 (Fig. 40).

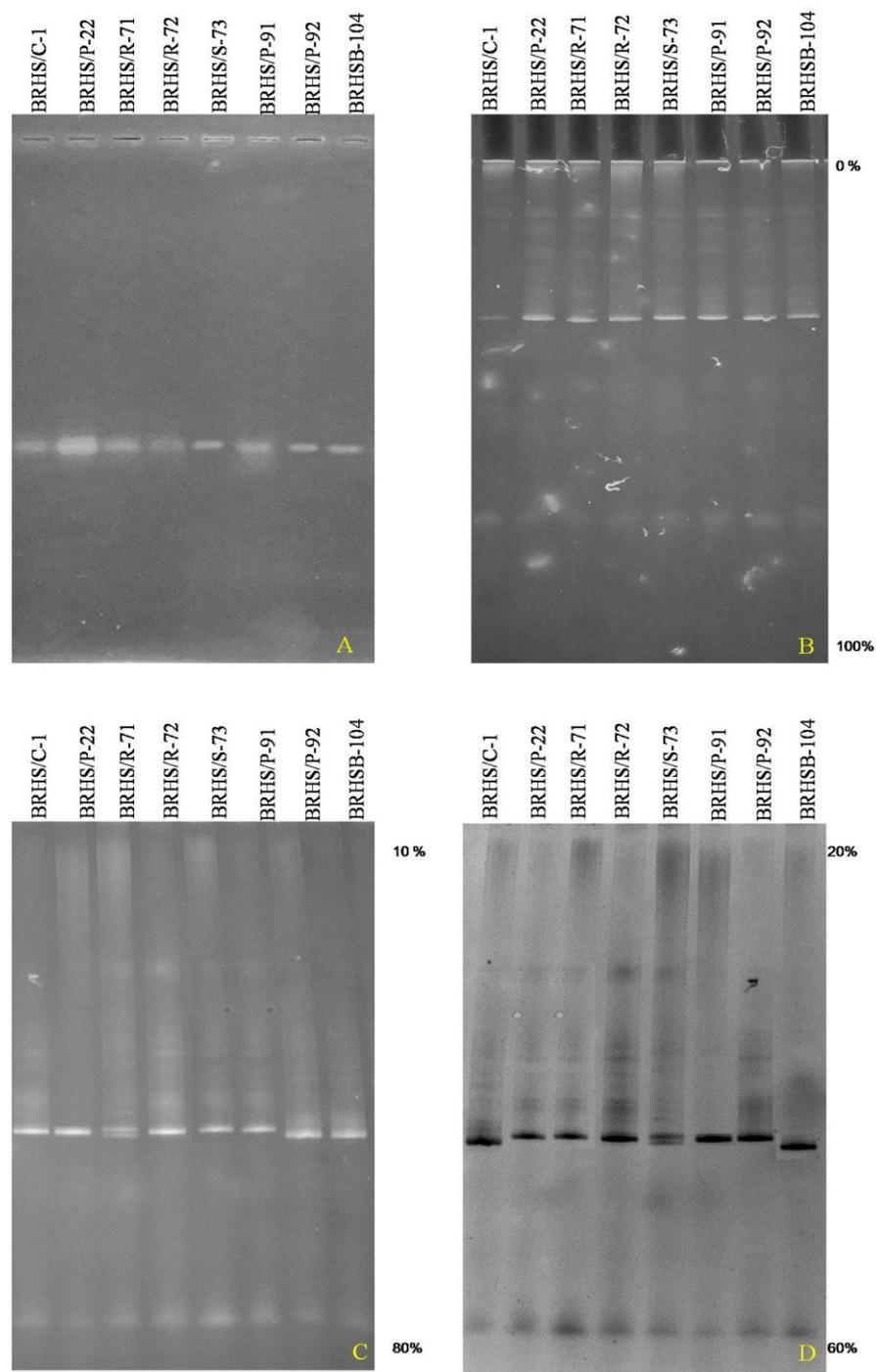


Fig. 39. Denature gradient gel electrophoresis of the ITS-PCR amplified products of PGPR isolates *Bacillus pumilus* (BRHS/C-1), *B. altitudinis* (BRHS/P-22), *Enterobacter cloacae* (BRHS/R-71), *Paenibacillus polymyxa* (BRHS/R-72), *B. altitudinis* (BRHS/S-73), *B. methylotrophicus* (BRHS/P-9), *Bukholderia symbiont* (BRHS/P-92) and *B. aerophilus* (BRHS/B-104). ITS-PCR products -250 bp (A); DGGE analysis- gradient 0-100 %, 12 hrs run, 100 V (B); Gradient 10-80%, 8 hrs run, 100 V; (C) gradient 20-60%, 8 hrs run, 100V (D).

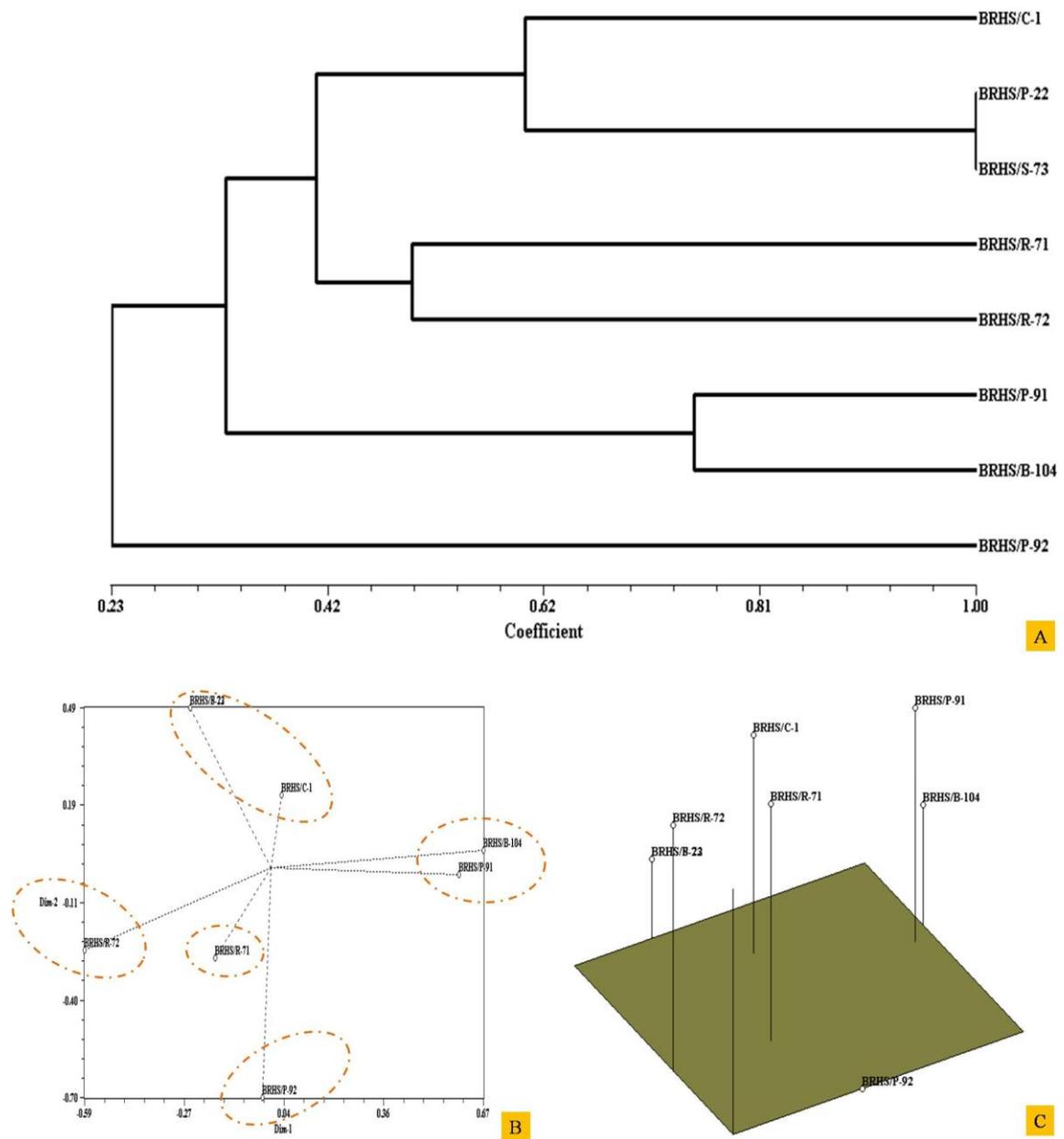


Fig. 40. Analysis of genetic relatedness among the PGPR isolates based on DGGE banding pattern. Dendrogram obtained based on the basis of UPGMA analysis showing four major clusters, coefficient ranges from 0.23-1 (A); Two and three dimensional plots of the UGMA analysis showing different isolates in different clades (B&C).

4.8.4. DGGE analysis of BCA isolates with reference to the known identified strains.

In a separate experiment an attempt was made to use the DGGE technique to compare the genetic make up of unknown samples isolated from different sources. For this once the denaturant concentration was standardized, the unknown samples were then analyzed with reference to the known ones. Different biocontrol isolates, RHS/ T-594, RHS/T-600 RHS/C-601, RHS/C-604, RHS/C-606, RHS/T- 626, RHS/P-572, RHS/A-481, RHS/A-482, RHS/A-483, FS/R-640, FS/R-641 obtained from different geographical locations of North Bengal were analyzed for their genetic relatedness based on their conserved sequences. A Uniform PCR product of 200 bp was obtained with the help of DGGE primer. The PCR product was subjected to 20-60 % denature gradient electrophoresis, run time 8h, 100 V, where a distinct banding pattern was obtained for each isolate (Fig. 41).

4.8.4.1. Analysis of polymorphism and genetic similarity value

The analysis of genetic similarity among the *Trichoderma* isolates was conducted on the basis of the reference strains. Isolates which were morphologically similar with the reference cultures showed highest degree of similarity. The degree of similarity between the reference strain *T. harzianum* RHS/S-559 and RHS/T-594, RHS/T-600, RHS/C-601, RHS/C-606, RHS/T-626, RHS/P-572, RHS/A-481, RHS/A-482, RHS/A-483 and FS/R-641 is 80 % (Less dissimilarity coefficient) whereas the degree of similarity between the reference strain *T. asperellum* RHS/S 561 and RHS/C-604 and FS/R-640 is 83 %. (Table 32).

4.8.4.2. Dendrogram Construction, PCA analysis, 2D and 3D plot

The Dendrogram was constructed by the UPGMA analysis based on the presence and absence of the bands in DGGE. The Dendrogram clearly shows that each individual isolates were separated however showed similarity with each other. The *T. harzianum* isolates were grouped in one single cluster and showed a similarity coefficient of 0.80 among themselves whereas, *T. asperellum* isolates were grouped in another cluster and showed a similarity coefficient of 0.85 among themselves. The overall similarity coefficient ranged from 0.53 to 1. Similarly the two dimensional and three dimensional plots of similarity coefficient were constructed with the help of NTSYS PC software. The 2 dimensional and three dimensional plots showed that there is a significant variation between the groups though the similarity coefficient is not less than 0.15. (Fig. 42).

Table 32. Genetic similarity matrix, obtained as the result of Simqual analysis of the DGGE bands of *Trichoderma* isolates

	FS/L-20	RHS/S-559	RHS/S-561	RHS/T-594	RHS/T-600	RHS/C-601	RHS/C-604	RHS/C-606	RHS/T-626	RHS/P-572	RHS/A-481	RHS/A-482	RHS/A-483	FS/R-640	FS/R-641
FS/L-20	1.0000000														
RHS/S-559	0.7500000	1.0000000													
RHS/S-561	0.2857143	0.4285714	1.0000000												
RHS/T-594	0.6000000	0.8000000	0.5714286	1.0000000											
RHS/T-600	0.6000000	0.8000000	0.5714286	0.6666667	1.0000000										
RHS/C-601	0.5000000	0.6666667	0.7142857	0.8333333	0.5714286	1.0000000									
RHS/C-604	0.3333333	0.5000000	0.8333333	0.6666667	0.4285714	0.8333333	1.0000000								
RHS/C-606	0.7500000	1.0000000	0.4285714	0.8000000	0.8000000	0.6666667	0.5000000	1.0000000							
RHS/T-626	0.6000000	0.8000000	0.5714286	0.6666667	1.0000000	0.5714286	0.4285714	0.8000000	1.0000000						
RHS/P-572	0.6000000	0.8000000	0.5714286	1.0000000	0.6666667	0.8333333	0.6666667	0.8000000	0.6666667	1.0000000					
RHS/A-481	0.7500000	1.0000000	0.4285714	0.8000000	0.8000000	0.6666667	0.5000000	1.0000000	0.8000000	0.8000000	1.0000000				
RHS/A-482	0.6000000	0.8000000	0.5714286	0.6666667	0.6666667	0.8333333	0.6666667	0.8000000	0.6666667	0.6666667	0.8000000	1.0000000			
RHS/A-483	0.6000000	0.8000000	0.5714286	0.6666667	0.6666667	0.8333333	0.6666667	0.8000000	0.6666667	0.6666667	0.8000000	1.0000000	1.0000000		
FS/R-640	0.2857143	0.4285714	1.0000000	0.5714286	0.5714286	0.7142857	0.8333333	0.4285714	0.5714286	0.5714286	0.4285714	0.5714286	0.5714286	1.0000000	
FS/R-641	0.6000000	0.8000000	0.5714286	0.6666667	1.0000000	0.5714286	0.4285714	0.8000000	1.0000000	0.6666667	0.8000000	0.6666667	0.6666667	0.5714286	1.0000000

" SIMQUAL: input=D:\DGGE Analysis\ Coeff=J; " by Cols, += 1.00000, -= 0.00000; 3 15L 15 0 ;

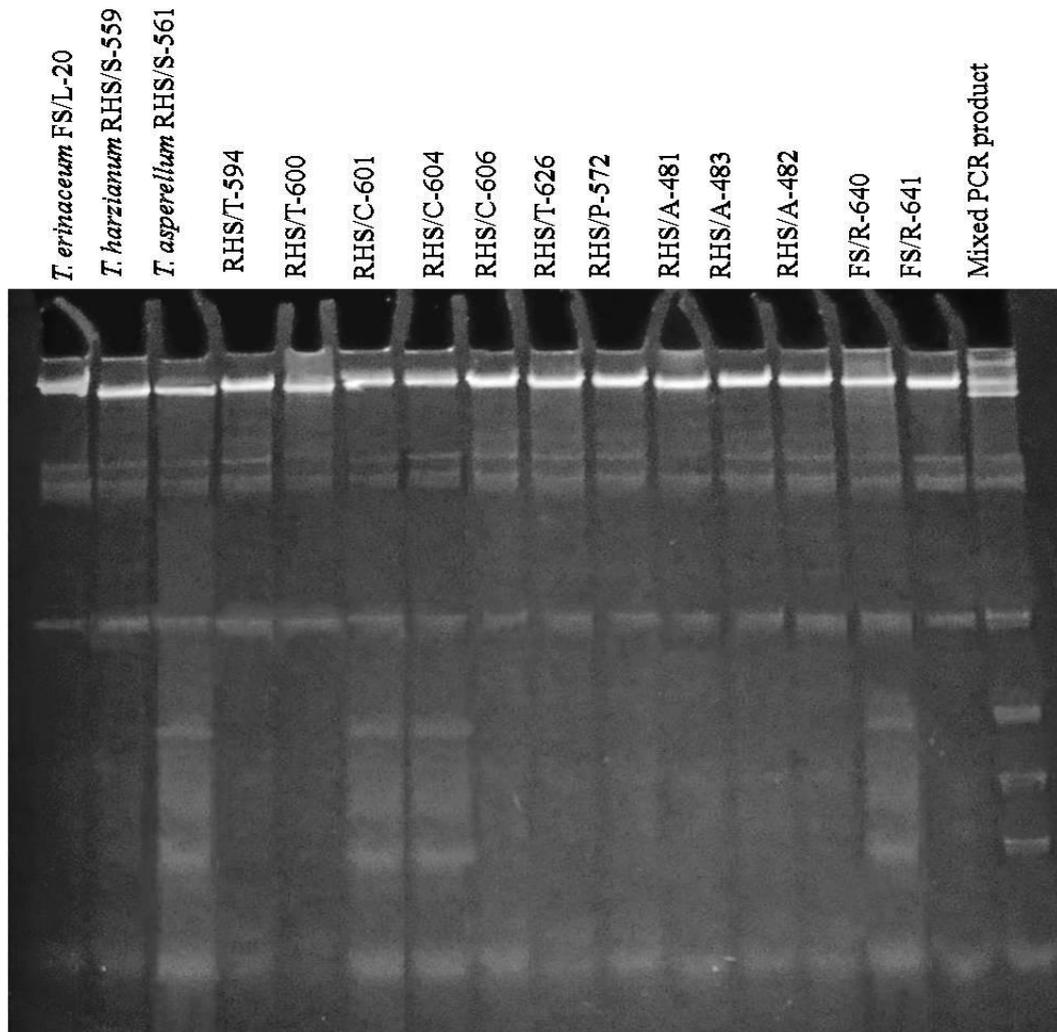


Fig. 41. Denature gradient gel electrophoresis of the ITS-PCR amplified products of *Trichoderma* isolates. Lane 1-*T. erinaceum* (FS/L-20); 2-*T. harzianum* (RHS/S-559); 3-*T. asperellum* (RHS/S-561); 4- RHS/ T-594; 5-RHS/T-600; 6-RHS/C-601;7-RHS/C-604; 8-RHS/C-606;9-RHS/T- 626;10-RHS/P-572; 11- RHS/A-481;12-RHS/A-482; 13-RHS/A-483;14-FS/R-640;15-FS/R-641; 16-Mixed PCR product.Gradient 20-60%, 8 hrs run, 100V.

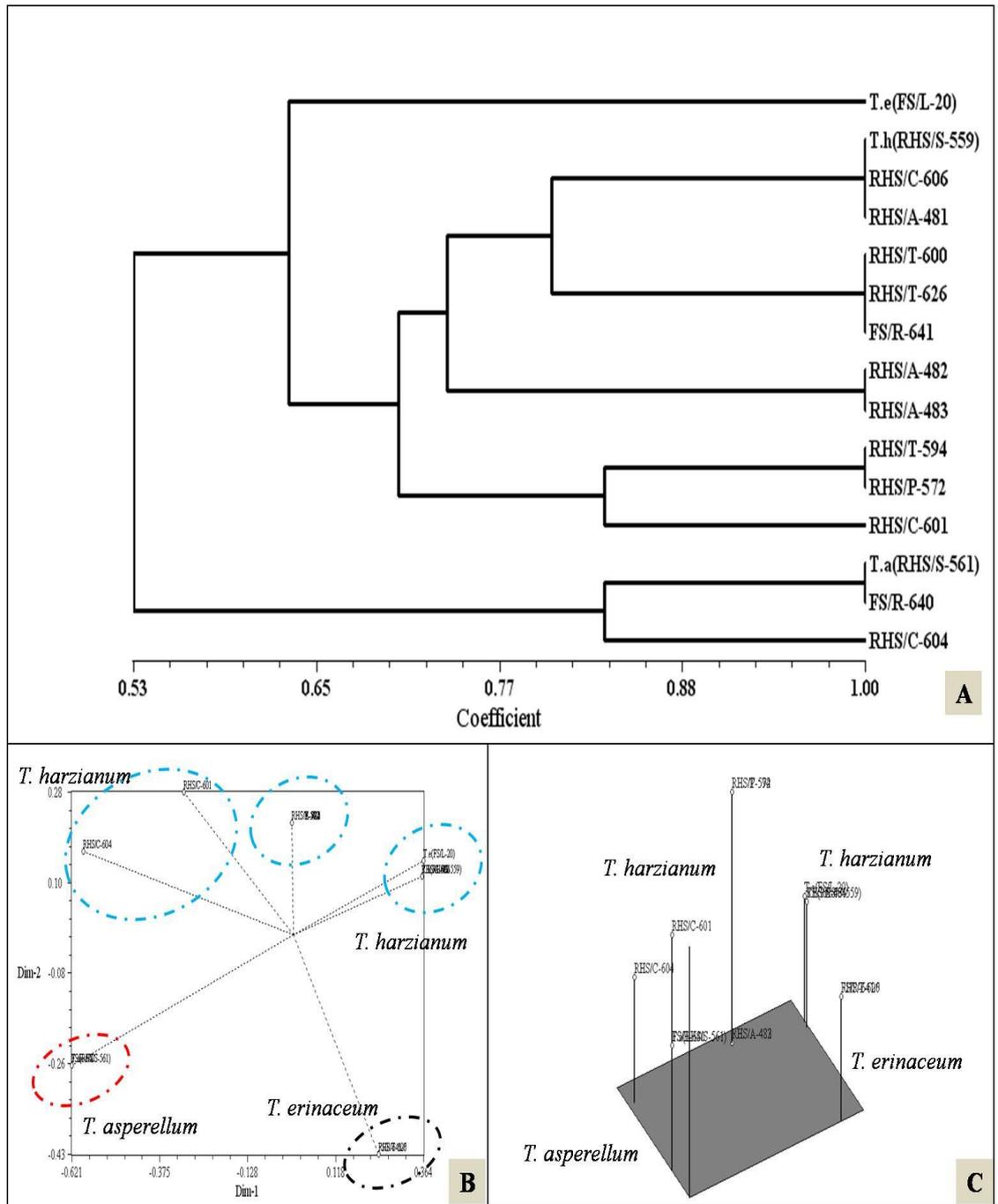


Figure 42. Analysis of genetic relatedness among the BCA isolates based on DGGE banding pattern. Dendrogram obtained based on the basis of UPGMA analysis showing three major clusters, coefficient ranges from 0.53-1 (A); Two and three dimensional plots of the UGMA analysis showing different isolates in different clades (B&C).

4.9. 18S rDNA sequence and phylogenetic analysis for identification of fungal isolates

The identities of all the fungal isolates (PSF, BCA and the pathogen) were confirmed with the help of analysis of rDNA sequences. The 18S rDNA sequences of all the potential isolates were amplified with the help of universal primers and sequenced. The sequenced products were then analyzed for conforming their identities and phylogenetic placements. The respective rDNA sequences have been deposited in NCBI gen bank database and an accession number have been provided.

4.9.1. Pathogen (*Thanatephorus cucumeris*/ NAIMCC-F-02903)

In this present investigation two important phyto-pathogens, *Sclerotium rolfsii* and *Thanatephorus cucumeris* were taken up for *in vivo* studies. *S. rolfsii* was obtained from the culture collection of Immuno-phytopathology Laboratory, Department of Botany, University of North Bengal whereas another pathogen, *T. cucumeris* was isolated from the stem of severely infected *Vigna radiata* growing in the experimental field. The isolate was designated as RHS/V-566. Initial morphological and microscopical characterization of the isolates was carried out and further identification of this pathogen was confirmed with the help of 18S rDNA sequence. ITS region of rDNA was amplified using ITS-1 and ITS-4 primers. A uniform product of size of 450 bp was obtained which was analyzed with the help of 1% Agarose gel electrophoresis and was sequenced. The obtained sequence was further used to query against NCBI Genbank sequences through BLAST. The analysis revealed isolate RHS/V-566 to have 99% homology with *Thanatephorus cucumeris*. The sequences were approved as 18S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,400). The accession number for isolate *T. cucumeris* RHS/V-566, provided by NCBI is **JN248540**. (Fig. 43)

4.9.1.1. Multiple sequence alignment and Phylogenetic analysis

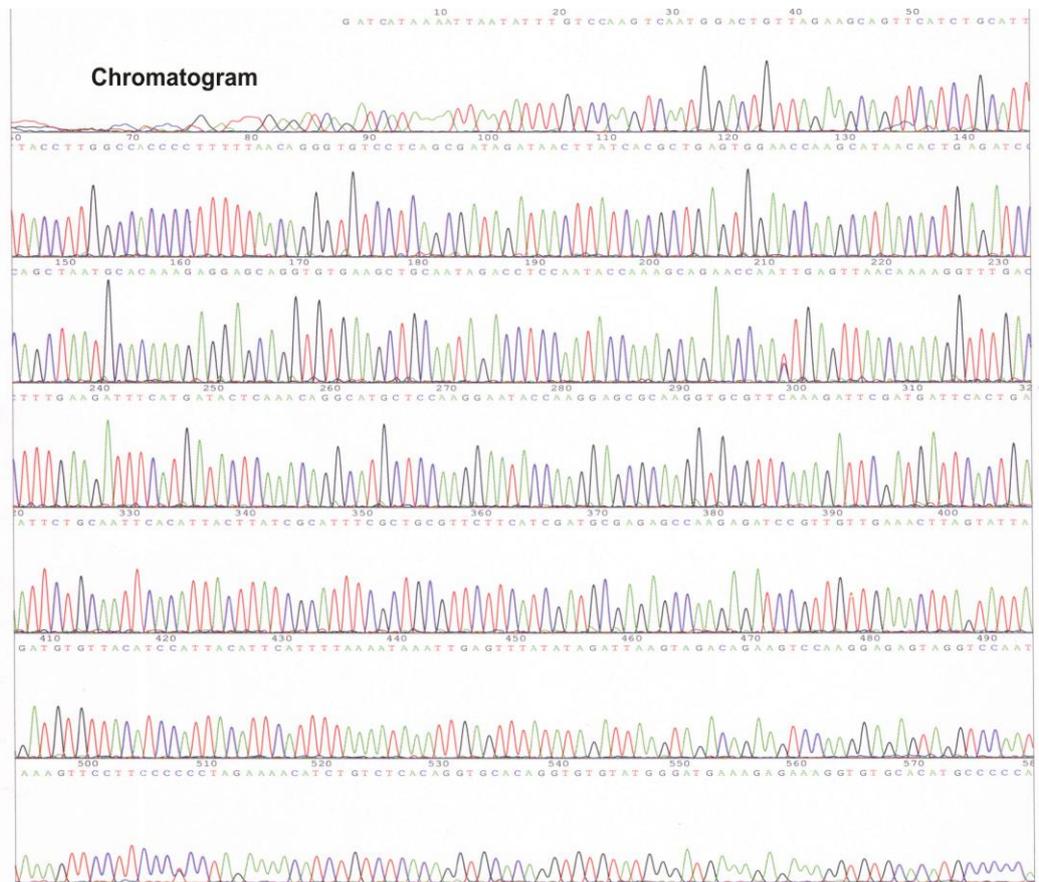
Multiple sequence alignment of the sequences showing maximum identity score (96-99 %) along with the sequence of *R. solani* was conducted to determine sequence homology using CLUSTAL-W software. The results of multiple sequence alignment shows a close match between the bases of the closely related species and also some variable regions which can be utilized for constructing strain specific primers for other studies (Table 33; Fig. 44). For phylogenetic analysis, 18S rDNA sequence of *T. cucumeris* RHS/V-566 was compared with ex-type sequences of the representative

species of the genus. The optimal tree with the sum of branch length = 0.53411025 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 276 positions in the final dataset. The strain (RHS/V-566) was found to be clustered with the other *T. cucumeris* strains thus confirming its identity. The *Eupenicellium* sequences was distantly related which was used as an out group (Fig. 45).

4.9.1.2. Analysis of Nucleotide frequency, ORF and DNA molecular weight of rDNA sequences

Combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html. The sequence of DNA fragments of ITS region for 650 residue sequence "*T. cucumeris*-RHS/V-566ITS4" starting with "TTGTAGCTGG" is presented in the Table 34. The guanine 'G' content of the sequence is maximum (18.00 %) with highest repetition of 40. Combinations like GC were also at the level 43.00 % which occurred at least 43 times in the entire sequence. The DNA Molecular Weight DNA residue sequence "560 ITS4" starting "TTGTAGCTGG" is 200285.29 Da.

A total of 2 open reading frames (ORF) for the designated sequence was calculated with the help of ORF finder available from http://www.ualberta.ca/~stothard/javascript/orf_find.html for residue sequence of *T. cucumeris* RHS/V-566 starting at " TTGTAGCTGG "



TTGTAGCTGGCCCCTAATTAACCTGGGGGCATGTCACACCTTTCTCTTTCATCCATACACACCTGTGCACCTGTGAGACAGATGTTTTCTAGGAGGGAAGGAACTTTATTGGACCTACTCTCCTTGGACTTCTGTCTACTTAATCTATATAAACCAATTTATTTTAAAATGAATGTAATGGATGTAACACATCTAATACTAAGTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAAAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGGCTCCTTGGTATTCTTGGAACATGCCTGTTTGAATATCATGAAATCTTCAAAGTCAAACCTTTTGTAACTCAATGGTCTGCTTGGTATTGGAGGCTATTGTCAGCTTACACCTGCTCCTTTGTGCATTAACCTGGATCTCAATGTTATGCTTGGTCCACTCAAGTGATAAATTATCTATCGCTGAGGACACCTGTTAAAAAAGGGTGGCCAAAGTAAATGCAGATGAACCTGCTCTAACAGTCCATTGACTTGCACAAATATTAATTTTATGATCTGATCTCAAATCACGTAGGACTACCGCTGAACTTAAAGCATATCATAAA

Sequence Deposited: NCBI

ACCESSION: JN248540

VERSION: JN248540.1

GI:359374341

DNA linear : 6509bp

Title : *Thanatephorus cucumeris* isolate RHS/V566 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

1 ttgtagctgg cccctaatta acttgggggc atgtgcacac ctttctcttt catccatac
61 acacctgtgc acctgtgaga cagatgtttt ctaggaggga aggaacttta ttggacctac
121 tctccttgga cttctgtcta cttaatctat ataaactcaa tttattttaa aatgaatgta
181 atggatgtaa cacatctaata actaagtctt aacaacggat cctctggctc tcgcatcgat
241 gaaaaacgca gcgaaatgca ataagtaatg tgaattgcaa aattcagatg atcatcgat
301 ctttgaacgc accttgcgct ccttggatatt ccttggaaac tgcctgtttg aatatcatga
361 aatcttcaaa gtaaacctt ttgttaactc aattggttct gcttggat tggaggctca
421 ttgcagcttc acacctgtc cttcttggc attaactgga tctcaatggt atgcttgggt
481 ccaactcaac tgataaatta tctatcgctg aggacacctt gttaaaaaag ggtggccaaa
541 gtaaatgcag atgaactgct tctaacagtc cattgacttg cacaatatt aattttatga
601 tctgatctca aatcacgtag gactaccgct gaacttaagc atatcataaa

Fig. 43. Chromatogram and sequence deposit of ITS region of *Thanatephorus cucumeris* RHS/V-566/NAIMCC-F-02903.

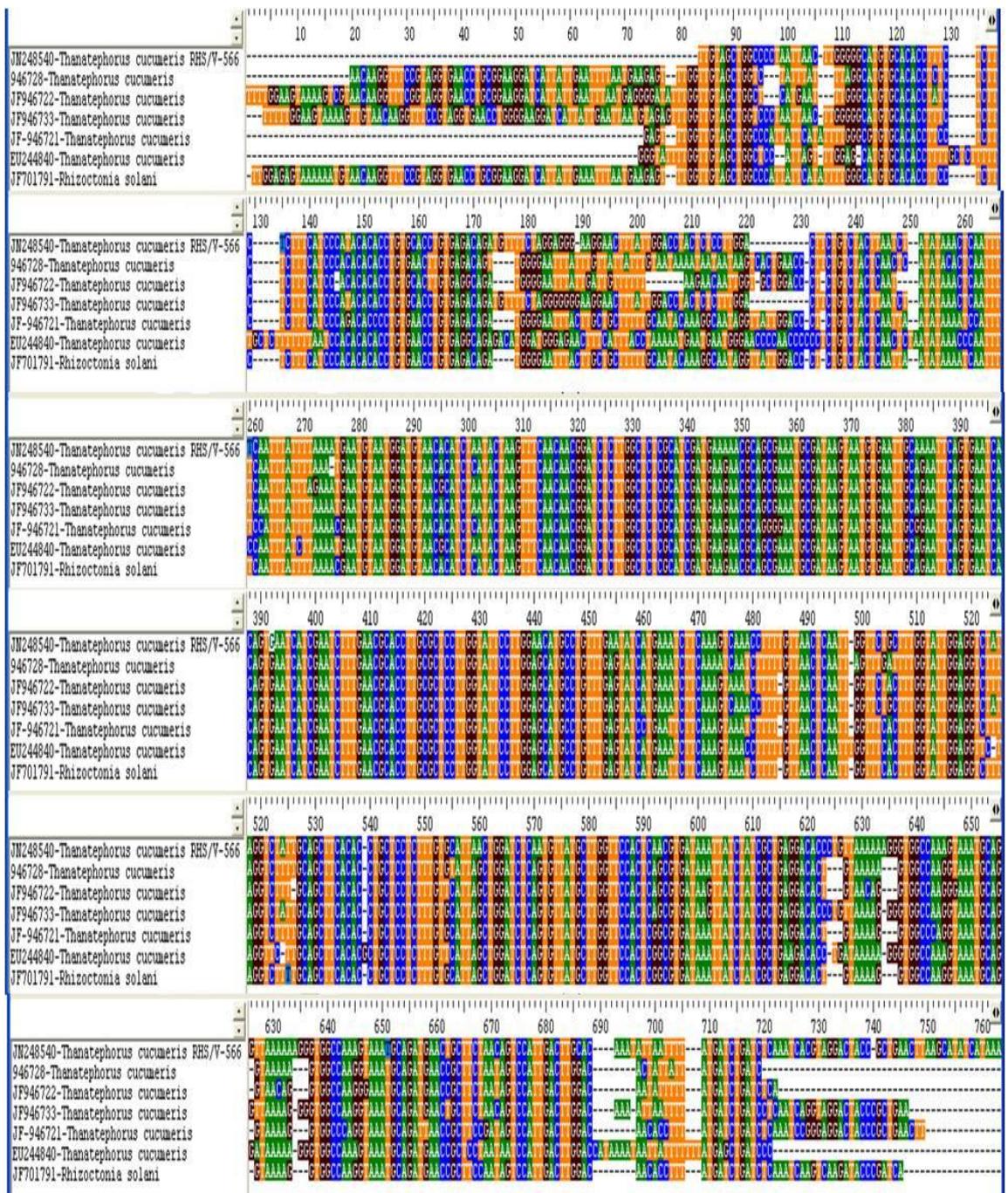


Fig. 44: Multiple sequence alignment of *T. cucumeris* RHS/V-566/ NAIMCC-F-02903 with ex-type strain sequences obtained from *NCBI genbank* database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

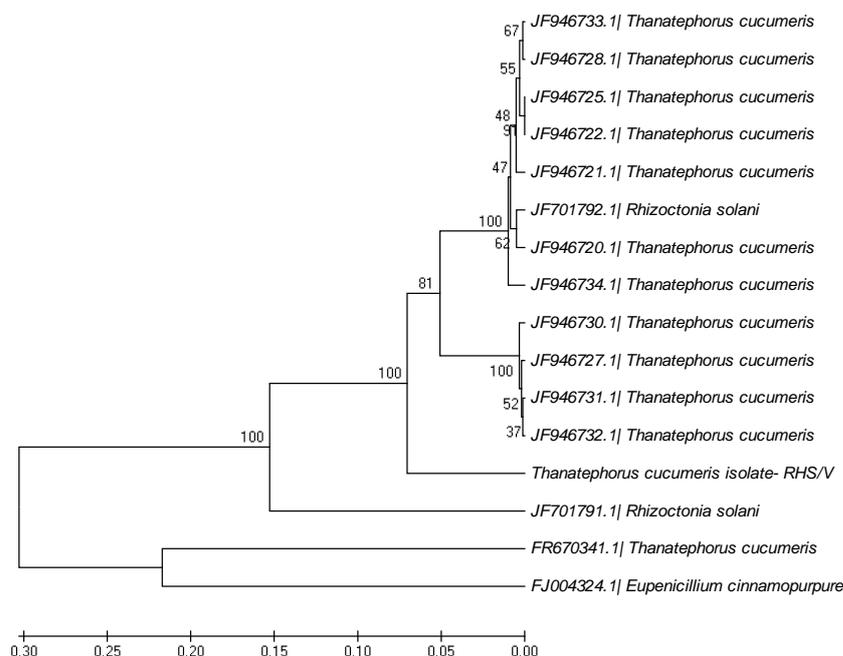


Fig. 45. The phylogenetic analyses of *T. cucumeris* / NAIMCC-F-02903 based on 18S rDNA sequences with the ex- type strains conducted using the UPGMA method of MEGA4.1 software. *Eupenicillium* 18S DNA sequences have been used as an out group.

Table 34. Nucleotide combinations and frequencies of different combinations of ITS sequences of *T. cucumeris* RHS/V-566, NAIMCC-F02912

Pattern	Times found	Percentage	Pattern	Times found	Percentage
G	117	18.00	AC	40	6.16
A	187	28.77	TG	57	8.78
T	208	32.00	TA	42	6.47
C	138	21.23	TT	62	9.55
GG	27	4.16	TC	47	7.24
GA	34	5.24	CG	13	2.00
GT	28	4.31	CA	43	6.63
GC	28	4.31	CT	59	9.09
AG	20	3.08	CC	23	3.54
AA	68	10.48	G,C	255	39.23
AT	58	8.94	A,T	395	60.77

Open Reading Frame of *Thanatephorus cucumeris* isolate RHS/V566 GenBank: JN248540.1

Results for 650 residue sequence "*T. cucumeris*-RHS/V-566/NAIMCC-F-02903, starting "TTGTAGCTGG"

>ORF number 1 in reading frame 1 on the direct strand extends from base 79 to base 264.

GACAGATGTTTTCTAGGAGGGAAGGAACTTTATTGGACCTACTCTCCTTG
GACTTCTGTCTACTTAATCTATATAAACTCAATTTATTTTAAAATGAATGT
AATGGATGTAACACATCTAATACTAAGTTTCAACAACGGATCTCTTGGCT
CTCGCATCGATGAAAAACGCAGCGAAATGCGATAA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

DRCFLGGKELYWTYSPWTSVYLIYINSIYFKMNVMDVTHLILSFNNGSLGSRI
DEKRSEM

R*

>ORF number 2 in reading frame 1 on the direct strand extends from base 388 to
base 525.

CTCAATTGGTTCTGCTTTGGTATTGGAGGTCTATTGCAGCTTCACACCTGC
TCCTCTTTGTGCATTAACTGGATCTCAATGTTATGCTTGGTTCCACTCAAC
GTGATAAATTATCTATCGCTGAGGACACCCTGTAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

LNWFCFGIGLLQLHTCSSLCINWISMLCLVPLNVINYLSLRTPC*

4.9.2. Phosphate Solubilizing Fungus-PSF, (*Talaromyces flavus* RHS/P-52, NAIMCC-F01948)

Among all the phosphate solubilizing isolate RHS-P-51 which was initially identified on the basis of morphological characters as *Talaromyces flavus*, was found to be the most potential PSF isolate. The identity of this isolate was further confirmed with help of 18S rDNA sequences with the help of universal primers.

A uniform product of size of 800 bp was obtained which was analyzed with the help of 1% Agarose gel electrophoresis and was sequenced. The obtained sequence was further used to query against NCBI Genbank sequences through BLAST. The analysis revealed isolate RHS/P-51 to have 100% homology with *Talaromyces flavus*. The sequences were approved as 18S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,230). The accession number for isolate *T. flavus* RHS/P-51, provided by NCBI is **GU324073** (Fig. 46).

4.9.2.1. Multiple sequence alignment and Phylogenetic analysis

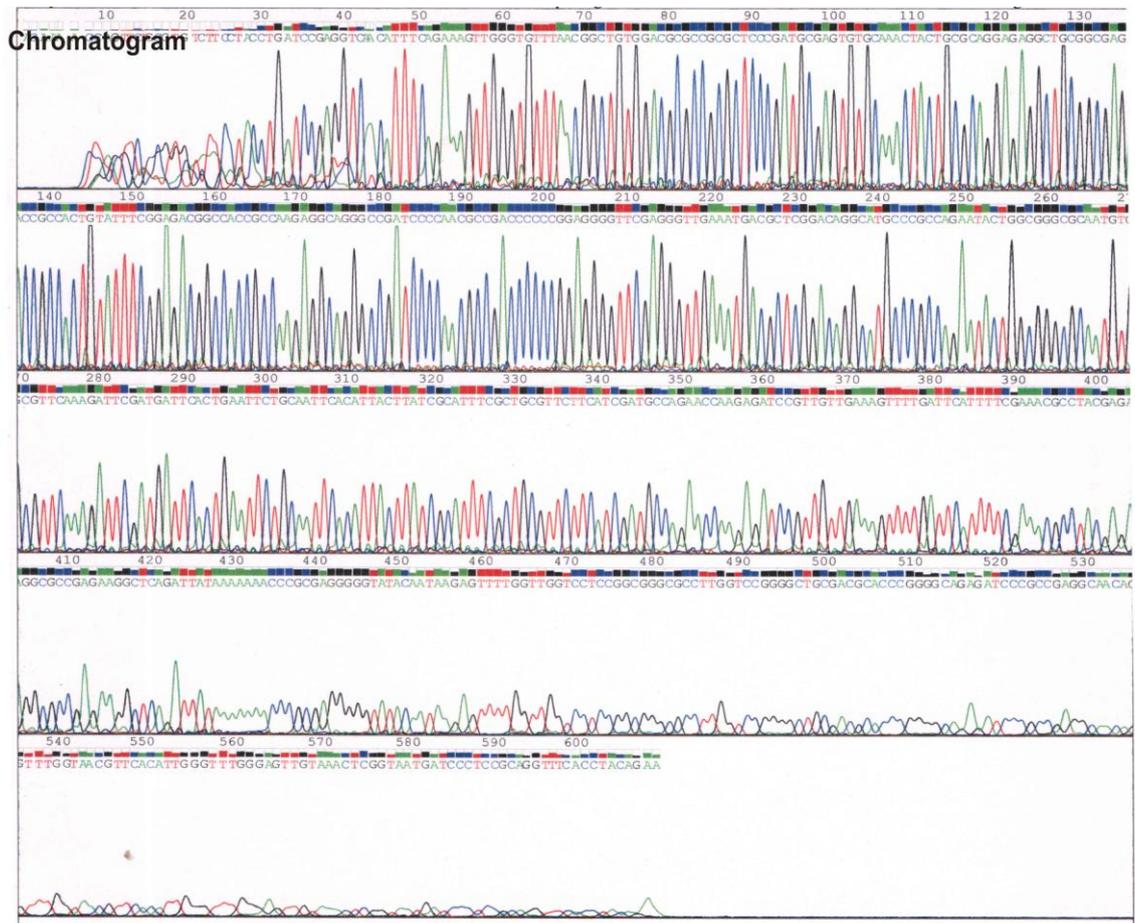
A multiple sequence alignment of ITS gene sequences of *T. flavus* (RHS/P-51) with the sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were

closely related and similar sequence indicated the relationship among the isolates. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 35; Fig. 47).

The 18S DNA sequence based phylogenetic analysis of *T. flavus* (RHS/P-51) was conducted with other ex-type strains presented in Table 36, obtained from NCBI-Genbank database as well as with the most commonly reported phosphate solubilizing isolates of other regions. The evolutionary history was inferred using the Neighbour joining (NJ) method. The optimal tree with the sum of branch length = 1.16899756 has been shown. The phylogenetic tree shows that *T. flavus* is closely related to *A. niger* group whereas distantly related to *A. clavatus* group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) has been shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option) (Fig. 48).

4.9.2.2. Analysis of Nucleotide frequency, ORF and DNA molecular weight of rDNA sequences

Combinations and percentage of occurrence of different nucleotide in the entire sequences were also calculated. The sequence of DNA fragments of ITS region for 565 residue sequence "*Talaromyces flavus* strain RHS/P-51- GU324073" starting with "TTGTTTTAAC" revealed that the guanine 'C' content of the sequence is maximum (29.20 %) with highest repetition of 165 (Table 37). Whereas DNA Molecular weight results for 565 residue sequence starting "TTGTTTTAAC" is 173567.12 Da. A total of 2 open reading frames for the designated sequence were calculated for 565 residue sequence "*Talaromyces flavus* strain RHS/P 51- GU324073" starting with "TTGTTTTAAC" (Fig. 49).



**TTGTTTTAACGTGAGTGTGCGGTCCTCACGGCTACATCTCACCCTTGTTCTCTTACCCTCGTGTTAGCTT
 TGGCGGGCCACCGCCGCCACCTGGTCCCCGGGGACTCACGTCCCCGGACCCGCGCCCGCGAAGCTC
 TCTTTGAACCCTGATGAAGATGGGCTGTCTCACAATCAAAC TTGTCTTAACTTTCAACATTACATCTC
 TTCATTCTGCCTCCATGAATAACGCAGCGAAATGTGATAATTAATGTACATTGAAATTTTGAATTTTCTC
 ATCTTAATCTTTAAATATCATTTCCTCCGGTCTTGGGGGATGCCTGTCCGAGAGACATTTTGTGCATC
 ATTCACGCTTTGTGTGTTAGGTGTAGTCCCCCGGGACCTGGCCTAAAGGCAGCTCCACCTCCCTCTGG
 TCCTCGAACGTGCGTGCCTCTGTCACTCGGTGGCCACGGAGGGGCGCGATAGCTCACCGCCATAATTTAC
 AACGGTGAAGTCGGATCAGGTAGAACTTACCCGCTAAATTTATCCTTCTCAAATTCGGAGGAAAA**

Sequence Deposited: NCBI

ACCESSION: GU324073

VERSION: GU324073.1

GI:284192449

DNA linear : 565bp

Title : *Talaromyces flavus* strain RHS/P 51 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```

1 ttgttttaac gtgagtgtgc ggtcctcacc gctacatctc acccttgttc tctctaccct
61 cgtgttagct ttggcggggc caccggccgc cacctgtgac cgggggact cacgtcccgc
121 gaccocgcgc ccgccaagc tctctttgaa ccctgatgaa gatgggctgt ctactactaa
181 tcaaacttgt ctaaacttcc aacattacat ctcttcattc ctgcctccat gaataacgca
241 gcgaaatgta ataattaatg tacattgaaa ttttgaattt tctcatctta atctttaa
301 atcattttcc ctccggtcct ggggggatgc ctgtccgaga gacatttttg tcatcattca
361 cgctttgtgt gttaggtgta gtcccccccg ggacctggcc taaaggcagc ctccacctcc
421 ctctggctct cgaacgtgcg tgcctctgtc actcgggtgc caccggaggg cggcgatagc
481 tcaccgccat aatttacaac ggtggaagtc ggcacagcta gaacttaccg gctaaattta
541 tccttctcaa attccggagg aaaaa

```

Fig. 46. Chromatogram and sequence deposit of ITS region of *Talaromyces flavus* RHS/P-51/ NAIMCC-F01948.

Table 35. Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of *T. flavus* used for analysis with ex-type strain sequence

```

Title T. flavus;!Format, DataType=Nucleotide CodeTable=Standard, NSeqs=7 NSites=765
Identical=. Missing=? Indel=-; !Domain=Data property=Coding CodonStart=1;

gi|284192449|gb|GU324073.1|_Tala GTGAGTGTGCGGTCACGGCTATCTCACCCCTTTTCTCTCT [114]
gi|254681489|gb|FJ537107.1|_Tala CC.....A.G.CC.G....C.C...C...CC..G....C [114]
gi|158138922|gb|EU021596.1|_Tala CC.....C.G.CC.G....C.C...C.A.CC..G.... [114]
gi|218938080|gb|FJ487931.1|_Tala CC.....C.G.CC.G....C.C...C.A.CC..G.... [114]
gi|305380912|gb|GU396595.1|_Tala CC.....C.G.CC.G....C.C...C.A.CC..G.... [114]
gi|305380913|gb|GU396596.1|_Tala CC.....C.G.CC.G....C.C...C.A.CC..G.... [114]
gi|639442|gb|U18354.1|TFU18354_T CC.....C.G.CC.G....C.C...C.A.CC..G.... [114]
gi|119369878|gb|EF123253.1|_Tala ..TG.A.G.GCC..G....C.C...C.A.CC..G.... [114]
gi|308055650|gb|HQ191279.1|_Tala AG.....G.G..C.G....C.C...C.A.CC..G.... [114]
gi|42741971|gb|AY532420.1|_Talar CC.....C.G.CCTG....C.C...C.A.CC..G.... [114]
gi|284192449|gb|GU324073.1|_Tala TCCCCGGGGGACTCCGTCCCGGACCCCGCGCCGCGAA [197]
gi|254681489|gb|FJ537107.1|_Tala ..G.....AT.....G.....G.....G.....G [197]
gi|158138922|gb|EU021596.1|_Tala ..G.....GT...T...G.....G.....G..... [197]
gi|218938080|gb|FJ487931.1|_Tala ..G.....GT.....GT.....G.....G..... [197]
gi|305380912|gb|GU396595.1|_Tala ..G.....G.....G.....G.....G.....G..... [197]
gi|305380913|gb|GU396596.1|_Tala ..G.....G.....G.....G.....G.....G..... [197]
gi|639442|gb|U18354.1|TFU18354_T ..G.....AT.....G.....G.....G.....G..... [197]
gi|119369878|gb|EF123253.1|_Tala ..G.....G.....G.....G.....G.....G..... [197]
gi|308055650|gb|HQ191279.1|_Tala ..G.....GT.....G.....G.....G.....G..... [197]
gi|42741971|gb|AY532420.1|_Talar ..G.....G.....G.....G.....G.....G..... [197]
gi|284192449|gb|GU324073.1|_Tala CAATCAAACCTGTCTTAACCTTCAACATTACATCTCTTC [275]
gi|254681489|gb|FJ537107.1|_Tala AT..G...A....AA.....A.GG.....G [275]
gi|158138922|gb|EU021596.1|_Tala .T.G...A....AA.....A.GG.....G [275]
gi|218938080|gb|FJ487931.1|_Tala .C.G...A....AA.....A.GG.....G [275]
gi|305380912|gb|GU396595.1|_Tala .T.G...A....AA.....A.GG.....G [275]
gi|305380913|gb|GU396596.1|_Tala .T.G...A....AA.....A.GG.....G [275]
gi|639442|gb|U18354.1|TFU18354_T TT.G...A....AA.....A.GG.....G [275]
gi|119369878|gb|EF123253.1|_Tala .T.G...A....AA.....A.GG.....G [275]
gi|308055650|gb|HQ191279.1|_Tala .T.G...A....AA.....A.GG.....G [275]
gi|42741971|gb|AY532420.1|_Talar .T.G...A....AA.....A.GG.....G [275]
gi|284192449|gb|GU324073.1|_Tala TAATGTACATFGAAATTTTGAATTTTCTCATCTTAATCT [357]
gi|254681489|gb|FJ537107.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|158138922|gb|EU021596.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|218938080|gb|FJ487931.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|305380912|gb|GU396595.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|305380913|gb|GU396596.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|639442|gb|U18354.1|TFU18354_T .....GA...G...C....CA..GA....TGAAC [357]
gi|119369878|gb|EF123253.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|308055650|gb|HQ191279.1|_Tala .....GA...G...C....CA..GA....TGAAC [357]
gi|42741971|gb|AY532420.1|_Talar .....GA...G...C....CA..GA....TGAAC [357]
gi|284192449|gb|GU324073.1|_Tala CCGAGAGACATTTTTGTTCATCATTACGCTTTGTGTGTT [436]
gi|254681489|gb|FJ537107.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|158138922|gb|EU021596.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|218938080|gb|FJ487931.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|305380912|gb|GU396595.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|305380913|gb|GU396596.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|639442|gb|U18354.1|TFU18354_T .....C.T....C.C.C...AG...GC..... [436]
gi|119369878|gb|EF123253.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|308055650|gb|HQ191279.1|_Tala .....C.T....C.C.C...AG...GC..... [436]
gi|42741971|gb|AY532420.1|_Talar .....C.T....C.C.C...AG...GC..... [436]
gi|284192449|gb|GU324073.1|_Tala CCACCTCCCTCTGGTCCCGAACGTGCGCTCTGTCA [514]
gi|254681489|gb|FJ537107.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|158138922|gb|EU021596.1|_Tala .G.GC.G.....G...AT.G.G..... [514]
gi|218938080|gb|FJ487931.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|305380912|gb|GU396595.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|305380913|gb|GU396596.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|639442|gb|U18354.1|TFU18354_T .G.G...G.....G...AT.G.G..... [514]
gi|119369878|gb|EF123253.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|308055650|gb|HQ191279.1|_Tala .G.G...G.....G...AT.G.G..... [514]
gi|42741971|gb|AY532420.1|_Talar .G.G...G.....G...AT.G.G..... [514]
;end
-----
dimensions ntax=10 nchar=462; format missing=? gap=- matchchar=. datatype=nucleotide interleave=yes;
matrix

```

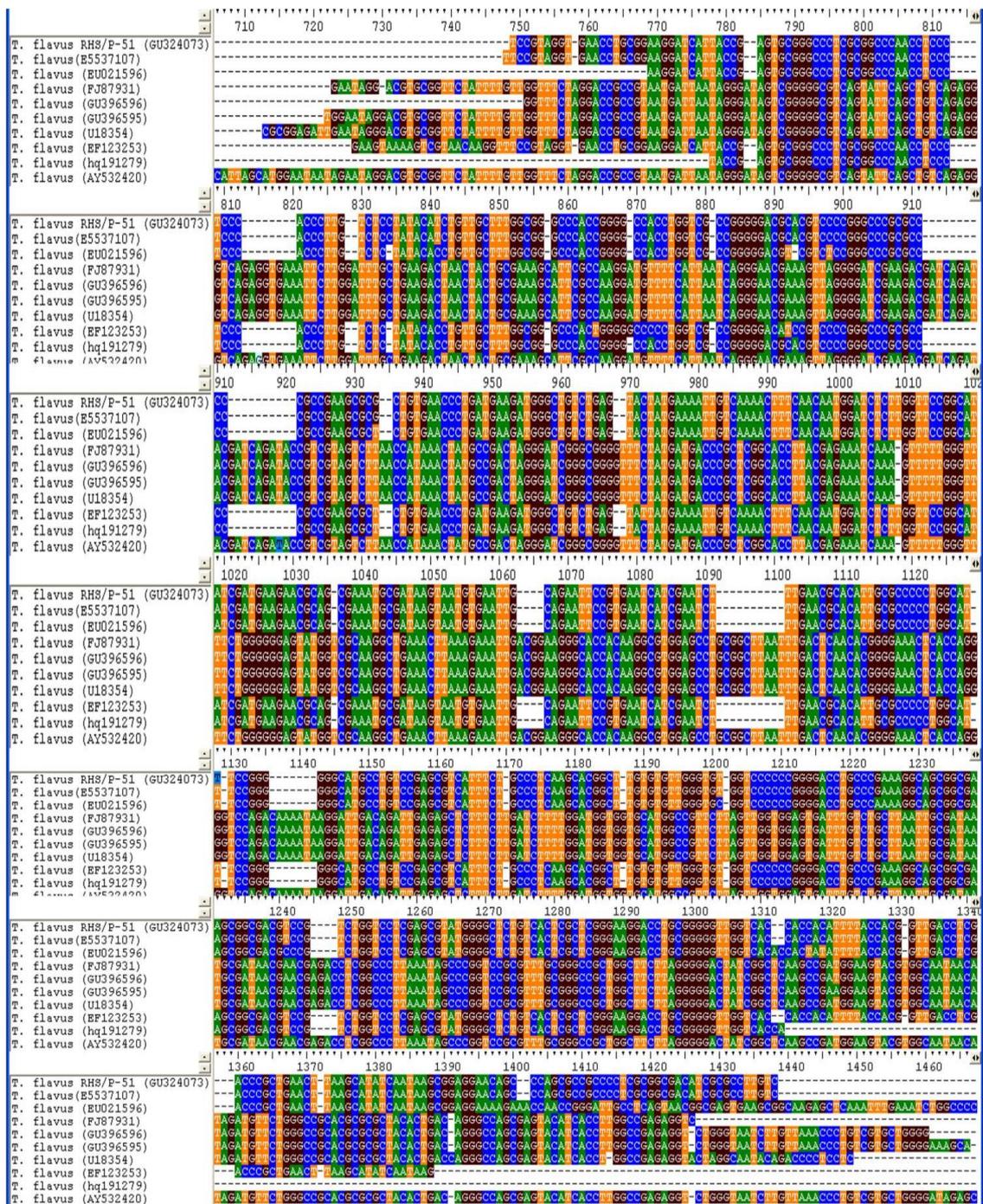


Fig. 47. Multiple sequence alignment of *T. flavus* RHS/P-51/ NAIMCC-F-01948 with ex-type strain sequences obtained from NCBI genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

Table 36. Genbank Accession numbers and geographic location of the Ex-Type strains of *Talaromyces flavus* that showed homology with isolate RHS/P-51 for identification.

Country of Origin	Strain No	GeneBank accession no	Country of origin	Strain No	GeneBank accession no
Australia	-	M83262	USA	S-16	AY532419
Austria	WB-239	AFY55513	USA	S-18	AY532418
Canada	-	U18354	USA	S-24	AY532417
China	SW- 0092	FJ537107	USA	S-26	AY532416
China	JMUPMD-3	HQ191279	USA	S-29	AY532415
China	-	EF123253	USA	S-2	AY532414
China	-	EF123253	USA	S-30	AY532413
China	ZJ4-B	FJ487931	USA	S-31	AY532412
China	XSD-46	EU273527	USA	TF1M	AY532411
China	Hn-50-1	EU287814	USA	W-1	AY532410
China	Jx-18-3	EU287815	USA	S-18	AY532409
France	LCP 2892	GU396596	USA	W-4	AY532408
France	LCP 3067	GU396595	USA	W-5	AY532407
India	RHS/P 51	GU324073	USA	W-13	AY532406
Japan	CBS-310.38	AB176618	USA	W-21	AY532405
USA	S-12	AY532420	USA	W-22	AY532404

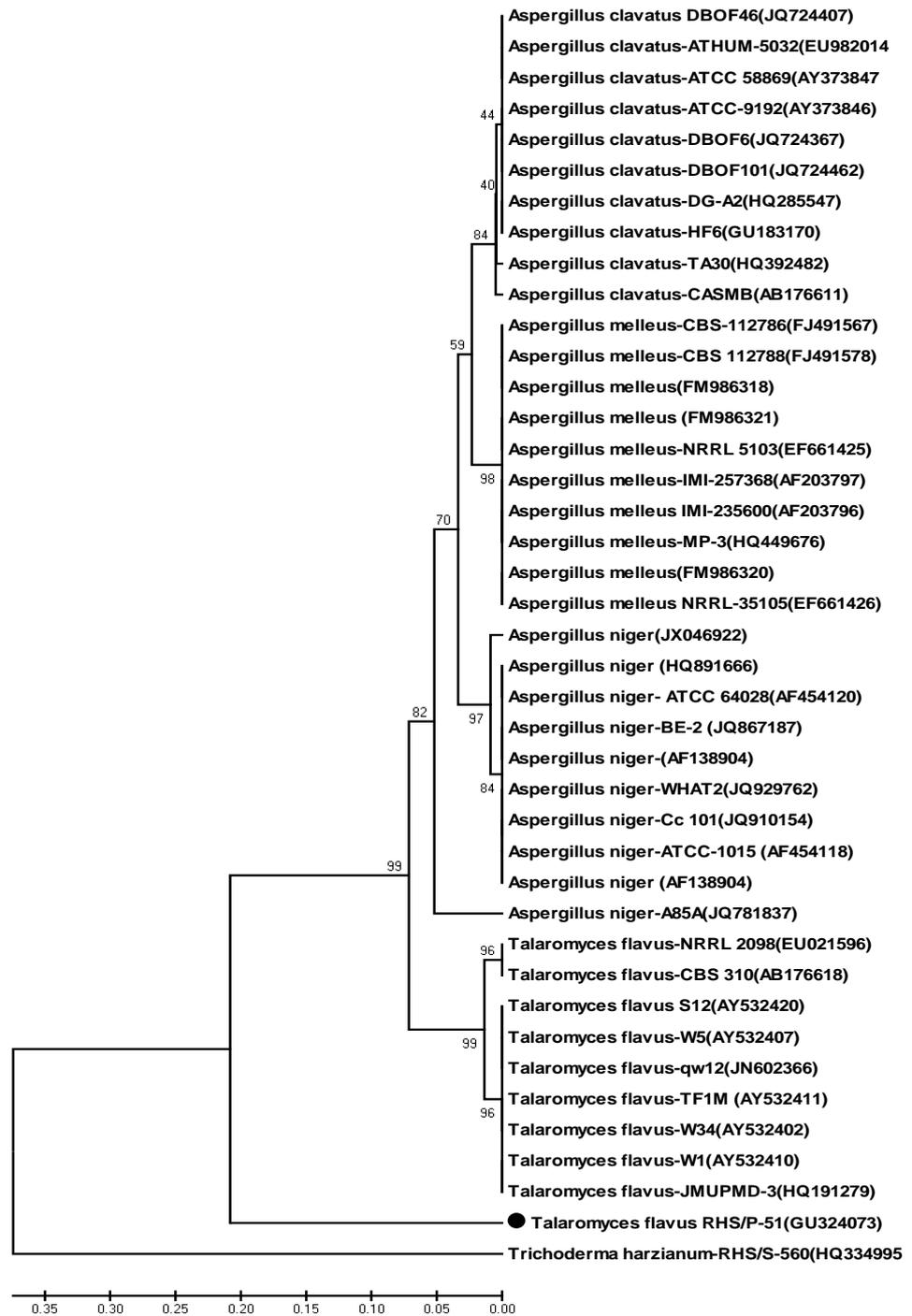


Fig. 48. Phylogenetic placement of *Talaromyces flavus* (RHS/P-51)/ NAIMCC-F-01948 among the members of other phosphate solubilizing fungi based on 16S rRNA gene. There were a total of 112 positions in the final dataset. *Trichoderma harzianum* was used as an out group.

Open Reading Frame of *Talaromyces flavus* strain RHS/P 51-GU324073/NAIMCC-F-01948'

Results for 650 residue sequence "*T. flavus*-RHS/P-51" starting "TTGTTTTAAC "

ORF number 1 in reading frame 1 on the direct strand extends from base 10 to base 156.

CGTGAGTGTGCGGTCTCACGGCTACATCTCACCTTGTCTCTCTACCCT
CGTGTTGCTTTGGCGGGCCACCGGCCGCCACCTGGTCCCCGGGGGACTC
ACGTCCCCGGACCCCGCGCCCGCCGAAGCTCTCTTTGAACCCTGA

Translation of ORF number 1 in reading frame 1 on the direct strand.

RECAVLTATSHPCSLYPRVSFGGPTGRHLVPGGLTSPDPAPAEALFEP*

ORF number 2 in reading frame 1 on the direct strand extends from base 382 to base 492.

TCCCCCGGGACCTGGCCTAAAGGCAGCCTCCACCTCCCTCTGGTCCTC
GAACGTGCGTGCCTCTGTCACTCGGTGGCCACGGAGGGGGCGGCGATAGC
TCACCGCCATAA

Translation of ORF number 2 in reading frame 1 on the direct strand.

SPPGPGLKAASTSLWSSNVRASVTRWPRRGGDSSPP*

Table 37. DNA Stats results for 565 residue sequence "*Talaromyces flavus* strain RHS/P-51- GU324073" starting "TTGTTTTAAC"

Pattern	Times found	Percentage	Pattern	Times found	Percentage
G	124	21.95	AC	31	5.50
A	117	20.71	TG	36	6.38
T	159	28.14	TA	24	4.26
C	165	29.20	TT	48	8.51
GG	39	6.91	TC	51	9.04
GA	26	4.61	CG	32	5.67
GT	31	5.50	CA	31	5.50
GC	28	4.96	CT	47	8.33
AG	17	3.01	CC	55	9.75
AA	36	6.38	G,C	289	51.15
AT	32	5.67	A,T	276	48.85

through BLAST. The analysis revealed isolates were identified on the basis of 100% homology with the reference sequence when analyzed by BLAST. The sequences were approved as 18S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,230)

The Accession number for all the selected isolates along with their identities is presented in table 38.

Table. 38. NCBI accession numbers of Identified *Trichoderma* isolated

Isolate No	Identified as	GenBank Acc. Number	% homology with reference sequence
FS/L-20	<i>Trichoderma erinaceum</i>	HM107419	100%
SF/S-474	<i>Trichoderma erinaceum</i>	GU187915	100%
FS/S-475	<i>Trichoderma erinaceum</i>	GU191829	100%
FS/S-478	<i>Trichoderma erinaceum</i>	HM117841	100%
RHS/S-559	<i>Trichoderma harzianum</i>	HQ334997	100%
RHS/S-560	<i>Trichoderma harzianum</i>	HQ334995	100%
RHS/S-561	<i>Trichoderma asperellum</i>	HQ334996	100%

4.9.3.1. Multiple sequence alignment

4.9.3.1.1. *Trichoderma harzianum* isolates

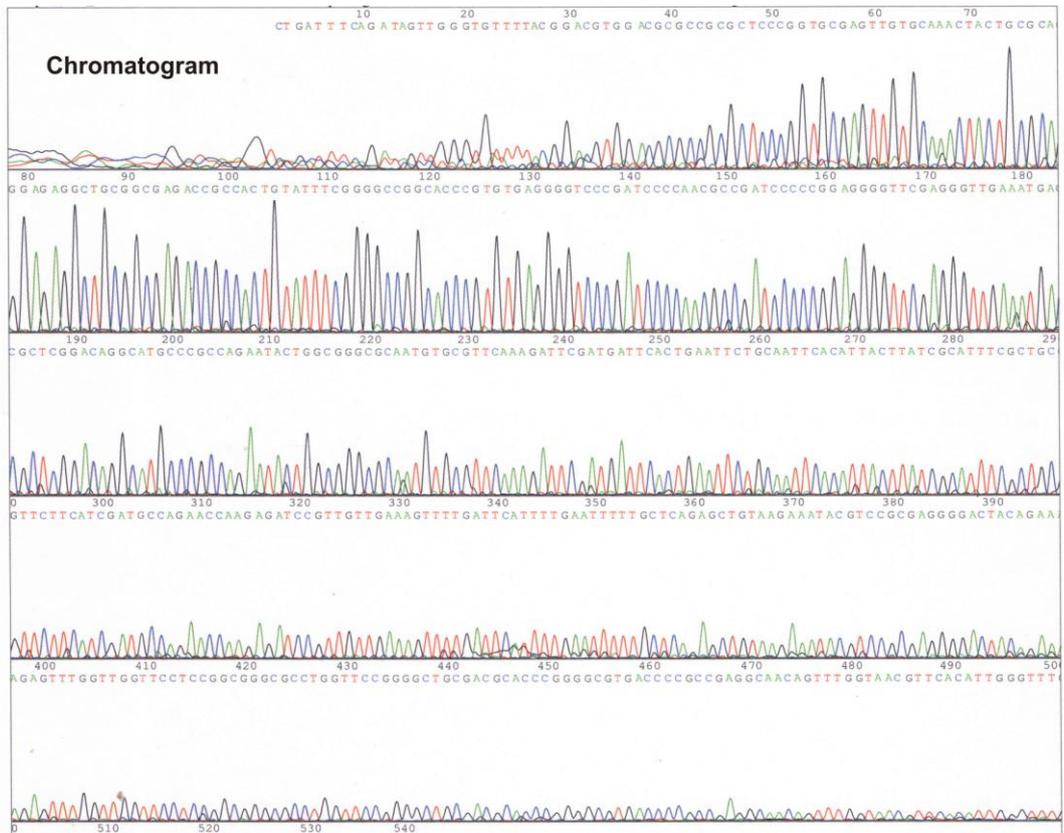
The gene rDNA sequences have been deposited in NCBI Genbank database with complete annotation and has been approved as rDNA sequences (**Fig. 50,51**). A multiple sequence alignment of these ITS gene sequences of the selected *Trichoderma harzianum* isolates (RHS/S-559 and RHS/S-560) were conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with our isolates CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 39, Fig. 52).

4.9.3.1.2. *Trichoderma asperellum* isolates

The rDNA sequence of the most potential *T. asperellum* isolate RHS/S-561 was approved as ITS sequence and has been deposited to NCBI Genbank database (**Fig. 53**). A multiple sequence alignment of ITS gene sequences of *Trichoderma asperellum* isolates (RHS/S-561) was conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with our isolates CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 40, Fig. 54).

4.9.3.1.3. *Trichoderma erinaceum* isolates

The 18S rDNA sequences of all the potential *T. erinaceum* were approved as ITS sequences and have been deposited in the NCBI genbank database (Fig. 55-58). A multiple sequence alignment of ITS gene sequences of all the selected *Trichoderma erinaceum* isolates (FS/L-20, FS/S-474, FS/S-475 and FS/S-478) were conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with our isolates CLUSTAL-W of MEGA-4.1 software. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 41, Fig. 59).



GTTTCGTAGGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACCTCCCAAACCCAATGTGAACGTTAC
 CAAACTGTTGCCTCGGCGGGGTCACGCCCGGGTGCCTCGCAGCCCCGGAACCAGGCGCCCGCGGAGGA
 ACCAACCAAACCTTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATG
 AATCAAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
 ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
 GCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCC
 TCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGACGCTCTCCTGCGCAGTAGTTTGCACA
 ACTCGCACGGGAGCGCGGCGGTCCACGTCCGTA AACACCCAACCTATCTGAAATCAG

Sequence Deposited: NCBI Title : *Hypocrea lixii* isolate RHS/S 559 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
 ACCESSION: HQ334997

VERSION: HQ334997.1

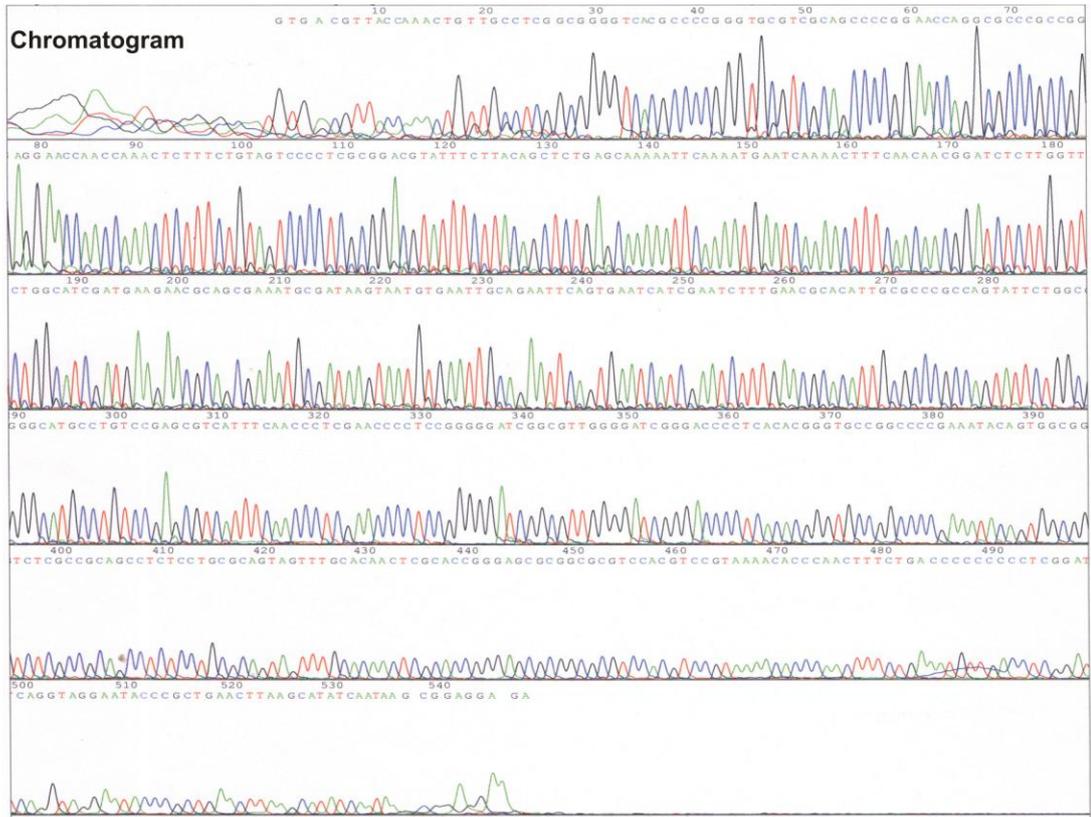
GI:315112488

DNA linear : 549bp

```

1 gtttcgtagg gtgaacctgc ggagggatca ttaccgagtt tacaactccc aaacccaatg
61 tgaacgttac caaactgttg cctcggcggg gtcacgcccc gggtagctcg cagccccgga
121 accagggcgc cgccggagga accaaccaaa ctctttctgt agtcccctcg cggacgtatt
181 tcttacagct ctgagcaaaa attcaaaatg aatcaaaaact ttcaacaacg gatctcttgg
241 ttctggcatc gatgaagaac gcagcgaat gcgataagta atgtgaattg cagaattcag
301 tgaatcatcg aatctttgaa cgcacattgc gcccgccagt attctggcgg gcatgcctgt
361 ccgagcgtca tttcaacctc cgaacccctc cgggggatcg gcgttgggga tcgggacccc
421 tcacacgggt gccggccccg aaatacagtg gcggtctcgc cgcagcctct cctgcgcagt
481 agtttgaca actcgcaccg ggagcggcgc gcgtccacgt cogtaaaaca cccaactatc
541 tgaaatcag
  
```

Fig. 50. Chromatogram and sequence deposit of ITS region of *Trichoderma harzianum* RHS/S-559/NAIMCC F-01968.



GTGACGTTACCAAAC TGTGCCTCGGGGGT CACGCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGAGGAACCAACCAAAC TCTTTCTGTAGTCCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAAT TCAAAATGAATCAAAC TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCC ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTC TGGCGGGCATGCCGTGCCGAGCGTCATTTCAACCCTCGAACCCTCCGGGGGATCGGCGTTGGGGATCGGG ACCCCTCACACGGGTGCCGCCCCGAAATACAGTGGCGGTCTCGCCGACGCTCTCCTGCGCAGTAGTTTG CACAAC TCGCACC GGGAGCGGGCGGTCCACGTCCGTAAAACACCCAAC TTTCTGACCCCCCCCCCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAGA

Sequence Deposited: NCBI

ACCESSION: HQ334995

VERSION: HQ334995.1

GI:315112486

DNA linear : 546bp

Title : *Hypocrea lixii* isolate RHS/S 560 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```

1  gtgacgttac  caaactgttg  cctcggcggg  gtcacgcccc  ggggtcgtcg  cagccccgga
61  accaggcgcc  cgccggagga  accaaccaaa  ctctttctgt  agtcccctcg  cggacgtatt
121  tcttacagct  ctgagcaaaa  attcaaatg  aatcaaaact  ttcaacaacg  gatctcttgg
181  ttctggcatc  gatgaagaac  gcagcgaat  gcgataagta  atgtgaattg  cagaattcag
241  tgaatcatcg  aatctttgaa  cgcacattgc  gcccgccagt  atctgtggcg  gcatgcctgt
301  ccgagcgtca  tttcaaccct  cgaaccctc  cgggggatcg  gegttgggga  tegggacccc
361  tcacacgggt  gccggccccg  aaatacagtg  ggggtctcge  cgcagcctct  cctgocgagt
421  agtttgaca  actcgcaccg  ggagcgcggc  gcgtccacgt  ccgtaaaaca  cccaactttc
481  tgaccccccc  cctcggatca  ggtaggaata  cccgctgaac  ttaagcatat  caataagcgg
541  aggaga

```

Fig. 51. Chromatogram and sequence deposit of ITS region of *Trichoderma harzianum* RHS/S-560/NAIMCC-F-01966.

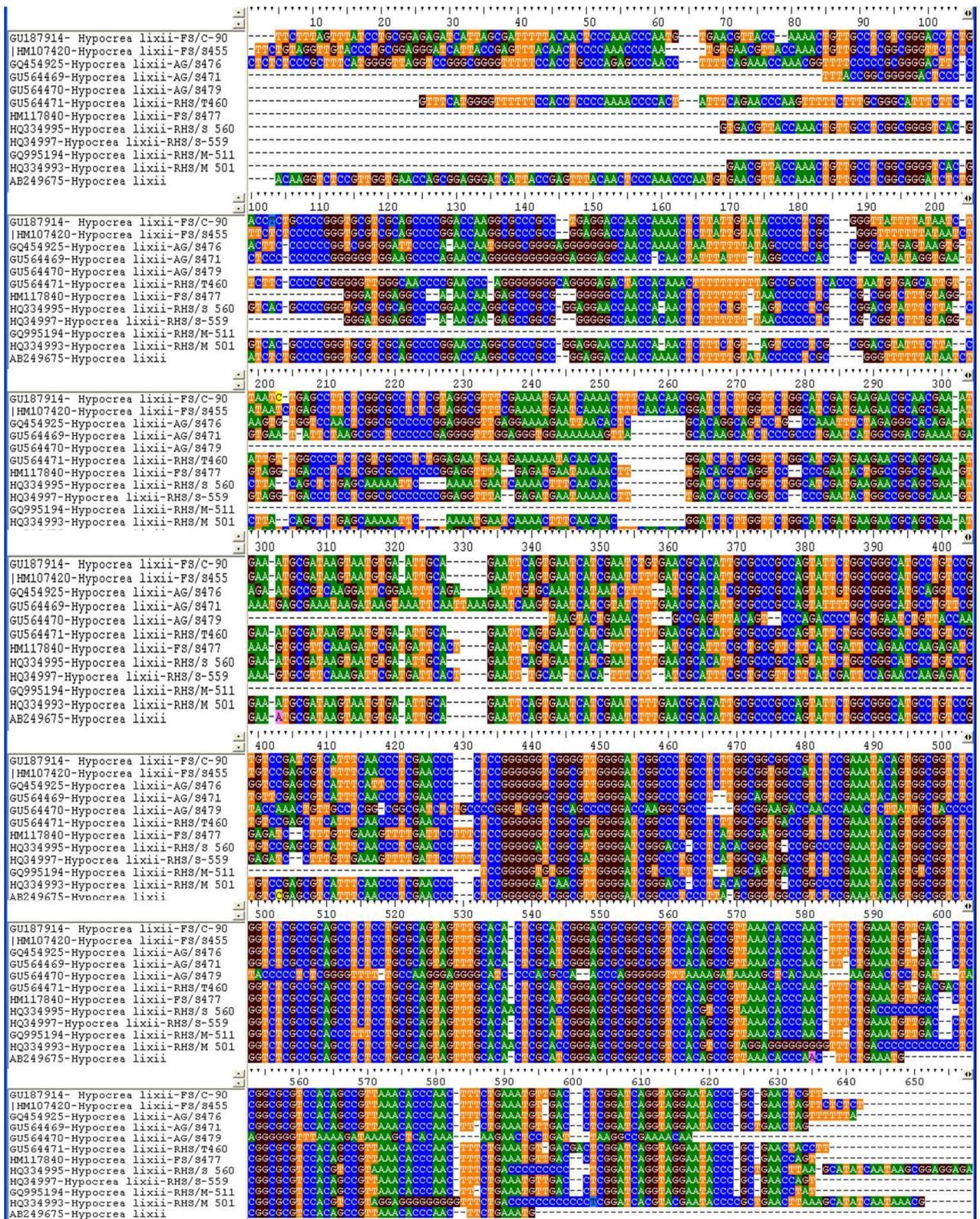
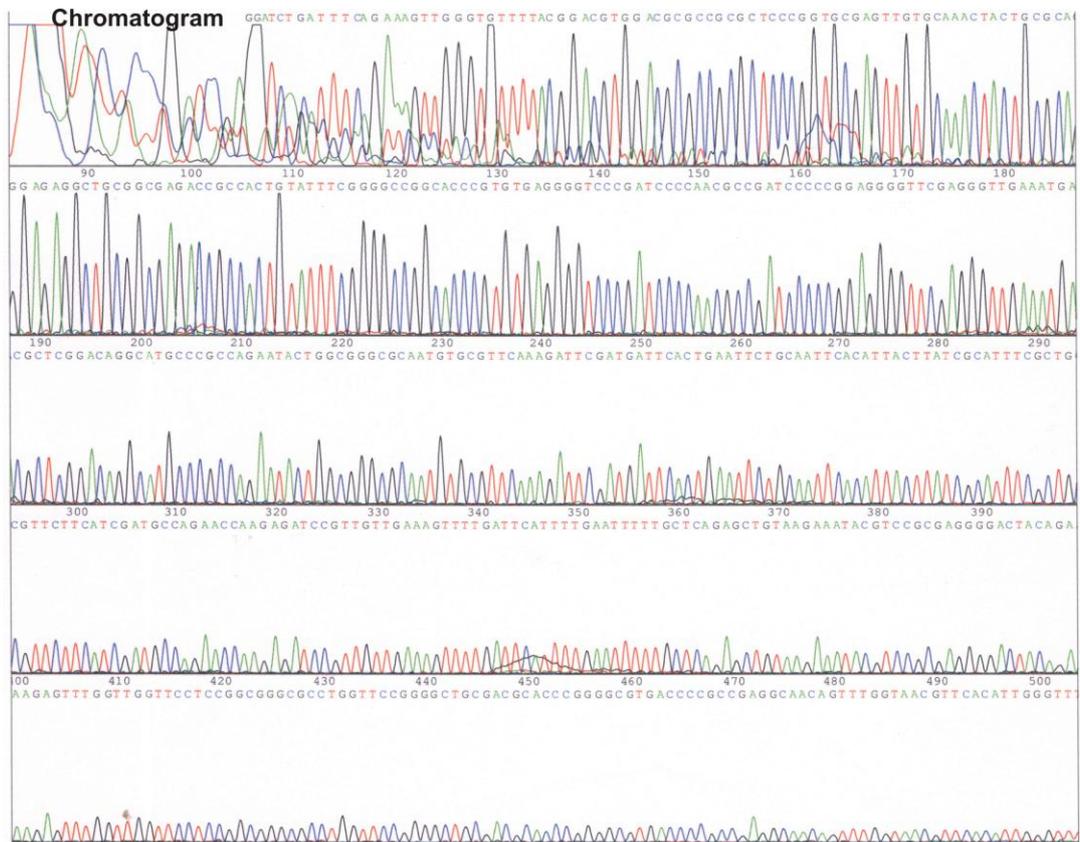


Fig. 52. Multiple sequence alignment of *T. harzianum* isolates with ex-type strain sequences obtained from NCBI genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.



ACGTTACCAAACCTGTTGCCCTCGGCGGGGTACGCCCCGGGTGCGTTCGACGCCCGGAACCAGGCGCCCGCC
GGAGGAACCAACCAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCA
AAATGAATCAAACCTTTCAACAACGGATCTCTTGGTTCGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGG
CGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGGCGTTGGGGATCGGGACC
CCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGACGCTCTCTGCGCAGTAGTTTGCAC
AACTCGCACCGGGAGCGCGGCGGTCCACGTCCGTAACACCCAACTTTCTGAAATGTTGACCTCGGATC
AGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGA

Sequence Deposited: NCBI

ACCESSION: HQ334996

VERSION: HQ334996.1

GI:315112487

DNA linear : 540 bp

Title : *Trichoderma asperellum* isolate RHS/S 561 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```

1 acgttaccaa actgttgctt cggcggggtc acgccccggg tgcgtgcgag ccccggaacc
61 aggcgccgcg cggaggaacc aaccaaactc tttctgtagt cccctcgcgg acgtatttct
121 tacagctctg agcaaaaatt caaaatgaat caaaactttc aacaacggat ctcttggttc
181 tggcatcgat gaagaacgca gcgaaatcgc ataagtaatg tgaattgcag aattcagtga
241 atcatogaat ctttgaacgc acattgcgcc cgccagtatt ctggcgggca tgcctgtccg
301 agcgtcattt caaccctcga acccctccgg gggatcggcg ttggggatcg ggaccctca
361 cacgggtgcc ggccccgaaa tacagtggcg gtctcgcgcg agcctctcct gcgcagtagt
421 ttgcacaact cgcaccggga gcgcggcgcg tccacgtccg taaaacaccc aactttctga
481 aatggtgacc toggatcagg taggaatacc cgctgaactt aagcatatca ataagcggag

```

Fig. 53. Chromatogram and sequence deposit of ITS region of *Trichoderma asperellum* RHS/S-561/NAIMCC-F-01967.

Table 40. Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of *T. asperellum* used for analysis with ex-type strain sequence

```

-----
Title T. asperellum alignemts; begin taxa; dimensions ntax= 7;tax labels
begin characters;
Domain=Data property=Coding
Codon Start=1;-
HQ265418_Trichoderma_asperellum_AACTGTTGCCTCGGCGGGGTACGCCCCGGGTGCGTCCCAGCCCC [141]
HQ334994_Trichoderma_asperellum_.TT.....G..... [141]
HQ334996_Trichoderma_asperellum_.A.....G..... [141]
FN396553_Trichoderma_asperellum....CC.....G..... [141]
AJ230669_Trichoderma_asperellum....CC.....G..... [141]
AJ230680_Trichoderma_asperellumT....T.....G..... [141]
AJ230668_Trichoderma_asperellum.....G..... [141]

HQ265418_Trichoderma_asperellum_GCCCCCGGAGGAACCAACCAACTCTTTCTGTAATCCCCTCGC [195]
HQ334994_Trichoderma_asperellum.....A.....G..... [195]
HQ334996_Trichoderma_asperellum.....A.....G..... [195]
FN396553_Trichoderma_asperellum.....A.....G..... [195]
AJ230669_Trichoderma_asperellum.....A.....G..... [195]
AJ230680_Trichoderma_asperellum.....A.....G..... [195]
AJ230668_Trichoderma_asperellum.....A.....G..... [195]

HQ265418_Trichoderma_asperellum_TCTTACAGCTCTGAACAAAAATCAAATGAATCAAACCTTCACAAC [254]
HQ334994_Trichoderma_asperellum.....G.....T..... [254]
HQ334996_Trichoderma_asperellum.....G.....T.....G...T.G..T.... [254]
FN396553_Trichoderma_asperellum.....G.....T.....C...T.... [254]
AJ230669_Trichoderma_asperellum.....G.....T.....C..... [254]
AJ230680_Trichoderma_asperellum.....G.....T.....T..... [254]
AJ230668_Trichoderma_asperellum.....G.....T.....A..... [254]

HQ265418_Trichoderma_asperellum_GGATACTTGGTTCAG [270]
HQ334994_Trichoderma_asperellum....C.A...A.T. [270]
HQ334996_Trichoderma_asperellum....C.A...A.T. [270]
FN396553_richoderma_asperellum....C.A...C.T. [270]
AJ230669_Trichoderma_asperellum....C.A...G.T. [270]
AJ230680_Trichoderma_asperellum....C.C...G.T. [270]
AJ230668_Trichoderma_asperellum....C.C....T. [270]
;end;

-----
dimensions nchar= 177;format missing= gap=- matchchar=.datatype=nucleotide
interleave=yes;matrix

```

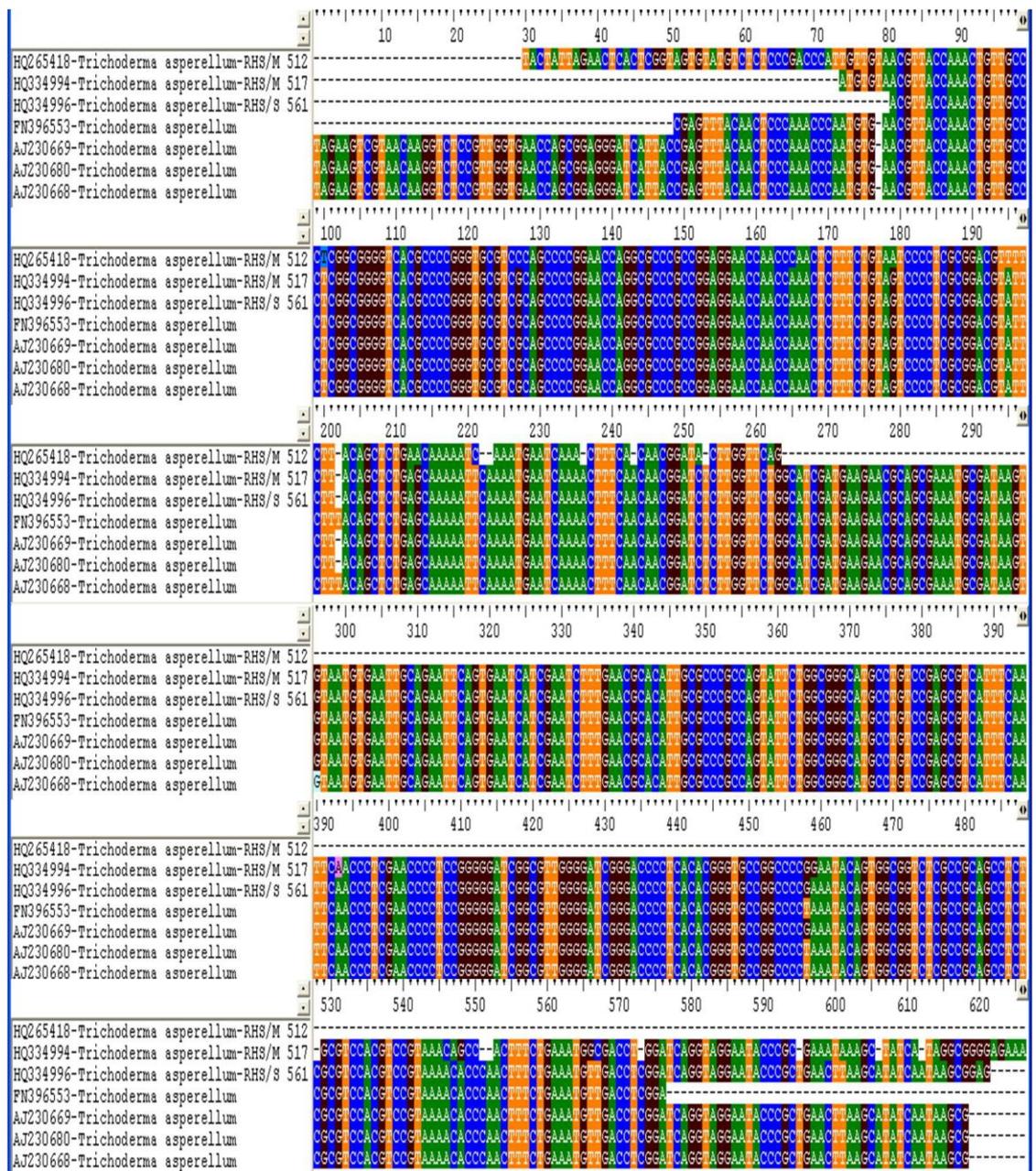
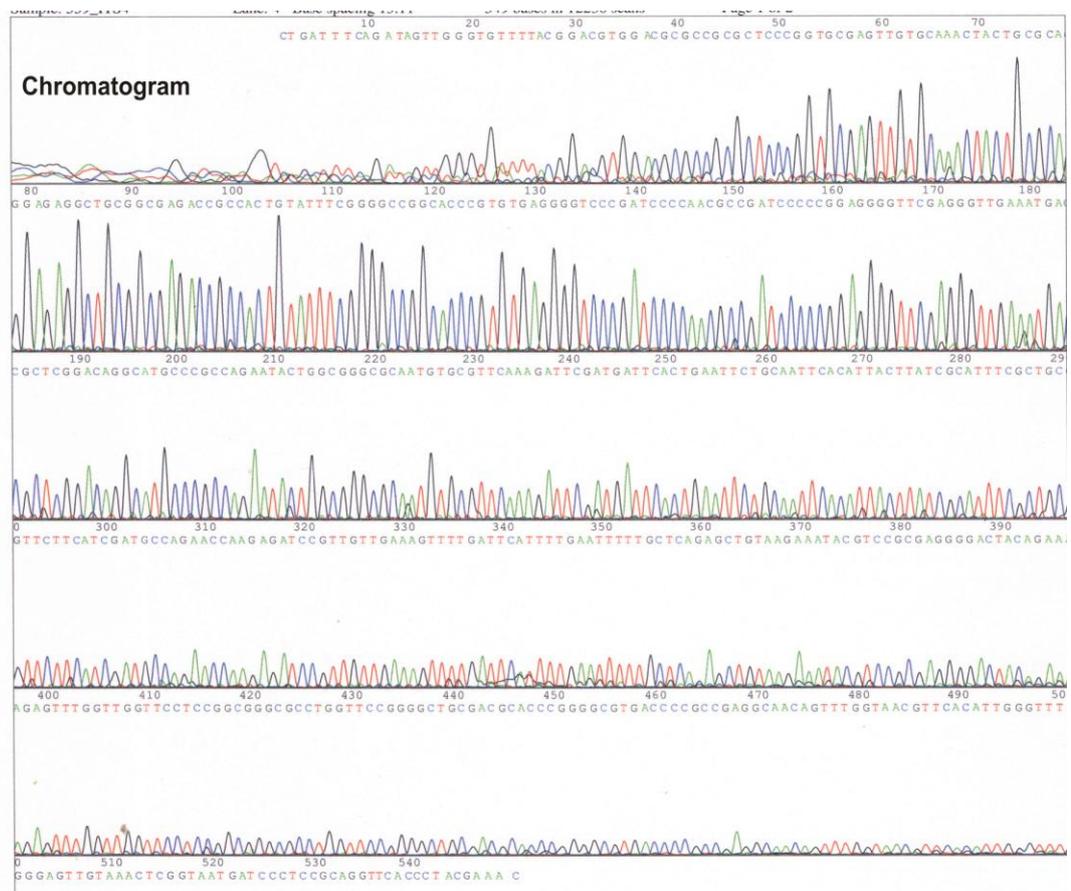


Fig.. 54. Multiple sequence alignment of *T. asperellum* isolates with ex-type strain sequences obtained from NCBI genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.



GTTTCGTAGGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACCTCCCAAACCAATGTGAACGTTAC
CAAACCTGTTGCCTCGGCGGGTCACGCCCCGGTGCCTGCGAGCCCCGGAACCAGGCGCCCGCGGAGGA
ACCAACCAAACCTCTTTCTGTAGTCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATG
AATCAAACTTTCAACAACGGATCTCTTGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTTCTGGCGG
GCATGCCGTGCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCC
TCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGAGCCTCTCCTGCGCAGTAGTTTGCACA
ACTCGCACCGGGAGCGCGCGGTCCACGTCCGTA AAAACACCAACTATCTGAAATCAG

Sequence Deposited: NCBI

ACCESSION: HQ334997

VERSION: HQ334997.1

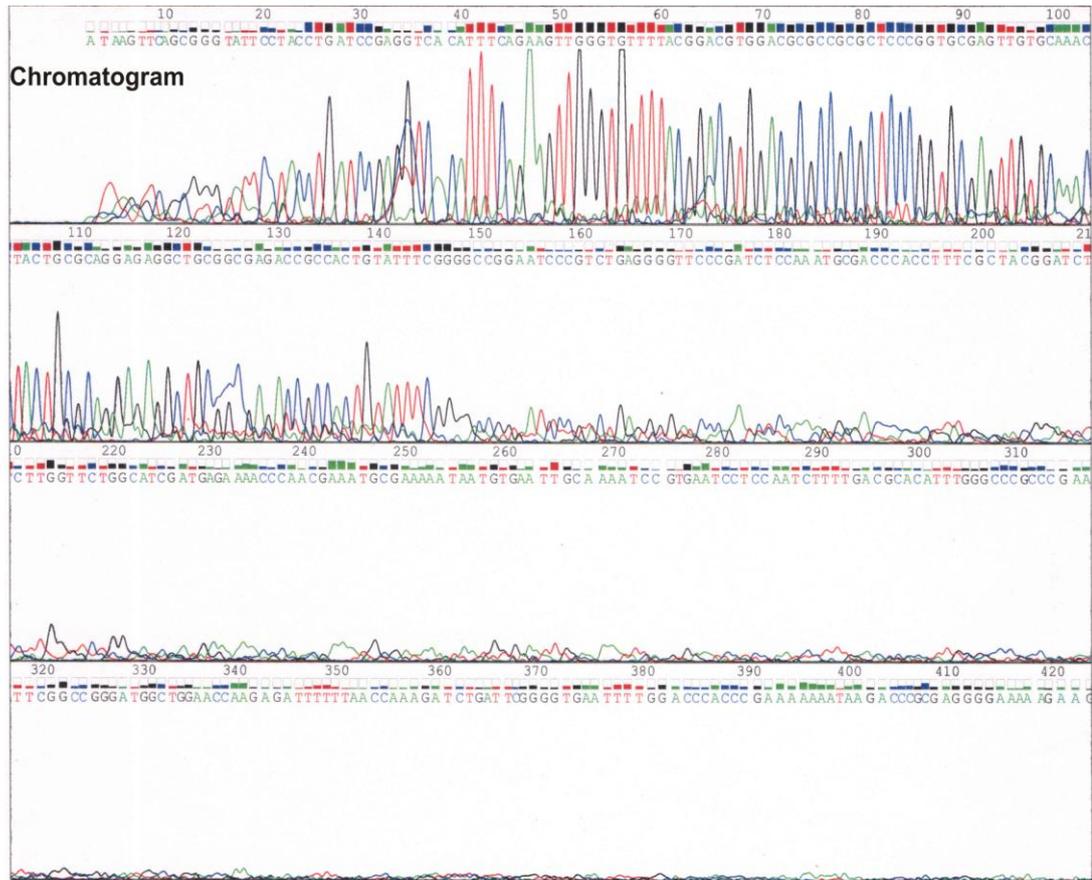
GI:315112488

DNA linear : 549bp

Title : *Trichoderma erinaceum* isolate FS/L20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

1 ttcaactct caaaccocaa tgtgaacat actaaactct tgcctcggcg gggtcacgcc
61 cgggtgctg cgcagcccc gaaccaggcg cagcgggag ggaccaacca aactcttac
121 ttagacccc tcgcgagct tattcttac agctctgagc aaaaattcaa aatgatcaa
181 aacttcaac aacggatctc ttggtcttg catcgatgaa gaacgcagcg aatgagata
241 agtaatgta attgcagaat tcagtgaatc atcgaatctt tgaacgcaca ttgcgcccgc
301 cagtattctg cggggcatgc ctgtccgagt gtcatttcaa cctcgaacc cctcggggg
361 gtcggcgttg gggatcggga accctcaga cgggaacccc gcccgaaat acagtggcgg
421 totcgcgca gctctcctg cgcagtagt tgcacaactc gcaccgggag cgcggcgct
481 ccagtcctg aaaaacocca actctgaaa tgtgacctg gatcaggtag gaatagccg
541 tgaagctgc g

Fig. 55. Chromatogram and sequence deposit of ITS region of *Trichoderma erinaceum* FS/L-20/NAIMCC-F-01949.



ATAGGTTCAGCGGGT**ATT**CCTACCTGATCCGAGGT**CAC**ATT**CAGA**AGTTGGGTGTTTTACGGA
CGTGGACGCGCCGCGCTCCC**GGT**GCGAGTTGTG**CAA**ACTACTGCGCAGGAGAGGCTGCGGCGAG
ACCGCCACTGTATTT**CGGG**CCCGGA**ATT**CCGTCTGAGGGGT**TCC**CGATCT**CCAA**ATCCGACCCA
CTTTTCGCTGCGGATCTCTTGGTTCTGGC**AT**CGATGAGAAA**ACCA**ACGAAATGCGAAAA**A**TAA
TGTGAATTGCAAATACGTGAATCCT**CCA**ATCTTTT**GAC**GCACATTTGGG**CC**CGCCGAATT**CG**
GCCGGATGGCTGGAACCAAGAGATTTTT**TA**ACCAAAGATCTGATTCGGGGTGAATTTTGGACC
CACCCGAAAAAATAAGACCCGCGAGGGG**AAAA**AGAAGGGGTTTTGGTTG

Sequence Deposited: NCBI Title : *Trichoderma erinaceum* strain FS/S-474 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA

ACCESSION: GU187915

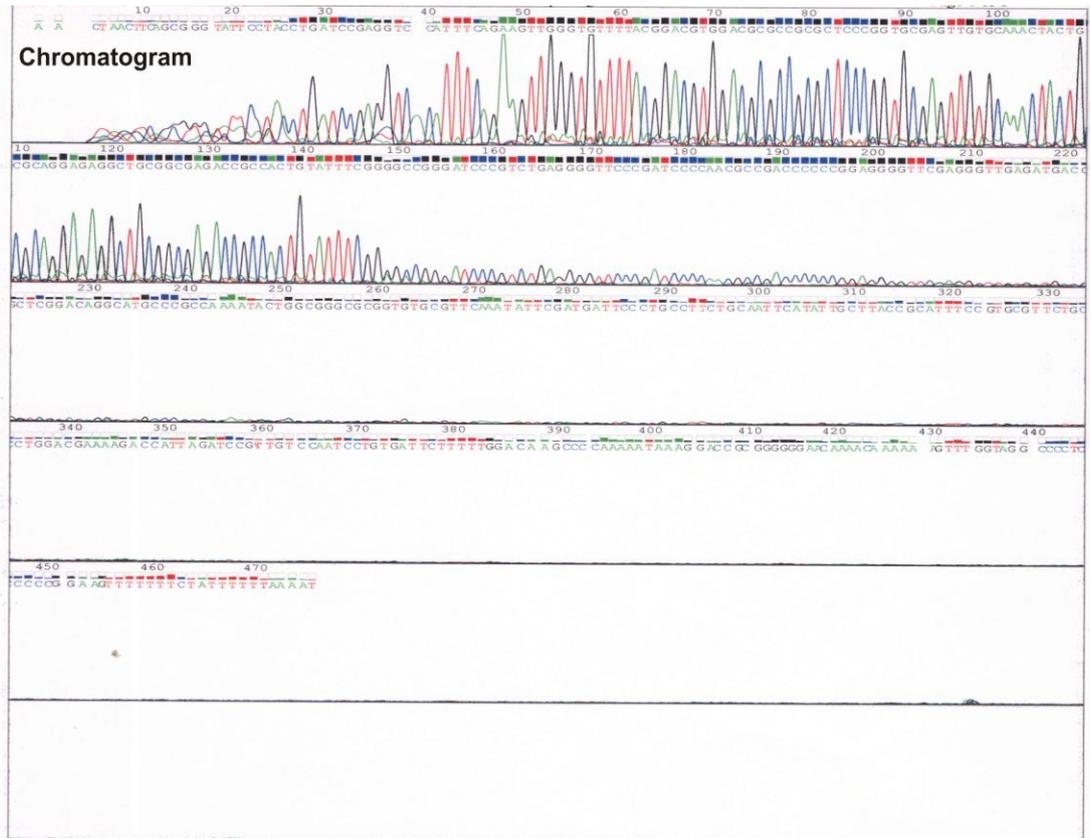
VERSION: GU187915

GI:270271229

DNA linear : 434bp

1 ataggttcag cgggtattcc tacctgatcc gaggtcacat ttcagaagtt ggggtgtttta
 61 cggacgtgga cgcgcgcgcg tcccgggtgog agttgtgcaa actactgcbc aggagaggtc
 121 gcgcgagac cgccactgta tttcggggcc ggaattccgt ctgaggggtt cccgatctcc
 181 aaatccgacc cacctttcgc tgcggatctc ttggttctgg catcgatgag aaaaccaac
 241 gaaatgcaa aaataatgtg aattgcaaaa tacgtgaatc ctccaatctt ttgacgcaca
 301 tttgggccc cccgaattcg gccgggatgg ctggaaccaa gagatTTTTT aaccaaagat
 361 ctgattcggg gtgaattttg gacccaccgc aaaaaataa gaccocgag gggaaaaaga
 421 aggggttttg gttg

Fig. 56. Chromatogram and sequence deposit of ITS region of *Trichoderma erinaceum* FS/S-474/NAIMCC-F-01960.



AAGGAACCGATCATTCCCGAGTTTACAGTCCCAACCCATGTGAACCATAACCAAAGTGTGCTCGGCGGGGTCACGCCCCGGGTGCGTGCAGCCCCGGAACCAGGCGCCCGCGGAGGGACCAACCAAAGTCTTTACTGTA GTCCCCTCGCGGATGTTATTTCTTACAGCTCTGAGCAAAAATCCAAAATGAATCAAAAACGTTCAACAACGG ATCTCTTGATTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTACAGAATTCAGTG GATCATCGAATCTTTGAACGCACATTCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCAAGCGTCATTT CAACCCTCGAACCCCTCCAGGGGTTCGGCGTGGGAAAAACAAAAAAAAAAAAAAAAAGCCACAAAAAAAAAAT AAAAAAAGTTTGAAGAAACAAAACAAAAAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTAACC CAACCAAAAAAAAAACAGAATATATAAGAAAAATAACGCCGATAGAAGGTAGAAAACCCGGTAAAATTTACCCA AAAAAAGGAAAAAAA

Sequence Deposited: NCBI

ACCESSION: GU191829

VERSION: GU191829.1

GI:270305454

DNA linear : 584bp

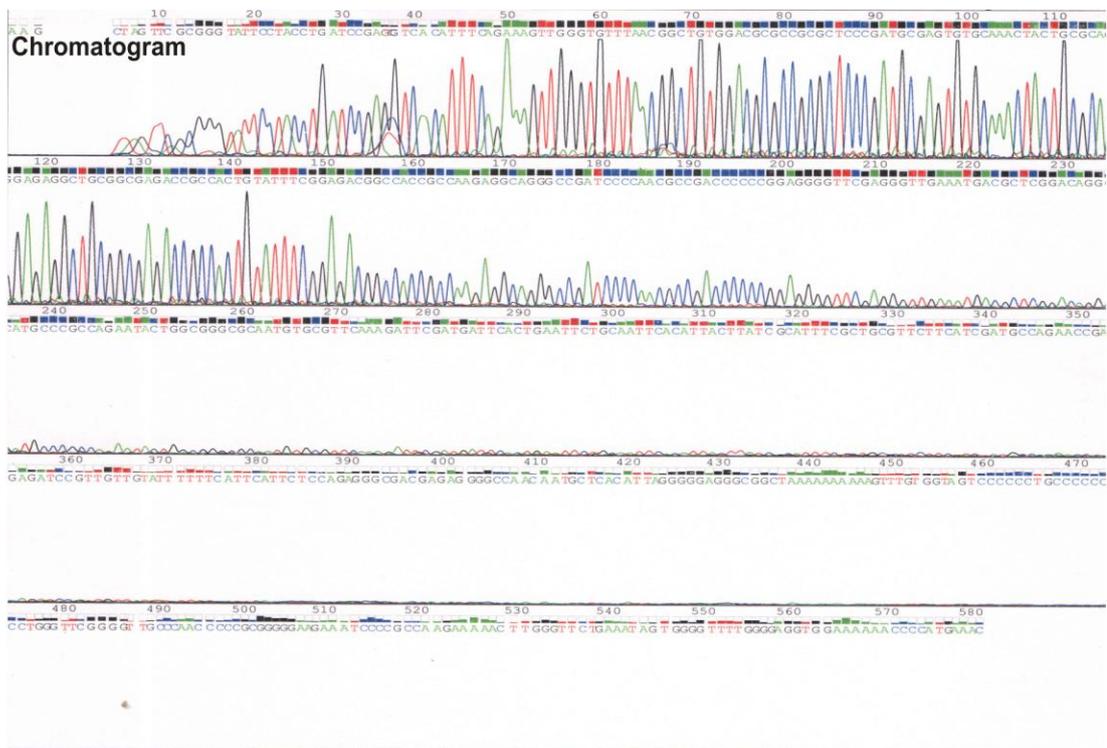
Title : *Trichoderma erinaceum* strain FS/S-475 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```

1 aaggaaccga tcattcccga gtttacagtc ccaaccatg tgaaccatac caaactgttg
61 ctcgcgggg gtcacgccc ggtgctgctg cagccccgga accaggcgcc cgccggagg
121 accaaccaaa ctctttactg tagtcccctc gcggatgtaa tttcttacag ctctgagcaa
181 aaatccaaaa tgaatcaaaa cgttaacaaa cggatctctt gattctgcca tcgatgaaga
241 acgcagcgaa atgcgataag taatgtgaat tacagaattc agtggatcat cgaatctttg
301 aacgcacatt ggcggccca gtattctggc gggcatgctt gtccaagcgt cattcaacc
361 ctogaacccc tccagggggt cggcgtggga aaaacaaaaa aaaaaaaaaa gcccacaaaa
421 aaaaataaaa aaaagttgga agaaacaaaa caaaaaaaaa aaaaaaaaaa aaaaaaaaaa
481 aaaaaaaaaa attaacccaa caaaaaaaaa cagaatatat aagaaataaa cgccgataga
541 aggtagaaaa cccgtaaaa tttaccctaa aaaaaggaaa aaaa

```

Fig. 57. Chromatogram and sequence deposit of ITS region of *Trichoderma erinaceum* FS/S- 475/NAIMCC-F-01953.



GGGGGGACGCCCCGCTTCTTTGAGACTCGGAAAACCATCTTCACCCCTGGGGGACCCATCGTTCGAT
 CGATCGATCAGGCCCCCGCCGCTTAATTTTTGGGGGGTTGCCCAACATTCACCCCGGAAGGGGTCT
 GGGGTCAAAAATTTCTTTGGGACAGACATCCCCCCAAAATTGGGGGGGGCCACATGGTGTGTTACAAA
 GTTTGGATGCTTCAATTATTTGTCAATCCCCCGTCTTCCCGCATTCGGGGGGCTTTTCTTTGGGACG
 GGCTCCTAGACCTCCGAACCCCTCCGGGGGGTGGCGTTGGGGATCGGGAACCCCTCAGACGGGATCCCA
 GCCCCGAAATACAGTGGCGGTCTCGCCGAGCCTCTCCTGCGCAGTAGTTTGACAACTCGCACCGGGAG
 CGCGGCGGTCCACGTGCCGTA AACACCCA ACTTCTGAAATGTGAAGACTCGGATCAGGTAGGAATACC
 CGCTGAACTAGCCTT

Sequence Deposited: NCBI

ACCESSION: HM117841

VERSION: HM117841.1

GI:298104180

DNA linear : 505 bp

Title : *Trichoderma erinaceum* isolate FS/S478 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

ORIGIN

```

1 ggggggacg cccctgcttt ctttgagact cggaaaacca ttttcacct tgggggaccc
61 atcgttcgat cgategatca ggccecccg cgccttaat ttttggggg ttgcccacac
121 attcccccg gaaggggtct ggggtcaaaa attcttttg gacagacatc cccccaaaa
181 ttggggggg cccacatggt gttttacaaa gtttgatgc tcaattatt tgttcaatcc
241 ccccgcttc cgcatttcg gggggcttt ctttgggac ggtcctaga cctcogaacc
301 cctcggggg gtcggcggtt gggatcgga acccctcaga cgggatccca gcccccgaat
361 acagtggcg tctcgcgca gctctcctg cgcagtagt tgcaaacct gcaccgggag
421 cgcggcgct ccactgccc taaaacacc aactctgaa atgtgaagac tcggatcagg
481 taggaatacc cgtgaacta gcctt
//

```

Fig. 58. Chromatogram and sequence deposit of ITS region of *Trichoderma erinaceum* FS/S- 478/NAIMCC-F-01954.

Table 41. Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of *T. erinaceum* used for analysis with ex-type strain sequence

```

-----
!Title Phylogenetic Analysis;!Format; DataType=Nucleotide CodeTable=Standard;
NSeqs=10; Domain=Data property=Coding CodonStart=1;

#HM107419_T._erinaceum_FS/L20 - - - - - [ 45]
#GU187915_T._erinaceum_FS/S-474 - - - - - [ 45]
#GU191829_T._erinaceum_FS/S-475 - - - - - [ 45]
#HM117841_T._erinaceum_FS/S478 - - - - - [ 45]
#DQ083009_T._erinaceum - - - - - [ 45]
#GQ249874_T._erinaceum_102/08 - - - - - [ 45]
#AY570797_T._erinaceum A GGG TAA GCC GTA CTT CGC CTC GAT TTC CCC AAT [ 45]
#HM107419_T._erinaceum_FS/L20 - - - - - [ 90]
#GU187915_T._erinaceum_FS/S-474 - - - - - [ 90]
#GU191829_T._erinaceum_FS/S-475 - - - - - [ 90]
#HM117841_T._erinaceum_FS/S478 - - - - - [ 90]
#DQ083009_T._erinaceum - - - - - [ 90]
#GQ249874_T._erinaceum_102/08 - - - - - [ 90]
#AY570797_T._erinaceum C GGG GGG GGC GCG TAG GGG TTT ATC TGG TGT GCG [ 90]
#HM107419_T._erinaceum_FS/L20 T ACT G-- TAG ACC CCT CGC GGA CGT TAT T-T CTT [ 270]
#GU187915_T._erinaceum_FS/S-474 - . . . .-- -- --T AT. TCG ..G .CG G.A -. .CG [ 270]
#GU191829_T._erinaceum_FS/S-475 . . . .-- . . . T. . . . . . . T. . . .- . . . [ 270]
#HM117841_T._erinaceum_FS/S478 G .TC .AT C. . G. . .C .CG CCG .C. .A -. T. . [ 270]
#DQ083009_T._erinaceum . . . .-- . . . T. . . . . . . . . . .- . . . [ 270]
#GQ249874_T._erinaceum_102/08 . . . .-- . . . T. . . . . . . . . . .- . . . [ 270]
#AY570797_T._erinaceum A CAC .TC .TC TT. G.C . . . .G .TC C.G GG. AGC [ 270]
#HM107419_T._erinaceum_FS/L20 G TTC TGG CAT CGA TGA -AG AAC GCA GCG AAA TGC [ 360]
#GU187915_T._erinaceum_FS/S-474 . . . . . . . . . . . G.A . . . C. . A. . . . . [ 360]
#GU191829_T._erinaceum_FS/S-475 A . . . . . . . . . . . - . . . . . . . . . . . [ 360]
#HM117841_T._erinaceum_FS/S478 . GA. A.A . . . .CC CCC -CA .A TTG .G. GGG GC. [ 360]
#DQ083009_T._erinaceum . . . . . . . . . . . - . . . . . . . . . . . [ 360]
#GQ249874_T._erinaceum_102/08 . . . . . . . . . . . - . . . . . . . . . . . [ 360]
#AY570797_T._erinaceum C ACG AAT T.C TTT .C GGA .A T-- . . . .C .G [ 360]
#HM107419_T._erinaceum_FS/L20 - GCG CCC GCC AGT ATT CT- GGC GGG CAT GCC TGT [ 450]
#GU187915_T._erinaceum_FS/S-474 T .G. . . . . C.A . . . .G- .C. . . . ATG .T G.A [ 450]
#GU191829_T._erinaceum_FS/S-475 - . . . . . . . . . . . - . . . . . . . . . . . [ 450]
#HM117841_T._erinaceum_FS/S478 - C. . T.T T. . C.C . . . TC- .G . . . .T. TT. .T. [ 450]
#DQ083009_T._erinaceum - . . . . . . . . . . . - . . . . . . . . . . . [ 450]
#GQ249874_T._erinaceum_102/08 - . . . . . . . . . . . - . . . . . . . . . . . [ 450]
#AY570797_T._erinaceum G .GC .G . . . .G G.C .AA . . . .A. .TC CAG G. . [ 450]
#HM107419_T._erinaceum_FS/L20 --- --- --- --- --- --- --- --- --- [867]
#GU187915_T._erinaceum_FS/S-474 --- --- --- --- --- --- --- --- --- [867]
#GU191829_T._erinaceum_FS/S-475 --- --- --- --- --- --- --- --- --- [867]
#HM117841_T._erinaceum_FS/S478 --- --- --- --- --- --- --- --- --- [867]
#DQ083009_T._erinaceum --- --- --- --- --- --- --- --- --- [867]
#GQ249874_T._erinaceum_102/08 --- --- --- --- --- --- --- --- --- [867]
#AY570797_T._erinaceum GGT TGA TTC TGC CAG TAG TCA TAT GCT TGT CTC [867]
;
end;

dimensions nchar= 177;format missing= gap=- matchchar=.datatype=nucleotide
interleave=yes;matrix

```

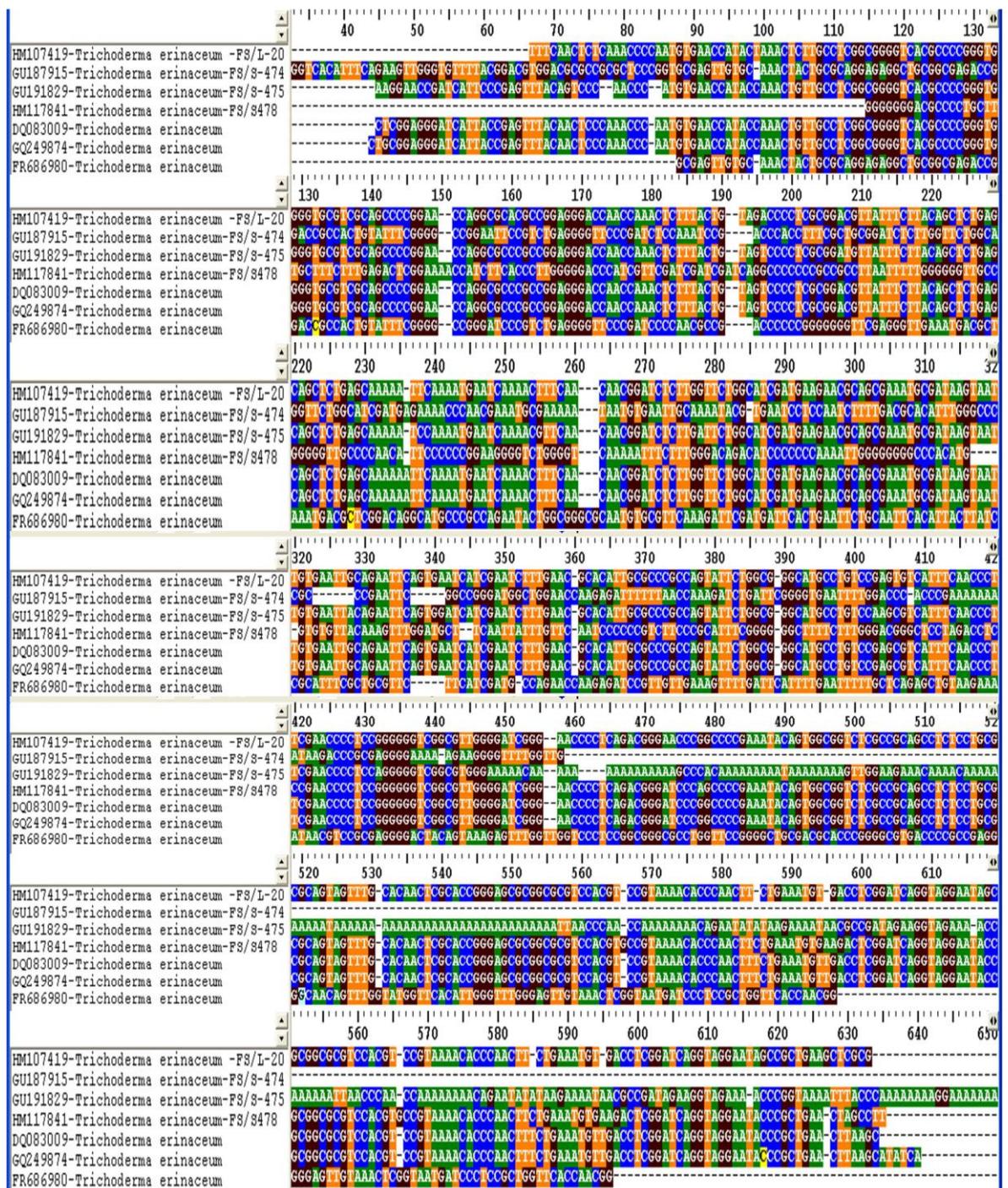


Fig. 59. Multiple sequence alignment of *T. erinaceum* isolates with ex-type strain sequences obtained from NCBI genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

4.9.3.2. Phylogenetic analysis of *Trichoderma* isolates

The 18S DNA sequence based phylogenetic analysis of *T. harzianum*, *T. asperellum* and *T. erinaceum* isolate was conducted with other ex-type strains obtained from NCBI-Genbank database which showed maximum homology (98-100 %) with the respective query sequences. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 5.65716586 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 72 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 99 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.2.

The phylogenetic tree represented in Fig. 60, shows that there is a geographical variation among the isolates. The isolates belonging to the same geographical zone were clustered in the same clade. Overall 18S rDNA sequences show that isolates of *T. harzianum* (Telomorph-*Hypocrea lixii*) is closely related to *T. erinaceum* which is indicated by their relative position in the phylogenetic tree. Whereas *T. asperellum* isolates showed comparatively lesser affinity with *T. harzianum* isolates. Though *T. viride* was used as an out group for this analysis they remained dispersed showing more or less affinities with all the three groups of *Trichoderma* spp. The analysis has been presented as a linear tree (NJ-tree) (Fig. 61) which shows the branch length (Phylogenetic distances) between individual isolates based on 18S rDNA sequences whereas the phylogenetic tree represented in circular (UPGMA-Tree) form describes the relative diversification of all the individual isolates across the clades (Fig. 62).

4.9.3.3. Analysis of Nucleotide frequency.

4.9.3.3.1. *Trichoderma harzianum* and *T. asperellum* isolates

Combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html. The sequence of DNA fragments of ITS region for all the *T. harzianum* isolates (RHS/S-559 and RHS/S-560) and *T. asperellum* isolate (RHS/S-561) is presented in Table. 42. The guanine ‘G’ content of the sequence of both the *T. harzianum* isolates was found to be 25 % with a highest repetitions of 141. Combinations like GC were also at the level 7.00-7.80 %. Whereas *T. asperellum* has G % of 25.74 and G, C% of 55.56.

Table 42. DNA stats results for 18S rDNA nucleotide frequencies of different isolates of *T. harzianum* and *T. asperellum*

Pattern	<i>T. harzianum</i> RHS/S 559		<i>T. harzianum</i> RHS/S 560		<i>T. asperellum</i> RHS/S-561	
	Times found	Percentage	Times found	Percentage	Times found	Percentage
G	141	25.68	141	25.82	139	25.74
A	131	23.86	129	23.63	130	24.07
T	112	20.40	108	19.78	110	20.37
C	165	30.05	168	30.77	161	29.81
GG	40	7.30	40	7.34	39	7.24
GA	30	5.47	32	5.87	30	5.57
GT	29	5.29	26	4.77	26	4.82
GC	41	7.48	43	7.89	43	7.98
AG	22	4.01	24	4.40	23	4.27
AA	48	8.76	45	8.26	47	8.72
AT	27	4.93	27	4.95	28	5.19
AC	34	6.20	32	5.87	32	5.94
TG	29	5.29	27	4.95	28	5.19
TA	14	2.55	15	2.75	15	2.78
TT	28	5.11	26	4.77	27	5.01
TC	41	7.48	40	7.34	40	7.42
CG	49	8.94	49	8.99	49	9.09
CA	39	7.12	37	6.79	37	6.86
CT	28	5.11	29	5.32	29	5.38
CC	49	8.94	53	9.72	46	8.53
G,C	306	55.74	309	56.59	300	55.56
A,T	243	44.26	237	43.41	240	44.44

4.9.3.3.2. *Trichoderma erinaceum* isolates

Combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html. The sequence of DNA fragments of ITS region for all the *T. erinaceum* isolates (FS/L-20, FS/S-474, FS/S-475 and FS/S-478) is presented in Table 43. The guanine ‘G’ content of the sequence was found to be highest in case of isolate FS/S-474 (28.11%) with highest repetitions of 122. Combinations like GC were also at the level 4.00-20 % in all the isolates.

Table. 43. DNA stats results for 18S rDNA nucleotide frequencies of different isolates of *T. erinaceum*

Pattern	<i>T. erinaceum</i> FS/L-20		<i>T. erinaceum</i> FS/S-474		<i>T. erinaceum</i> FS/S-475		<i>T. erinaceum</i> FS/S-478	
	Times found	Percentage	Times found	Percentage	Times found	Percentage	Times found	Percentage
G	140	25.41	122	28.11	111	19.01	10	20.00
A	134	24.32	108	24.88	240	41.10	10	20.00
T	111	20.15	100	23.04	98	16.78	10	20.00
C	166	30.13	104	23.96	135	23.12	10	20.00
GG	40	7.27	40	9.24	30	5.15	10	20.00
GA	32	5.82	37	8.55	32	5.49	8	16.33
GT	25	4.55	19	4.39	22	3.77	2	4.08
GC	42	7.64	25	5.77	27	4.63	10	20.00
AG	23	4.18	42	9.70	26	4.46	10	20.00
AA	50	9.09	26	6.00	147	25.21	8	16.33
AT	26	4.73	21	4.85	34	5.83	8	16.33
AC	35	6.36	0	0.00	32	5.49	2	4.08
TG	29	5.27	29	6.70	23	3.95	2	4.08
TA	14	2.55	10	2.31	21	3.60	8	16.33
TT	26	4.73	37	8.55	25	4.29	2	4.08
TC	42	7.64	24	5.54	29	4.97	2	4.08
CG	48	8.73	34	7.85	32	5.49	8	16.33
CA	38	6.91	18	4.16	39	6.69	1	2.04
CT	33	6.00	18	4.16	17	2.92	8	16.33
CC	47	8.55	34	7.85	47	8.06	8	16.33
G,C	306	55.54	226	52.07	246	42.12	20	40.00
A,T	245	44.46	208	47.93	338	57.88	20	40.00

4.9.3.4. Analysis of DNA molecular weight of rDNA sequences

The DNA Molecular Weight DNA residue of all the isolates was calculated with the help of online DNA molecular weight calculator http://www.ualberta.ca/~stothard/javascript/dna_mw.html. The molecular weight of DNA of *T. harzianum* isolates ranged from 16.00 to 59.00 KDa, similarly in case of

T. erinaceum the DNA molecular weight ranged from 15.50 to 18.00 KDa and in case of *T. asperellum* isolates, the DNA molecular weight ranged from 16.50 to 70.00 KDa (Table 44).

Table 44. DNA molecular weight of different *Trichoderma* isolates calculated on the basis of 18S rDNA sequences

Isolate	Strain	Gen Bank Acc.No	Starting sequence	DNA mol weight (Da)
<i>T. harzianum</i>	RHS/S-559	HQ334997	GTTTCGTAGG	168275.55
	RHS/S-560	HQ334995	GTGACGTTAC	169251.23
<i>T. erinaceum</i>	FS/L20	HM107419	TTTCAACTCT	169846.63
	FS/S-474	GU187915	ATAGGTTTCAG	180580.62
	FS/S-475	GU191829	AAGGAACCGA	163442.62
	FS/S-478	HM117841	GGGGGGGACG	155686.26
<i>T. asperellum</i>	RHS/S-561	HQ334996	ACGTTACCAA	166514.48

4.9.3.5. Analysis of Open Reading Frame (ORF) of rDNA gene sequences

Open Reading Frames (ORF) for the designated sequence was calculated with the help of ORF finder available from http://www.ualberta.ca/~stothard/javascript/orf_find.html. The detail description of individual isolates has been given as under:

1. NCBI Acc. No. HQ334997, *Trichoderma harzianum* RHS/S-559/NAIMCC-F-01968

Results for 549 residue sequence "*T. harzianum*- RHS/S 559" starting "GTTTCGTAGG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 64 to base 162.

ACGTTACCAA ACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAG
CCCCGGAACCAGGCGCCCGCGGAGGAACCAACCAA ACTCTTTCTGTAG

>Translation of ORF number 1 in reading frame 1 on the direct strand.

TLPNCC LGGVTPRVRRSPGTRRPPEEPTKLFL*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 163 to base 255.

TCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

SPRGRISYSSEQKFKMNQNFQQRISWFWHR*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 304 to base 543.

ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGGC
TTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGC
GGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACCTCGCACCGGG

>Translation of ORF number 3 in reading frame 1 on the direct strand.

IIESLNAHCARQYSGGHACPSVISTLEPLRGIGVGDRDPSHGCRPRNTVAVSP
QPLLRSSLHNSHRERGA STSVKHPTI*

2. NCBI ACC.No. HQ34994; *Trichoderma harzianum* RHS/S-560/ NAIMCC-F-01966

Results for 546 residue sequence " *T. harzianum*- RHS/S 560" starting "GTGACGTTAC"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 102.

GTGACGTTACCAAACCTGTTGCCTCGGGCGGGGTCACGCCCGGGTGC GTCG
CAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACCTCTTTCTG

>Translation of ORF number 1 in reading frame 1 on the direct strand.

VTLPNCCLGGVTPRVRRSPGTRRPPEEPTKLFL*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 103 to base 195.

TCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

SPRGRISYSSEQKFKMNQNFQQRISWFWHR*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 244 to base 483.

ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGGC
TTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGC
GGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACCTCGCACCGGG
AGCGCGGCGCGTCCACGTCCGTAAAACACCCAACCTTTCTGA

>Translation of ORF number 3 in reading frame 1 on the direct strand.

IIESLNAHCARQYSGGHACPSVISTLEPLRGIGVGDRDPSHGCRPRNTVAVSP
QPLLRSSLHNSHRERGA STSVKHPTF*

3. NCBI Acc. No. GUHM107419; *Trichoderma erinaceum*-FS/L-20/ NAIMCC-F-01949

Results for 551 residue sequence "*T. erinaceum* FS/L-20" starting "TTTCAACTCT"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 219.

TTTCAACTCTCAAACCCCAATGTGAACCATACTAAACTCTTGCCTCGGCG
GGGTCACGCCCCGGGTGCGTCGCAGCCCCGAACCAGGCGCACGCCGGA
GGGACCAACCAAACCTTTACTGTAGACCCCTCGCGGACGTTATTTCTTA
CAGCTCTGAGCAAAAATTCAAATGAATCAAACCTTTCAACAACGGATCT

>Translation of ORF number 1 in reading frame 1 on the direct strand.
FQLSNPNVNHTKLLPRRGHAPGASQPRNQAHAAGGTNQTLYCRPLADVISYSS
EQKFKMNQNFQQRISWFWHR*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 268 to base 492.

ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTCCGAGTGTCATTTCAACCCTCGAACCCCTCCGGGGGGTTCGGCG
TTGGGGATCGGGAACCCCTCAGACGGGAACCCGGCCCCGAAATACAGTG
GCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACCTCGCACCG

>Translation of ORF number 2 in reading frame 1 on the direct strand.
IIESLNAHCARQYSGGHACPSVISTLEPLRGVGVGDREPLRREPGPEIQWRSR
RSLSCAVVCTTRTGSAARPR*

4. NCBI Acc. No. GU187915; *Trichoderma erinaceum* FS/S-474/ NAIMCC-F-01960

Results for 434 residue sequence " *T. erinaceum* FS/S-474 " starting
"ATAGGTTTCAG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 28 to base 261.

TCCGAGGTCACATTTTCAGAAGTTGGGTGTTTTACGGACGTGGACGCGCCG
CGCTCCCGGTGCGAGTTGTGCAAACCTACTGCGCAGGAGAGGCTGCGGCG
AGACCGCCACTGTATTTTCGGGGCCGGAATTCCGTCTGAGGGGTTCCCGAT
CTCCAAATCCGACCCACCTTTCGCTGCGGATCTCTTGTTCTGGCATCGAT

>Translation of ORF number 1 in reading frame 1 on the direct strand.
SEVTFQKLGVLRTWTRRAPGASCANYCAGEAAARPPPLYFGAGIPSEGFPISKS
DPPFAADLLVLASMRKPNEMRKIM*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 295 to base 432.

CGCACATTTGGGCCCCGCCGAATTCGGCCGGGATGGCTGGAACCAAGAG
ATTTTTTAACCAAAGATCTGATTCGGGGTGAATTTTGGACCCACCCGAAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.
RTFGPARIRPGWLEPRDFLTKDLIRGEFWTHPKKIRPARGKRRGFG

5. NCBI Acc. No. GU191829; *Trichoderma erinaceum* FS/S-475/ NAIMCC-F-01953

Results for 584 residue sequence "*T. erinaceum*- FS/S-475" starting
"AAGGAACCGA"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 222.

AAGGAACCGATCATTCCCGAGTTTACAGTCCCAACCCATGTGAACCATAC
CAAACCTGTTGCCTCGGCGGGGTACGCCCCGGGTGCGTCGCAGCCCCGGA
ACCAGGCGCCCGCCGGAGGGACCAACCAAACCTTTACTGTAGTCCCCTC
GCGGATGTTATTTCTTACAGCTCTGAGCAAAAATCCAAAATGAATCAAAA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

KEPIIPEFTVPTHVNHTKLLPRRGHAPGASQPRNQAPAGGTNQTLYCSPLADV
ISYSSEQKSKMNQNVQQRIS*

>ORF number 2 in reading frame 1 on the direct strand extends from base 238 to base 462.

AGAACGCAGCGAAATGCGATAAGTAATGTGAATTACAGAATTCAGTGGA
TCATCGAATCTTTGAACGCACATTGCGCCCCGCCAGTATTCTGGCGGGCAT
GCCTGTCCAAGCGTCATTTCAACCCTCGAACCCTCCAGGGGGTTCGGCGT
GGGAAAAACAAAAAAAAAAAAAAAAAGCCACAAAAAAAAAATAAAAAAAAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

RTQRNAISNVNYRIQWIIESLNAHRCARQYSGGHACPSVISTLEPLQGVGVGKT
KKKKKPTKKNKKKLEETKQKK*

6. NCBI Acc. No. HM117841; *Trichoderma erinaceum*-FS/S 478

Results for 505 residue sequence "*Trichoderma erinaceum* isolate FS/S478 "starting "GGGGGGGACG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 99.

GGGGGGGACGCCCTGCTTTCTTTGAGACTCGGAAAACCATCTTCACCCT
TGGGGGACCCATCGTTCGATCGATCGATCAGGCCCCCGCCGCCTTAA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

GGDAPAFFETRKTIFTLGGPIVRSIDQAPPPP*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 100 to base 459.

TTTTTGGGGGGTTGCCCAACATTCCCCCGGAAGGGGTCTGGGGTCAAA
AATTTCTTTGGGACAGACATCCCCCAAATTGGGGGGGGGCCACATGG
TGTGTTACAAAGTTTGGATGCTTCAATTATTTGTTCAATCCCCCGTCTTC
CCGCATTTTCGGGGGGCTTTTCTTTGGGACGGGCTCCTAGACCTCCGAACC
CCTCCGGGGGGTTCGGCGTTGGGGATCGGGAACCCTCAGACGGGATCCC

>Translation of ORF number 2 in reading frame 1 on the direct strand.

FLGGCPNIPPGRGLGSKISLGQTSPPKLGGAHMVCYKVWMLQLFVQSPRLPA
FRGAFLWDGLLDLRTPPGRRWGSQTPQTGSQPRNTVAVSPQPLLRSSLHNS
HRERGASTCRKTPNF*

7. NCBI Acc. No. HQ334996; *Trichoderma asperellum*-RHS/S-561/ NAIMCC-F-01967

Results for 540 residue sequence "*T. asperellum*-RHS/S 561" starting "ACGTTACCAA"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 99.

ACGTTACCAAACACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAG
CCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACACTCTTTCTGTAG

>Translation of ORF number 1 in reading frame 1 on the direct strand.

TLPNCCLLGGVTPRVRRSPGTRRPPEEPTKLFL*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 100 to base 192.

TCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

SPRGRISYSSEQKFKMNQNFQQRISWFWHR*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 241 to base 480.

ATCATCGAATCTTTGAACGCACATTGCGCCCCGCCAGTATTCTGGCGGGCA
TGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCG
TTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGC

>Translation of ORF number 3 in reading frame 1 on the direct strand.

IIESLNAHCARQYSGGHACPSVISTLEPLRGIGVGDRDPSHGCRPRNTVAVSP
QPLLRSSLHNSHRERGASTSVKHPTF*

4.10. 16S rDNA sequence analysis for identification of PGPR isolates

From the total collection of 135 bacterial isolates, at least 48 isolates were found to solubilize phosphate however only eight bacterial isolates *viz* BRHS/C-1, BRHS/P-22, BRHS/R-71, BRHS/R-72, BRHS/S-73, BRHS/P-91, BRHS/P-92 and BRHS/B-104 were found to be most promising PGPR isolates based on their *in vitro* characterization. All these isolates were initially characterized on the basis of their morphological and biochemical characterizations. However the identities of each individual PGPR isolate was confirmed on the basis of 16S rDNA sequences. The ITS region of the bacterial isolates were amplified with the help of universal primer pair. After direct sequencing of the PCR product 16S rRNA gene sequence of approximately 800 to 1,400 base pairs were obtained. The obtained sequence was further used to query against NCBI Genbank sequences through BLAST. The analysis revealed isolate BRHS/C-1 had 99 % similarity with *Bacillus pumilus*, BRHS/P-22 showed 98% with *Bacillus altitudinis*, BRHS/R-71 showed 98 % similarity with *Enterobacter cloacae*, BRHS/R-72 had 99 % similarity with *Paenibacillus polymyxa*, BRHS/S-73 had 99% homology with *Bacillus*

altitudinis, BRHS/P-91 had 99 % similarity with *Bacillus methylotrophicus*, BRHS/P-92 had 99 % similarity with *Burkholderia sp.* and BRHS/B-104 had 99 % similarity with *Bacillus aerophilus*. The sequences were approved as 16S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,321) The NCBI Accession numbers for each isolate has been provided in Table 45.

Table 45. NCBI Genbank Accession number of PGPR isolates

Sl.No	Isolate	Identified as	NCBI GenBank Acc. No.	Homology with the query sequence
1	BRHS/C-1	<i>Bacillus pumilus</i>	JF836847	99 %
2	BRHS/P-22	<i>Bacillus altitudinis</i>	HQ849482	98%
3	BRHS/R-71	<i>Enterobacter cloacae</i>	KC703974	98%
4	BRHS/R-72	<i>Paenibacillus polymyxa</i>	KC703775	99%
5	BRHS/S-73	<i>Bacillus altitudinis</i>	JF899300	99%
6	BRHS/P-91	<i>Bacillus methylotrophicus</i>	JQ765577	99%
7	BRHS/P-92	<i>Burkholderia sp.</i>	JQ765578	99%
8	BRHS/B-104	<i>Bacillus aerophilus</i>	KC603894	99%

4.10.1. 16S rDNA sequencing, Multiple sequence alignment and phylogenetic analysis¹

4.10.1.1. *Bacillus* isolates

The 16S rDNA sequences of all the Bacilli isolates were approved as ITS sequences and have been deposited in NCBI Genbank database (Fig. 62-66). A multiple sequence alignment of ITS gene sequences of all the selected *Bacilli* isolates (*B. pumilus*, *B. altitudinis*, *B. methylotrophicus*, *B. aerophilus*) were conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. The difference in this highly conserved regions are shown in different colours (Fig. 67). The text delineated version of the same alignment presented in Table 46 provides the information to design specific primers for detection and identification.

4.10.1.2. *Enterobacter cloacae*

16S rDNA sequence of *E. cloacae* has been approved as ITS sequence and has been deposited in NCBI Genbank database (**Fig. 68**). A multiple sequence alignment of 16S rDNA sequences of *Enterobacter cloacae* (BRHS/R-71) were conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with CLUSTAL-W algorithm. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 47, Fig. 69).

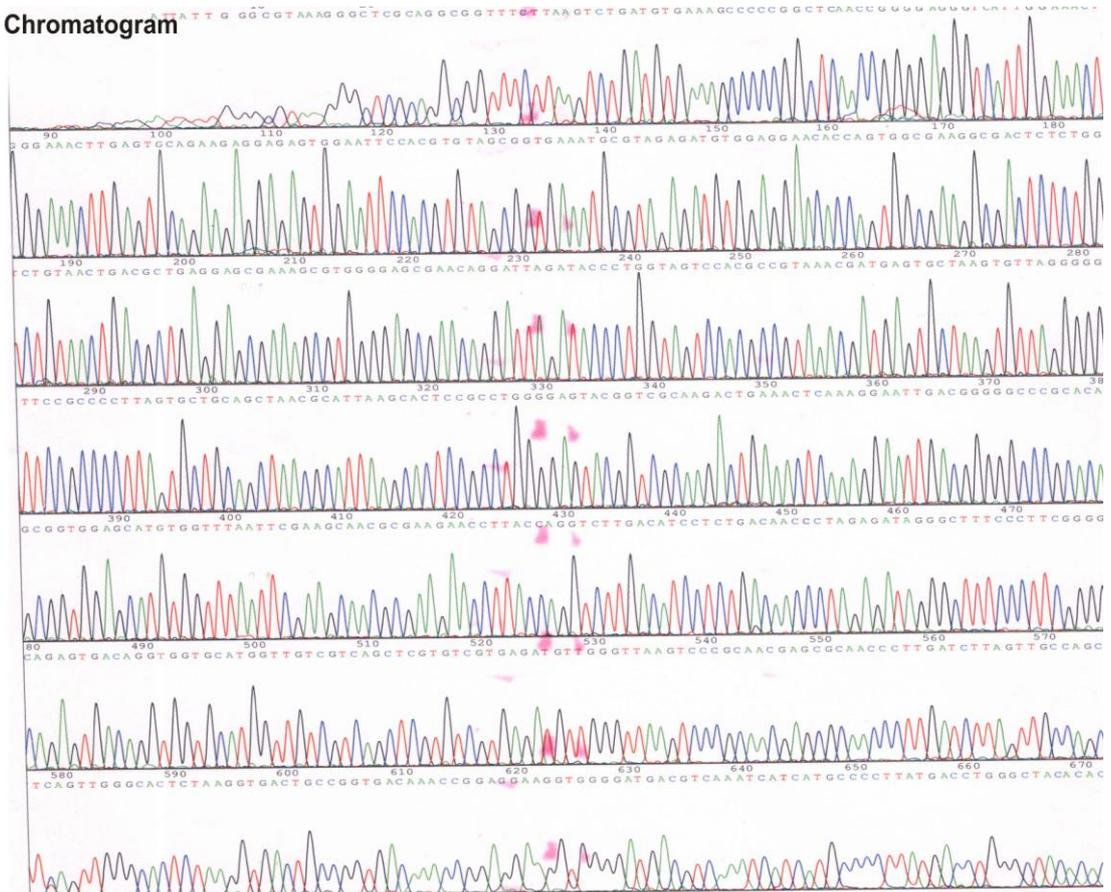
4.10.1.3. *Paenibacillus polymyxa*

16S rDNA sequence of *P. polymyxa* has been approved as ITS sequence and has been deposited in NCBI Genbank Database (Fig. 70). A multiple sequence alignment of 16S rDNA sequences of *Paenibacillus polymyxa* (BRHS/R-72) were conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with CLUSTAL-W algorithm. Analysis of the same regions of conserved sequences also highlighted the portions of this sequence which was not identical to that of the other related species. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 48; Fig. 71).

4.10.1.4. *Burkholderia symbionts*

16S rDNA sequence of *Burkholderia* sp. has been approved as ITS sequence and has been deposited in NCBI Genbank Database (**Fig. 72**). A multiple sequence alignment of 16S rDNA sequences were conducted with the sequences of ex-type strains obtained from NCBI Genbank database showing maximum homology with CLUSTAL-W algorithm. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates. This difference in the sequences will provide an important information for developing strain specific primers which have been highlighted in the alignment result (Table 49, Fig. 73).

Chromatogram



GAAAAGGGGGCTTGCTCCCGGATGTTAGCGGGCAGCGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGAAACCGGAGCTAATA
 CCGATAGTTCCTTGAACCGCATGGTCAAGGATGAAAGACGGTTTCGGCTGCTCCTACAGATGGACCCCGGGCGCATTAGCTAGTTGGTGGGGTAAATGGCTC
 ACCAAGCGACGATGCGTAGCCGACCTGAAAGGGTGTACGGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGACGACGATAGGGAATCTTCCGC
 AATGGAGAAAGTCTGACGGAGCAACGCCGCTGAGTGATGAAGTTTTTCGGATCGTAAGCTCTGTTGTTAGGGAAGAACAAGTGCAGAGTAACCTGCTCGCA
 CCTTGACGTACTAACAGAAAGCCACGGCTAACCTGACGTCAGCAGCCCGGTAATACGAGGTGGCAAGCGTTGTCGGAAATATTGGCGTAAAGGGCTC
 GCAGCGGTTCTTAAGTCTGATGTAAAGCCCCGGCTTACCAGGGAGGCTATTGAAACGGGAAACTTGAGTGCAGAAGAGGAGATGGAATCCACGT
 GTAGCGGTGAATGCGTAGAGATGTGGAGAACACCAAGTGGCAAGCGGACTCTCTGCTGTAAGTCTGACTGACGCTGAGGAGCAAGCGTGGGAGCCGAACGAT
 TAGATACCTGGAGTCCACGCCGTAACGATGAGTGTAGTGTAGGGGTTTTCCGCCCTTATCTGCAGCTAACGCATTAGCACTCCGCCTGGGGAGTA
 CGGTTCGAAGACTGAACCAAGGAATTGACGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAAATCGAAGCAACCGGAAGAACCTTACCAGGTCTTGACA
 TCCTCTGACAAACCTAGGATAGGGCTTTCCTTCGGGACAGAGTGCAGGTGGTGCATGGTTGTCGTCGCTCGTGTGAGATGTTGGGTTAAGTCCCGC
 AACGAGCGCAACCTTGATTAGTTGCCAGCATTAGTTGGGCACTTAAGTGTACTGCCGGTACAAACCGGGAAAGTGGGGATGACGTCAAATCATCATG
 CCCCTTATGACCTGGGCTACACCGTGTACAATGGACAGAACAAGGGCTCGGAGACCGCAAGGTTAGCCAAATCCATAAATCTGTTCTCATTTCGATCGCA
 GTCTGCAATCGACTGCGTGAAGCTGAACGCTAGAATCGGAACAGCATGCCGGGTGATTACGTTCCGGGCTGTCCCACGCCGTACCCGAAAGTTTGTA
 CCCC

Sequence Deposited: NCBI Title : *Bacillus pumilus* strain BRHS/C1 16S ribosomal RNA gene, partial sequence

ACCESSION: JF836847

VERSION: JF836847.1

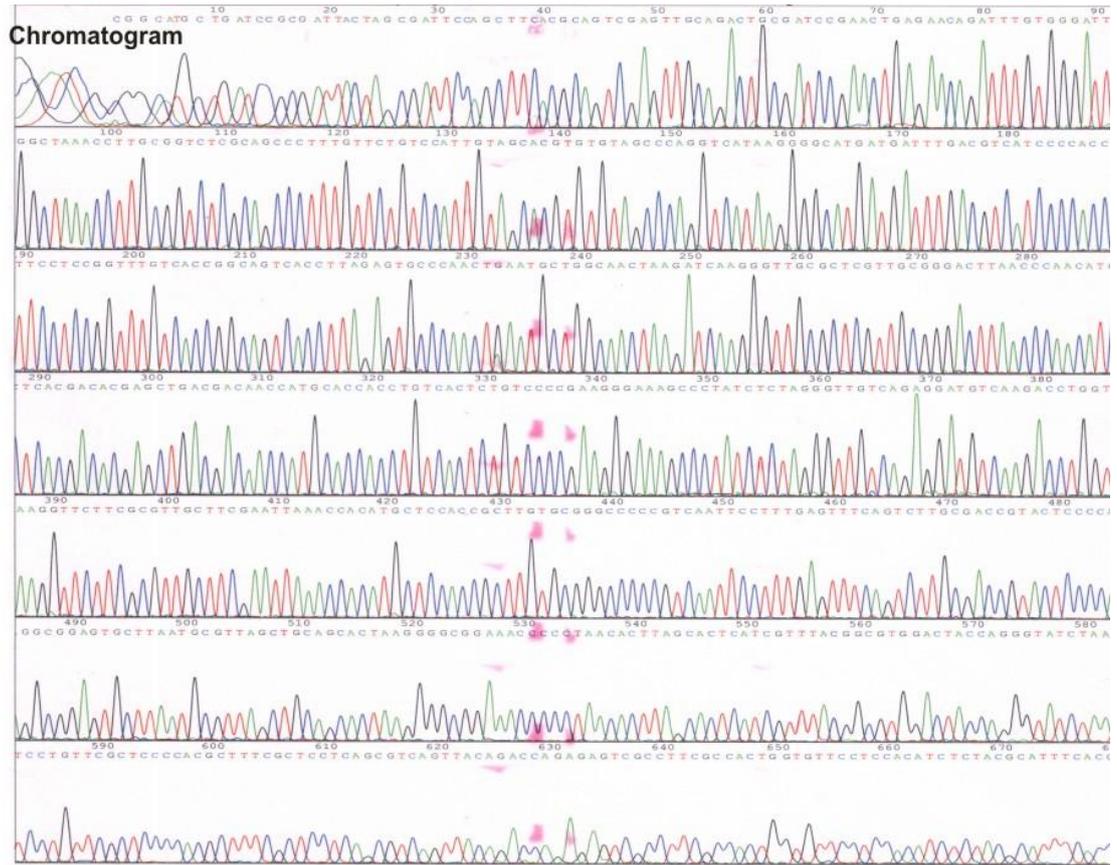
GI:347978040

DNA linear : 1361bp

```

1 ctcccgggaa tgttagggc ggaacgggtga gtaaacacgtg ggtaaacctgc ctgtaagact
61 gggataaactc cgggaaacccg gagctaatac cggatagttc cttgaaacccg atgggttcaag
121 gatgaaagac ggtttcggct gtaactaaca gatgaaacccg cgggcaatc gctagtgtg
181 gaggtaaacg ctcaacaaag cgaacgatgag tagcagaccc gagaggtgga tcggcaaac
241 tgggaactgag acacggccca gactcctacg ggaggcagca gtaggaaatc ttcccgaatg
301 gacgaaagtc tgaaggagca acgcccgggtg agtggatgag gttttcggat cgttaaagtc
361 tgttgttagg gaaagaaacg tgaagagta actgcttgc cttgaaacccg acctaaccac
421 aaagccacgg ctaactacgt gccagcagcc cgggtaatac gtaggggggc aagcgtttgc
481 cggaaattatt gggcggtgaa aggggctcgc agggggttt ctaagctcg atgtgaaagc
541 cccccggctt caaccggggg aggggcaatt ggaaacttg gaaacctga gtgcaaaaga
601 agggaggtgg aatttccacg gtagcgggtg aaattgctga aagatgtgga aggaacacca
661 gtggcgaagg cgaactctctg gttctgtaact gaocctgagg agcgaagccg tggggagca
721 acaggattag ataccctggt agtccacgcc gtaaacgatg agtgcataag tttaggggtg
781 ttcgccccct tagtgctgca gtaaacgcat taagcaactcc gctggggag taoggttcga
841 agactgaaac tcaaggaat tgaacggggc cgcacaacg ggtggagcat gtaggtttaa
901 tcgaaagaac gegaagaac ttaccagtc ttgacatcct ctgcaaaccc tagaagtagg
961 gcttccctt cgggacaaga gtagcaggtg gtagatggtt gtagcagct cgtgtcgtga
1021 gatgtgggt taagtcccgc aacgagcga accctgac ttagttgca geattcaagt
1081 gggcaactca agtgactgc cgtgacaaa ccggaggaag gtaggggatga cgtcaaatca
1141 tcatgcccc tatgacctg gctacacac tgctacaatg tagcaaacca agggctgcga
1201 gaccgcaagg ttagccaat ccaacaaatc tttctcagt tcgagtcgca gttcgaact
1261 cgaactgctg aagctgaaat cgtagtaat ccggatcag catgcccgg gtgaataact
1321 tccccggcct tgtttaacc atccccgca aa
  
```

Fig. 62. Chromatogram and sequence deposit of ITS region of *Bacillus pumilus* BRHS/C-1.



AGGGTATGTTAGCCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGTAACCTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAA
 CCCGATGGTTCAGGATGAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCCATAGCTAGTTGGTGGGTAAGGCTCACCAGGCGACGATGC
 GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTCCGCAATGGACGAAAGTTGA
 CGGAGCAACGCCCGCTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTGTGTAGGGAAGAACAAGTGCAAGAGTAACCTGTCACCTTACCGGTACCTA
 ACCAGAAAGCCACGGCTAATACGTGCAGCAGCCCGGTAATACGTAGTGGCAAGCCTTGTCCGGAATATTGGGCGTAAAGGGCTCGCAGCCGGTTTCT
 TAAGTCTGATGTAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAACCTGGGAACTTGAGTGCAGAAGAGGAGAGTGGAAATCCACGCTGTAGCGGTGAA
 ATCGTATAGATGTGGAGGAACACCAGTGGCGAAGCGGACTCTCTGGTCTGTAACCTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
 TGGTAGTCCACGCCGTAACGATGAGTCTAAGTGTAGGGGGTTTCCGCCCTTAGTGTCTGAGCTAACGCATTAAGCACTCCGCTGGGGAGTACGGTCCG
 CAAGACTGAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAATCGAAGCAACCGGAAGAACCTTACCAGGTCTTGACATCTC
 CTGACAAACCTTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAA
 CGAGCGCAACCTTGTATCTAGTGTCCAGCATTAGTGGGCACTTAAGGTGACTGCCGGTGACAAACCGGAGGAAGTGGGGATGACGTCAAATCATCAT
 GCCCCTTATGACCTGGGCTACACAGTGTACAATGGACAGAAACAAAGGGCTCGGAGACCGCAAGGTTTAGCCAATCCCAAAATCTGTTCTCAGTTCGGAT
 CGCAGTCTGCACCTGACTGCGTGAAGCTGGAATCGTAGTAATCGCGGATCAGCATGCCCGGTGAATACGTTCCAGGCTGTGTACACATCGCCCAT

Sequence Deposited: NCBI Title : *Bacillus altitudinis* strain BRHS/S73 16S ribosomal RNA gene, partial sequence

ACCESSION: JF899300

VERSION: JF899300.1

GI:350542699

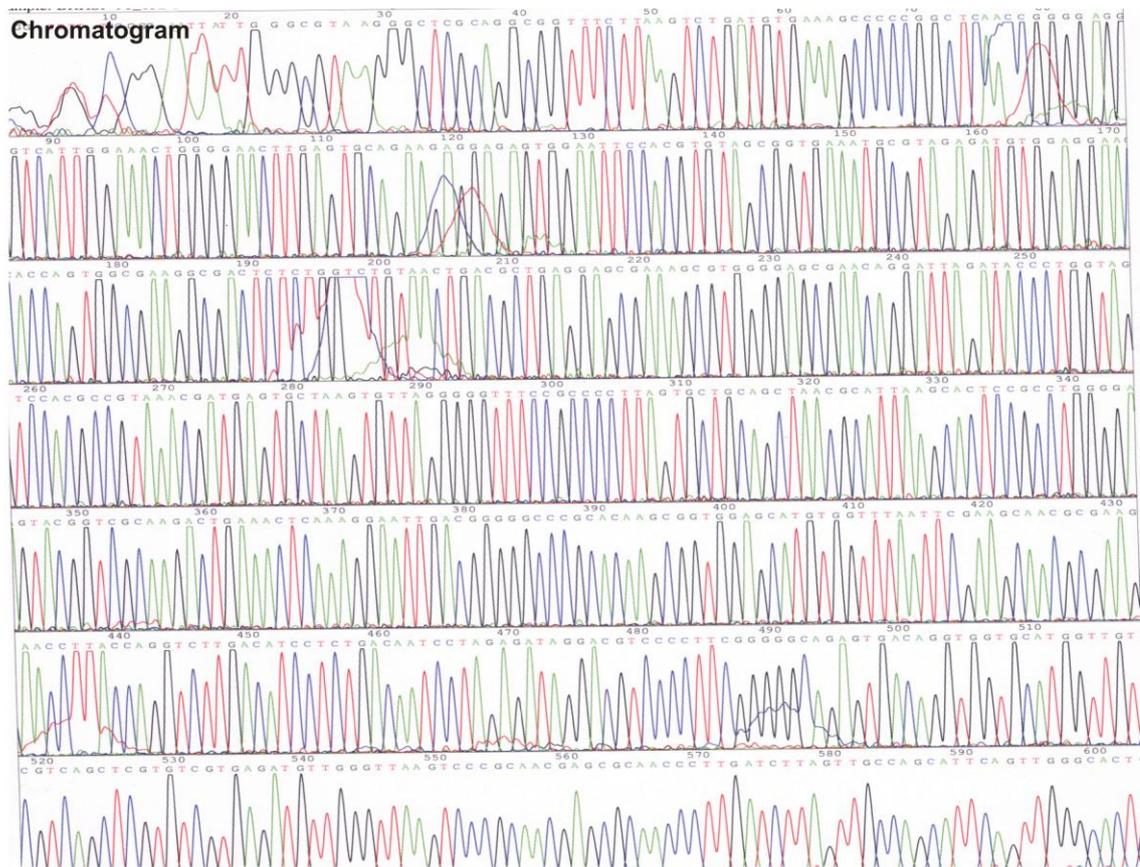
DNA linear : 1321bp

```

1 agggataggt tagccggggg acgggtgagt aacaogtggg taacctgocct gtaagactgg
61 taaactccggg aaaccgggagc taataccgga tagttcccttg aaccgcatgg tccaaggatg
121 aaagacggtt togggtgtca cttacagatg gaccocgggc gcattagcta gttggtgagg
181 taaggctcac caaggcgacg atgcttagcc gacctgagag ggttagcggc cacactggga
241 ctggacacgg cccagactcc tacgggaggc agcagtaggg aatcttccgc aatggacgaa
301 agttgacgga gcaacgcccgc gtgagtgatg aaggttttcc gatcgtaaag ctctgttgtt
361 agggaaagaac aagtgaaga gtaactgott gcaocttgac ggtacctaac cagaaagcca
421 cggctaacta cgtgccagca gccogogttaa tacgtaggtg gcaagcgttg tccggaatta
481 ttgggctgaa aggctcgca ggcggttctc taagtctgat gtgaaagccc ccggtcgaac
541 cgggaggggt catggaaac tgggaaactt gagtgcagaa gaaggagtg gaattccacg
601 tgtagcggtg aaatgcgtag agatgtggag gaaccaccgt ggcgaaggcg acctctggtt
661 ctgtaactga ccctgaggag cgaaaagcgtg gggagcgaac aggttagat acctctgtag
721 tccacgcogt aaacgatgag tgcataagtg tagggggttt ccgcccotta gtgtgcgacg
781 taacgcaatta agcaactccgc ctggggagta cggtcgcaag actgaaactc aaagaaattg
841 acgggggccc gcacaagcgg tggagcatgt ggtttaatto gaagcaaacg gaagaacott
901 accaggtcct gacatccctc gacaacccta gagataggcg ttccctctcg gggacagagt
961 gacaggtggg gcattggtgt cgtcaagctcg tgtcgtgaga tgttgggta agtcccga
1021 cggagcgaac ccttgatctt agttgccagc attcagttgg gcactctaa gttgactggc
1081 gtgcaaaccc ggaggaaagt ggggatgacg tcaaatcctc atgcccctta tgacctgggc
1141 tacacacggt ctacaatgga cagaacaaag ggotgcgaga ccgcaagttt tagccaatcc
1201 cacaaaatgc ttctcagttc ggatcgcagt ctgcactcga ctgcgtgaag ctggaatcgc
1261 tagtaatogc ggatcagcat gccogogtga atacgttcca ggctgtgtac acatogocaa
1321 t

```

Fig. 64. Chromatogram and sequence deposit of ITS region of *Bacillus altitudinis* BRHS/S-73.



GGGGGGCGGGCCCTATAATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGCTGGGATAAATCCGGAAACCCGGGCTAATACCGGATGGTGTCTGAACCGCATGGTTCAGACATAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGGCGGCATTAGCTAGTTGGTGAGGTAACGGCTCACAAAGCGCAGCATGCCGACCTGAGAGGGTATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGGAATCTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCTGAGTGATGAAGGTTTTCGGATCGTAAGCTCTGTTGTAGGGAAGAACAAGTCCGCTTCAAATAGGGGGCACCTTGACGGTACC TAACCAGAAAGCCACGGCTAACTACGTGCAGCAGCCGGTAAACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGA AACTGGGAACCTGAGTCGAGAAGAGGAGAGTGAATTCACGCTGATAGCGGTGAAATGCGTAGAGATGTGGAGGAACCCAGTGGCGAAGGGCAGCTCTCTGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGGGAGCGAAGCAGGATTAGATACCTTGGTAGTCCACGCCGTAACAGTAGTGTAAGTGTAGGGGGTTTCCGCCCTTAGTGTGACGCTAACGCATTAAGCACTCCGCTGGGGAGTACGGTCCGAAAGCTGAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGAGCATGTGGTTAATTCGAAGCAACGCGAAGAACCTTACAGGCTTTGACATCCTTGAACAATCTAGAGATAGGACGTCCTCCTCGGGGGCAGAGTGACA GGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGTTAAGTCCCGCAACGAGCGCAACCCCTTGATCTTAGTTGCCAGCATTCAAGTTGGGCAC TCTAAGGTGACTGCCGCTGACAAACCGGAGGAAGTGGGGATGACCTCAAAATCATGCCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAAC AAAGGGCAGCGAAACCGGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTGCAGTGCCTGAGCTGGAATCGCTAGTAAT CGCGGA

Sequence Deposited: NCBI Title : *Bacillus methylotrophicus* strain BRHS/P91 16S ribosomal RNA gene, partial sequence

ACCESSION: JQ765577

VERSION: JQ765577.1

GI:339899801

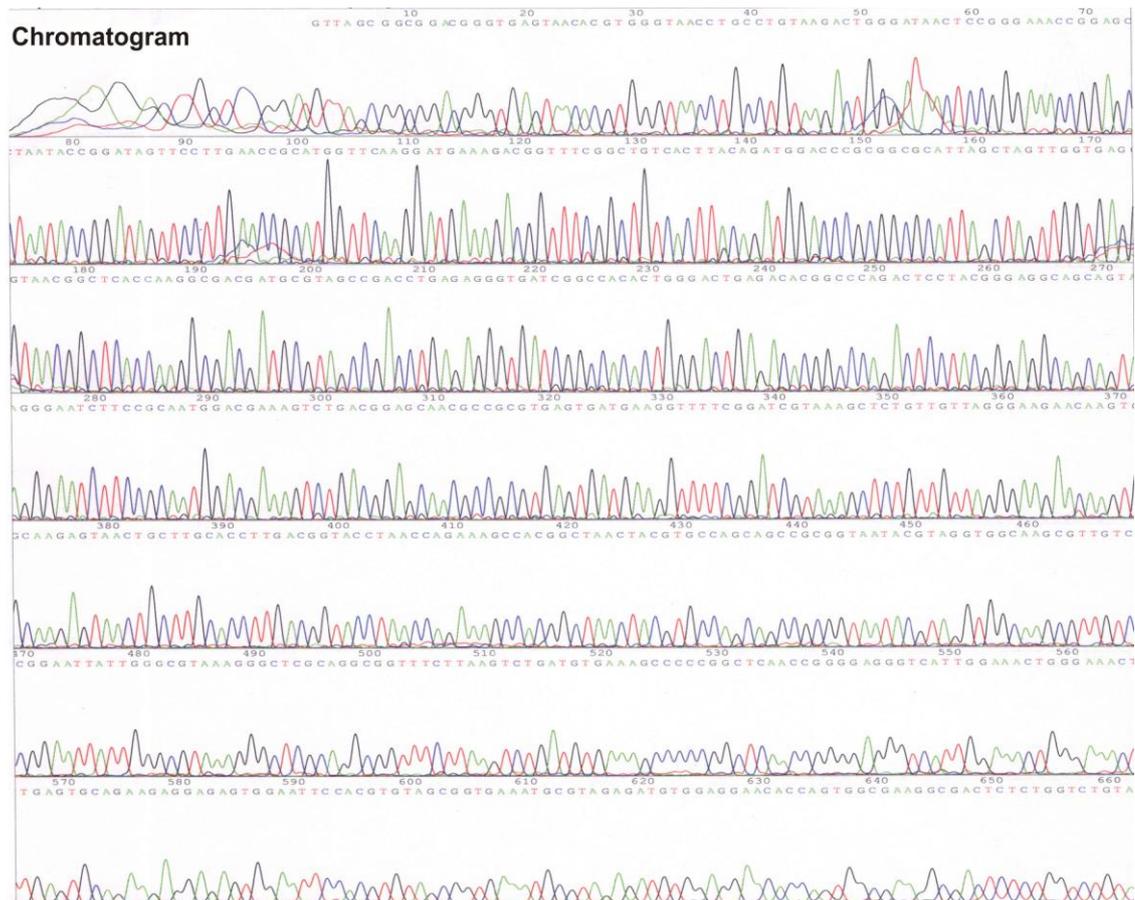
DNA linear : 1452bp

```

1  gggggggcgg  gggccctataa  tgcaagtcga  ggggacagat  gggagcttgc  tccctgatgt
61  tagcggcgga  cggggtgagta  acacgtgggt  aaactgcctg  taagctggg  ataaactcgg
121  gaaaccgggg  ctaaatccgg  atggttgtct  gaaccgcatg  gttcagacat  aaaaggtggc
181  ttcggctacc  acttacagat  ggaccgcggg  cgcattagct  agttggtgag  gtaaccggctc
241  accaaggcga  cgatccgtag  ccgacctgag  aggtgatcgc  gccacactgg  gactgagaca
301  cggcccagac  toctacggga  ggcagcagta  gggaaatctc  cgcaatggac  gaaagtctga
361  cggagcaacg  ccgctgtagt  gatgaagttt  ttcggatcgt  aaagctctgt  tgttagggaa
421  gaacaagtgc  cgttcaataa  gggcggcacc  ttgacgggtac  ctaaccagaa  agccaaggct
481  aactacgtgc  cagcagcggc  ggaataactg  atgtggcag  cgttgcctcg  aattattggg
541  cgtaaagggc  tcgcaggcgg  tttcttaagt  ctgatgtgaa  agcccccgcc  tcaaccgggg
601  aggtcattg  gaaactgggg  aacttgagt  cagaagagga  gagtggaatt  tccgtgttag
661  cggtgaaatg  cgtagagatg  tggaggaaac  cccagtgggc  aaggcgaact  tctgttctgt
721  aactgacgtc  gaggagcga  agcgtgggga  gcgaacagga  ttagataccc  tggtagtcca
781  cgccgtaaac  gatgagtctc  aagtgttagg  gggtttccgc  cccttagtgc  tgcagctaac
841  gcatcaagca  ctccgcttgg  ggaatcgggt  cgaagactg  aaactcaag  gaattgacgg
901  gggcccgcac  aagcgtgga  gcatgtgggt  taattcgaag  caacgggaag  aaacttaca
961  ggtcttgaca  toctctgaca  atcctagaga  taggactgcc  ccttcggggg  cagagtgaca
1021  ggttaagtc  gtttgcctc  agctcgtctc  gtgagatgtt  ggttaagtc  ccgcaacgag
1081  cgcaaccctt  gatcttagtt  gccagcattc  agttgggacc  tctaagtgta  ctgcccgtga
1141  caaacgggag  gaaggtgggg  atgacgtcaa  atcaatcgtc  cccttatgac  ctggcttaca
1201  cagctgtctc  aatggacaga  acaaaaggga  gcgaaacccg  gaggttaagc  caatccaca
1261  aatctgtctc  agtctcggat  cgcagctctg  aactcgaact  cgtgaagct  gaattcctag
1321  aatocggga  tcagatgac  gcggtgaata  cgttccgggg  cctgtcacg  accgcccgtc
1381  acaccagag  agttttagta  acccgaagtc  ggtgaggtaa  ccttttagga  gccagcccgc
1441  caaacgggag  gaaggtgggg  atgacgtcaa  atcaatcgtc  cccttatgac  ctggcttaca

```

Fig. 65. Chromatogram and sequence deposit of ITS region of *Bacillus methylotrophicus* BRHS/P-91.



GTTAGCGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGAAACCGGAGCTAATACCGGATAGTTCCTGAAACCGCATG
 GTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGGCGCATTAGCTAGTTGGTGAGGTAACCGGTCACCAAGGCCACGATGCGTAGCC
 GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCAGACTCTACGGGAGGAGCAGTAGGGAACTCTCCGCAATGGACGAAAGTCTGACGGA
 GCAACCCCGCTGAGTGATGAAGGTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAAGTGAAGAGTAACCTGCTTGCACCTTGACGGTACCTAACCA
 GAAAGCCACGGCTAACCTACGTGCCAGCAGCCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATGGGCGTAAAGGGCTCCGAGGCGGTTTCTTAAG
 TCTGTAGTGAAAGCCCCGGCTCAACCGGGGAGGTCATTGGAAGTGGGAACTTGAGTGCAGAAGAGGAGTGGAAATCCACGCTGAGCGGTGAAATGC
 GTAGAGATGTGGAGAACACCAAGTGGCGAAGCGCACTCTCTGTTCTGTAACCTGACCGTGAAGGACGAAAGCGTGGGGAGCAACAGGATAGATACCCCTGGT
 AGTCCACCGCGTAAACGATGAGTGAAGTGTAGGGGTTTCGGCTGTAACTGACCGTGAAGGACGAAAGCGTGGGGAGCAACAGGATAGATACCCCTGGT
 ACTGAAACTCAAAGGAATGACGGGGCCCGCACAAAGCGGTGAGCATGTGGTTAATTGCAAGCAACGGAAGAACTTACCAGGTCTTGACATCCTCTGA
 CAACCCTAGAGATAGGGCTTTCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGCTGCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAG
 CGCAACCCCTGATCTTAGTTGCCAGCATTAGTTGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAGGTTGGGATGACGTCGAATCATCATGC
 CCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCCAATCCCAAAATCTGTTCTCAGTTCGGATCC
 CAGTCTGCAACTGACTGCGTGAAGCTGGAATCG

Sequence Deposited: NCBI Title : *Bacillus aerophilus* strain BRHS/B-104 16S ribosomal RNA gene, partial sequence

ACCESSION: KC603894
 VERSION: KC603894.1
 GI:471184500
 DNA linear : 1256 bp

N
 1 gtttagcggcg gacgggtgag taacacgtgg gtaacctgcc tgaagactg ggataactcc
 61 gggaaacccg agctaatacc ggatagttcc ttgaaccgca tggttcaagg agtgaagacg
 121 gtttcggtg tcaacttacg atggaccggc ggcgattag ctagtgtggg aggtaaacgg
 181 tcaccaagcg gacgatgctg agccgacctg adagggatg ctgcccactc gggactgaga
 241 cacggcccaq actcctacgq gaggcagcag tagggaatct tccgcaatg acgaaagtct
 301 gacggagcaa cgcgccgtga gtgatgaagg ttttcggatc gtaaaactct gttgttagg
 361 aagaacaagt gcaagagtaa ctgcttgcaq cttgacggtg ctaaccaga aagccacggc
 421 taactacgtg ccagcagccg cggtaatacg taggtggcaa cggttgtccg gaattattgg
 481 cgtataaagg ctgcagggcg gtttcttaag tctgatgtga aagccccggc ctcaaccggg
 541 gagggtcatt ggaactcggg aaacttgagt gcagaagagg agagtggaat tccacgtgta
 601 cgggtgaaat gcgtagagat gttgaggaac accagtggcg aaggcgactc tctggtctgt
 661 aactgacgct gaggagcgaq agcgtgggga gcaaacagga ttgatatacc tggtagtcca
 721 cgccgtaaac gatgagtctc aagtgttagg gggtttccg ccttagtgc tgcagtaaac
 781 gcattaaaga ctccgcctgg ggagtacggt cgcaagactg aaactcaag gaattgacgg
 841 gggccccgac aagcgggtga gcatgtggtt taattcgaag caacccgag aaccttaca
 901 ggtcttgaca tcctctgaca accctagaga tagggcttc ccttcgggga cagagtaca
 961 ggtggtgcat ggttctgctc agctcgtgtc gtgagatggt gggtaagtc ccgcaacgag
 1021 cgcaaccctt gatcttagtt gccagcattc agttgggcac tctaaggatg ctgcccgtga
 1081 caaacccggg gaaggtgggg atgacgtcaa atcatcatgc cccttatgac ctgggtaca
 1141 cacgtgctac aatggacaga caaaagggct gcgagaccgc aaggtttagc caatcccaca
 1201 aatctgttct cagttcggat cgcagttcgc aactgcactg cgtgaagctg gaatcgc

Fig. 66. Chromatogram and sequence deposit of ITS region of *Bacillus aerophilus* BRHS/B-104.

Table 46. Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different Bacilli isolates

```

-----
!Title Phylogenetic Analysis;!Format   DataType=Nucleotide CodeTable=Standard
-----
#JF836847_Bacillus_pumilus-BRHS/C-1      CGG ACG GGT GAG TAA CAC GTG GGT [ 99]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 .C. G.. C.. AC. ... .. .A. .C. [ 99]
#JF899300_Bacillus_altitudinis-BRHS/S-73 .C. T.. A.. AA. ... A.. .A. .A. [ 99]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 .TC C.. A.. AC. ... .. .A. .A. [ 99]
#KC603894_Bacillus_aerophilus-BRHS/B-104 .TA C.. T.. CT. ... .. .A. .C [ 99]
#JF836847_Bacillus_pumilus-BRHS/C-1      AGG ATG AAA GAC GGT TTC GGC TGT [ 198]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .. . [ 198]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. . [ 198]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 GAC .A ... .GT .C ... .. .AC [ 198]
#KC603894_Bacillus_aerophilus-BRHS/B-104 ... .. . [ 198]
#JF836847_Bacillus_pumilus-BRHS/C-1      AGC AAC GCC GCG TGA GTG ATG AAG [ 396]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .. .A. ... .. . [ 396]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. .A. ... .. . [ 396]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 ... .. .T. ... .. . [ 396]
#KC603894_Bacillus_aerophilus-BRHS/B-104 ... .. . [ 396]
#JF836847_Bacillus_pumilus-BRHS/C-1      AAC CAG AAA GCC ACG GCT AAC TAC [ 495]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .A ... .. . [ 495]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. . [ 495]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 ... .. . [ 495]
#KC603894_Bacillus_aerophilus-BRHS/B-104 ... .. . [ 495]
#JF836847_Bacillus_pumilus-BRHS/C-1      CGG TTT -CT TAA GTC TGA TGT GAA [ 594]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .. T.. ... .. . [ 594]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. -.. ... .. . [ 594]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 ... .. -.. ... .T. ... .. . [ 594]
#KC603894_Bacillus_aerophilus-BRHS/B-104 ... .. -.. ... .T. ... .. . [ 594]
#JF836847_Bacillus_pumilus-BRHS/C-1      GGG GAG CGA ACA GGA TTA GAT ACC [ 792]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .. . [ 792]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. . [ 792]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 ... .. .C ... .. . [ 792]
#KC603894_Bacillus_aerophilus-BRHS/B-104 ... .. .C ... .. . [ 792]
#JF836847_Bacillus_pumilus-BRHS/C-1      TAA GCA CTC CGC CTG GGG AGT ACG [ 891]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .. . [ 891]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. .A. ... .. . [ 891]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 C.. ... .A. ... .. . [ 891]
#KC603894_Bacillus_aerophilus-BRHS/B-104 C.. ... .A. .G ... .. . [ 891]
#JF836847_Bacillus_pumilus-BRHS/C-1      AAA CCG GAG GAA GGT GGG GAT GAC [1188]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 T.. ... .A ... .. . [1188]
#JF899300_Bacillus_altitudinis-BRHS/S-73 T.. ... .A ... .. .C. ... .. [1188]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 C.. ... .A ... .. . [1188]
#KC603894_Bacillus_aerophilus-BRHS/B-104 G.. ... .T ... .. .A. ... .. [1188]
#JF836847_Bacillus_pumilus-BRHS/C-1      CGC GG- TGA ATA CGT TCC CGG GCC [1386]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 ... .G ... .. . [1386]
#JF899300_Bacillus_altitudinis-BRHS/S-73 ... .. -.. ... .. .-A. .T [1386]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 ... .. -.. ... .. . [1386]
#KC603894_Bacillus_aerophilus-BRHS/B-104 --- --- --- --- --- --- --- [1386]
#JF836847_Bacillus_pumilus-BRHS/C-1      A GTC GTA ACA AGG TAG CCG TAT CG [1514]
#HQ849482_Bacillus_altitudinis_BRHS/P-22 - -A- A-- --G T-- -C- --- -A- C- [1514]
#JF899300_Bacillus_altitudinis-BRHS/S-73 - -A- -C- --G --- -C- --- --- G- [1514]
#JQ765577_Bacillus_methylotrophicus_BRHS/P-91 - -C- -G- C-T C-- -C- --- -T- A- [1514]
#KC603894_Bacillus_aerophilus-BRHS/B-104 - -G- AC- --T C-- -A- --- -T- T- [1514]
End;
-----
NSeqs=5 NSites=1514   Identical=. Missing=? Indel=-; !Domain=Data property=Coding
CodonStart=1

```

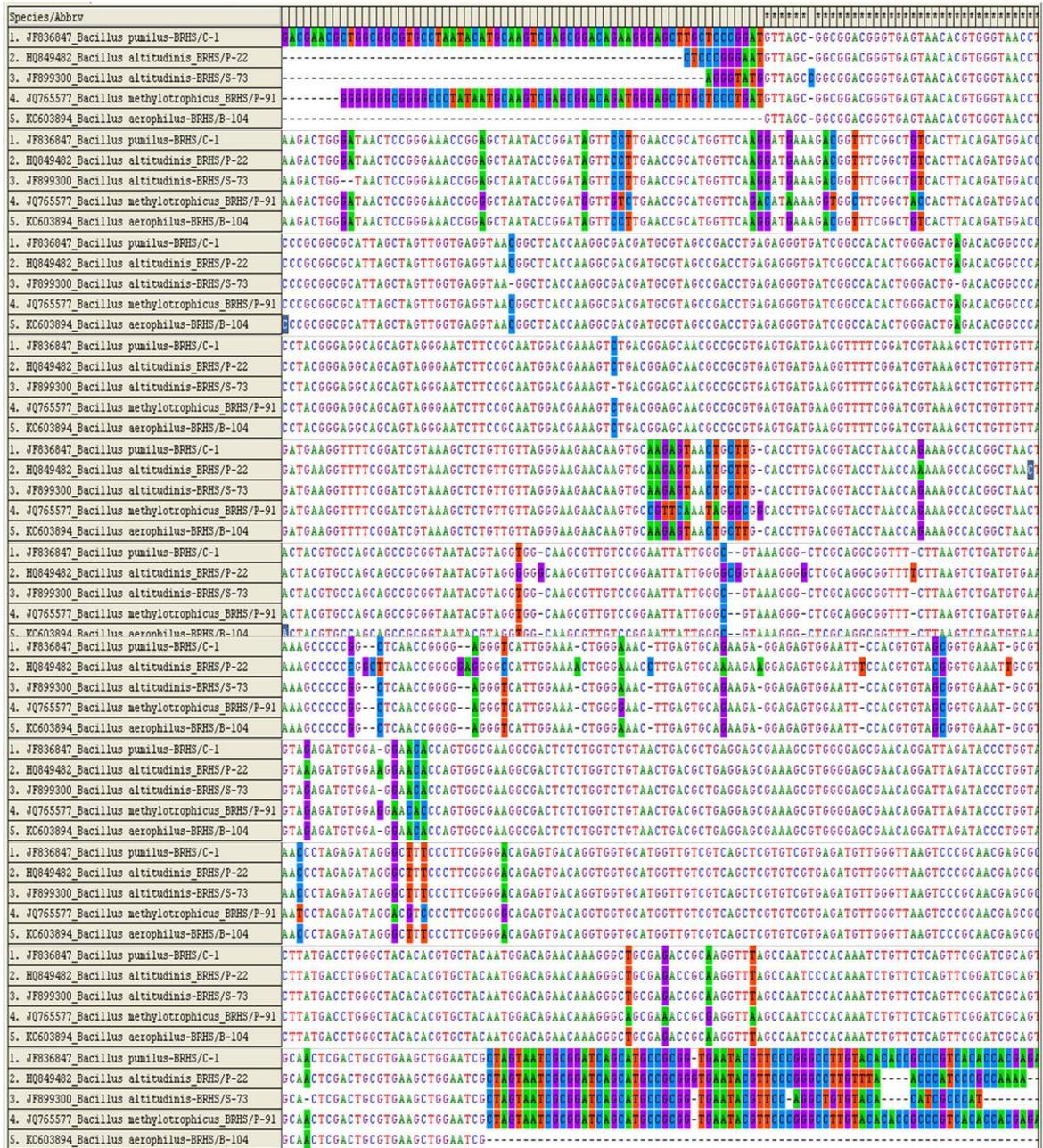
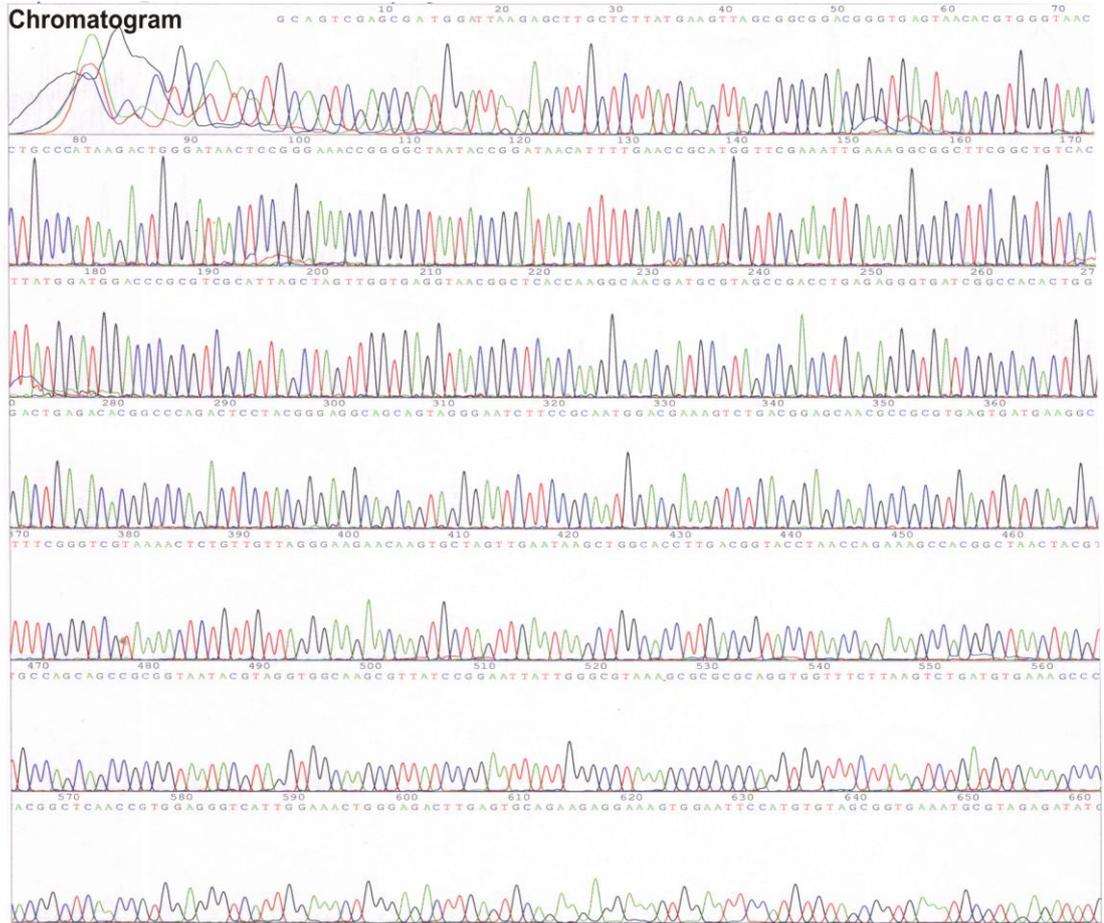


Fig. 67. Multiple sequence alignment of Bacillii isolates. Different colours shows different bases. Difference in conserved regions are indicated by different colours.



GCAGTCGAGCGATGGATTAAGAGCTTGCCTTTATGAAGTTAGCGCGGACGGGTGAGTAAACGTTGGTAACTGCCCATAGACTGGGATAACTCCGGGAAACCGGGCTAATACCGGATAACATTTGAACCGCATGGTTCCGAAATTTGAAAGCGCGCTTCGGCTGTCACCTTATGGATGGACCCCGCTCGCATTAGCTAGTTGTGAGGTAACGGCTCACCAAGGCACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCAGACTCTACGGGAGGACGAGTAGGGAATCTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAAGTGAAGGCTTTCCGGTTCGTAATAACTCTGTTGTTAGGGAAGAACAGTGTAGTTGAAATAAGCTGGCACCCTGACCGTACCTAACCAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGCCAAGCGTTATCCGGAATTATTGGGCGTAAAGCCGCGCAGGTGTTCTTAAGTCTGATGTGAAAGCCACCGCTCAACCGTGGAGGTCATGGAACTGGGAGACTGAGTGCAGAAAGAAAAGTGGAAATCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGAACACCAAGTGGCGAAGGGCACTTTCTGGTCTGTAAGTGAACCTGACACTGAGGCGCGAAGCGTGGGAGCAAAACAGGATTAGATACCTTGGTAGTCCACGCCGTAACGATGAGTCTAAGTGTAGAGGGTTTCCGCCCTTATGCTGAAGTTAACGCTAAGCACTCCGCCCTGGGAGTACGCCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCTTTGACATCCTTGAAACCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGATCTTAGTTGCCATCATTAAAGTTGGGCACTTAAGTGACTGCCGGTGACAAAACCGGAGGAAGTGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGTACACACGCTGCTACAATGGACGGTACAAGAGCTGCAAGACCCGCGAGGTGAGCTAATCTATAAAACCGTTCTCAGTTCGGATT

Sequence Deposited: NCBI Title : *Enterobacter cloacae* strain BRHS/R-71 16S ribosomal RNA gene, partial sequence

ACCESSION: KC703974
 VERSION: KC703974.1
 GI:473093975
 DNA linear : 1260bp

```

1 gcagtcgagc gatgattaa gagcttgctc ttatgaagt agcggcgagc gggtagtaa
61 cacgtgggta acctgcccac aagactggga taactccggg aaacgggggc taataccgga
121 taacattttg aaccgcatgg ttcgaaattg aaagggcgct tcggctgtca cttatggatg
181 gacccgctgc gcattagcta gttggtgagg taaccgctca ccaaggcaac gatcgctagc
241 cgacctgaga gggtagtcgg ccacactggg actgagacac ggcccagact cctaccggag
301 gcagcagtag ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgagt
361 atgaaggctt tcgggtcgta aaactctgtt gttagggaag aacaagtgtc agttgataaa
421 gctggccact tgacggtaac taaccagaaa gccacggcta actacgtgcc agcagccgcg
481 gtaatacgta gttggcaacg gttatccgga attattgggc gtaaaagcgc cgcaggtggt
541 ttcttaagtc tgatgtgaaa gccaccggct caaccgtgga gggtcattgg aaactgggag
601 acttgagtgc agaagaggaa agtggaattc catgtgtagc ggtgaaatgc gttagagat
661 ggaggaacac cagtggcgaa ggcgacttcc tgggtctgta ctgacactga ggcgcaaa
721 cgtggggagc aaacaggatt agataccctg gtagtccacg ccgtaaacga tgagtgttaa
781 gtgtagagag gtttccgccc ttagtgctg aagttaacgc attaagcact ccgctggggg
841 agtacggccg caaggtgtaa actcaagga attgacgggg gcccgcaaac cgggtggagc
901 atgtggttta atcgaagca acgcgaagaa ccttaccagc tcttgacatc cctgaaaaac
961 ctagagata gggctctccc tccgggagca gagtaccagg tgggtgatgt ttgtgtcag
1021 ctctgtctgt gtagtgttgg gtttaagtc gcaacgagcg caaccctgga tcttagttgc
1081 catcattaag ttgggcactc taaggtgact gccggtgaca accgggagga aggtgggat
1141 gacgtcaaat catctgccc cttatgacct gggctacaca cgtgctacaa tggacggtac
1201 aaagagctgc aagaccgca ggtggagcta atctcataaa accgttctca gttcggattg

```

Fig. 68. Chromatogram and sequence deposit of ITS region of *Enterobacter cloacae* BRHS/R-71.

Table 47. Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different *Enterobacter* isolates

```

-----
!Title Phylogenetic Analysis;!Format   DataType=Nucleotide CodeTable=Standard

#KC703974_Enterobacter_cloacae_BRHS/R-71  --- --- --- --- --- --- --- --- [ 45]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [ 45]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [ 45]
#ESU39556_Enterobactee_cloacae            CTC AGA TTG AAC GCT GGC GGC AGG CCT [ 45]
#AJ508302_Enterobacter_cloacae            --- --- --- --- --- GGC GGC AGG CCT [ 45]
#AM947037_Enterobacter_cloacae            CTC AGA TTG AAC GCT GGC GGC AGG CCT [ 45]
#AM947038_Enterobacter_cloacae            CTC AGA TTG AAC GCT GGC GGC AGG CCT [ 45]
#AM947039_Enterobacter_cloacae            CTC AGA TTG AAC GCT GGC GGC AGG CCT [ 45]
#AM947040_Enterobacter_cloacae            CTC AGA TTG AAC GCT GGC GGC AGG CCT [ 45]
#AB680426_Enterobacter_cloacae            --- --A TTG AAC GCT GGC GGC AGG CCT [ 45]
#KC703974_Enterobacter_cloacae_BRHS/R-71  GAT GGA TTA AG- -AG CTT GCT -CT TAT [ 90]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [ 90]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [ 90]
#ESU39556_Enterobactee_cloacae            .G. A.C AC. GAG -. . . . . . . . CGG [ 90]
#AJ508302_Enterobacter_cloacae            .G. AAC AGG .AG C.R Y. . . . . G. . .CG [ 90]
#AM947037_Enterobacter_cloacae            .G. AAC AGG .AG C. . . . . . . . .YG [ 90]
#AM947038_Enterobacter_cloacae            .G. AAC AGG .AG C. . . . . . . . .YG [ 90]
#AM947039_Enterobacter_cloacae            .G. AAC AGG .AG C. . . . . . . . .TG [ 90]
#AM947040_Enterobacter_cloacae            .G. AAC AGG .AG C. . . . . . . . .TG [ 90]
#AB680426_Enterobacter_cloacae            .G. A.C AC. GAG AG- -C. TGC T. . CGG [ 90]
#KC703974_Enterobacter_cloacae_BRHS/R-71  --T AAG TTG GGC ACT CTA AGG TGA CTG [1170]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#ESU39556_Enterobactee_cloacae            -TC CG. CC. .A . . . .A. . . .A. .A [1170]
#AJ508302_Enterobacter_cloacae            AT. .G. CC. .A . . . .A. . . .A. . . [1170]
#AM947037_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#AM947038_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#AM947039_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#AM947040_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1170]
#AB680426_Enterobacter_cloacae            -TC CG. CC. .A . . . .A. . . .A. . . [1170]
#KC703974_Enterobacter_cloacae_BRHS/R-71  AAG GTG GGG ATG ACG TCA AAT CAT CAT [1215]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#ESU39556_Enterobactee_cloacae            . . . . . . . . . . . . .G. . . . . [1215]
#AJ508302_Enterobacter_cloacae            . . . . . . . . . . . . .G. . . . . [1215]
#AM947037_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#AM947038_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#AM947039_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#AM947040_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1215]
#AB680426_Enterobacter_cloacae            . . . . . . . . . . . . .G. . . . . [1215]
#KC703974_Enterobacter_cloacae_BRHS/R-71  --- --- --- --- --- --- --- --- [1350]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#ESU39556_Enterobactee_cloacae            CTG CAA CTC GAC TCC ATG AAG TCG GAA [1350]
#AJ508302_Enterobacter_cloacae            CTG CAA CTC GAC TCC ATG AAG TCG GAA [1350]
#AM947037_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#AM947038_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#AM947039_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#AM947040_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1350]
#AB680426_Enterobacter_cloacae            CTG CAA CTC GAC TCC ATG AAG TCG GAA [1350]
#AB680426_Enterobacter_cloacae            CAG AAT GCT ACG GTG AAT ACG TTC CCG [1395]
#KC703974_Enterobacter_cloacae_BRHS/R-71  --- --- --- --- --- --- --- --- [1522]
#AJ276652_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#HF674994_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#ESU39556_Enterobactee_cloacae            GA GTC GTA ACA AGG TAA CCG T [1522]
#AJ508302_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#AM947037_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#AM947038_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#AM947039_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#AM947040_Enterobacter_cloacae            --- --- --- --- --- --- --- --- [1522]
#AB680426_Enterobacter_cloacae            GA G-- --- --- --- --- --- --- [1522]
-----

```

NSeqs=5 NSites=1514 Identical=. Missing=? Indel=-; !Domain=Data property=Coding CodonStart=1



Fig. 69. Multiple sequence alignment of *Enterobacter cloacae* with other ex-type strain sequences obtained from Genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

Table 48. Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different *Paenibacillus* isolates

```

-----
!Title Phylogenetic Analysis;!Format   DataType=Nucleotide CodeTable=Standard
-----
#JC8240_Paenibacillus_polymyxa      -- --- --- --- --- -TC GAG CGG A-- [ 45]
#HE981792_Paenibacillus_polymyxa    CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72  CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#KC389912_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#HE981790_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#NRP44328_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#HE981787_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#HE981786_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#EU781785_Paenibacillus_polymyxa     CC TAA TAC ATG CAA G.. ... .. GGT [ 45]
#JC8240_Paenibacillus_polymyxa      CT GTA AGA CTG GGA TAA CTC CGG GAA [ 135]
#HE981792_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 .C AC. ... .A. ... .. .A .C. ... [ 135]
#KC389912_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#HE981790_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#NRP44328_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#HE981787_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#HE981786_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#EU781785_Paenibacillus_polymyxa     .C AC. ... .A. ... .. .A .C. ... [ 135]
#JC8240_Paenibacillus_polymyxa      AG TTC CTT GAA CCG CAT GGT TCA AGG [ 180]
#HE981792_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 .C A.. ... TTC .T. ... .G AG. ... [ 180]
#KC389912_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#HE981790_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#NRP44328_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#HE981787_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#HE981786_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#EU781785_Paenibacillus_polymyxa     .C A.. ... TTC .T. ... .G AG. ... [ 180]
#JC8240_Paenibacillus_polymyxa      GT CAC TTA CAG ATG GAC CCG CGG CGC [ 225]
#HE981792_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 .. ... .G TG. ... .G. .T. ... .. [ 225]
#KC389912_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#HE981790_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#NRP44328_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#HE981787_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#HE981786_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#EU781785_Paenibacillus_polymyxa     .. ... .G TG. ... .G. .T. ... .. [ 225]
#JC8240_Paenibacillus_polymyxa      AA TAC GTA GGT GGC AAG CGT TGT CCG [ 540]
#HE981792_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#KC389912_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#HE981790_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#NRP44328_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#HE981787_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#HE981786_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#EU781785_Paenibacillus_polymyxa     .. ... ..G ..G ..G ..G ..G ..G ..G [ 540]
#JC8240_Paenibacillus_polymyxa      TG AGG AGC GAA AGC GTG GGG AGC GAA [ 765]
#HE981792_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#KC389912_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#HE981790_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#NRP44328_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#HE981787_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#HE981786_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#EU781785_Paenibacillus_polymyxa     .. ... C.. ... ..G ..G ..G ..G ..G ..G ..G [ 765]
#JC8240_Paenibacillus_polymyxa      - - - - - - - - - - [1478]
#HE981792_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#KC703975_Paenibacillus_polymyxa_BRHS/R-72 T AGA TGA TTG GGG TG [1478]
#KC389912_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#HE981790_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#NRP44328_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#HE981787_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#HE981786_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
#EU781785_Paenibacillus_polymyxa     T AGA TGA TTG GGG TG [1478]
-----
NSeqs=5 NSites=1514   Identical=. Missing=? Indel=-; !Domain=Data property=Coding
CodonStart=1

```

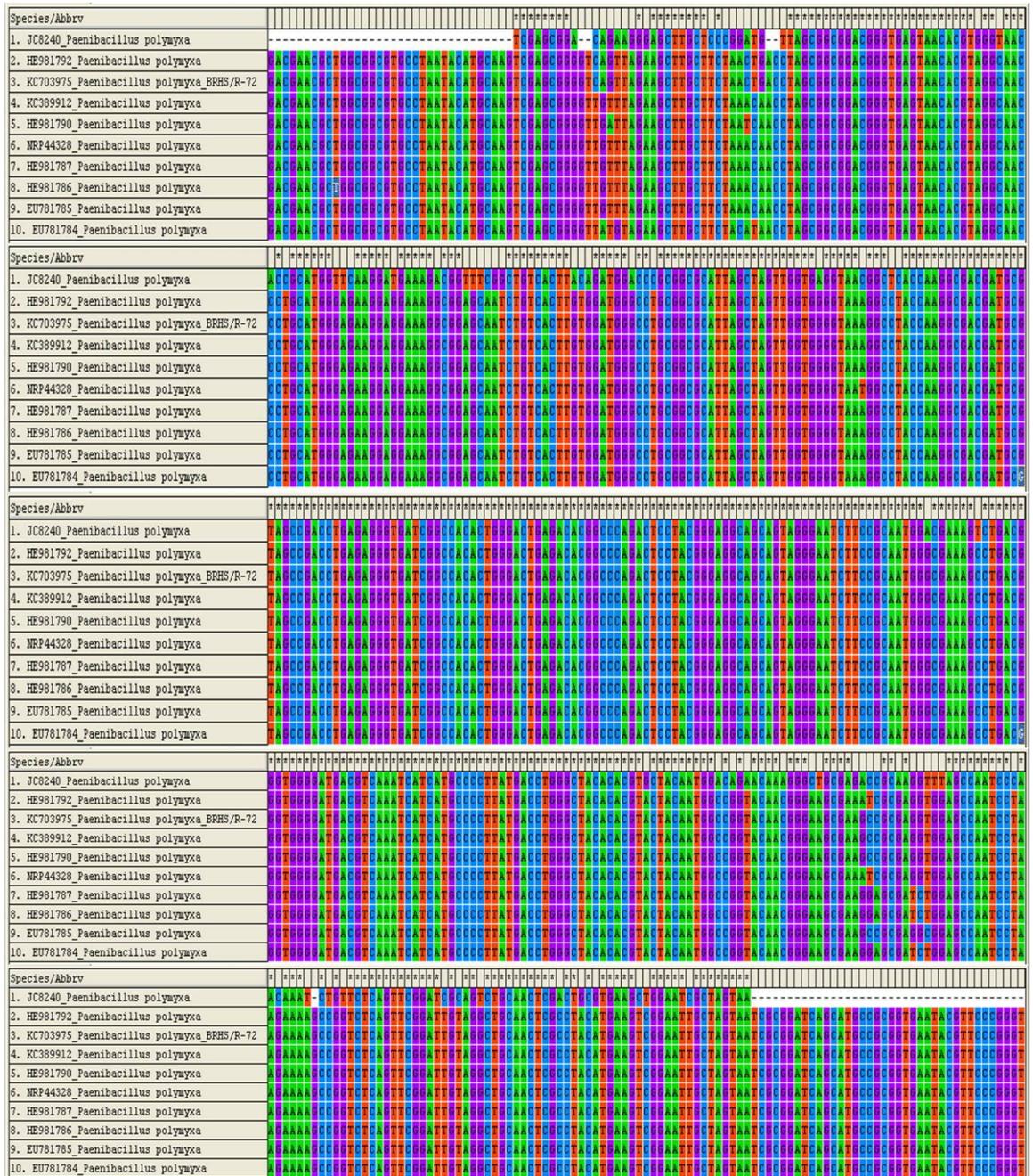


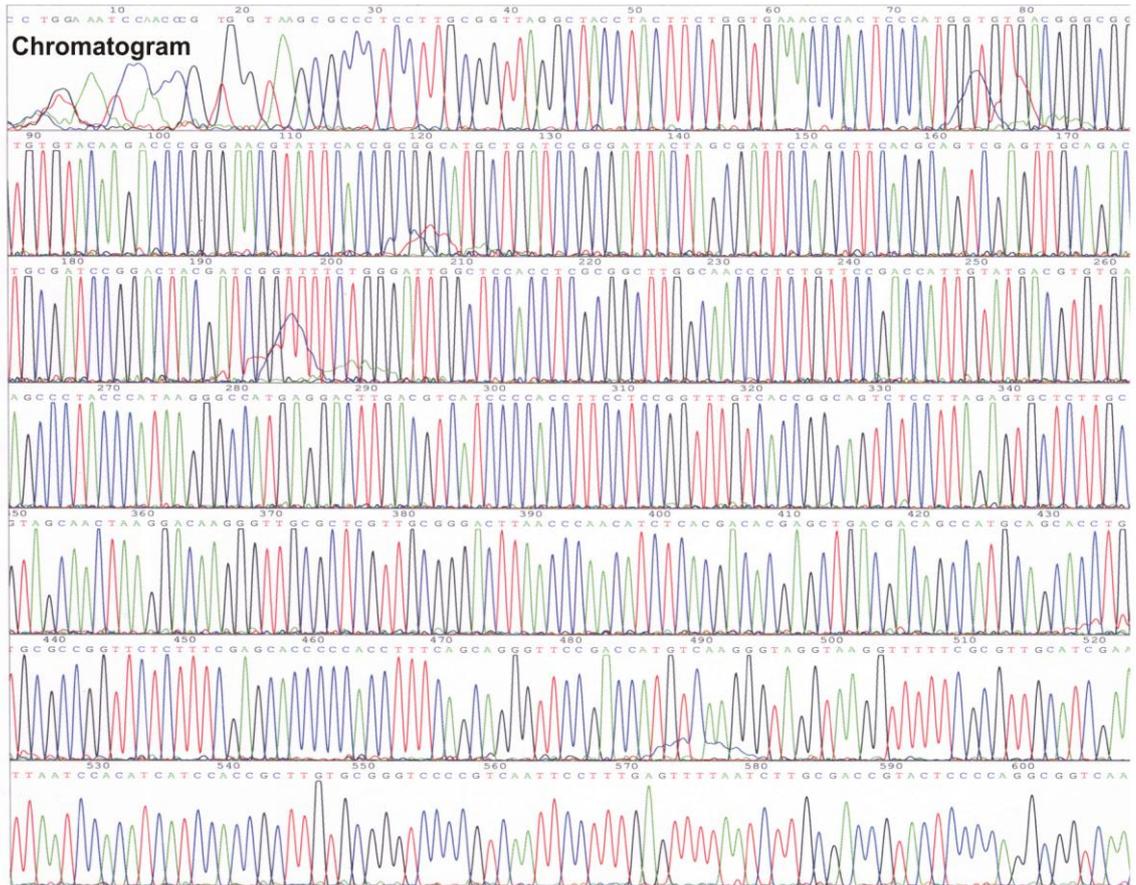
Fig. 71. Multiple sequence alignment of *Paenibacillus polymyxa* with other ex-type strain sequences obtained from Genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

4.10.2. Phylogenetic analysis of PGPR isolates based on 16S rDNA sequences.

Phylogenetic placements of PGPR isolates was carried out with the other representatives of members of *Bacillus* spp. as well as with ex type sequences obtained from gen bank database that showed maximum homologies in case of non bacilli isolates. The phylogenetic tree obtained with UPGMA method shows the optimal tree with the sum of branch length = 7.99112791. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 26 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Two types of phylogenetic tree have been obtained, the linear tree (NJ-Tree) which shows relationship among all the tested isolates (**Fig. 74**) and the radial (UPGMA) representation of the same tree which gives us their relative diversification positions across the clades. *Burkholderia symbionts* and *Enterobacter cloacae* were placed outside the *Bacilli* clade (Fig. 75).

4.10.3. Analysis of and Nucleotide frequency and DNA molecular weight of 16S rDNA sequences of PGPR isolates

Combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html. The sequence of 16S rDNA fragments of ITS region for all the PGPR isolates belonging to *Bacillus* spp. is presented in **Table 50 & 48**. The “GC” content was the highest in case of isolate *B. methylotrophicus* (107 times), similarly combinations like “CG” was also found highest in this isolate (103 times). Analysis of nucleotide sequences was also carried out among the non bacilli isolates as well. The “GC” content in *Burkholderia symbionts* (BRHS/P-92) was found to be comparatively higher than the other two PGPR isolates. The results also revealed that there is a considerable amount of variations in the nucleotide pattern of all the PGPR isolates. Similarly, molecular weight of 16S rDNA sequences deposited in NCBI genbank of all the PGPR isolates were calculated and have been presented in Table 51.



ACTGTGGGGCATTCTTTTAAACATGCAAGTCGAACGGCAGCACGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGTCTCG
 TAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGACCTGAGGGAGAAAGCGGGGATCTTCGGACCTCGCGCTATAGGGGCGGCCGATGGCA
 GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAAGCCACTGGGACTGAGACACGGCCAGACTCCT
 ACGGGAGGAGCAGTGGGGAATTTGGCAATGGGGGCAACCTGATCCAGCAATGCCGCGTGTGTGAAGAGGCCTTCGGGTGTAAGACACTTTTGTCCG
 GAAAGAAAACCTTCGTCCCTAATATGGATGGAGGATGACGGTACCAGGAAGAAAGAACCCGGCTAACTACGTGCCAGCAGCCGGTAAATCGTAGGGTGGCA
 CGGTTAATCGGAATTACTGGGCGTAAAGCGTGGCAGCGGTCTGTAAAGCCGATGGAATCCCGGGCTTAACCTGGGAACGTGATGGACTGGCGAG
 GCTTTGAGTGTGGCAGAGGGAGGTAGAATCCACGTGTAGCAGTGAATCGGTAGAGATGTGGAGGAATACCGATGGCGAAGCAGCCCTCTGGGCCAACAC
 TGACGCTCATGCACGAAAGCGTGGGAGCAACAGGATAGATACCCCTGGTGTAGTCCAGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCTTAG
 TAACGTAGCTAACCGCTGAAGTTGACCGCTGGGAGTACGGTCCGCAAGATTAAACCTCAAGGAATTGACGGGACCCGCAAGCCGGTGGATGATGTGGA
 TTAATTCGATGCAACCGCAAAAACCTTACCTACCTTACATGGTCCGAACCTGCTGAAAGGTGGGGTGTCTGAAAGAGAACCGGCCACAGGTGCTGCA
 TGGCTGTCTCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAAGCAGGCGCAACCCCTTGTCTAGTTGCTACGCAAGAGACTCTAAGGAGACTGC
 CGGTGACAAACCGGAGAAAGTGGGGATGACGTCAGTCTCATGGCCCTTATGGGTAGGGTTCCACCGTATACAATGGTCCGGAAGAGGGTTGCCAAG
 CCGCGAGGTGGAGCCATCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTCGTGAAGCTGGAATCGTGTAGTAACTCGGGATCAGCATG
 CCGCGG

Sequence Deposited: NCBI Title : Burkholderia sp. BRHS/P92 16S ribosomal RNA gene, partial sequence

ACCESSION: JQ765578

VERSION: JQ765578.1

GI:386804555

DNA linear : 1444bp

```

1 actgtggggg cattcctttt aacatgcaag tcgaacggca gcaacggggc aaccctgggt
61 gcgagtggcg aacgggtgag taatacatcg gaacgtgtcc tgtagtgggg gatagcocgg
121 cgaagocgg attaataccg catacgacct gagggagaaa gcgggggac ttocggacctc
181 gcgctatagg ggcggccgat ggcagattag ctagtgtggt gggtaaaagg ctaccaaggc
241 gaogtctgt agctggtctg agaggaacgac cagccacact gggactgaga cagcgccag
301 actcctacgg gaggcagcag tggggaattt tggcaaatgg gggcaacct gatccagcaa
361 tgcogogtgt gtgaagaagg ccttcggggt gtaaaagca tttgtccgga aagaaaaactt
421 cgtccctaat atggatggag gatgacggta ccggaagaat aagcaccggc taactacgtg
481 ccagcagcgg cgtgtaaacg tagggtgoga gogttaatcg gaataactgg gogttaaog
541 tgcgacggcg gtcgtgtaag accgatgtga aatccccggg cttaacctgg gaactgatt
601 ggtgactggc agctttaga tgtggcagag ggaggtaga ttccacgtgt agcagtga
661 tgcgtagaga tgtgaggaa tacogtatggc gaagcgagcc tcctggggcca acactgaogc
721 tcatgcaaga aagcgtgggg agcaaacagg attagatacc ctggtagtcc acgcccataa
781 cgatgtcaac tagttgttgg ggattcaatt ccttagtaac gtagtacaog cgtgaagt
841 accgcctggg gactacggtc gcaagattaa aactcaaaag aattgacggg gaccocgaca
901 agcgtggat gatgtggat aattogatgc aacgggaaaa accttaacta ccttgaca
961 ggtcggaacc ctgtgaaag gtgggggtgc tcgaaagaga accggcgac aggtgctga
1021 tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaaacg gcgcaacct
1081 tgtccctagt tgctacgcaa gagcaactota aggagactgc cgttgacaaa ccggaggaag
1141 gtgggatgta cgtcaagtcc tcatggccct tatgggtagg gcttcacaag ctatacaatg
1201 gtcggaacag aggggtgcaa agcccgaggg tggagccaat cccagaaaaa cgtcgtagt
1261 ccggatcgca gtctcaact cgactcgtg aagctggaat ccgtagttaac ccggatcag
1321 catgocggcg tgaatacgtt cccgggtott gtacacacog cgtcaacac catgggatg
1381 gtttcaaca gaagttagta gcctaacccg aaggaggggc cttaccaacg gttggattc
1441 cagg

```

Fig. 72. Chromatogram and sequence deposit of ITS region of *Burkholderia sp.* BRHS/P-92.

Table 49. Nucleotide sequence alignments of the parts of the 16S rDNA repeats encoding ITS region of different isolates of PGPR

```

-----
!Title Phylogenetic Analysis;!Format; DataType=Nucleotide CodeTable=Standard;
NSeqs=15; NSites=1466; Identical=. Missing=? Indel=-; !Domain=Data property=Coding
CodonStart=1

#JQ765578_Burkholderia_symbiont_BRHS/P92 TGG CGA ACG GGT GAG TAA TAC ATC GGA [ 99]
#AB558211_Burkholderia_sp                ... .. [ 99]
#AB558210_Burkholderia_sp                ... .. [ 99]
#AB558209_Burkholderia_symbiont         ... .. [ 99]
#EU665363_Burkholderia_symbiont         ... .. [ 99]
#AB665362_Burkholderia_symbiont         ... .. [ 99]
#AB558209_Burkholderia_sp                ... .. [ 99]
#EU548332_Burkholderia_symbiont         ... .. [ 99]
#AB665361_Burkholderia_symbiont         A. GC. .C ... .G ... .AC .-. ... [ 99]
#KC665360_Burkholderia_symbiont         A. GC. .C ... .G ... .AC .-. ... [ 99]
#JQ765578_Burkholderia_symbiont_BRHS/P92 GGAG GAC GAC CAG CCA CAC TGG GAC TGA [ 297]
#AB558211_Burkholderia_sp                ... .. [ 297]
#AB558210_Burkholderia_sp                ... .. [ 297]
#AB558209_Burkholderia_symbiont         ... .. [ 297]
#EU665363_Burkholderia_symbiont         ... .. [ 297]
#AB665362_Burkholderia_symbiont         ... .. [ 297]
#AB558209_Burkholderia_sp                ... .. [ 297]
#EU548332_Burkholderia_symbiont         ... .. [ 297]
#AB665361_Burkholderia_symbiont         ... .. [ 297]
#KC665360_Burkholderia_symbiont         ... .. [ 297]
#JQ765578_Burkholderia_symbiont_BRHS/P92 GTAA GCA CCG GCT AAC TAC GTG CCA GCA [ 495]
#AB558211_Burkholderia_sp                ... .. [ 495]
#AB558210_Burkholderia_sp                . .CA TCG ... ..T G.T G. ... [ 495]
#AB558209_Burkholderia_symbiont         ... .. [ 495]
#EU665363_Burkholderia_symbiont         ... .. CAT [ 495]
#AB665362_Burkholderia_symbiont         ... .. [ 495]
#AB558209_Burkholderia_sp                ... .. [ 495]
#EU548332_Burkholderia_symbiont         ... .. [ 495]
#AB665361_Burkholderia_symbiont         ... .. [ 495]
#KC665360_Burkholderia_symbiont         ... .. [ 495]
#JQ765578_Burkholderia_symbiont_BRHS/P92 G GTT AAG TCC CGC AAC GAG CGC AAC CCT 1089]
#AB558211_Burkholderia_sp                . ... 1089]
#AB558210_Burkholderia_sp                . ... 1089]
#AB558209_Burkholderia_symbiont         . ... 1089]
#EU665363_Burkholderia_symbiont         . ... 1089]
#AB665362_Burkholderia_symbiont         . ... 1089]
#AB558209_Burkholderia_sp                . ... 1089]
#EU548332_Burkholderia_symbiont         . ... 1089]
#AB665361_Burkholderia_symbiont         . ... 1089]
#KC665360_Burkholderia_symbiont         . ... 1089]
#JQ765578_Burkholderia_symbiont_BRHS/P92 ACG GGT TGG ATT TCC AGG --- --- --- 1466]
#AB558211_Burkholderia_sp                ... -. G.. ... CAT GAC TGG GGT G-- -- 1466]
#AB558210_Burkholderia_sp                ... -. G.. ... CAT GAC TGG GGT G-- -- 1466]
#AB558209_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT G-- -- 1466]
#EU665363_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT GAA GT 1466]
#AB665362_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT GAA GT 1466]
#AB558209_Burkholderia_sp                ... -. G.. ... CAT GAC TGG GGT G-- -- 1466]
#EU548332_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT GAA GT 1466]
#AB665361_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT GAA GT 1466]
#KC665360_Burkholderia_symbiont         ... -. G.. ... CAT GAC TGG GGT GAA GT 1466]
End;//

-----
NSeqs=5 NSites=1514 Identical=. Missing=? Indel=-; !Domain=Data property=Coding
CodonStart=1

```



Fig. 73. Multiple sequence alignment of *Burkholderia symbiont* with other ex-type strain sequences obtained from Genbank database. Different colours shows different bases. Difference in the conserved regions are indicated by different colours.

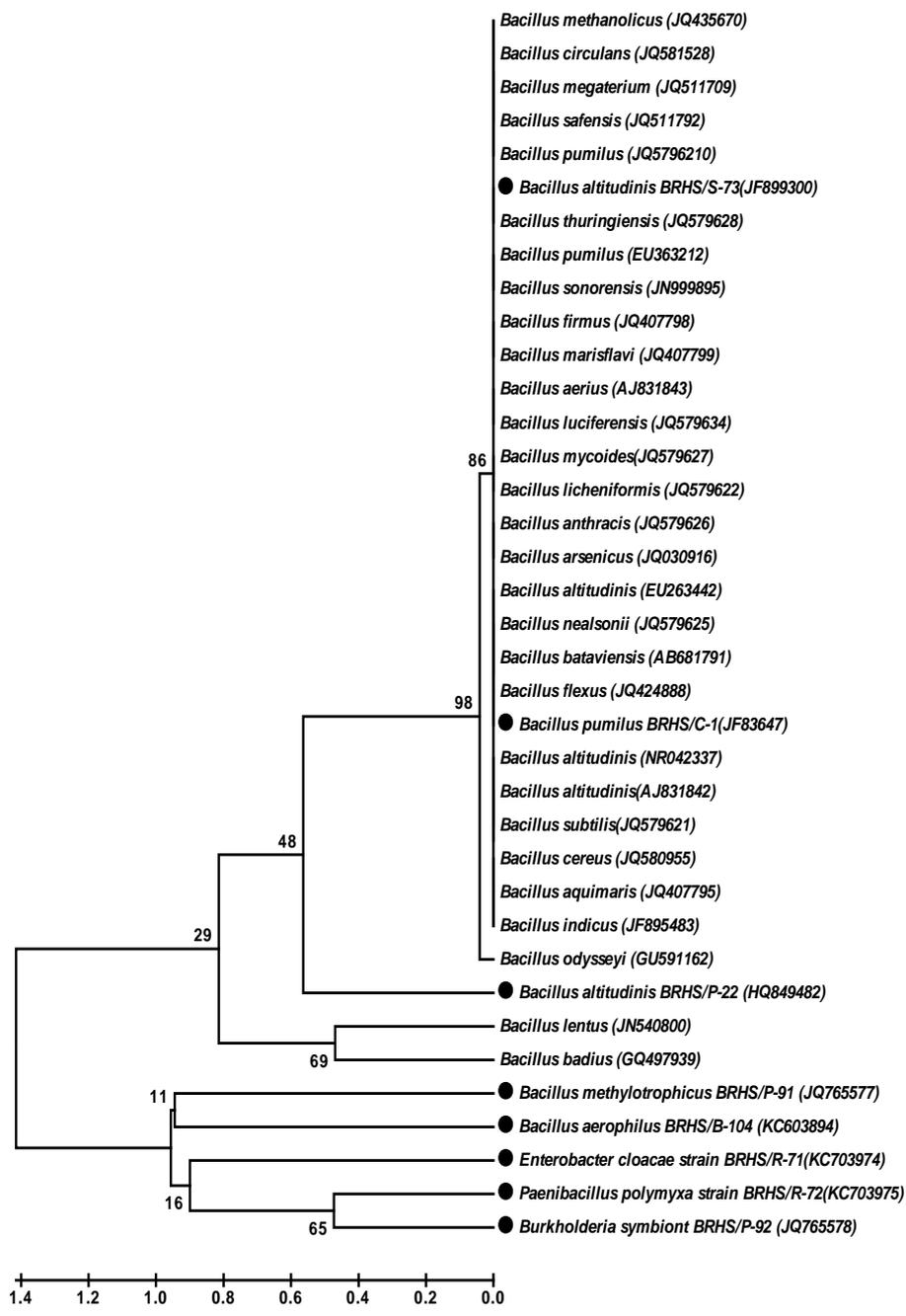


Fig. 74. Neighbour Joining analysis based Phylogenetic placement of PGPR isolates on the basis of 16S rDNA gene sequences. Sum of branch length = 7.99112791 is shown There were a total of 99 positions in the final dataset (Linear form).

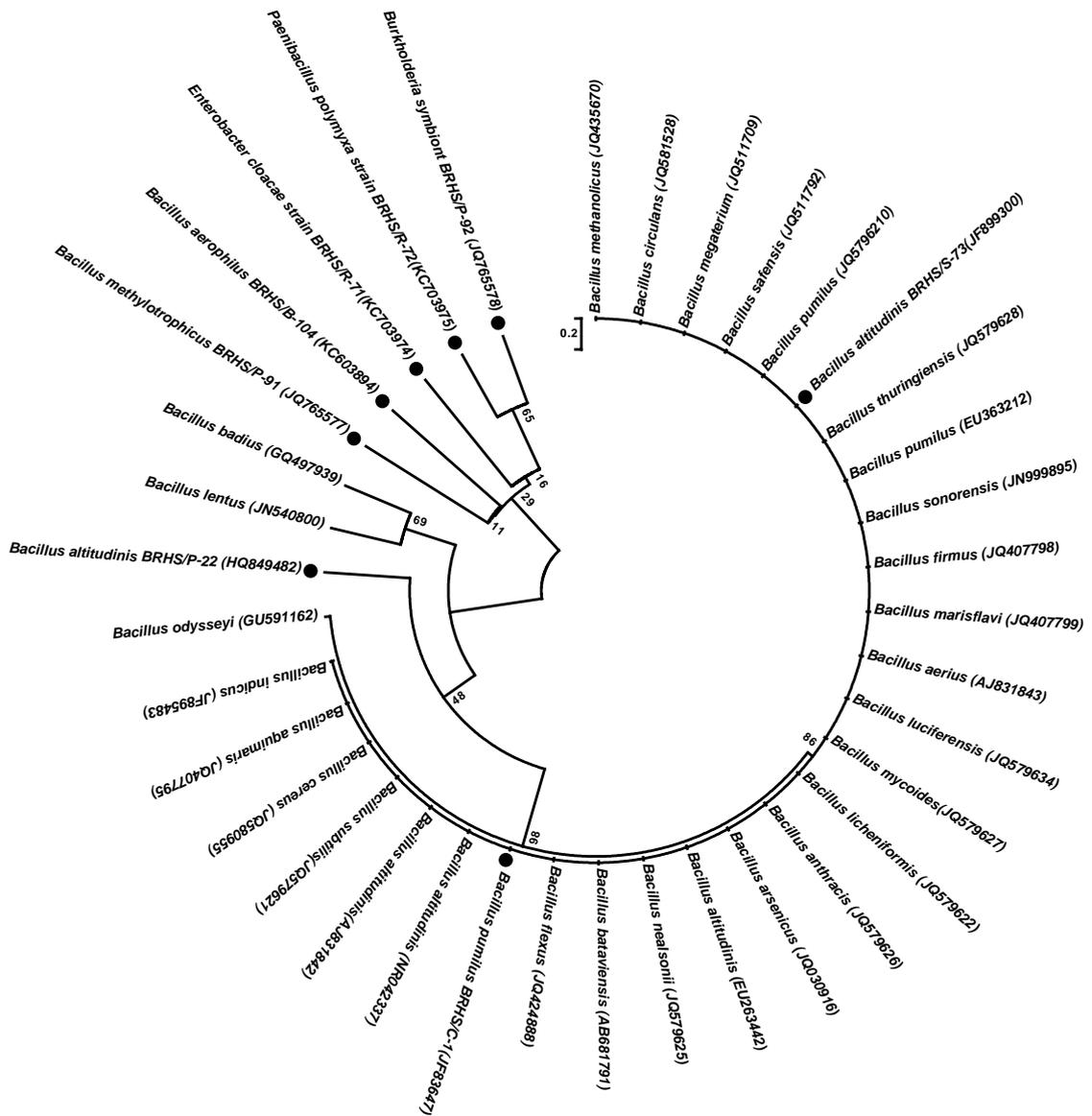


Fig. 75. UPGMA analysis based Phylogenetic placement of PGPR isolates on the basis of 16S rDNA gene sequences. Sum of branch length = 6.2258873 is shown There were a total of 99 positions in the final dataset (radial form).

Table 50. Nucleotide combinations and frequencies of different combinations of 16S rDNA sequences of PGPR isolates belonging to Bacilli group

Pattern	<i>Bacillus pumilus</i> BRHS/C-1		<i>Bacillus altitudinis</i> BRHS/P-22		<i>Bacillus altitudinis</i> BRHS/S-73		<i>Bacillus methylotrophicus</i> BRHS/P-91		<i>Bacillus aerophylus</i> BRHS/B-104	
	Times found	Percentage	Times found	Percentage	Times found	Percentage	Times found	Percentage	Times found	Percentage
G	424	31.15	419	30.99	414	31.34	463	31.89	395	31.45
A	333	24.47	341	25.22	330	24.98	361	24.86	317	25.24
T	278	20.43	273	20.19	270	20.44	283	19.49	254	20.22
C	326	23.95	319	23.59	307	23.24	345	23.76	290	23.09
GG	128	9.41	135	9.99	125	9.47	149	10.27	119	9.48
GA	111	8.16	103	7.62	103	7.80	113	7.79	103	8.21
GT	91	6.69	88	6.51	93	7.05	98	6.75	86	6.85
GC	94	6.91	93	6.88	93	7.05	103	7.10	86	6.85
AG	99	7.28	97	7.18	103	7.80	110	7.58	99	7.89
AA	95	6.99	107	7.92	92	6.97	103	7.10	91	7.25
AT	52	3.82	50	3.70	50	3.79	54	3.72	44	3.51
AC	87	6.40	86	6.37	85	6.44	93	6.41	83	6.61
TG	99	7.28	96	7.11	98	7.42	101	6.96	93	7.41
TA	58	4.26	56	4.15	57	4.32	62	4.27	52	4.14
TT	61	4.49	62	4.59	56	4.24	57	3.93	54	4.30
TC	60	4.41	59	4.37	58	4.39	63	4.34	55	4.38
CG	97	7.13	91	6.74	88	6.67	102	7.03	83	6.61
CA	69	5.07	75	5.55	77	5.83	83	5.72	71	5.66
CT	74	5.44	73	5.40	71	5.38	74	5.10	70	5.58
CC	85	6.25	80	5.92	71	5.38	86	5.93	66	5.26
G,C	750	55.11	738	54.59	721	54.58	808	55.65	685	54.54
A,T	611	44.89	614	45.41	600	45.42	644	44.35	571	45.46

Table 51. Nucleotide combinations and frequencies of different combinations of 16S rDNA sequences of PGPR isolates (Non-Bacilli)

Pattern	<i>Enterobacter cloacae</i> BRHS/R-71		<i>Paenibacillus polymyxa</i> BRHS/R-72		<i>Burkholderia symbionts</i> BRHS/P-92	
	Times found	Percentage	Times found	Percentage	Times found	Percentage
G	391	31.03	408	31.51	467	32.34
A	327	25.95	327	25.25	359	24.86
T	264	20.95	261	20.15	282	19.53
C	278	22.06	299	23.09	336	23.27
GG	117	9.29	123	9.51	158	10.95
GA	101	8.02	108	8.35	110	7.62
GT	84	6.67	87	6.72	95	6.58
GC	88	6.99	90	6.96	103	7.14
AG	97	7.70	104	8.04	102	7.07
AA	99	7.86	93	7.19	103	7.14
AT	53	4.21	45	3.48	64	4.44
AC	78	6.20	84	6.49	90	6.24
TG	94	7.47	95	7.34	105	7.28
TA	60	4.77	54	4.17	65	4.50
TT	59	4.69	55	4.25	55	3.81
TC	51	4.05	57	4.40	57	3.95
CG	82	6.51	86	6.65	102	7.07
CA	67	5.32	72	5.56	80	5.54
CT	68	5.40	73	5.64	68	4.71
CC	61	4.85	68	5.26	86	5.96
G,C	669	53.10	707	54.59	803	55.61
A,T	591	46.90	588	45.41	641	44.39

Table 52. DNA molecular weight of different *Trichoderma* isolates calculated on the basis of 18S rDNA sequences

Isolate	Strain	Gen Bank Acc.No	Starting sequence	DNA mol weight (Da)
<i>B. pumilus</i>	BRHS/C-1	JF836847	GAAAAGGGGG	422741.26
<i>B. altitudinis</i>	BRHS/P-22	HQ849482	CTCCCGGGAA	420055.63
<i>E. cloacae</i>	BRHS/R-71	KC703974	GCAGTCGAGC	391858.63
<i>P. polymyxa</i>	BRHS/R-72	KC703775	TCGAGCGGAC	402615.38
<i>B. altitudinis</i>	BRHS/S-73	JF899300	AGGGTATGGT	410581.51
<i>B. methylotrophicus</i>	BRHS/P-91	JQ765577	GGGGGGGCGG	451365.75
<i>Burkholderia sp.</i>	BRHS/P-92	JQ765578	ACTGTGGGGG	449149.35
<i>B. aerophylus</i>	BRHS/B-104	KC603894	GTTAGCGGCG	390471.53

4.10.4. Analysis of Open Reading Frame (ORF) of 16S rDNA gene sequences of PGPR isolates

Open Reading Frames (ORF) for the designated 16S rDNA sequences of all the PGPR isolates were calculated with the help of ORF finder available from http://www.ualberta.ca/~stothard/javascript/orf_find.html. The detail description of individual isolates has been given as under:

1. NCBI Acc. No. JF836847; *Bacillus pumilus* -BRHS/C1

Results for 1361 residue sequence "JF836847" starting "GAAAAGGGGG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 295 to base 399.

GGAATCTTCCGCAATGGAGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTTCGGATCGTAAGCTCTGTTGTTAGGGAAGAACAAGTGCGA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

GIFRNGESLTEQRRVSDEGFRIVSSVVREEQVRE*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 532 to base 630.

TGTGAAAGCCCCGGCTCTACCGGGGAGGGTCATTGGAAACGGGAAACT
TGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAATGCGTA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

CESPRLYRGGSLLETGNLSAEEESGIPRVAVNA*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 883 to base 1038.

TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCC
TAGGATAGGGCTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTG
TCGTCGCTCGTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAAC

>Translation of ORF number 3 in reading frame 1 on the direct strand.

SKQREEPYQVLTSSDNPRIGLSLRGQSDRWCMVVVARVVRCWVKSERNP
*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 1258 to base 1359.

AATCGCGAACAGCATGCCGCGGTGATTACGTTCCGGGCCTGTCCCACGCC
GTACACCCGAAAGTTTGTACCCCGAGTCGGTAGTGACCTTTTGACTCCCC

>Translation of ORF number 4 in reading frame 1 on the direct strand.

NREQHAAVITFRACPTYTRKFPVPRVGSDDLTPP

2. NCBI Acc. No. HQ849482.1; *Bacillus altitudinis*-BRHS/P-22

Results for 1352 residue sequence "HQ849482 " starting "CTCCCGGGAA"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 250 to base 369.

GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCG
GATCGTAAAGCTCTGTTGTTAG

>Translation of ORF number 1 in reading frame 1 on the direct strand.

DTAQTPTGGSSRESSAMDESLTEQRRVSDEGFRIVKLCC*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 370 to base

525.GGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAA
CCAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GGGGCAAGCGTTGTCCGGAATTATTGGGGCGGTAAAGGGGCTCGCAGGC
GGTTTTCTTAA

>Translation of ORF number 2 in reading frame 1 on the direct

strand.GRTSARVTACTLTVPNQKATANYVPAAAVIRRGASVVRNYWGGKGA
RRFS*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 532 to base

768.TGTGAAAGCCCCCGGCTTCAACCGGGGGAGGGGCCATTGGAAA
GGGAAACCTTGAGTGCAAAAGAAGGAGAGTGGAATTTCCACGTGTACGG
GTGAAATTGCGTAAAGATGTGGAAGGAACACCAGTGGCGAAGGCGACTC
TCTGGTCTGTA
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA

>Translation of ORF number 3 in reading frame 1 on the direct

strand.CESPPASTGGGAIGKLG NLECKRRRVEFPRVRV KLRKDVEGTPVAKAT
LWSVTDAEERKRGERTGLDTLVVHAVNDEC*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 814 to base 984.

GCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGG

>Translation of ORF number 4 in reading frame 1 on the direct

strand.ALRGSTVARLKLK GIDGGPHKRWSMWFNSKQREEPYQVLTSSDNPR
DRAFPSGTE*

3. NCBI Acc. No. JF899300; *Bacillus altitudinis*-BRHS/S73

Results for 1321 residue sequence " JF899300" starting "AGGGTATGGT"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 520 to base 666.

TGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

CESPRLNRGGSLETGKLECRERGEWNSTCSGEMRRDVEEHQWRRRLSGL

>**ORF number 2** in reading frame 1 on the direct strand extends from base 913 to base 1011.

CATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.

HPLTTLEIGLSLRGQSDRWCMVVVSSCREMLG*

>ORF number 3 in reading frame 1 on the direct strand extends from base 1075 to base 1248.

CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGG
CTGCGAGACCGCAAGGTTTAGCCAATCCACAAATCTGTTCTCAGTTCGG

>Translation of ORF number 3 in reading frame 1 on the direct

strand.LPVTNRRKVGMTSNHHAPYDLGYTRATMDRTKGCETARFSQSHKSV
LSSDRSLHSTA*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 1256 to base 1314.

CCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGGTGA

>Translation of ORF number 6 in reading frame 1 on the direct strand.

PIPQICSQFGSQSATRLREAGIASNRGSACRG*

4. NCBI Genbank Acc. No. JQ765577; *Bacillus methylotrophicus*-BRHS/P-91

Results for 1452 residue sequence " JQ765577" starting "GGGGGGGCGG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 115 to base 222.

CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTC
AGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGC

>Translation of ORF number 1 in reading frame 1 on the direct strand.

LRETGANTGWLSEPHGSDIKGGFGYHLQMDPRRIS*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 235 to base 360.

CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGG

>Translation of ORF number 2 in reading frame 1 on the direct strand.

RLTKATMRSRPERVIGHTGTETRPRLREAAVGNLPQWTKV*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 442 to base 579.

GGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGG
GCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGA

>Translation of ORF number 3 in reading frame 1 on the direct strand.

GGTLTPVNQKATANYVPAAAVIRRWQALSGIIGRKGLAGGFSLM*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 796 to base 897.

GTGCTAAGTGTTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATT
AAGCACTCCGCTGGGGAGTACGGTCGCAAGACTGAACTCAAAGGAAT

>Translation of ORF number 4 in reading frame 1 on the direct strand.

VLSVRGFPPLSAAANALSTPPGEYGRKTETQRN*

>**ORF number 5** in reading frame 1 on the direct strand extends from base 994 to base 1092.

GACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGA

>Translation of ORF number 5 in reading frame 1 on the direct strand.

DVPFGGRVTGGAWLSSARVVRWCWVKSRNERNP*

5. NCBI Genbank Acc No; **KC603894**; *Bacillus aerophilus*-BRHS/B-104

Results for 1256 residue sequence " KC603894" starting "GTTAGCGGCG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 487 to base 666.

AGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAAC
CGGGGAGGGTCATTGGAACTGGGAACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA

>Translation of ORF number 1 in reading frame 1 on the direct strand.

RARRRFLKSDVKAPGSTGEGHWKLGNSAEESGIPRVAVKCVEMWRNTSG
EGDSLIV*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 736 to base 837.

GTGCTAAGTGTTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATT
AAGCACTCCGCTGGGGAGTACGGTCGCAAGACTGAACTCAAAGGAAT

>Translation of ORF number 2 in reading frame 1 on the direct strand.
VLSVRGFPPLSAAANALSTPPGEYGRKTETQRN*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 934 to base 1032.
GGCTTTCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGA

>Translation of ORF number 3 in reading frame 1 on the direct strand.
GFPFGDRVTTGGAWLSSARVVRCWVKSRNERNP*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 1105 to base 1245.
CGTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATG
GACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCCAATCCCACAAAT

>Translation of ORF number 4 in reading frame 1 on the direct strand.
RQIIMPLMTWATHVLQWTEQRAARPQGLANPTNLFSVRIAVCNSTA*

6. NCBI Acc. No. JQ765578; *Burkholderia* sp.-BRHS/P-92

Results for 1444 residue sequence " JQ765578" starting "ACTGTGGGGG"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 1 to base 105.
ACTGTGGGGGCATTCCTTTTAACATGCAAGTCGAACGGCAGCACGGGGG
CAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGT

>Translation of ORF number 1 in reading frame 1 on the direct strand.
TVGAFLLTCKSNGSTGATLVASGERVSNTSERVL*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 289 to base 429.
GACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAA
TGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTTGTCCGAAAGAAAACCTTCGTCCCTAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.
DTAQTPTGGSSGEFWTMGATLIQQRVCEEGLRVVKHFPERKLRP*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 463 to base 570.
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAG
CGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTCTGTAAAGA

>Translation of ORF number 3 in reading frame 1 on the direct strand.
APANYVPAAAVIRRVRALIGITGRKACAGLLRPM*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 586 to base 717.
CCTGGGAACTGCATTGGTGAAGTGGCAGGCTTTGAGTGTGGCAGAGGGAG
GTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATAC
CGATGGCGAAGGCAGCCTCCTGGGCCAACACTGA

>Translation of ORF number 4 in reading frame 1 on the direct strand.
PGNCIGDWQALSVAEGGRIPRVAVKCVEMWRNTDGEGLLQGH*

>**ORF number 5** in reading frame 1 on the direct strand extends from base 718 to base 816.
CGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTTCATTCCTTAG

>Translation of ORF number 5 in reading frame 1 on the direct strand.
RSCTKAWGANRIRYPGSPRPRKRCQLVVGDSFP*

7. NCBI Gen Bank Acc. No: KC703974; *Enterobacter cloacae* -BRHS/R-71

Results for 1260 residue sequence " KC703974" starting "GCAGTCGAGC"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 214 to base 339.
CGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGA

>Translation of ORF number 1 in reading frame 1 on the direct strand.
RLTKATMRSRPERVIGHTGTETPRLLREAAVGNLQPWTKV*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 421 to base 558.
GCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA

>Translation of ORF number 2 in reading frame 1 on the direct strand.
AGTLTVPNQKATANYVPAAAVIRRWQALSGIIGRKARAGGFSLM*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 655 to base 780.
AGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAGTG
ACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAA

>Translation of ORF number 3 in reading frame 1 on the direct strand.
RYGGTPVAKATFWSVTDTEARKRGEQTGLDTLVVHAVNDEC*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 826 to base 996.
GCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCCTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGG
CTTCTCCTTCGGGAGCAGAGTGA

>Translation of ORF number 4 in reading frame 1 on the direct strand.
ALRLGSTAARLKLKGIDGGPHKRWSMWFNSKQREEPYQVLTSSSENPRDRAS
PSGE*

8. NCBI Gen Bank Acc. No. KC703975; *Paenibacillus polymyxa*- BRHS/R-72

Results for 1295 residue sequence " KC703975 " starting "TCGAGCGGAC"

>**ORF number 1** in reading frame 1 on the direct strand extends from base 271 to base 390.

GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCG
GATCGTAAAGCTCTGTTGTTAG

>Translation of ORF number 1 in reading frame 1 on the direct strand.
DTAQTPTGGSSRESSAMDESLTEQRRVSDEGFRIVKLCC*

>**ORF number 2** in reading frame 1 on the direct strand extends from base 391 to base 552.

GGAAGAACAAGTGCAAGAGTAACTGCTTGACCTTGACGGTACCTAACC
AGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTT

>Translation of ORF number 2 in reading frame 1 on the direct strand.
GRTSARVTACTLVVPNQKATANYVPAAAVIRRWQALSGIIGRKGLAGGFLSL
M*

>**ORF number 3** in reading frame 1 on the direct strand extends from base 649 to base 774.

AGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGT
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAA

>Translation of ORF number 3 in reading frame 1 on the direct strand.
RCGGTPVAKATLWSVTDAEERKRGERTGLDTLVVHAVNDEC*

>**ORF number 4** in reading frame 1 on the direct strand extends from base 820 to base 990.

GCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGG

>Translation of ORF number 4 in reading frame 1 on the direct strand.
ALRLGSTVARLKLKIDGGPHKRWSMWFNSKQREEPYQVLTSSDNPRDRAF
PSGTE*

>**ORF number 5** in reading frame 1 on the direct strand extends from base 1027 to base 1161.

GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCA
GCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCTTATGA

>Translation of ORF number 5 in reading frame 1 on the direct strand.

DVGLSPATSATLDLSCQHSVGHKVTAGDKPEEGGDDVKSSCPL*

4.11. Immunological characterization of *T. flavus* (PSF) *T. harzianum* (BCA) and *T. cucumeris* (Pathogen)

Immunological formats of one potential PFS isolate *Talaromyces flavus* RHS/P-51, BCA isolate *Trichoderma harzianum* RHS/S-559 and the pathogen *Thanatephorus cucumeris* RHS/-566 was standardized for easy detection.

4.11.1. Soluble protein

Initially Total soluble protein of *Talaromyces flaus* RHS/P-51, *Trichoderma harzianum* (RHS/S-559, RHS/S-560, RHS/T-460) *T. asperellum* (RHS/S-560) *T. erinaceum* (FS/L-20, FS/S-474, FS/S-475) and *Thanatephorus cucumeris* RHS/-566 was quantified and then analysed by SDS-PAGE. The total soluble protein content of *T. flavus* was 236.45 µg/g fresh weight. Similarly protein content of all the three isolates of *T. harzianum* was 246.52, 212.33 and 273.44 µg/g respectively and the protein content of the *T. asperellum* isolates was 246.42 and 233.58 whereas the protein content of *T. erinaceum* isolates was 217.44 and 216.45 the total protein content of the pathogen *T. cucumeris* was 274.18 µg/g fresh weight. Total soluble protein were further analysed by SDS-PAGE (Fig. 76). Mycelial protein of *T. flavus* exhibited an average of 18 bands with the molecular weight ranging from 14.36 to 188.73 kda. Similarly isolates of *T. harzianum* exhibited an aerge of 13 bands in SDS-PAGE and ranged from 12.23 to 103.37 kda. Isolates of *T. asperellum* exhibited an average of 12 bands reaging from 18.17.to 126.40 kda, isolates of *T. ericenum* exhibited an average of 14 bands which ranged from 12.14 to 117.63. Whereas in case of the pathogen *T. cucumeris* a total of 14 bands were found to resolve in the SDS-PAGE gel which ranged from 14.44 to 103.28 kda (Table 53).

4.11.2. Serological Assays of *Talaformyces flavus* RHS/P-51

Polyclonal antibodies (PABs) were raised in rabbit against mycelial proteins as described previously and these were used in various immunological formats. The effectiveness of the purified antigen of *T. flavus* (RHS/P-51) in raising PABs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction was done with PAB raised against mycelial protein yielded sharp bands which was stained blue. (Fig. 77 A). Total soluble proteins of *T. flavus* was extracted and was used as homologous antigen source, similarly for heterologous bonding fresh antigen from *Aspergillus niger*, *A. melleus*, *A. clavatus* and *T.harzianum*. Dot immunobinding assayes performed using all these antigen

preparation with PAb of *T. flavus*. Antigens were carefully spotted on nitrocellulose paper and probed with Ab of *T. flavus*. Results have been presented in Fig. 77 C and Table 54. Western blot analyses using polyclonal antibody of *T. flavus* RHS/P-51 revealed that all the PABs collected at four different intervals and coded as 1st, 2nd, 3rd and 4th bleed could show different levels of homologous reactions with the antigen of *T. flavus*. In case of first bleed a total of 8 bands were observed ranging from 42 to 100 kDa. In case of second bleed there were a total of 11 bands ranging from 43 to 100 kDa whereas there were a total of 10 bands in the third bleed ranging from 48 to 100 kDa. Lastly, when fourth bleed was used then a total of thirteen bands were observed ranging from 14 to 100 kDa (Fig. 77 D, Table 55)

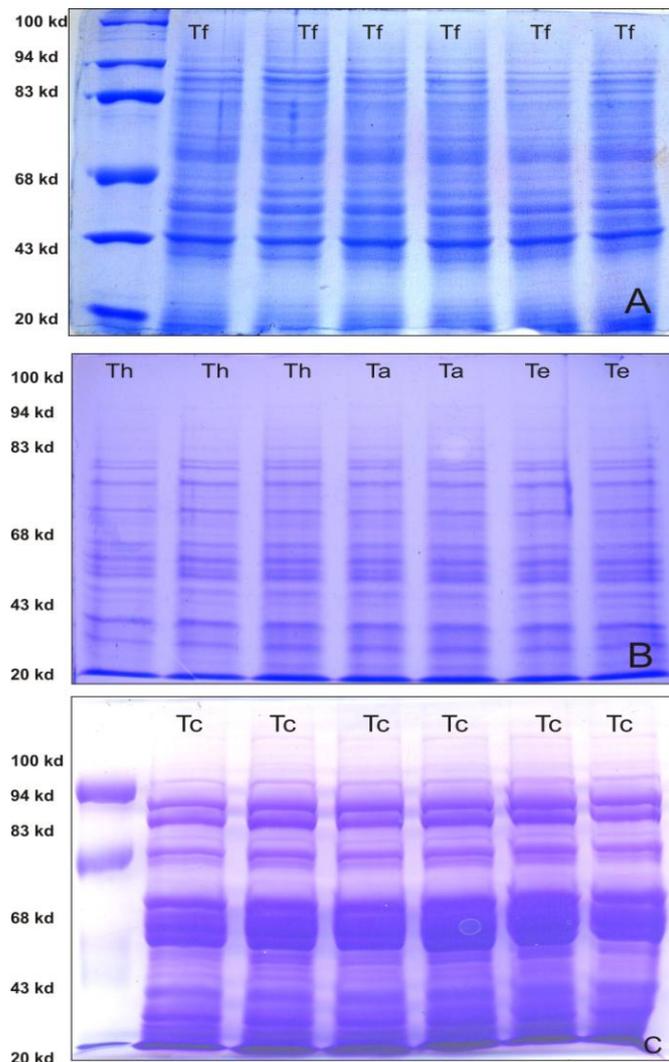


Fig. 76. SDS-PAGE profiles of fungal. *Talaromyces flaus* RHS/P-51 (A); *Trichoderma harzianum* (RHS/S-559, RHS/S-560, RHS/T-460) *T. asperellum* (RHS/S-560, RHS/M-512) *T. erinaceum* (FS/L-20 & FS/S-474) (B); *Thanatephorus cucumeris* RHS/-566 (C).

Table 53. SDS-PAGE analysis of total soluble proteins of *T. flavus*, *T. harzianum*, *T. asperellum*, *T. erinaceum* and *T. cucumeris*

Isolates	Code	Protein content (µg/gm)	Molecular weight (kDa)
<i>Talaromyces flavus</i>	RHS/P-51	236.45	14.36, 22.43, 27.88, 33.16, 44.15, 45.51, 53.77, 58.82, 62.47, 68.24, 75.15, 83.13, 97.32, 105.27, 140.22, 145.76, 174.25, 188.73 (18)
	RHS/S-559	246.52	12.23, 16.96, 17.18, 18.33, 19.35, 25.22, 28.11, 41.45, 59.34, 73.28, 86.15, 92.12, 103.20 (13)
<i>Trichoderma harzianum</i>	RHS/S-560	212.33	12.73, 16.24, 17.42, 18.24, 19.15, 25.44, 28.18, 41.43, 59.37, 73.43, 86.17, 92.25, 103.17 (13)
	RHS/T-460	273.44	12.33, 16.16, 17.22, 18.18, 19.42, 25.32, 28.73, 41.12, 59.22, 73.47, 86.21, 92.37, 103.37 (13)
	RHS/S- 561	246.42	18.17, 19.77, 25.26, 28.20, 41.44, 59.38, 73.18, 86.55, 92.42, 103.18, 116.43,126.44 (12)
<i>Trichoderma asperellum</i>	RHS/M-512	233.58	18.33, 19.43, 25.16, 28.10, 41.23, 59.71, 73.23, 86.44, 92.16, 103.15, 116.16,126.40 (12)
	FS/L-20	217.44	12.44, 16.30, 17.15, 18.21, 19.12, 25.40, 28.34, 41.42, 59.62, 73.87, 86.15, 92.13, 103.44,117,82 (14)
<i>Trichoderma erinaceum</i>	FS/S-474	216.45	12.14, 13.26, 14.55, 18.18, 19.34, 25.48, 28.27, 41.26, 59.25, 73.17, 86.16, 92.36, 103.26,117.63 (14)
<i>Thanatephorus cucumeris</i>	RHS/V-566	274.18	14.44, 16.30, 17.15, 18.21, 19.12, 41.42, 44.43, 46.73, 59.62, 62.11, 73.87, 86.15, 92.13, 103.28 (14)

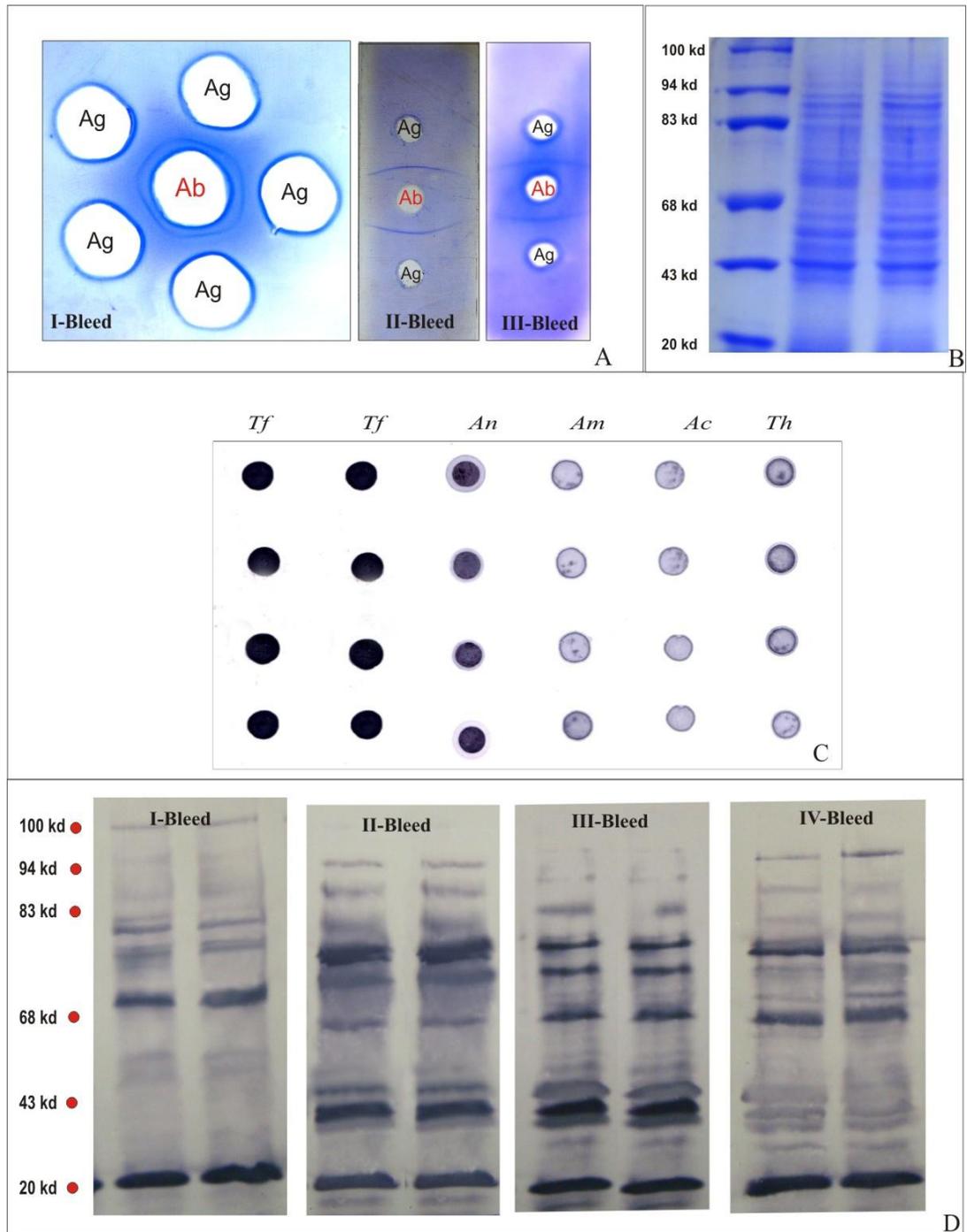


Figure 77. Serological assays of *T. flavus*. Agar gel double diffusion tests of homologous antigen of *T. flavus* and polyclonal antibody raised against *T. flavus* (A). SDS PAGE electrophoresis of *T. flavus* antigen. Dot immuno binding assay of homologous antigen of *T. flavus* and other heterologous antigens and probed with antibody raised against *T. flavus* (C). Western Blot analysis of *T. flavus* antigens probed with homologous antibody (D).

Table 54. Scoring of dots obtained with DIBA of *T. flavus* and other heterologous antigens

Isolates	Colour intensity ^a Using mycelial PAb (4 th bleed)
<i>Talaromyces flavus</i> (RHS/P-51)	++++
<i>Aspergillus niger</i>	++
<i>Aspergillus melleus</i>	+
<i>Aspergillus clavatus</i>	±
<i>Trichoderma harzianum</i>	±

^a Fast red colour intensity : + + + + = Deep purple; + + light purple; ± Faint purple, Antigen concentration 100 µg/ml, antibody concentration 40µg/ ml.

Table 55. Analysis of molecular weights of bands obtained in western blot analysis of *T. flavus* antigen and probed with its homologous antibody

1 st bleed Molecular weight (kDa)	2 nd bleed Molecular weight (kDa)	3 rd bleed Molecular weight (kDa)	4 th bleed Molecular weight (kDa)
42, 74, 78,76, 80, 83, 94, 100 (8)	43, 50, 54, 60, 75, 76, 83, 90, 94, 98, 100 (11)	48,60,65, 76,80, 83, 90, 94, 98, 100 (10)	43, 50, 54, 60, 65, 76, 80, 83, 90, 94, 98, 100 (13)

4.11.3. Serological assay of *Trichoderma harzianum* RHS/S-559

Total soluble proteins of the BCA isolates, *T. harzianum* (RHS/S-559 and RHS/S-560) were extracted and was used as homologous antigen source. Initially The effectiveness of the purified antigen of *T. harzianum* (RHS/S-559) in raising PABs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction was done with PAb raised against mycelial protein yielded sharp bands which was stained blue (Fig. 81 E). DOT immunobinding assay

was performed using fresh antigen from *T. harzianum*, *T. asperellum* and *T. erinaceum*. Dot immunobinding assays performed using all these antigen preparation with PAb of *T. harzianum*. Antigens were carefully spotted on nitrocellulose paper and probed with Ab of *T. harzianum*. Results have been presented in Fig. 81, A and Table 56. Clear and intense color reactions were observed in case of homologous antigen (Th). Antigens of *T. asperellum* and *T. erinaceum* showed lesser color intensities.

Table 56. Scoring of dots obtained with DIBA of *T. harzianum* and other heterologous antigens

Isolates	Code	Colour intensity ^a Using mycelial PAb (4 th bleed)
	RHS/S-559	++++
<i>T. harzianum</i>	RHS/S-560	+++
	RHS/T-460	+++
	RHS/S-561	+
<i>T. asperellum</i>	RHS/M-512	+
	RHS/M-517	+
	FS/L-20	±
<i>T. erinaceum</i>	FS/S-474	±
	FS/S-475	±

^a Fast red colour intensity : + + + + = Deep purple; += light purple; ± Faint purple, Antigen concentration 100 µg/ml, antibody concentration 40µg/ ml.

Western blot analyses using polyclonal antibody of *T. harzianum* RHS/S-559 revealed that all the PAbS collected at three different intervals and coded as 1st, 2nd and 3rd bleed could show different levels of homologous reactions with the antigen of *T. harzianum*. In case of first bleed a total of 5 bands were observed ranging from 65 to 100 kDa. In case of second bleed there were a total of 4 bands ranging from 44 to 93 kDa whereas there were a total of 9 bands in the third bleed ranging from 40 to 93 kDa (Fig. 78 C ;Table 57). In an another set of experiment, fresh antigens of *T. harzianum* (RHS/S-559, RHS/S-560 and RHS/T-460) *T. asperellum* (RHS/S-561

and RHS/M-512) and *T. erinaceum* (FS/L-20 and FS/S-474) were probed with PABs of *T. harzianum* (3rd bleed) to verify the specificity of the antibody. The results presented in Table 58 and Fig. 78, D shows that In case of *T. harzianum* an average of 9 bands ranging from 40- 93 kDa were observed, whereas in case of *T. asperellum* antigens and average of 8 bands ranging from 40- 83 kDa was obtained and in case of *T. erinaceum* an average of 7 bands ranging from 40-83 kDa were obtained.

Table 57. Analysis of molecular weights of bands obtained in western blot analysis of *T. harzianum* antigen and probed with its homologous antibody

1 st bleed	2 nd bleed	3 rd bleed
Molecular weight (kDa)	Molecular weight (kDa)	Molecular weight (kDa)
65, 70, 83, 97, 100 (5)	44, 70, 83, 93 (4)	40, 44, 58, 62, 63, 65, 70, 83, 93 (9)

Table 58. Analysis of molecular weights of bands obtained in western blot analysis of *T. harzianum*, *T. asperellum* and *T. erinaceum* antigens and probed with PAB of *T. harzianum* (3rd bleed)

Isolate	Code	Molecular weight (kDa)
	RHS/S-559	40, 44, 58, 62, 63, 65, 70, 83, 93 (9)
<i>T. harzianum</i>	RHS/S-560	40, 44, 58, 62, 63, 65, 70, 83, 93 (9)
	RHS/T-460	40, 44, 58, 62, 63, 65, 70, 83, 93 (9)
<i>T. asperellum</i>	RHS/S-561	40, 44, 58, 62, 63, 65, 70, 83 (8)
	RHS/M-512	40, 44, 58, 62, 63, 65, 70, 83 (8)
<i>T. erinaceum</i>	FS/L-20	40, 44, 58, 62, 65, 70, 83 (7)
	FS/S-474	40, 44, 58, 62, 65, 70, 83 (7)

4.11.4. Serological assays of *Thanatephorus cucumeris* RHS/V-566

Initially The effectiveness of the purified antigen of *T. cucumeris* in raising PABs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction was done with PAB raised against mycelial protein yielded sharp bands which was stained blue (Fig. 79 A).

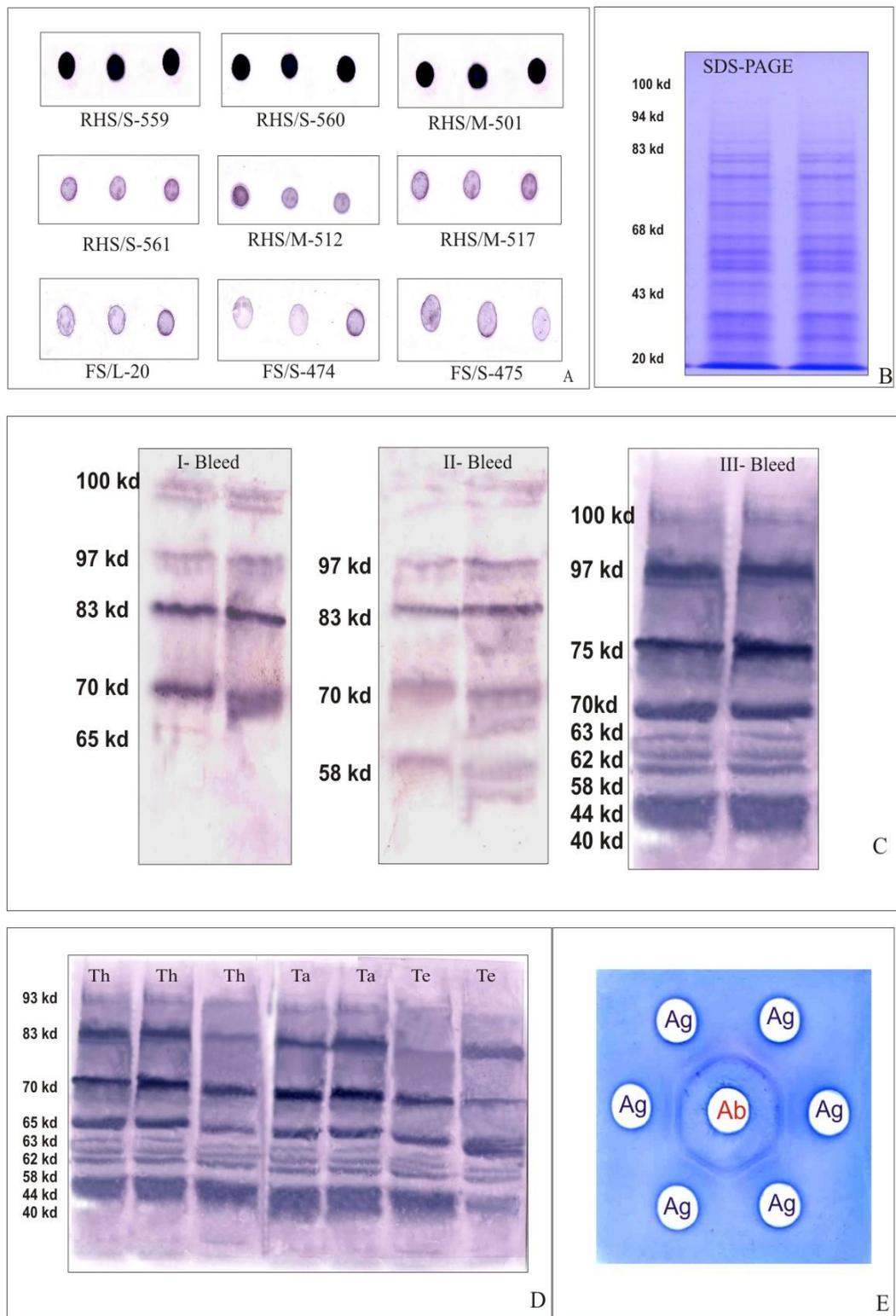


Fig. 78. Serological assays of *Trichoderma harzianum* RHS/S-559. Dot immunobinding assay (A); Western blot analysis with homologous antigen and antibodies of *T. harzianum* (C); Western blot analysis of *T. harzianum*, *T. asperellum* and *T. erinaceum* isolates probed with PAbs of *T. harzianum* (D), Double immune diffusion test of *T. harzianum*.

Total soluble proteins of *T. cucumeris* was extracted and was used as homologous antigen source, similarly for heterologous binding fresh antigen from *Rhizoctonia solani*, *Sclerotium rolfii* and *Talaromyces flavus* were used. Dot immunobinding assays performed using all these antigen preparation with PAb of *T. cucumeris*. Antigens were carefully spotted on nitrocellulose paper and probed with Ab of *T. harzianum* Results have been presented in Fig. 79 C and Table 59. Clear and intense color reactions were observed in case of homologous antigen (Tc). In case of *R. solani* antigens, the colour intensity was comparatively lower than *T. cucumeris*. However, antigens of *S. rolfii* and *T. flavus* showed lesser color intensities.

Western blot analyses using polyclonal antibody of *T. cucumeris* RHS/V-566 revealed that all the PABs collected at three different intervals and coded as 1st, 2nd and 3rd bleed could show different levels of homologous reactions with the antigen of *T. cucumeris*. In case of first bleed a total of 7 bands were observed ranging from 60 to 94 kDa. In case of second bleed there were a total of 9 bands ranging from 60 to 98 kDa were obtained whereas there were a total of 11 bands in the third bleed ranging from 40 to 91 kDa (Fig. 79 D, Table 60).

Table 59 Scoring of dots obtained with DIBA of *T. cucumeris* and other heterogonous antigens

Isolate	Colour intensity ^a Using mycelial PAb (4 th bleed)
<i>Thanatephorus cucumeris</i>	++++
<i>Rhizoctonia solani</i>	++
<i>Sclerotium rolfii</i>	±
<i>Talaromyces flavus</i>	±

^a Fast red colour intensity : + + + + = Deep purple; ++= light purple; ± Faint purple, Antigen concentration 100 µg/ml, antibody concentration 40µg/ ml.

Table 60. Analysis of molecular weights of bands obtained in western blot analysis of *T. cucumeris* antigen and probed with its homologous antibody

1 st bleed Molecular weight (kDa)	2 nd bleed Molecular weight (kDa)	3 rd bleed Molecular weight (kDa)
60, 70, 74, 80, 85, 91, 94	60, 70, 74, 80, 85, 91, 94, 98	40, 43, 54, 60, 70, 74, 80, 85, 91

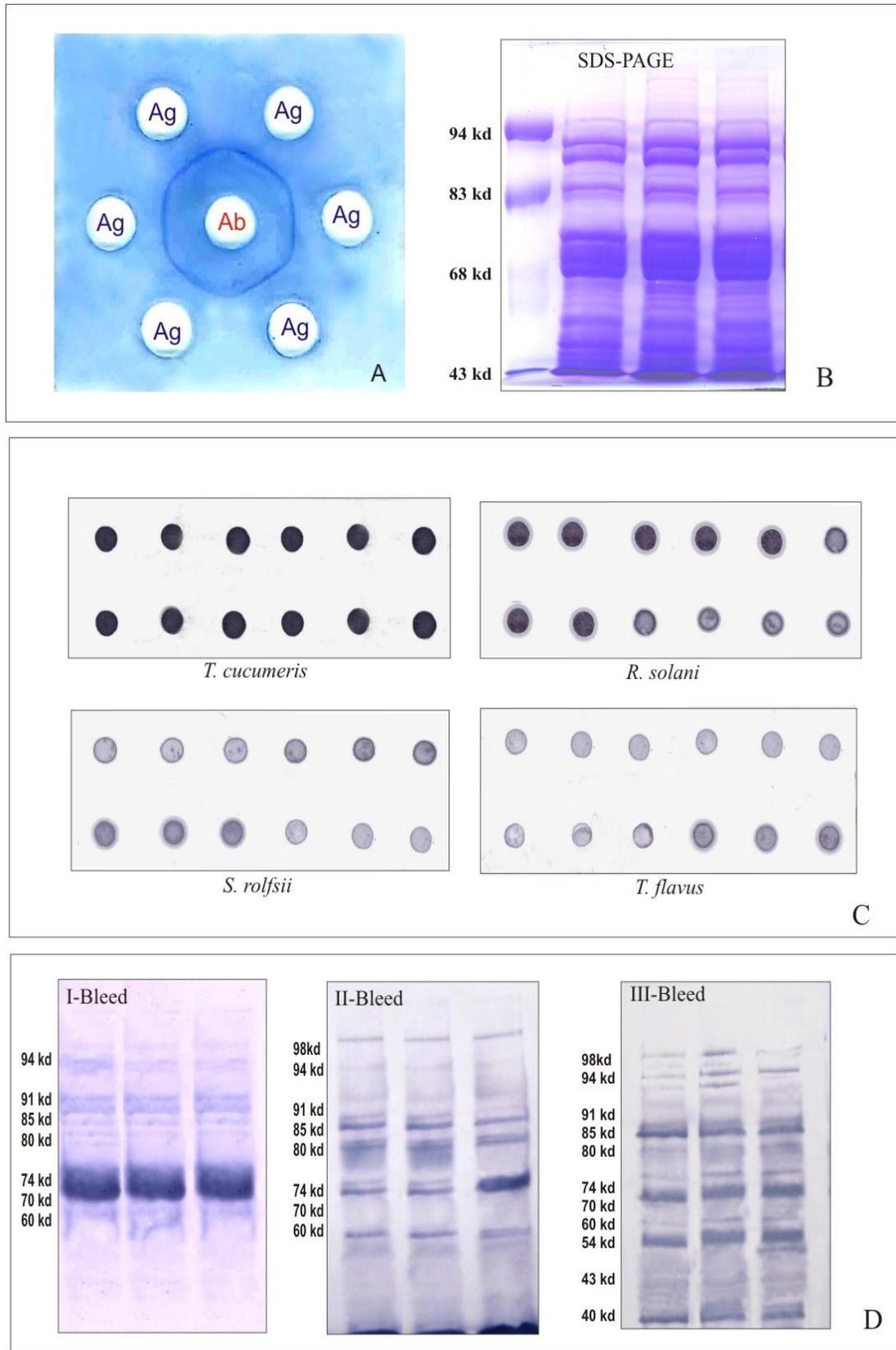


Fig. 79. Serological assays of *Thanatephorus cucumeris* RHS/V-566. Agar gel double diffusion test (A), Dot immuno binding assay (B); SDS-PAGE analysis of antigen of *T. cucumeris*. Western blot analysis with homologous antigen and antibodies of *T. cucumeris* (D).

4.11.5. Indirect immunofluorescence

Indirect immune fluorescence of hyphae and spores of *T. harzianum* RHS/S-559 was conducted with homologous polyclonal antibody. The fungal mycelium and the spores along with phialides treated with PABs and labeled with FITS showed apple green fluorescence (Fig. 80). Similarly the mycelium of *T. cucumeris* treated with its homologous PABs showed apple green fluorescence. Mycelia of both fungal isolates neither showed auto-fluorescence nor they showed any fluorescence when treated with normal serum followed by FITC (Fig. 81). Treatment of mycelia of *T. cucumeris* with homologous antiserum followed by FITC also showed apple green fluorescence that was more intense on young hyphal tips apart from this the intercalary chlymodospores also showed bright green fluorescence.

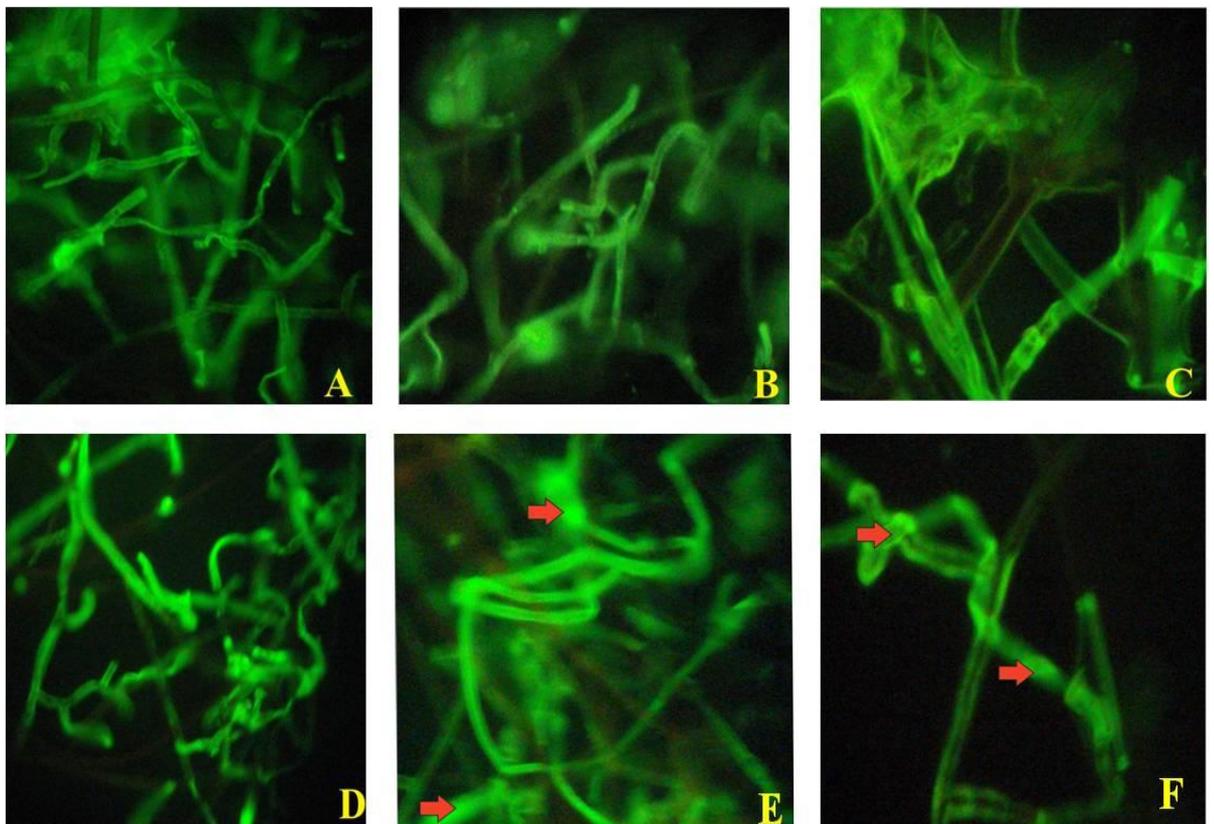


Fig. 80. Indirect immuno-fluorescence of hyphae and chlymodospores of *T. cucumeris* treated with homologous PABs and reacted with FITC conjugate IgG.

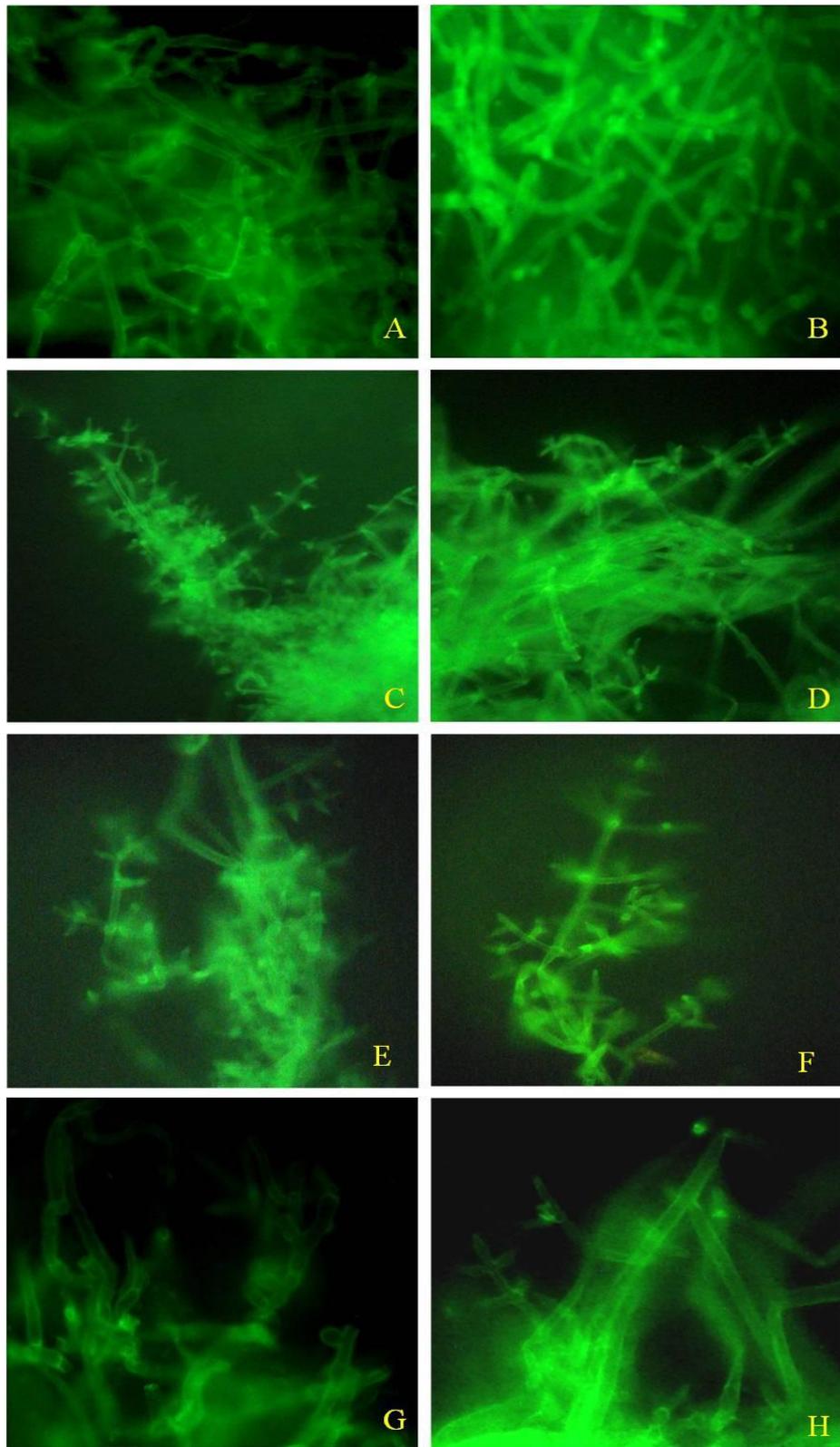


Fig. 81. Indirect immuno-fluorescence of hyphae and phialides of *Trichoderma harzianum* treated with homologous PABs and reacted with FITC conjugate IgG.

4.12. Effect of selected PSF on plant growth

On the basis of initial screening of fungal isolates for phosphate solubilization, *A. niger* FS/L-04, *A. melleus* FS/L-17, *A. clavatus* RHS/P-38 and *T. flavus* RHS/P-51 were found to be most efficient phosphate solubilizers. Evaluation of these isolates for enhancement of growth of six different crop plants viz. *Phaseolus vulgaris*, *Glycine max*, *Cicer arietinum*, *Vigna radiata*, *Pisum sativum* and *Oryza sativa* in green house condition was done in terms of root shoot dry weight, height and number of leaves over similar increase in control. Results revealed that *T. flavus* could enhance growth of all the tested crops more efficiently than the *Aspergillus* isolates. Differences among the different tested crops were not significant indicating that all tested crops responded to the PSF isolates. The overall results showed that these isolates had significant positive effect on the growth of all the tested crops in pot condition evaluated in terms of height of the plants, number of leaves per plant and root and shoot dry weight. The maximum effect of the PSF isolates was noticed in *Glycine max* (Fig. 82 & Fig. 83).

4.12.1. Effect of amendment of soil with PSF isolates on phosphate mobilization

4.12.1.1. Soil, root and leaf phosphate contents

All the PSF isolates could solubilize phosphate *in vitro* as evident by the appearance of halo zone around the colony in PVK medium. The phosphate solubilizing potential was quantified in modified Pikovskaya broth supplemented with tricalcium phosphate and rock phosphate. The potential of these isolates were further tested in the pot conditions where they were amended in the soil. The total residual phosphate in un-inoculated soil was found to be much higher than the soil amended with PSF isolates while root and leaf phosphate contents significantly increased in plants grown in PSF amended soil comparison to control. The total soil phosphate content was reduced by almost 50% by *T. flavus* compared to other PSF isolates. Similarly, the total phosphate content of the roots and leaves was also higher in all the crops treated with *T. flavus*. Among the test crops, soil phosphate content of *Glycine max* was reduced to a greater extent, however root and leaf phosphate content was found to be maximum in case of *Pisum sativum*. In all the cases the difference between the treated and control was found to be significant at the level $P=0.5$ when tested with students' *t* test. (Fig. 84).

4.12.1.2. Acid and alkaline phosphatase content

Enhancement of total phosphate content was found to be correlated with the enhancement of alkaline and acid phosphatase activities of the rhizosphere soil amended with PSF isolates. The alkaline phosphatase activities of the un-amended soil was found to lie between the range of 15 to 21 $\mu\text{g p-nitrophenyl/ g/h}$, however this activity increased significantly in the amended soil where the highest activity was found to be exhibited with the soil amended with *T. flavus* (38-48 $\mu\text{g p-nitrophenyl/ g/h}$). The acid phosphatase activity of the un-amended soil found to be ranging between 60-82 $\mu\text{g p-nitrophenyl/ g/h}$, which increased to a maximum of 154-172 $\mu\text{g p-nitrophenyl/ g/h}$ in *T. flavus* amended soils. Though the difference between the control and treated sets in case of each crop was significant ($P=0.05$), no any significant differences among the different tested crops were noted (Table 61).

Table 61. Alkaline and acid phosphatase content of soil treated with PSF isolates

Crop	Alkaline phosphatase ($\mu\text{g p-nitrophenyl/g/h}$)				
	Non Amended	<i>A. niger</i>	<i>A. melleus</i>	<i>A. clavatus</i>	<i>T. flavus</i>
<i>V. radiata</i>	15.44 ± 1.735	38.54 ± 1.256	31.25 ± 1.448	32.35 ± 1.118	48.50 ± 2.663
<i>P. sativum</i>	17.65 ± 0.980	31.33 ± 2.44	35.35 ± 2.78	30.35 ± 1.15	48.25 ± 2.113
<i>G. max</i>	14.86 ± 1.033	25.76 ± 2.76	20.74 ± 1.18	22.12 ± 1.167	35.43 ± 2.893
<i>C. arietinum</i>	11.18 ± 1.166	23.52 ± 1.18	18.35 ± 1.15	20.37 ± 1.226	30.18 ± 2.163
<i>P. vulgaris</i>	12.36 ± 0.933	27.50 ± 2.09	18.95 ± 1.11	25.44 ± 1.030	33.26 ± 1.227
<i>O. sativa</i>	21.23 ± 1.116	32.33 ± 2.55	28.75 ± 2.48	29.45 ± 1.270	46.45 ± 1.770
Crop	Acid phosphatase ($\mu\text{g p-nitrophenyl/ g/h}$)				
	Non Amended	<i>A. niger</i>	<i>A. melleus</i>	<i>A. clavatus</i>	<i>T. flavus</i>
<i>V. radiata</i>	72.49 ± 6.334	155.37 ± 9.433	133.22 ± 5.767	155.35 ± 8.336	178.55 ± 8.263
<i>P. sativum</i>	60.14 ± 8.445	135.35 ± 8.176	125.25 ± 6.882	145.55 ± 7.335	163.25 ± 6.443
<i>G. max</i>	68.45 ± 7.828	87.16 ± 8.225	80.15 ± 6.103	95.33 ± 5.824	114.65 ± 7.658
<i>C. arietinum</i>	65.18 ± 7.166	92.82 ± 8.162	83.49 ± 7.165	88.15 ± 8.117	128.17 ± 7.412
<i>P. vulgaris</i>	72.78 ± 6.376	114.10 ± 7.335	94.45 ± 7.238	98.36 ± 6.174	128.82 ± 6.335
<i>O. sativa</i>	82.30 ± 8.442	122.25 ± 6.721	106.25 ± 6.473	135.66 ± 9.212	172.35 ± 8.191

Data of three replicate experiments and \pm =SE.



Fig. 82. Effect of PFS isolates (*A. niger*, *A. melleus*, *A. clavatus* and *T. flavus*) on growth of crop plants in pot conditions.

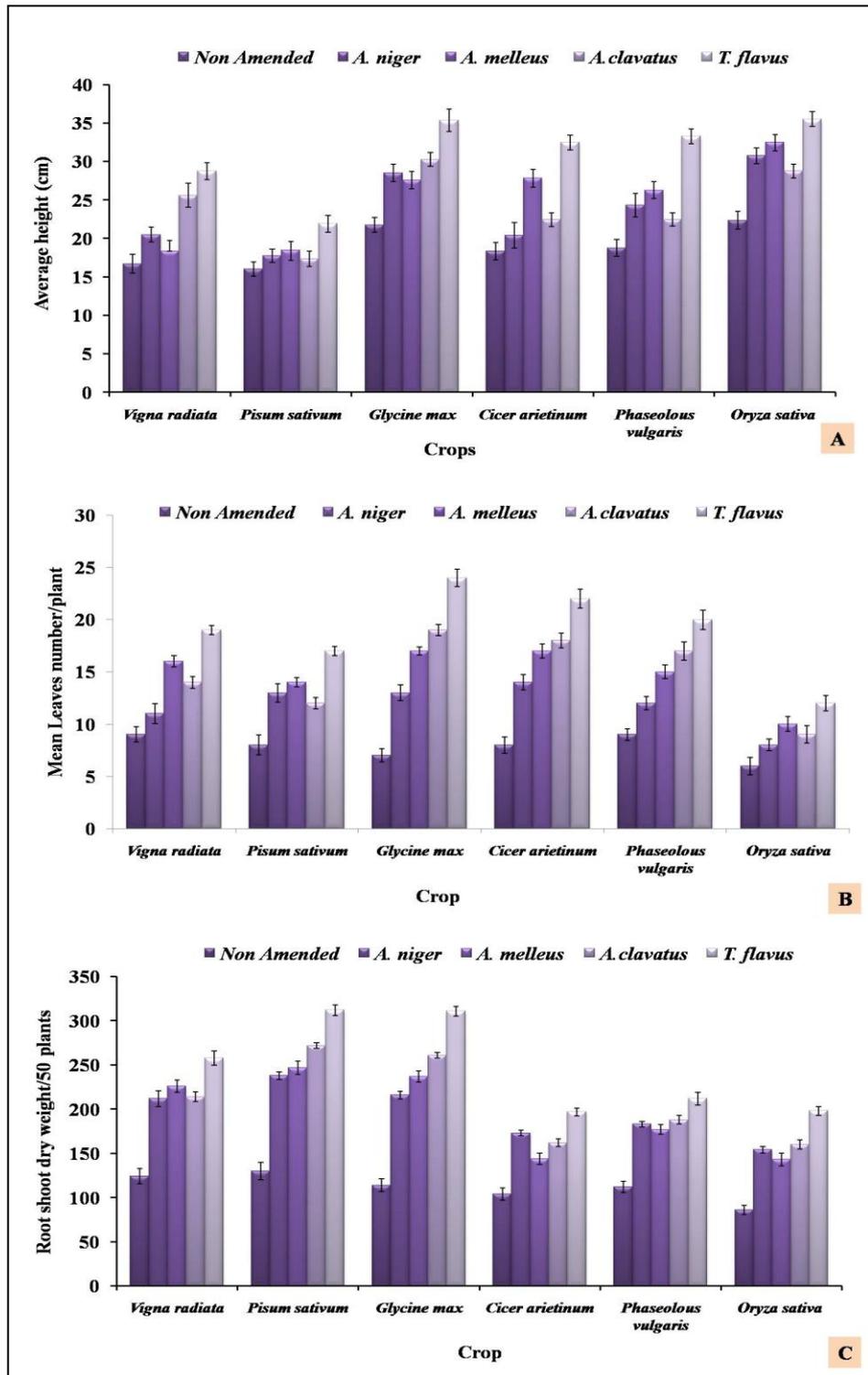


Fig. 83. Effect of PSF isolates on growth of different crops in green house condition measured in terms of average height (A); Mean leaves number per plant (B) and Root and shoot dry weight (C).

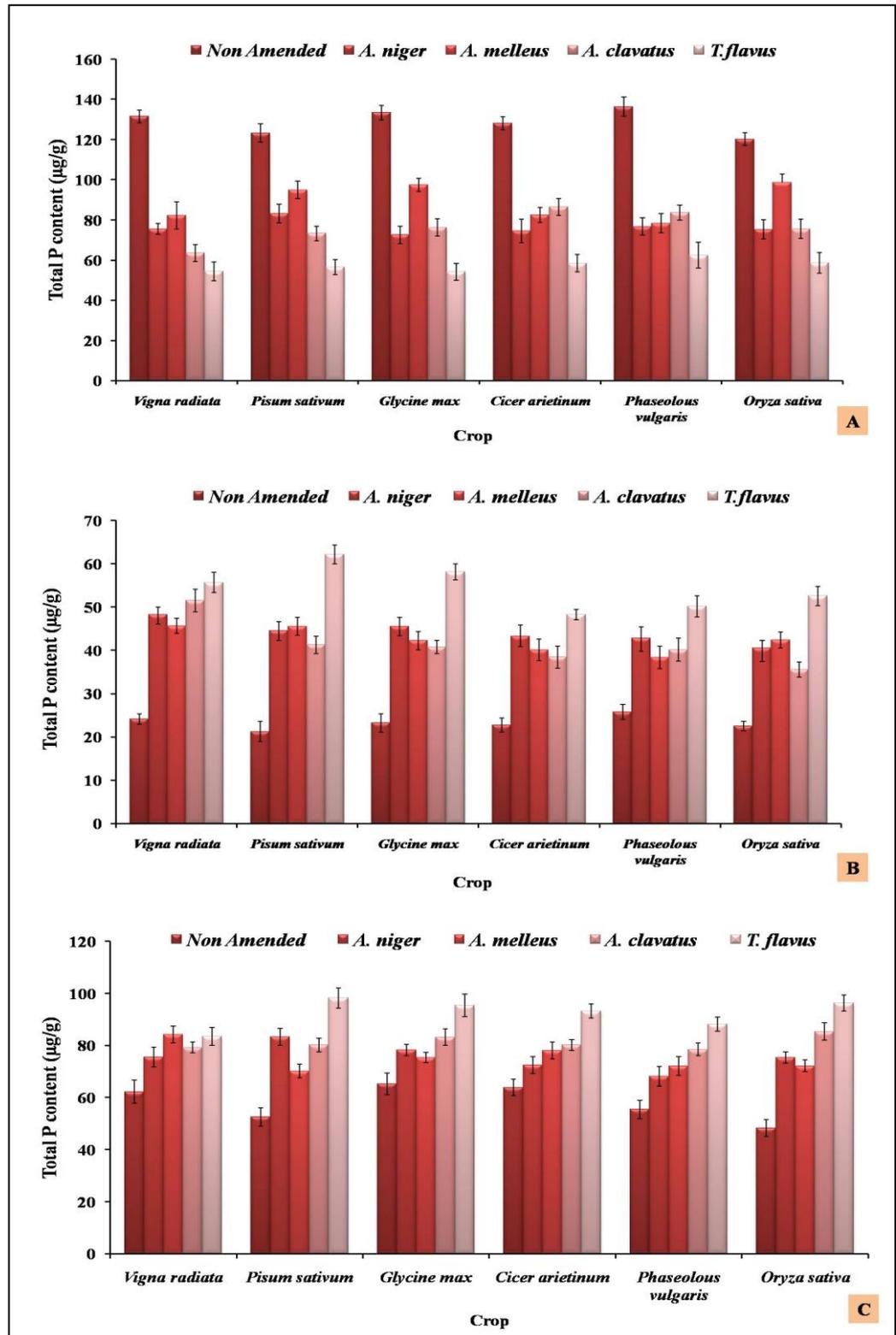


Fig. 84. Effect of PSF on total phosphate contents. Soil (A); Root (B) and Leaves (C).

4.13. Evaluation of selected PGPR isolates on plant growth promotion

Among the several isolates of PGPR obtained from different regions of North Bengal seven PGPR isolates, *Bacillus pumilus* BRHS/C-1, *Enterobacter cloacae* BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. altitudinis* BRHS/S-73, *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* BRHS/P-92 and *B. aerophilus* BRHS/B-104 were selected for *in vivo* evaluation of their effects on growth of different crop plants.

4.13.1. Growth promotion of *Cicer arietinum*, *Vigna radiata*, *Glycine max* and *Triticum aestivum* in field conditions

For the first set of experiment of field evaluation of PGPR isolates on plant growth promotion, potential PGPR isolates viz. *Bacillus pumilus* BRHS/C-1, *Enterobacter cloacae* BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. altitudinis* BRHS/S-73 and *B. methylotrophicus* BRHS/P-91 were selected to evaluate their effect in growth of *Vigna radiata*, *Cicer arietinum*, *Glycine max* and *Triticum aestivum* in field condition in comparison to control sets. Evaluation of their effect on overall growth and development of the test crops were computed in terms of shoot length, root length and shoot and root fresh weight. Results revealed that *B. altitudinis* BRHS/S-73 could enhance growth of all the tested crops more efficiently followed by *B. pumilus* BRHS/C-1 than the other PGPR isolates. Differences among the different tested crops were not significant indicating that all tested crops responded to the PGPR isolates (Fig. 85, 86, 87, 88 & 89).

4.13.2. Effect of application of PGPR isolates on phosphate mobilization

4.13.2.1. Soil, root and leaf phosphate contents

The total residual phosphate in un-inoculated soil was found to be much higher than the soil amended with PGPR isolates while root and leaf phosphate contents significantly increased in comparison to control. The total soil phosphate was found to be reduced to a greater extent in the all the soil treated with *B. altitudinis* BRHS/S-73. Similarly the reduction in the soil content was found to be correlated with the increase in root and leaf phosphates. Overall, *B. altitudinis* and *B. pumilus* were found to influence growth of the test plants. In all the cases the difference between the treated and control was found to be significant at the level $P=0.5$ when tested with students' *t*' test (Fig. 90).

4.13.2.2. Acid and alkaline phosphatase content

Enhancement of total phosphate content was found to be correlated with the enhancement of alkaline and acid phosphatase activities of the rhizosphere soil amended with PGPR isolates. The alkaline phosphatase activities of the un-amended soil was found to lie between the range of 0.039 to 0.075 $\mu\text{g p-nitrophenyl/ g/h}$, however the activity increased significantly in the amended soil where the highest activity was found to be exhibited with the soil amended with *B. altitudinis* (0.21-0.28 $\mu\text{g p-nitrophenyl/ g/h}$). The acid phosphatase activity in the un-amended soil found to be ranging between 0.027 to 0.044 $\mu\text{g p-nitrophenyl/ g/h}$, which increased to a maximum of 0.15-0.19 $\mu\text{g p-nitrophenyl/ g/h}$ in *B. altitudinis* amended soils followed by *B. pumilus* amended soil. Though the difference between the control and treated sets in case of each crop was significant ($P=0.05$), no any significant differences among the different tested crops were noted (Table 62).

Table 62. Effect of PGPR on Alkaline and Acid phosphatase activities of rhizosphere soil

Alkaline phosphatase activity ($\mu\text{g-paranitrophenyl/g/h}$)						
Crop	Control	<i>B. pumilus</i>	<i>B. altitudinis</i>	<i>B. methylotrophicus</i>	<i>P. polymyxa</i>	<i>E. cloacae</i>
<i>V. radiata</i>	0.045 ± 0.003	0.18 ± 0.003	0.21 ± 0.007	0.15 ± 0.008	0.12 ± 0.008	0.11 ± 0.003
<i>C. arietinum</i>	0.051 ± 0.003	0.20 ± 0.004	0.26 ± 0.005	0.18 ± 0.005	0.15 ± 0.007	0.16 ± 0.007
<i>G. max</i>	0.039 ± 0.003	0.17 ± 0.004	0.22 ± 0.004	0.11 ± 0.006	0.15 ± 0.006	0.13 ± 0.007
<i>T. aestivum</i>	0.075 ± 0.008	0.24 ± 0.008	0.28 ± 0.005	0.22 ± 0.006	0.18 ± 0.003	0.17 ± 0.003
Acid phosphatase activity ($\mu\text{g-paranitrophenyl/g/h}$)						
Crop	Control	<i>B. pumilus</i>	<i>B. altitudinis</i>	<i>B. methylotrophicus</i>	<i>P. polymyxa</i>	<i>E. cloacae</i>
<i>V. radiata</i>	0.033 ± 0.008	0.089 ± 0.008	0.15 ± 0.006	0.077 ± 0.004	0.063 ± 0.004	0.077 ± 0.003
<i>C. arietinum</i>	0.044 ± 0.006	0.15 ± 0.007	0.18 ± 0.008	0.097 ± 0.007	0.095 ± 0.006	0.088 ± 0.006
<i>G. max</i>	0.027 ± 0.004	0.13 ± 0.006	0.17 ± 0.005	0.12 ± 0.003	0.098 ± 0.003	0.087 ± 0.005
<i>T. aestivum</i>	0.037 ± 0.006	0.15 ± 0.008	0.19 ± 0.008	0.098 ± 0.007	0.088 ± 0.005	0.093 ± 0.005

Values are average of three replicate experiments. \pm =SE



Fig. 85. Evaluation of growth promotion of *Vigna radiata* by selected PGPR isolates in field condition.



Fig. 86. Evaluation of growth promotion of *Cicer arietinum* by selected PGPR isolates in field condition.



Fig. 87. Evaluation of growth promotion of *Triticum aestivum* by selected PGPR isolates in field condition.



Fig. 88. Evaluation of growth promotion of *Glycine max* by selected PGPR isolates in field condition.

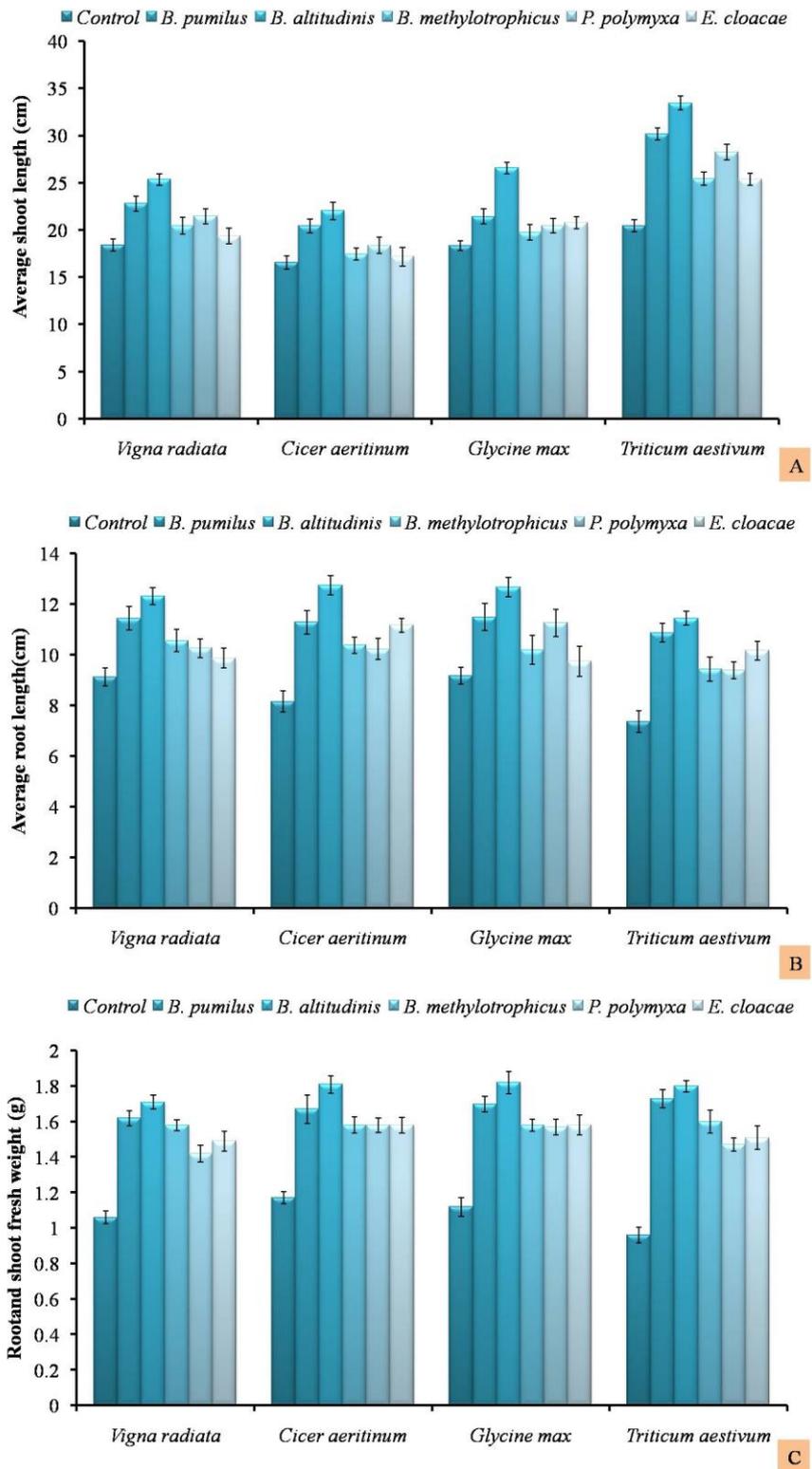
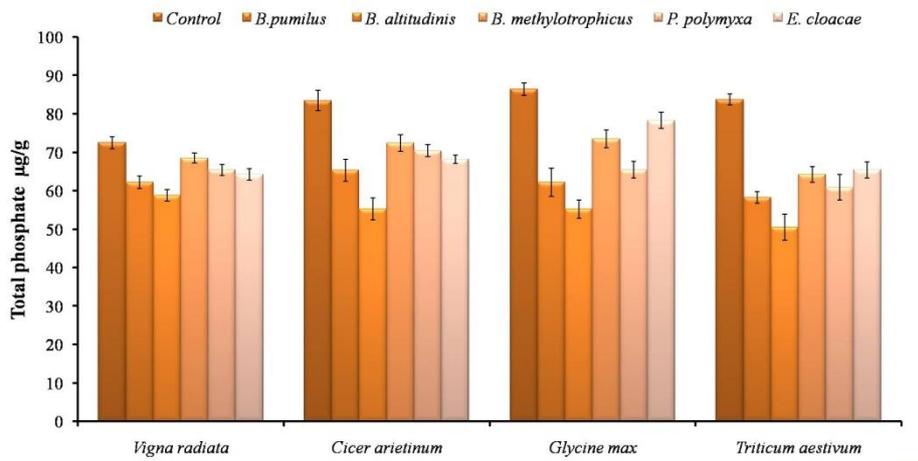
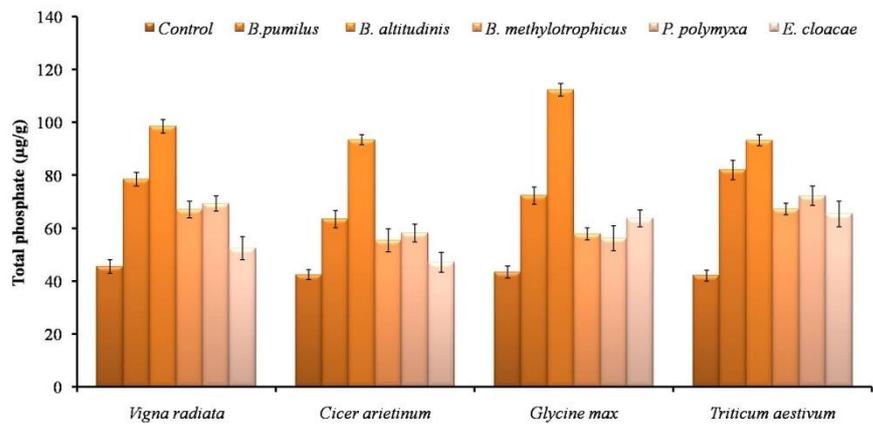


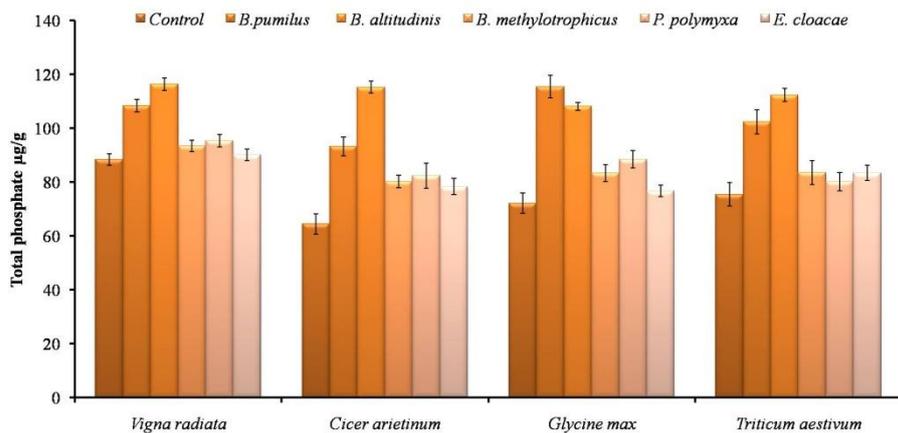
Fig. 89. Evaluation of growth promotion of *V. radiata*, *C. aeritinum*, *G. max* and *T. aestivum* by PGPR isolates in field conditions. Average shoot height (A); Average root length (B) and Root and Shoot fresh weight (C).



A



B



C

Fig. 90. Effect of PGPR application on total phosphate content. Soil (A), root (B) and leaves (C) of test plants grown in field conditions.

4.13.3. Growth promotion of Tea saplings by *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73

B. altitudinis BRHS/S-71 and *B. pumilus* BRHS/C-1 were found to be most efficient plant growth promoters in field conditions. Both these efficient PGPR isolates were further tested for their effect in enhancing the growth of four different varieties of tea sapling (TV-9, TV-20, TV-25 and TV-26) in nursery conditions where they were applied to the rhizosphere of six month old tea plants in nursery conditions at a regular interval of 15 days for one month. Growth promotion of different varieties by individual bacterium was noted as compared to untreated control in terms of increase in height, leaf fresh and dry mass. Results revealed an increase in all the parameters by single as well as dual application of bacteria. The overall result reveals that the growth of tea seedlings grown under same environmental and physical conditions was enhanced to a greater extent when both the bacterial isolates were applied jointly (1:1) (Fig. 91 & 92). Statistical analysis (ANOVA) revealed that increases in all three parameters were significantly enhanced by the treatments. Initial differences among the plants selected for the study were not significant.

4.13.4. Effects of *B. pumilus* and *B. altitudinis* P content

4.13.4.1. Soil, root and leaf phosphate contents

Both the PGPR isolates could solubilize phosphate *in vitro* as evident by the appearance of halo zone around the colony in PVK medium. All these selected PGPR isolates could also solubilize maximum amount of tricalcium and rock phosphate. The total residual phosphate in un-inoculated soil was found to be much higher than the soil amended with PGPR isolates while root and leaf phosphate contents significantly increased in comparison to control. The overall result reveals that the soil P content decreased following application of *B. pumilus* and *B. altitudinis* singly or jointly in treated nursery grown tea plants in comparison to control. Changes in phosphate contents following the treatments were statistically significant (Fig. 93). Increase in root and leaf phosphate contents was observed following the application of bacteria in all the four varieties of tea seedlings. Maximum phosphate content was obtained in the leaf tissues. However total P content of both the roots and the leaves of the tea seedlings were found to be higher in those plants which were jointly treated with both the bacteria.

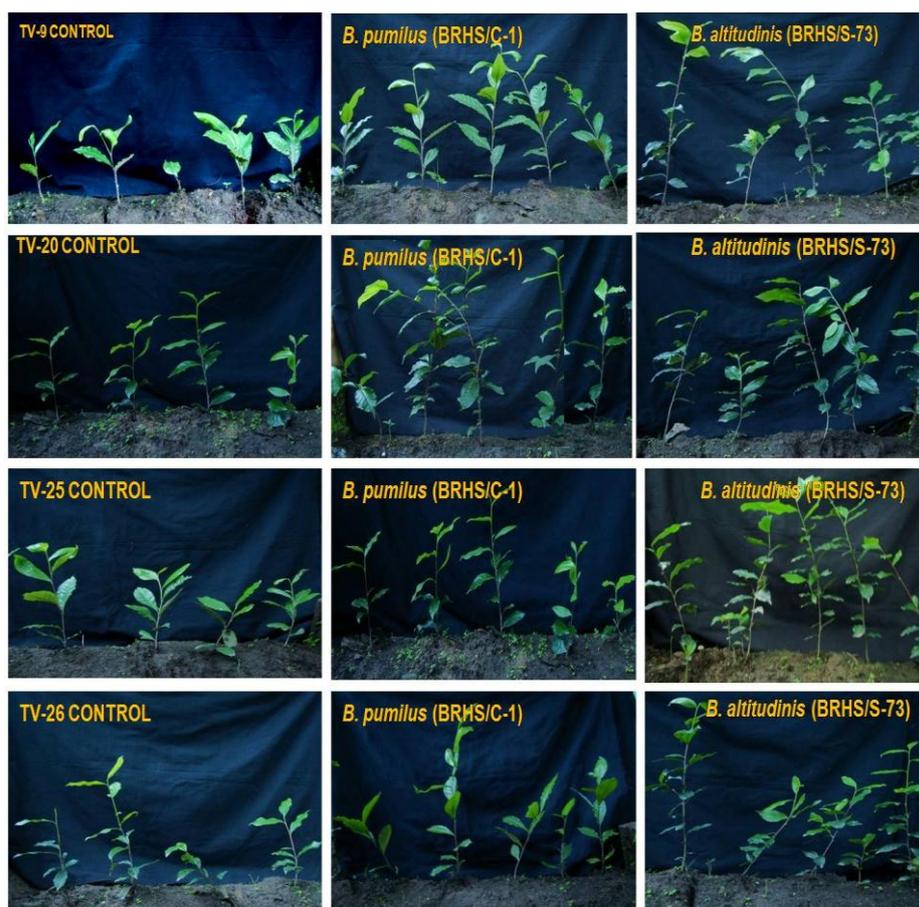


Fig. 91. Effect of *B. pumilus* and *B. altitudinis* on the growth of four tea varieties in nursery conditions.

4.13.4.2. Acid and alkaline phosphatase content

Enhancement of total phosphate content was found to be correlated with the enhancement of alkaline and acid phosphatase activities of the rhizosphere soil amended with both *B. pumilus* and *B. altitudinis*. Alkaline phosphatase activities was found to be much lesser than the acidic phosphatase activities in all the tea varieties. Over all result revealed that the activities of both acid and alkaline phosphatase activities were enhanced greatly when both the isolates were applied jointly (Table 63). The difference between the control and treated sets in case of each crop was significant ($P=0.05$), no any significant differences among the different tested crops were noted. Statistical analysis (ANOVA) revealed that increases in both the acid and alkaline phosphate were significantly enhanced by the treatments. Initial differences among the plants selected for the study were not significant (Table 63 a & b).

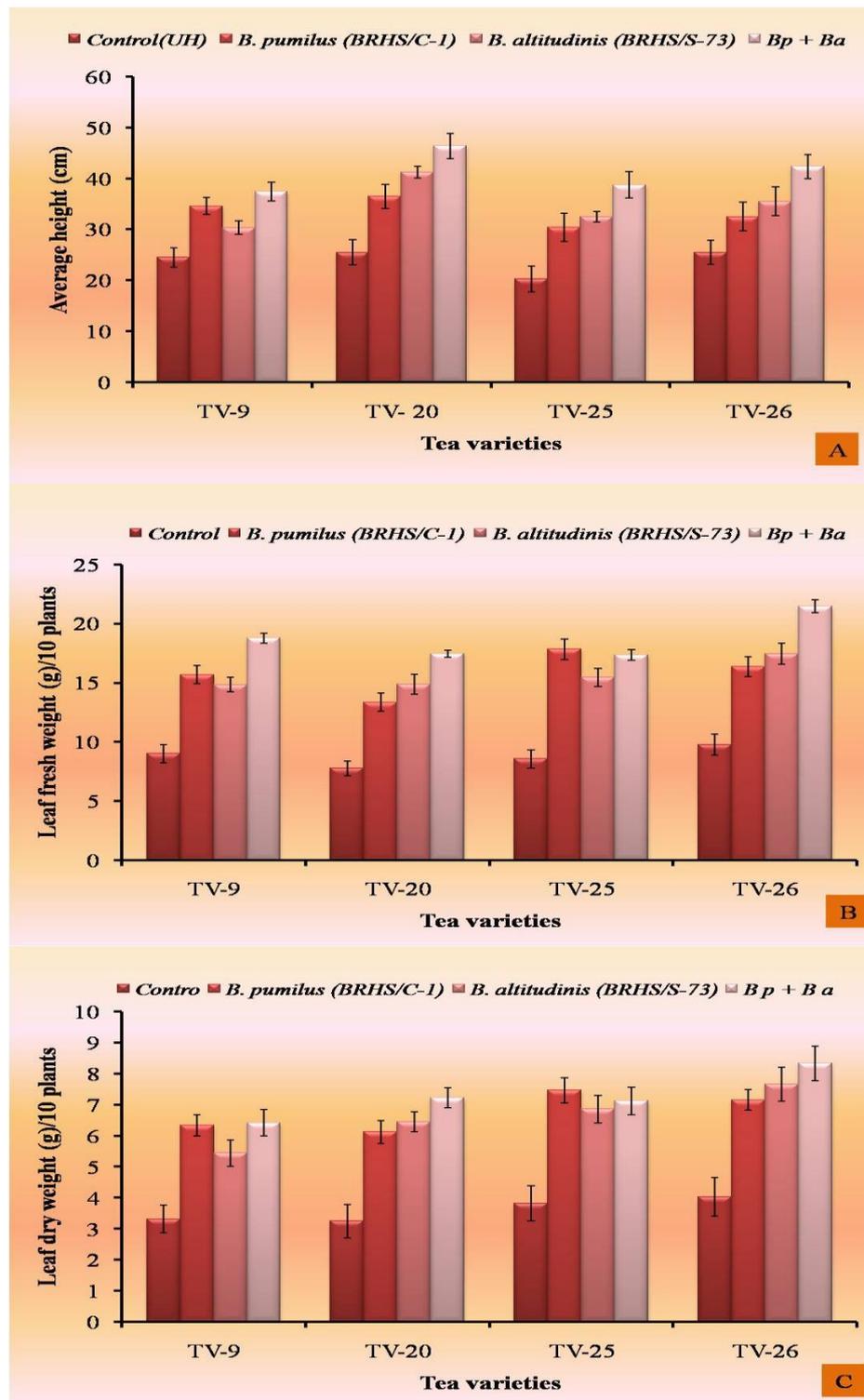


Fig. 92. Evaluation of growth promotion of tea saplings by *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73 grown in nursery conditions. Average height (A), leaf fresh weight (B) and dry weight (C).

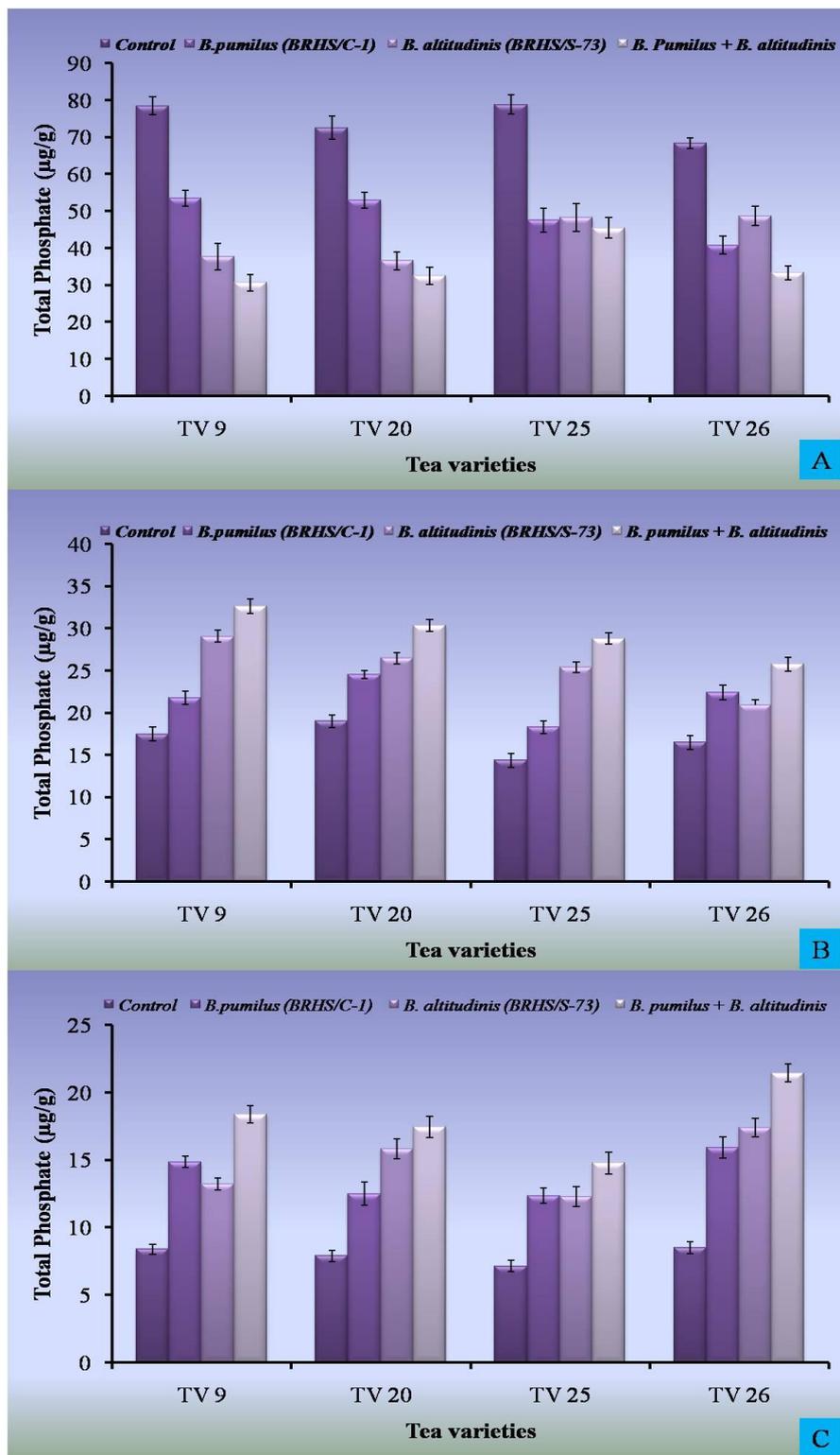


Fig. 93. Evaluation of Total P-content of soil (A), root (B) and leaves (C) of tea saplings following treatment by *B. pumilus* and *B. altitudinis*.

Table 63. Acidic and alkaline phosphatase activities of the soil following application of the *B. pumilus* and *B. altitudinis*

		Phosphatase activities ($\mu\text{g p-nitrophenyl/g/h}$)	
Tea Varieties	Treatments	Acid Phosphatase	Alkaline phosphatase
TV-9	Control	62.36 \pm 3.66	17.44 \pm 2.16
	<i>B. pumilus</i>	87.45 \pm 4.36	28.35 \pm 2.18
	<i>B. altitudinis</i>	83.46 \pm 4.42	22.35 \pm 1.22
	<i>B. p + B. a</i>	93.76 \pm 3.06	37.48 \pm 1.06
TV-20	Control	60.82 \pm 3.26	15.36 \pm 1.14
	<i>B. pumilus</i>	85.32 \pm 6.32	27.89 \pm 1.22
	<i>B. altitudinis</i>	80.56 \pm 4.84	22.33 \pm 2.17
	<i>B. p + B. a</i>	92.88 \pm 4.10	32.74 \pm 1.18
TV-25	Control	60.44 \pm 4.12	18.97 \pm 2.15
	<i>B. pumilus</i>	73.21 \pm 3.44	25.12 \pm 2.23
	<i>B. altitudinis</i>	76.46 \pm 3.23	28.36 \pm 2.26
	<i>B. p + B. a</i>	87.45 \pm 4.08	36.28 \pm 1.32
TV-26	Control	58.26 \pm 3.12	14.36 \pm 2.18
	<i>B. pumilus</i>	75.46 \pm 4.28	28.55 \pm 2.14
	<i>B. altitudinis</i>	78.34 \pm 4.16	31.75 \pm 2.28
	<i>B. p + B. a</i>	93.25 \pm 5.15	35.82 \pm
CD(P=0.05)	(Treatments)	16.229	5.426
	(Varieties)	14.303	3.770

Values are average three replicate sets (10 plants each) \pm Standard Error.

Table 63 a. ANOVA of the data presented in table 63 (Acid Phosphatase activity)

Source of Variation	SS	df	MS	F
Rows	134.01985	3	44.67328333	5.166907518
Columns	2028.6021	3	676.2007	78.20930587
Error	77.81435	9	8.646038889	
Total	2240.4363	15		

Table 63 b. ANOVA of the data presented in table 63 (Alkaline Phosphatase activity)

Source of Variation	SS	df	MS	F
Rows	21.61736875	3	7.205789583	0.84915792
Columns	731.3327188	3	243.7775729	28.72768547
Error	76.37225625	9	8.48580625	
Total	829.3223438	15		

4.14. Effect of BCA isolates on inhibiting root diseases

Evaluation of effect of biocontrol agents (BCA) in suppressing root diseases in pot conditions of selected legumes was carried out. *Talaromyces flavus* (RHS/P-51), *Trichoderma harzianum* (RHS/S-559) and *T. asperellum* (RHS/S-561) were found to be most efficient pathogen inhibitors *in vitro*, experiments were further conducted to determine whether these could also control diseases caused by *Sclerotium rolfsii* and *Thanatephorus cucumeris*.

4.14.1. Influence of *T. flavus* and *T. harzianum* on Sclerotial blight of *Vigna radiata*

4.14.1.1. Disease development

Effect of the two biocontrol agents on development of sclerotial blight of *Vigna radiata*, caused by *S. rolfsii* was determined (Fig. 94). Seedlings of *Vigna radiata* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *S. rolfsii* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of these BCA as soil amendments was effective in reducing sclerotial blight incidence when applied prior to artificial inoculation of the pathogen. *T. harzianum* was found to control disease development more efficiently than *T. flavus* when applied singly. However, the severity of the disease was much lesser (1.25) when the soil was jointly amended with both *T. harzianum* and *T. flavus* (Table 64, Fig. 95A).

4.14.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *V. radiata* inoculated with only *S. rolfsii* increased with time, reaching a maximum of 73.33% at the end of 30 d. In contrast, when the soil was pre treated with *T. harzianum* and *T. flavus*, the maximum disease severity was only 20 and 23 % respectively which were much reduced when applied jointly (8%). Biocontrol efficacy of both *T. harzianum* and *T. flavus* under green house condition after 30 d of pathogen challenge was found to be 50 and 45%. Regardless of the values expressed, the biocontrol efficacy (BE %) was found to be as high as 83.33% when both *T. harzianum* and *T. flavus* were applied jointly (Fig. 95B).

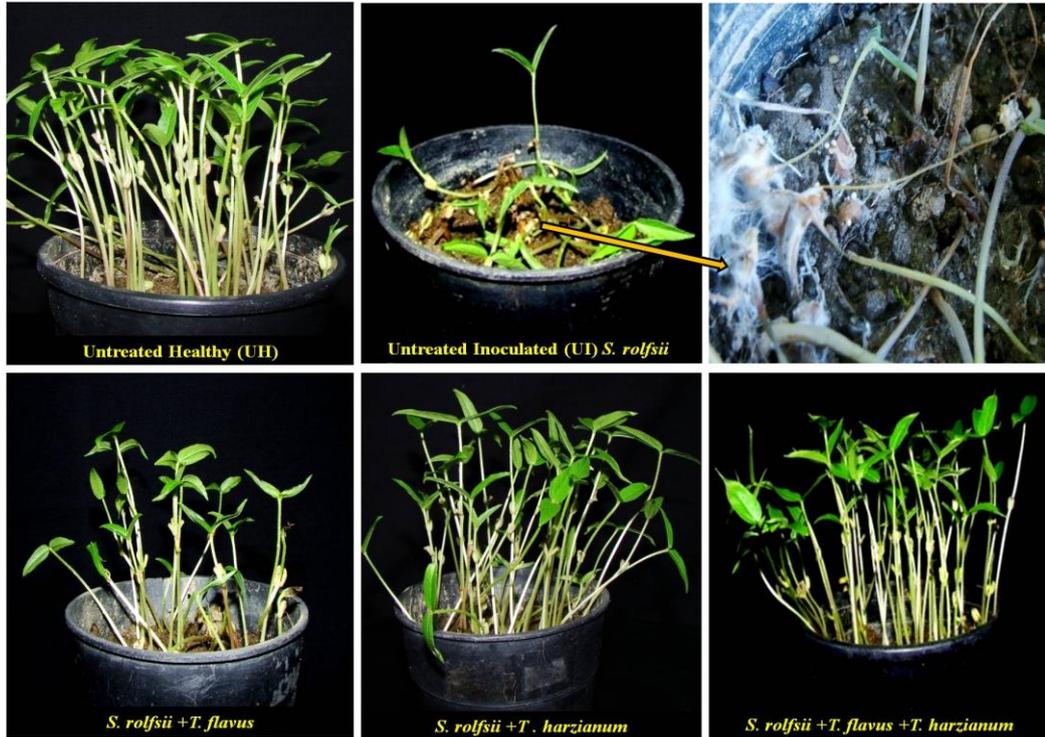


Fig. 94. Sclerotial blight development of *Vigna radiata* in presence and absence of *T. harzianum* and *T. flavus* in pot conditions.

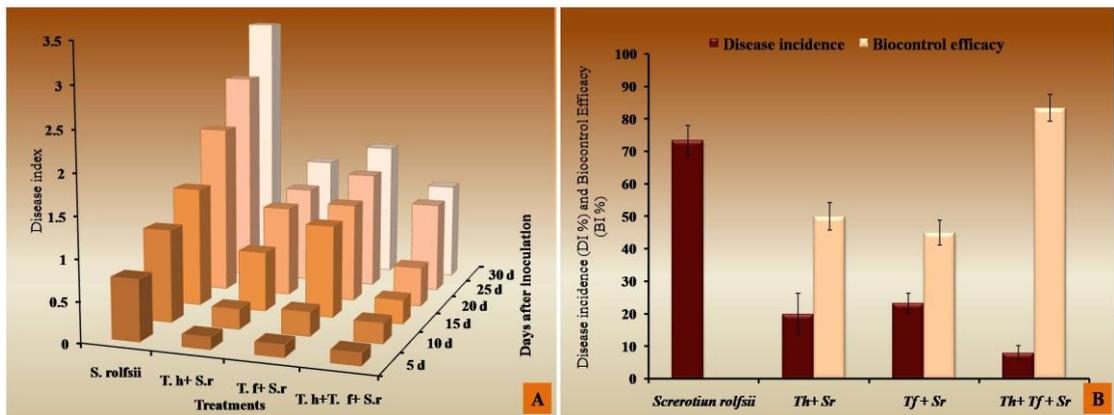


Fig. 95. Sclerotial blight development in the roots of *Vigna radiata* in presence and absence of *T. harzianum* and *T. flavus* in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of *T. harzianum* and *T. flavus* in inhibiting Sclerotial blight disease of *V. radiata* (B).

Table 64. Sclerotial blight development in the roots of *Vigna radiata* in presence and absence of *T. harzianum* and *T. flavus* in pot conditions

Days after inoculation	Untreated Inoculated (<i>S. rolfsii</i>)	<i>T. harzianum</i> + <i>S. rolfsii</i>	<i>T. flavus</i> + <i>S. rolfsii</i>	<i>T. harzianum</i> + <i>T. flavus</i> + <i>S. rolfsii</i>
5	0.75	0.15	0.15	0.15
10	1.15	0.25	0.30	0.25
15	1.50	0.75	1.15	0.30
20	2.15	1.15	1.25	0.50
25	2.75	1.25	1.50	1.15
30	3.45	1.50	1.75	1.25

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80%of the root effected 80-85% of the shoot and leaves withered; 6-whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

4.14.1.3. Biochemical changes

Application of *T. harzianum* and *T. flavus* to soil was found to affect the biochemical responses of plants. Disease establishment is also known to cause biochemical changes in the host. Hence, in another series of experiments, biochemical responses of *Vigna radiata* following application of biocontrol agents and challenge inoculated with the pathogen- *Sclerotium rolfsii* were determined. The reduction in disease incidence of *V. radiata* by pretreatment of soil by *T. harzianum* and *T. flavus* was noted and the conference of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU in roots of *Vigna radiata* after 2, 4, 6, 8 days of pathogen challenge. The results showed that the activities of POX, PAL, and GLU were significantly higher after six days of pathogen challenge which started declining after eight days of pathogen challenge. In contrary peroxidase activity was significantly higher even after eight days of pathogen challenge. Among all the treatments the plants which were grown in soil jointly treated with *T. harzianum* and *T. flavus* showed significantly higher defense enzyme activities than those treated singly (Fig. 96).

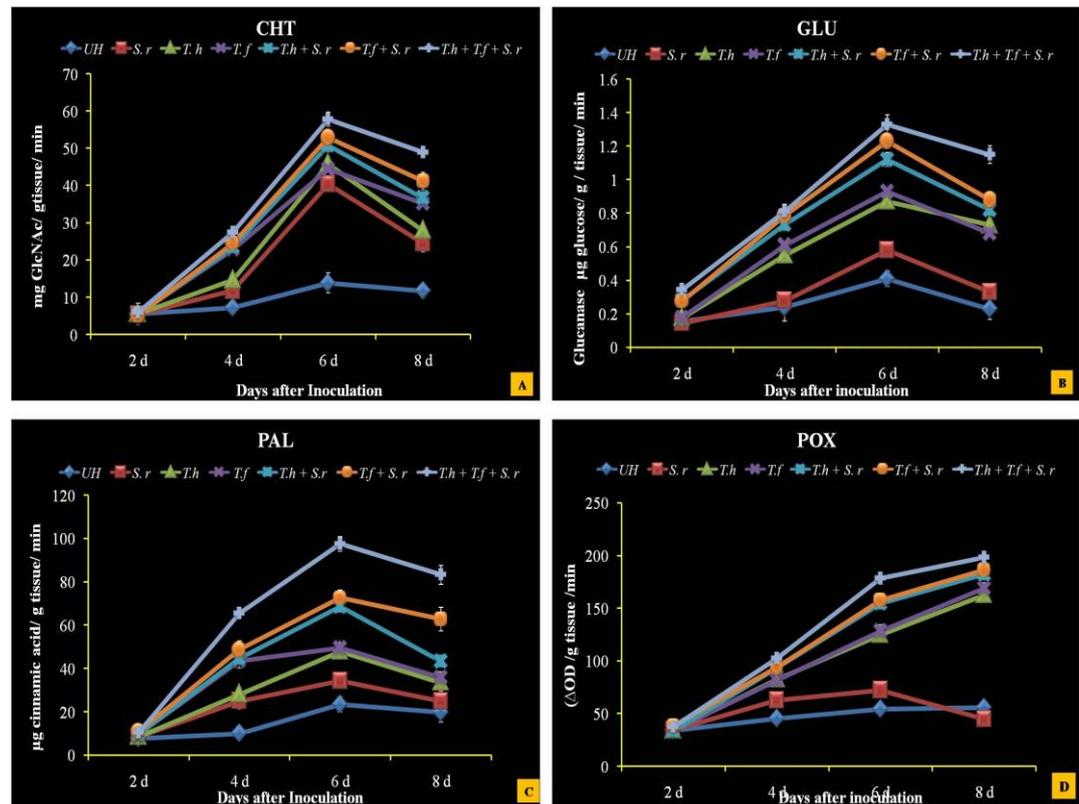


Fig. 96. Defense enzyme activities in the roots of *Vigna radiata* following inoculation with *S. rolfisii* and treatment with *T. harzianum* and *T. flavus*. Chitinase (A), β -1,3 Glucanase (B), Phenylalanine ammonia lyase(C) and peroxidase (D).

4.14.2. Influence of *T. flavus*, *T. harzianum* and *T. asperellum* on root rot of *Cicer aeritinum* caused by *Thanatephorus cucumeris*

4.14.2.1. Disease development

Effect of the three biocontrol agents (*T. flaus*, *T. harzianum* and *T. asperellum*) on development of root rot caused by *T. cucumeris* was determined (Fig. 98). Seedlings of *Cicer aeritinum* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *T. cucumeris* recorded in terms of disease index of the plants challenged by the pathogen as well as total seedling survival percentage calculated at the end of 30 days. It was observed that application of these BCA as soil amendments was effective in reducing root rot incidence when applied prior to artificial inoculation of the pathogen. The disease severity in *C. aeritinum* inoculated with only *T. cucumeris* increased with time, reaching a maximum of 6 at

the end of 30 days. In contrast, when the seeds were pre treated with BCA followed by soil treatment the disease severity was significantly reduced. Among all the three BCA isolates, *T. harzianum* was found to control disease more efficiently than *T. flavus* and *T. asperellum* when applied singly. However, the severity of the disease was much lesser (1.25) when the soil was jointly amended with *T. harzianum*, *T. flavus* and *T. asperellum* (Table 65, Fig. 97 A).

4.14.2.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *C. arietinum* inoculated with only *T. cucumeris* increased with time, reaching a maximum of 85.45% at the end of 30 d. In contrast, when the soil was pre treated with *T. harzianum*, *T. flavus* and *T. asperellum* the maximum disease severity was only 10, 12 and 16% respectively which were much reduced when applied jointly (4.16%). Biocontrol efficacy of *T. harzianum*, *T. flavus* and *T. asperellum* under green house condition after 30 d of pathogen challenge was found to be 58, 55 and 54%. Biocontrol efficacy (BE %) was found to be as high as 85.45% when all the three BCA isolates were applied jointly (Fig. 97B).

4.14.2.3. Biochemical changes

Application of *T. harzianum*, *T. flavus* and *T. asperellum* to soil was found to affect the biochemical responses of plants. Disease establishment is also known to cause biochemical changes in the host. Hence, in another series of experiments, biochemical responses of both the roots and leaves of *C. arietinum* following application of biocontrol agents and challenge inoculated with the pathogen-*Thanatephorus cucumeris* were determined. The conference of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU in both the roots and leaves of *Cicer arietinum* after pathogen challenge. The results showed that the activities of POX, PAL, and GLU were significantly higher in plants treated with BCA isolates followed by pathogen challenge. In contrary peroxidase activity was found to be higher in the leaves than the roots. Among all the treatments the plants which were grown in soil jointly treated with *T. harzianum*, *T. flavus* and *T. asperellum* showed significantly higher defense enzyme activities than those treated singly (Table 66).

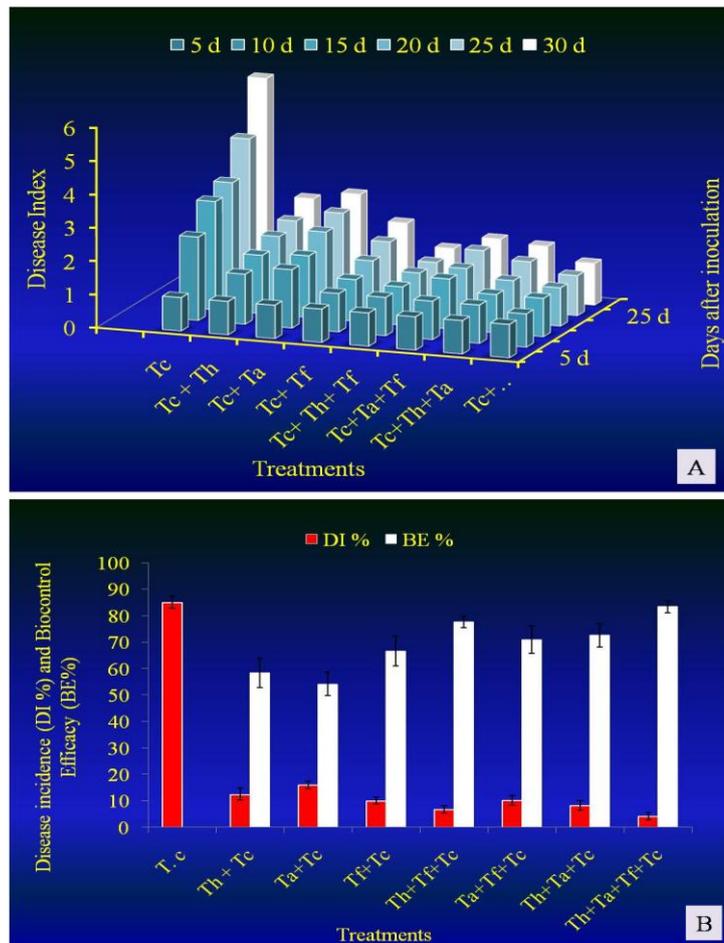


Fig. 97. Seedling survival percentage (A), Disease index (B) of *Cicer arietinum* and Biocontrol efficacy % of BCA isolates following inoculation with *T. cucumeris*.

Table 65. Percent survival of *C. aeritinum* seedlings in presence and absence of *T. harzianum*, *T. flavus* and *T. asperellum* in pot conditions

Treatments	Seedling Survival %					
	Days after inoculation					
	5d	10d	15d	20d	25d	30d
<i>Untreated healthy</i>	100	98.45	98.00	98.00	98.00	98.00
<i>Thanatephorus cucumeris (T. c)</i>	94.6	82.3	31.6	23.00	12.34	4.20
<i>T. h + T. c</i>	92.5	76.3	75.6	74.3	72.55	70.55
<i>T. a + T. c</i>	93.55	78.5	77.55	75.6	72.3	71.3
<i>T.f + T. c</i>	92.55	81.5	80.43	76.60	75.35	74.32
<i>T. h + T. f + T.c</i>	93.45	83.5	81.25	81.42	81.33	80.25
<i>T. a + T. f + T.c</i>	92.35	81.25	80.25	79.55	78.25	77.75
<i>T. .h + T. a+ T. c</i>	92.35	82.44	80.12	77.46	75.42	74.23
<i>T. h + T. a + T. f +T.c</i>	93.25	91.55	88.25	86.25	85.55	84.75

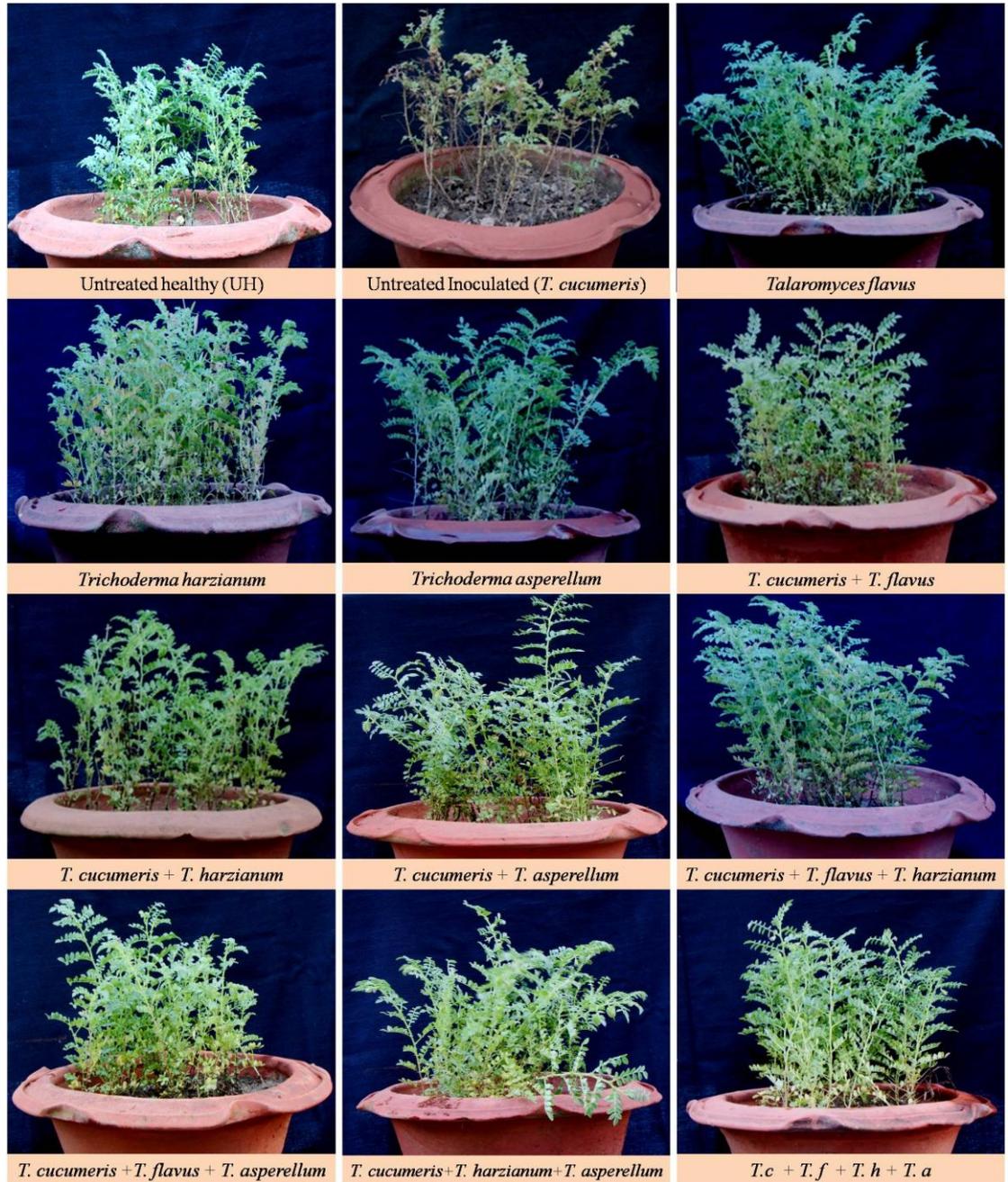


Fig. 98. Sclerotial blight development in *Cicer arietinum* in presence and absence of *T. harzianum*, *T. flavus* and *T. asperellum* in pot conditions.

Table 66. Defense enzyme activities in the shoots and roots of *Cicer arietinum* following inoculation with *T. cucumeris* and treatment with *T. harzianum*, *T. flavus* and *T. asperellum*

Treatments	CHT (mg GlcNAc/ g tissue/ min)		GLU (µg glucose/ g tissue/ min)	
	Shoot	Root	Shoot	Root
Untreated healthy	15.50±1.2	12.35±0.8	08.85±0.7	11.25±0.5
<i>Thanatephorus cucumeris</i> (<i>T. c</i>)	22.25±1.5	18.75±1.4	12.56±0.8	14.35±0.4
<i>Trichoderma harzianum</i> (<i>T. h</i>)	35.52±1.6	24.35±1.7	18.56±0.9	22.35±0.8
<i>Trichoderma asperellum</i> (<i>T.a</i>)	28.25±1.5	20.76±1.4	15.56±0.9	18.36±0.7
<i>Talaromyces flavus</i> (<i>T. f</i>)	40.35±1.3	35.25±2.3	16.35±0.8	25.35±0.7
<i>T. h</i> + <i>T. c</i>	65.5±1.7	45.25±2.4	28.43±0.7	27.75±0.6
<i>T. a</i> + <i>T. c</i>	55.25±1.1	45.50±2.4	25.24±0.8	22.43±0.3
<i>T.f</i> + <i>T. c</i>	75.12±2.5	57.36±3.1	36.35±0.7	48.75±0.6
<i>T. h</i> + <i>T. f</i> + <i>T.c</i>	87.50±2.5	73.20±2.7	56.58±.9	73.26±0.8
<i>T. a</i> + <i>T. f</i> + <i>T.c</i>	88.23±2.2	75.50±3.4	48.33±0.4	83.45±0.7
<i>T. .h</i> + <i>T. a</i> + <i>T. c</i>	86.54±3.3	72.43±2.1	55.34±0.7	87.42±0.8
<i>T. h</i> + <i>T. a</i> + <i>T. f</i> + <i>T.c</i>	103.18±3.4	92.45±3.7	73.26±0.8	112.34±0.8
Treatments	PAL (µg cinnamic acid/g tissue / min)		POX (ΔOD/ g tissue/ min)	
	Shoot	Root	Shoot	Root
Untreated healthy	82.43±3.4	112.42±5.7	112.35±2.6	55.35±3.2
<i>Thanatephorus cucumeris</i> (<i>T. c</i>)	95.74±3.2	132.53±6.8	212.35±3.8	58.55±3.3
<i>Trichoderma harzianum</i> (<i>T. h</i>)	158.44±5.7	184.35±7.2	213.50±3.5	82.34±3.2
<i>Trichoderma asperellum</i> (<i>T.a</i>)	165.36±6.4	195.55±7.1	211.25±4.8	73.54±5.1
<i>Talaromyces flavus</i> (<i>T. f</i>)	193.25±6.3	211.45±6.4	282.35±5.8	95.52±5.2
<i>T. h</i> + <i>T. c</i>	187.55±6.2	235.35±5.1	274.25±3.6	112.50±5.8
<i>T. a</i> + <i>T. c</i>	146.45±7.1	213.45±6.2	288.96±2.8	98.35±3.3
<i>T.f</i> + <i>T. c</i>	206.44±7.1	226.45±6.8	324.44±4.8	124.50±4.4
<i>T. h</i> + <i>T. f</i> + <i>T.c</i>	203.47±8.3	243.55±7.1	468.75±3.9	118.35±5.7
<i>T. a</i> + <i>T. f</i> + <i>T.c</i>	213.14±7.1	277.14±6.4	413.55±3.9	156.75±6.6
<i>T. .h</i> + <i>T. a</i> + <i>T. c</i>	220.12±8.3	264.35±6.3	422.43±4.8	126.42±4.8
<i>T. h</i> + <i>T. a</i> + <i>T. f</i> + <i>T.c</i>	315.76±7.4	383.45±6.4	495.53±5.8	183.25±6.8

Values are average of three replicate experiments; ±= Standard Error

4.15. Effect of PGPR isolates on inhibiting root diseases

Evaluation of effect of plant growth promoting isolates (PGPR) in suppressing root diseases in pot conditions of selected legumes and plantation crop were carried out. *Bacillus pumilus* (BRHS/C-1), *Paenibacillus polymyxa* (BRHS/R-71), *Enterobacter cloacae* (BRHS/R-72), *B. altitudinis* (BRHS/S-73), *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* (BRHS/P-92) and *B. aerophilus* (BRHS/B-104) were found to be most efficient pathogen inhibitors *in vitro*, experiments were further conducted to determine whether these could also control collar rot diseases caused by *Sclerotium rolfsii* and root rot disease caused by *Thanatephorus cucumeris* in legumes and tea seedlings in pot and nursery conditions.

4.15.1. Root rot disease caused by *Thanatephorus cucumeris*

4.15.1.1. Inhibition of Root rot of *Glycine max* by *Bacillus pumilus*, *Paenibacillus polymyxa*, *Enterobacter cloacae* and *B. altitudinis*

4.15.1.1.1. Disease development

Effect of the four PGPR isolates *Bacillus pumilus* (BRHS/C-1), *Paenibacillus polymyxa* (BRHS/R-71), *Enterobacter cloacae* (BRHS/R-71) and *B. altitudinis* (BRHS/S-73), in development of root rot disease of *Glycine max* was determined (Fig. 99). Seedlings of *Glycine max* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *T. cucumeris* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of these PGPR isolates as soil drench prior were effective in reducing root rot incidence when applied prior to artificial inoculation of the pathogen. The disease severity in *Glycine max* increased with time, reaching a maximum of 6 at the end of 30 days. In contrast, when the seeds were pre treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. Among all the bacterial isolates tested, *B. altitudinis* was found to be most effective followed by *B. pumilus*. The disease development between the treated and the control untreated pots were significant however it was not significant among different treatments. (Table 67, Fig. 100A).



Fig. 99. Root rot development in *Glycine max* in presence and absence of PGPR isolates in pot conditions.

4.15.1.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *G. max* inoculated with only *T. cucumeris* increased with time, reaching a maximum of 90% at the end of 30 d. In contrast, when the soil was pre treated with PGPR isolates the maximum disease severity was only 12.5 %. The lowest severity was noticed when isolate *B. altitudinis* was added prior to pathogen inoculation (5.75 %). Biocontrol efficacy of *B. pumilus*, *P. polymyxa*, *E. cloacae* and *B. altitudinis* was calculated and has been presented in Fig. 100 B, *B. altitudinis* was found to have the highest BE% (70.83 %).

Table 67. Root rot development in the roots of *Glycine max* in presence and absence of PGPR isolates in pot conditions

Days after inoculation	<i>T. cucumeris</i>	<i>T. cucumeris</i>		<i>T. cucumeris</i>	
		+	+	+	+
		<i>B. pumilus</i>	<i>P. polymyxa</i>	<i>E. cloacae</i>	<i>B. altitudinis</i>
5 d	1.15	0.25	0.50	1.00	0.20
10 d	1.50	0.50	1.15	1.25	0.35
15 d	2.75	0.75	1.25	1.50	0.50
20 d	4.25	1.15	1.50	1.75	0.75
25 d	5.50	1.25	1.75	2.00	1.15
30 d	6.00	1.75	2.00	2.15	1.50

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80% of the root effected 80-85% of the shoot and leaves withered; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.15.1.1.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of *Glycine max* following application of biocontrol agents and challenge inoculated with the pathogen- *Thanatephorus cucumeris* were determined. The reduction in root rot incidence of *G. max* by pretreatment of soil by PGPR isolates were noted and the conferrence of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU both in the roots and leaves after 12, 24 and 36 h of bacterial application to the rhizosphere. The results showed that the activities of CHT, GLU and PAL were significantly higher in the leaves than the roots of the plant treated with PGPR isolates prior to pathogen challenge. Contrary to the

activities of defense enzymes activities in the leaves, POX activity was found to be significantly higher in roots than the leaves even after 36 h of pathogen challenge. Over all *B. altitudinis* followed by *B. pumilus* was found to induce resistance against *T. cucumeris* more effectively than the other two PGPR isolates (Fig. 104).

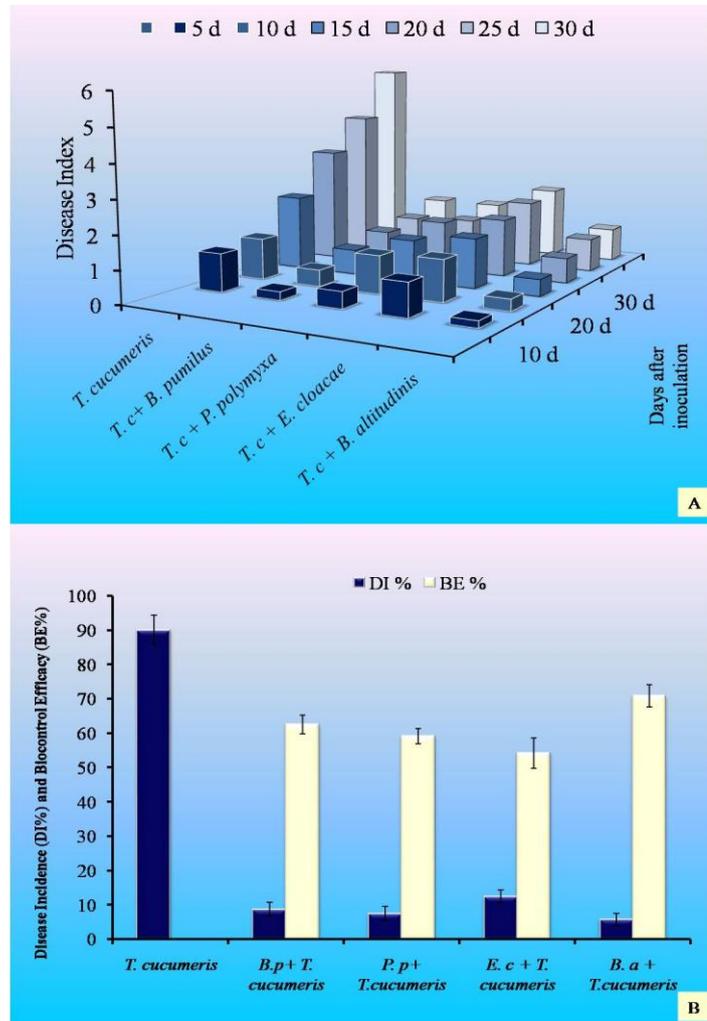


Fig. 100. Development of root rot disease of *Glycine max* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of PGPR isolates in inhibiting root rot disease of *Glycine max* (B).

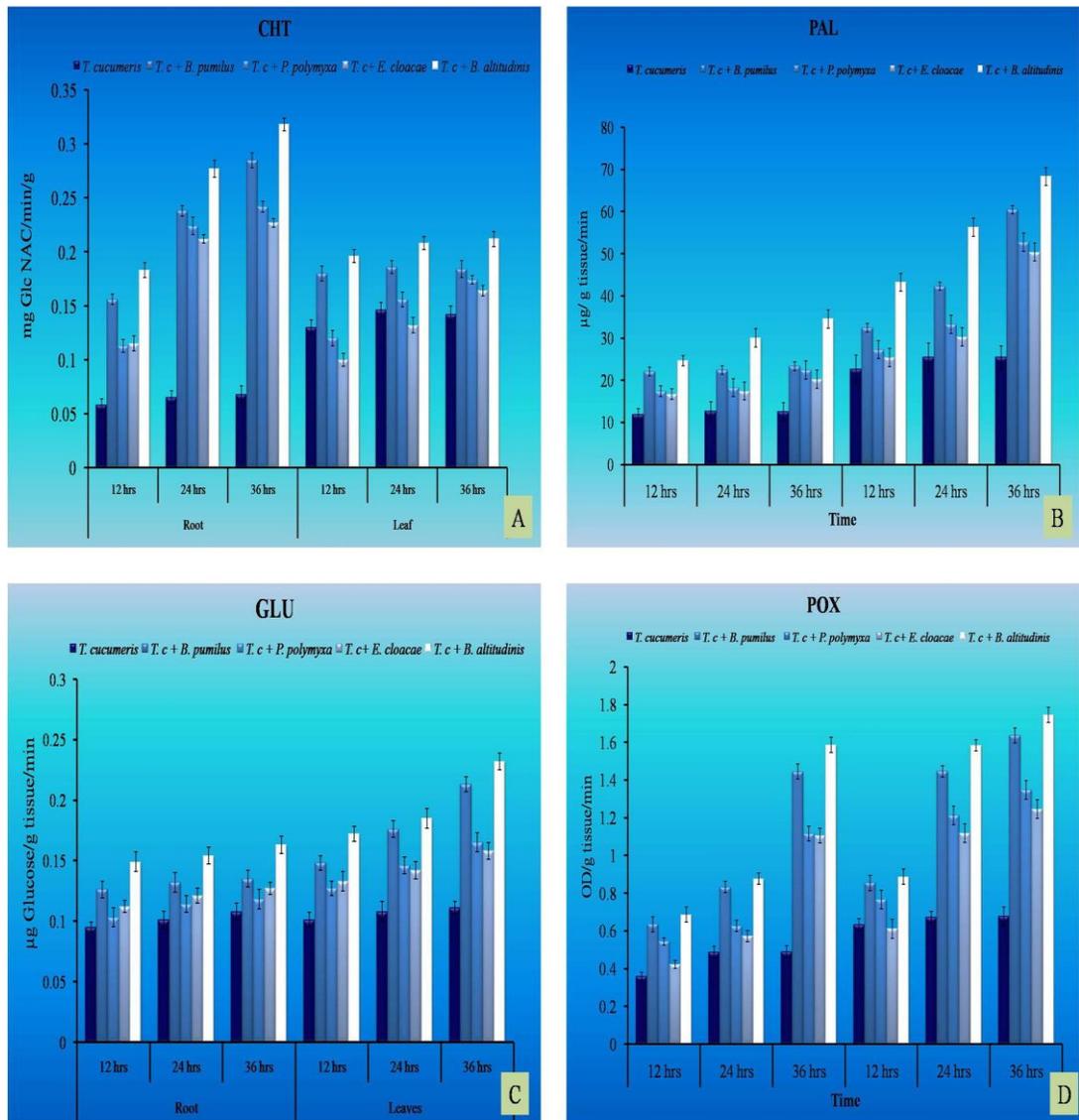


Fig. 101. Defense enzyme activities in the roots and leaves of *Glycine max* following treatment with PGPR isolates and pathogen challenge Chitinase (A), β -1,3 Glucanase (B), Phenylalanine ammonia lyase(C) and peroxidase (D).

4.15.1.1.4. Tissue and cellular location of chitinase enzyme by FITC labeling

Apart from the enzymatic assessment of the defense enzymes through spectrophotometric analysis, an attempt was also made to conduct fluorescent immunocytochemical studies to locate the sites of chitinase (CHT) enzyme expression following treatment with bacterial isolate and pathogen inoculation within the tissue. *B. altitudinis* was found to elicit chitinase enzyme expression more

efficiently than rest of the other isolates, therefore leave, stem and root sections of *B. altitudinis* treated plants were selected for this study. Plant sections treated with homologous antisera (PAb-CHT) and then reacted with FITC developed bright apple green fluorescence that was distributed throughout the leaf, stem and root tissues. Of much significance was the strong reaction of PAb-CHT towards the epidermis and within the vascular bundle of the plants treated with the bacterium which was evident by much brighter fluorescence than the adjoining tissues. Fluorescence of very low intensity was observed in case of control plants (Fig. 102).

4.15.1.2. Inhibition of root rot of *Lycopersicon esculentum* by *Bacillus methylotrophicus*, *Burkholderia symbionts* and *Bacillus aerophilus*

4.15.1.2.1. Disease development and Disease incidence (DE %) and biocontrol efficacy (BE %)

Effect of the three PGPR isolates *B. methylotrophicus* BRHS/P-91, *Burkholderia symbionts* (BRHS/P-92) and *Bacillus aerophilus* (BRHS/B-104) on development of root rot disease of *Lycopersicon esculentum* caused by *Thanatephorus cucumeris* in pot conditions was determined (Fig. 103). Seedlings of *Lycopersicon esculentum* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *T. cucumeris* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of these PGPR isolates as soil drench prior were effective in reducing root rot incidence when applied prior to artificial inoculation of the pathogen. The disease severity increased with time, reaching a maximum of 6 at the end of 30 days. In contrast, when the seeds were pre-treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. Among all the bacterial isolates tested, *B. aerophilus* BRHS/B-104 was found to be most effective when applied singly. However the combined effect of all the isolates was much more effective when all the three isolates were applied jointly. Biocontrol efficacy of *B. methylotrophicus*, *Burkholderia symbiont* and *B. aerophilus* were calculated and has been presented in Table 68 & Fig. 104A. *Bacillus aerophilus* BRHS/B-104 was found to have the highest BE% of 66.50 when applied singly. Whereas the biocontrol efficacy of all the isolates in joint application was found to be 97.15 % (Fig. 104 B).

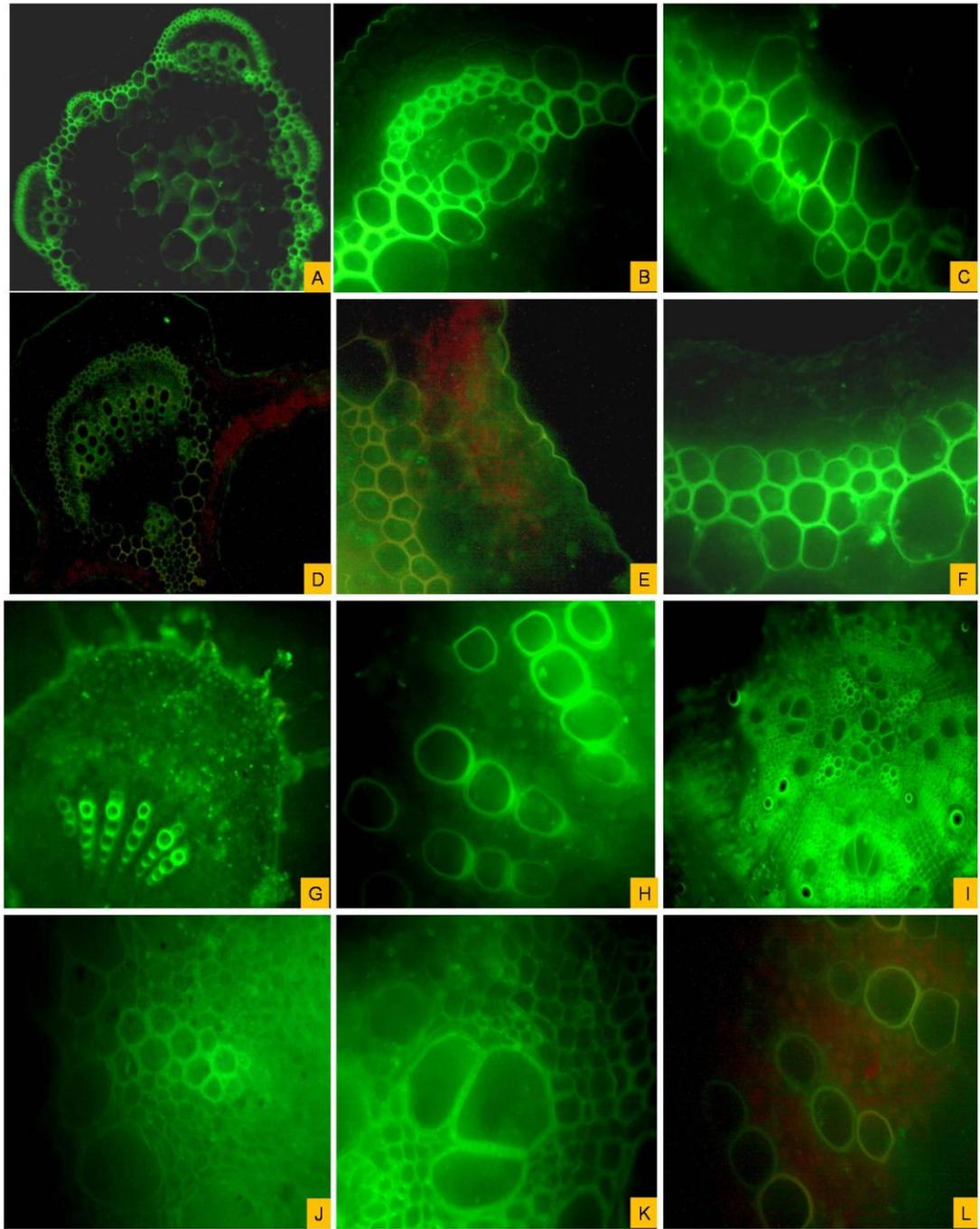


Fig. 102. FITC Labeling of stem, leaf and root tissues of *Glycine max* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73. TS of midrib (A-C); TS of leaf lamina (D-F); TS of stem showing vascular regions (G&H); TS of root showing centrally located xylem, phloem tissues and cortex (I-K); TS of stem of untreated control plant (L).

Table 68. Root rot development in the roots of *Lycopersicon esculentum* in presence and absence of PGPR isolates in pot conditions

Treatemtns	5d	10d	15d	20d	25d	30d
<i>T. c</i>	1.50	2.50	3.75	4.50	5.50	6.00
<i>B. m</i> + <i>Tc</i>	1.15	1.5	1.50	1.75	1.75	2.00
<i>B. s</i> + <i>Tc</i>	1.15	1.25	1.50	1.50	1.50	1.75
<i>B. a</i> + <i>Tc</i>	1.15	1.15	1.15	1.25	1.25	1.50
<i>B. m</i> + <i>B.s</i> + <i>Tc</i>	0.50	0.50	0.75	1.00	1.15	1.15
<i>B.m</i> + <i>B.a</i> + <i>Tc</i>	0.50	0.50	0.75	1.15	1.25	1.25
<i>B.a</i> + <i>B. s</i> + <i>Tc</i>	0.50	0.50	0.50	0.75	1.15	1.15
<i>B. m</i> + <i>B. s</i> + <i>B.a</i> + <i>Tc</i>	0.25	0.25	0.50	0.50	1.00	1.00

T.c= *Thanatephorus cucumeris*, *B. m*= *Bacillus methylotrophicus*, *B. s*= *Burkholderia symbiont*, *B.a*= *Bacillus aerophilus*.

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80% of the root effected 80-85% of the shoot and leaves withered; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.15.1.2.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of Tomato seedlings following application of biocontrol agents and challenge inoculated with the pathogen- *Thanatephorus cucumeris* were determined. The reduction in root rot incidence of *L. esculentum* by pretreatment of soil by PGPR isolates were noted and the conferrence of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes both in the roots and leaves after 12, 24 and 36 h of bacterial application to the rhizosphere. The results showed that the activities of CHT, GLU and PAL were significantly higher in the leaves than the roots of the plant treated with PGPR isolates prior to pathogen challenge. Contrary to the activities of defense enzymes activities in the leaves, PAL activity was found to be significantly higher in roots than the leaves even after 36 h of pathogen challenge. Over all *B. aerophilus* followed by *Burkholderia symbiont* was found to induce resistance against *T. cucumeris* more effectively when applied singly. However the activities of all the tested defense enzymes were found to be maximum when all the three PGPR isolates were applied jointly (Fig. 105).



Fig. 103. Root rot development in *Lycopersicon esculentum* in presence and absence of PGPR isolates in pot conditions.

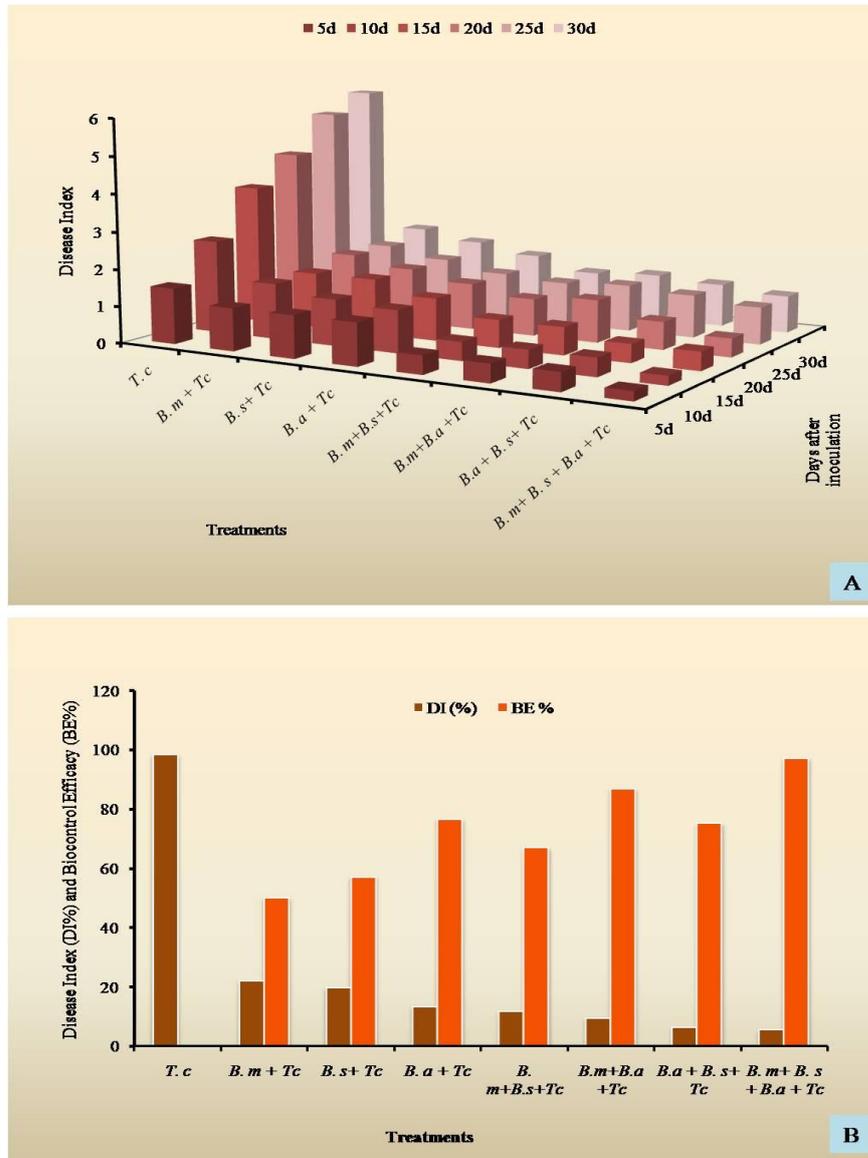


Fig. 104. Development of root rot disease of *Lycopersicon esculentum* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of PGPR isolates in inhibiting root rot disease of *L. esculentum* (B).

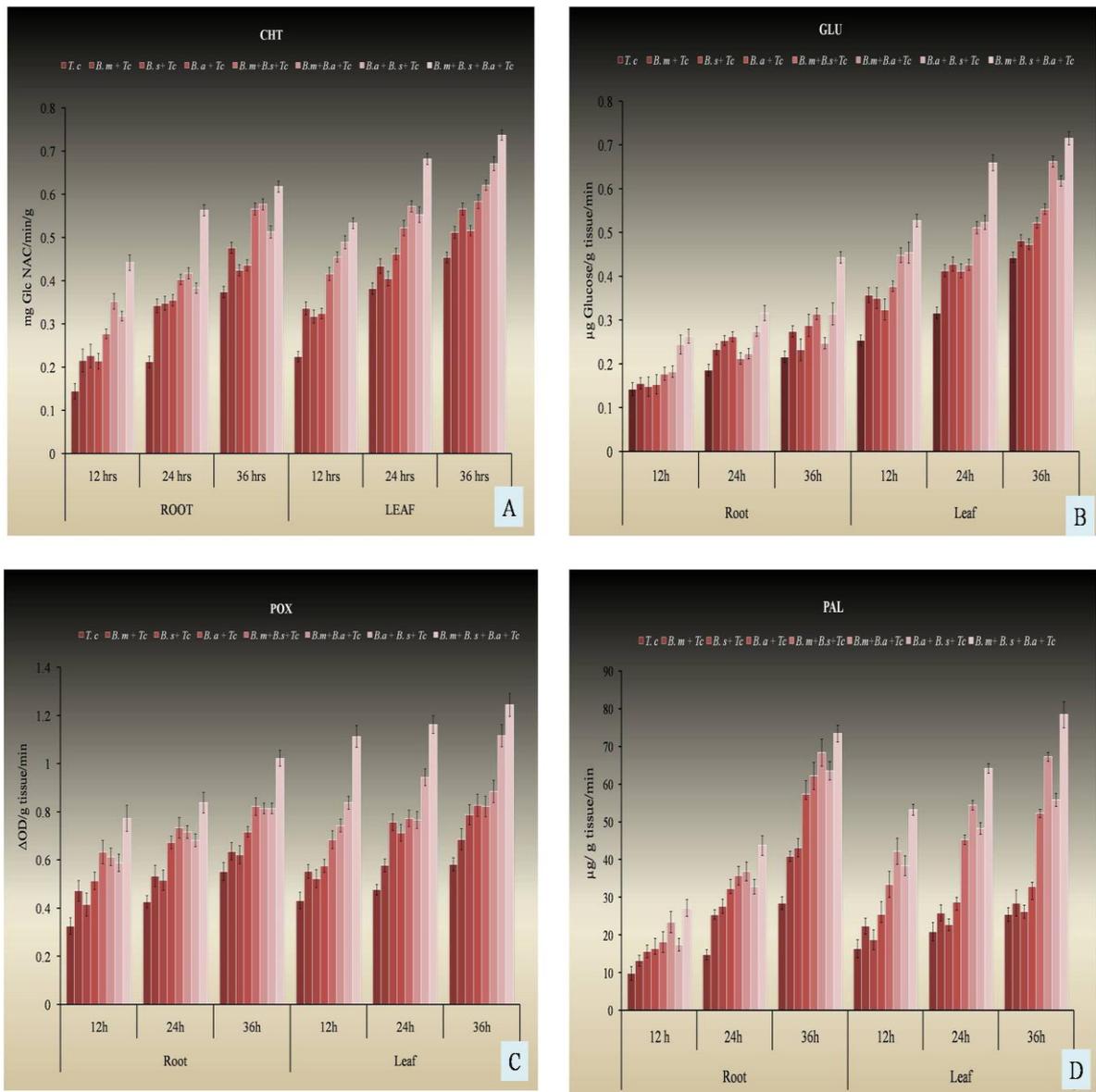


Fig. 105. Defense enzyme activities in the roots and leaves of *Lycopersicon esculentum* following treatment with PGPR isolates and pathogen challenge Chitinase (A), β -1,3 Glucanase (B), Phenylalanine ammonia lyase (C) and peroxidase (D).

4.15.1.3. Inhibition of root rot of *Brassica juncea* by PGPR isolates

4.15.1.3.1. Disease development

Effect of the seven PGPR isolates *Bacillus pumilus*, *Paenibacillus polymyxa*, *Enterobacter cloacae*, *Bacillus altitudinis*, *Bacillus methylotrophicus*, *Burkholderia symbiont* and *Bacillus aerophilus* on development of root rot disease of *Brassica juncea* caused by *Thanatephorus cucumeris* in pot conditions was determined. Seedlings of *B. juncea* were inoculated and disease assessment was done after 2, 4, 6, 8, 10, 12 and 14 days of inoculation with *T. cucumeris* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of these PGPR isolates as soil drench prior were effective in reducing root rot incidence when applied prior to artificial inoculation of the pathogen. The disease severity increased with time, reaching a maximum of 6 at the end of 14 days. In contrast, when the seeds were pre treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. Among all the bacterial isolates tested, *B. pumilus*, *B. altitudinis* and *B. aerophilus* was found to be most effective when applied singly. The disease development between the treated and the control untreated pots were significant however it was not significant among different treatments (Table 69, Fig. 106 A).

4.15.1.3.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *B. juncea* inoculated with only *T. cucumeris* increased with time, reaching a maximum of 86% at the end of 14 d. In contrast, when the soil was pre treated with PGPR isolates the maximum disease severity was only 20%. The lowest severity was noticed when isolate *B. aerophilus* was added prior to pathogen inoculation (5.00 %) followed by *B. altitudinis* (6.66%) and *B. pumilus* (7.66%). Biocontrol efficacy of all the tested PGPR isolates were calculated and has been presented in Fig. 106 B, *B. aerophilus* was found to have the highest BE% (66.16 %).

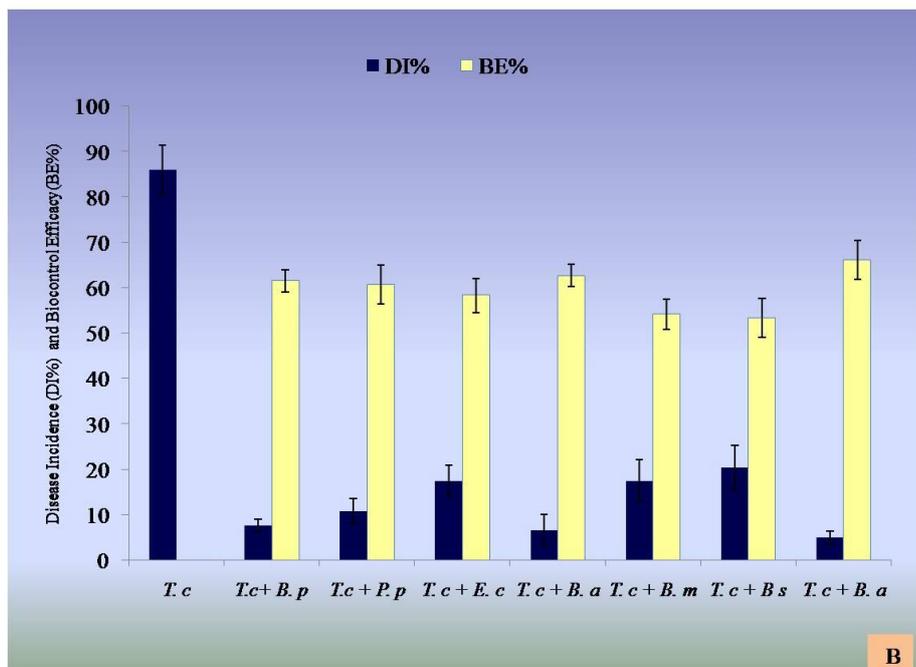
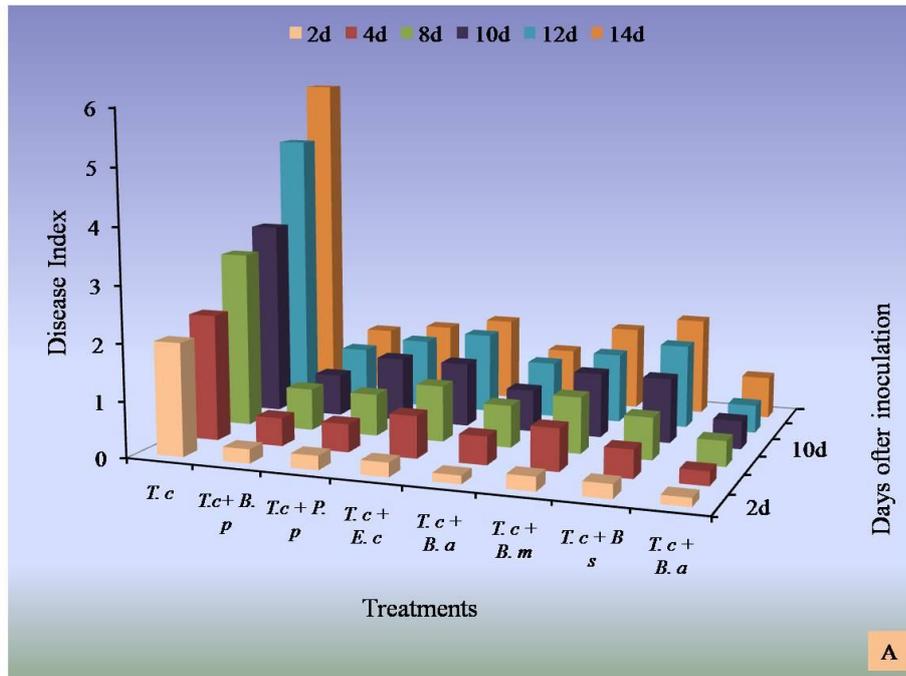


Fig. 106. Development of root rot disease of *B. juncea* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index up to 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of PGPR isolates in inhibiting root rot disease of *B. juncea* (B).

Table 69. Root rot development in the roots of *Brassica juncea* in presence and absence of PGPR isolates in pot conditions

TREATMENTS	Days after Inoculation						
	2d	4d	6d	8d	10d	12d	14d
<i>Thanatephorus cucumeris</i>	2.00	2.25	3.00	3.15	3.50	5.00	6.00
<i>T. cucumeris</i> + <i>Bacillus pumilus</i>	0.25	0.50	0.50	0.75	0.75	1.00	1.15
<i>T. cucumeris</i> + <i>Paenibacillus polymyxa</i>	0.25	0.50	0.50	0.75	1.15	1.25	1.30
<i>T. cucumeris</i> + <i>Enterobacter cloacae</i>	0.25	0.75	1.00	1.00	1.15	1.45	1.50
<i>T. cucumeris</i> + <i>Bacillus altitudinis</i>	0.15	0.50	0.50	0.75	0.75	1.00	1.00
<i>T. cucumeris</i> + <i>B. methylotrophicus</i>	0.25	0.75	0.85	1.00	1.15	1.25	1.50
<i>T. cucumeris</i> + <i>Burkholderia symbiont</i>	0.25	0.50	0.50	0.75	1.15	1.50	1.75
<i>T. cucumeris</i> + <i>Bacillus aerophilus</i>	0.15	0.25	0.35	0.45	0.50	0.50	0.75

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80% of the root effected 80-85% of the shoot and leaves withered; 6-whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

4.15.1.3.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of *B. juncea* seedlings following application of biocontrol agents and challenge inoculated with the pathogen- *Thanatephorus cucumeris* were determined. The reduction in root rot incidence by pretreatment of soil by PGPR isolates were noted and the conferrence of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- CHT, GLU, PAL and POX in the seedlings after 12, 24 and 36 h of bacterial application to the rhizosphere. The results showed that the activities of all the teste enzymes was higher in plants treated with PGPR isolates prior to pathogen challenge. Over all *B. aerophilus* followed by *B. pumilus* and *B. altitudinis* was found to induce resistance against *T. cucumeris* more effectively when applied singly (Fig. 107).

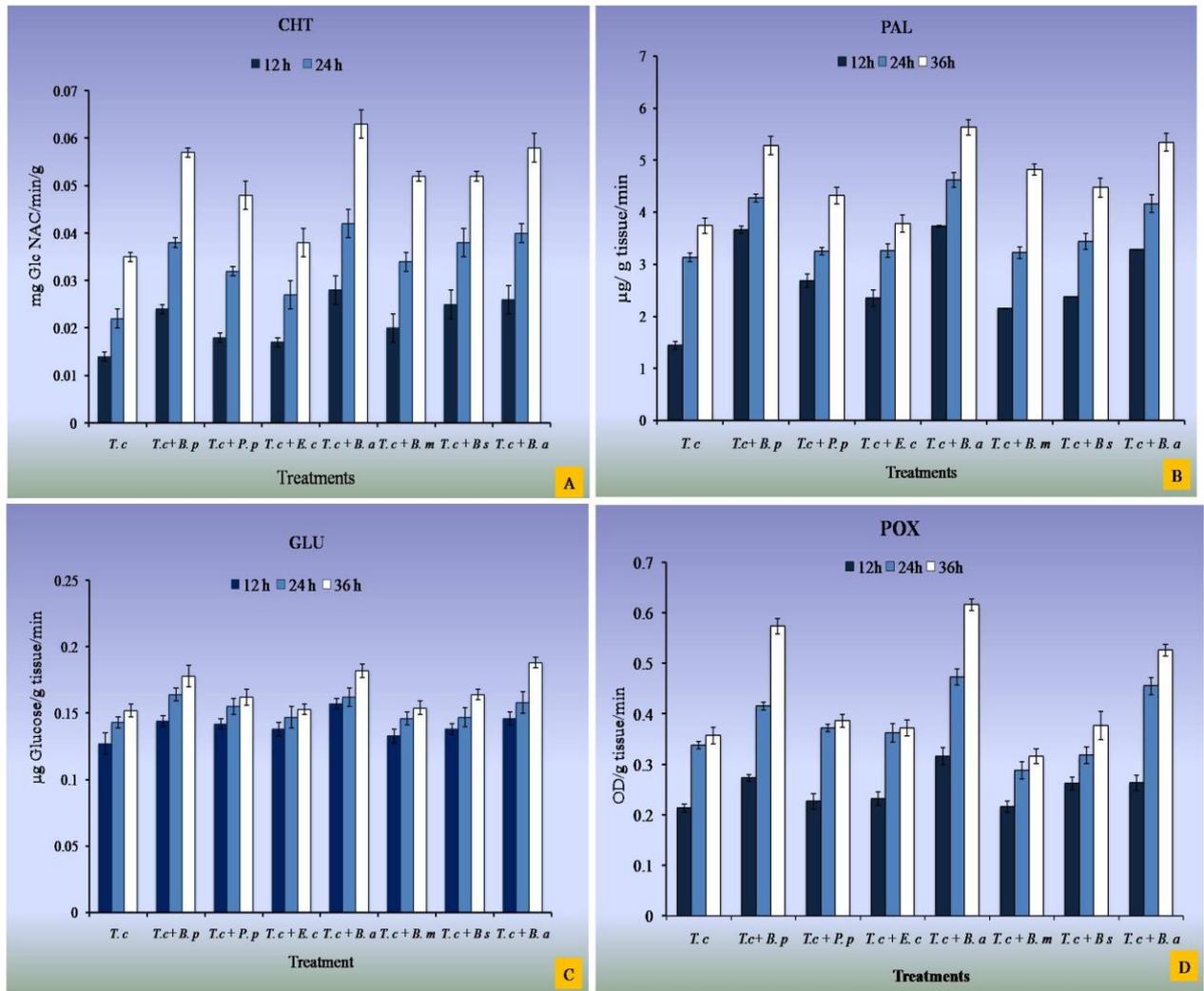


Fig. 107. Defense enzyme activities in the roots and leaves of *B. juncea* following treatment with PGPR isolates and pathogen challenge Chitinase (A), β -1,3 Glucanase (C), Phenylalanine ammonia lyase (B) and peroxidase (D).

4.15.2. Sclerotial blight disease caused by *Sclerotium rolfsii*

4.15.2.1. Inhibition of Sclerotial rot of *Glycine max* by *Bacillus pumilus* and *B. altitudinis*

4.15.2.1.1. Disease development

Effect of the two PGPR isolates *Bacillus pumilus* (BRHS/C-1) and *B. altitudinis* (BRHS/S-73) in development of sclerotial blight disease of *Glycine max* caused by *Sclerotium rolfsii* was determined (Fig. 108). Seedlings of *Glycine max* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *S. rolfsii* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of these both *B. pumilus* and *B. altitudinis* both singly and in combination as soil drench prior were effective in reducing root rot incidence when applied prior to artificial inoculation of the pathogen. The disease severity in *Glycine max* increased with time, reaching a maximum of 6 at the end of 30 days. In contrast, when the seeds were pre treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. Among all the bacterial isolates teste, *B. altitudinis* (BRHS/S-73) was found to be most effective. However the disease severity was much reduced when both the isolates were applied jointly. The disease development between the treated and the control untreated pots were significant however it was not significant among different treatments (Table 70, Fig. 109A).

4.15.2.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *G. max* inoculated with only *S. rolfsii* increased with time, reaching a maximum of 85% at the end of 30 d. In contrast, when the soil was pre treated with PGPR isolates the maximum disease severity was only 14.28 % in case of *B. altitudinis* in single combination and 8% when applied jointly. Biocontrol efficacy of *B. pumilus* and *B. altitudinis* was calculated and has been presented in Fig. 109B.

Table 67. Sclerotial blight development in the roots of *Glycine max* in presence and absence of PGPR isolates in pot conditions

Days after inoculation	<i>S. rolfsii</i>	<i>B. pumilus</i>	<i>B. altitudinis</i>	<i>B. pumilus</i> +
		+ <i>S. rolfsii</i>	+ <i>S. rolfsii</i>	+ <i>S. rolfsii</i>
5d	1.15	0.25	0.25	0.15
10d	1.25	0.5	0.5	0.25
15d	2.25	0.75	0.5	0.5
20d	3.5	1	0.75	0.75
25d	4.5	1.15	1.15	1
30d	6	1.5	1.25	1.15

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80% of the root effected 80-85% of the shoot and leaves withered; 6-whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.15.2.1.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of *Glycine max* following application of biocontrol agents and challenge inoculated with the pathogen- *Sclerotium rolfsii* were determined. The reduction in root rot incidence of *G. max* by pretreatment of soil by PGPR isolates were noted and the conferrence of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU both in the roots and leaves after 12, 24 and 36 h of bacterial application to the rhizosphere. The results showed that the activities of CHT and GLU and were significantly higher in the leaves than the roots of the plant treated with PGPR isolates prior to pathogen challenge. Contrary to the activities of defense enzymes activities in the leaves, PAL and POX activity was found to be significantly higher in roots than the leaves even after 36 h of pathogen challenge. Over all *B. altitudinis* followed was found to induce resistance against *S. rolfsii* more effectively than *B. pumilus* however the defense enzyme activities was much more enhanced when both the isolates were applied jointly (Fig. 110).

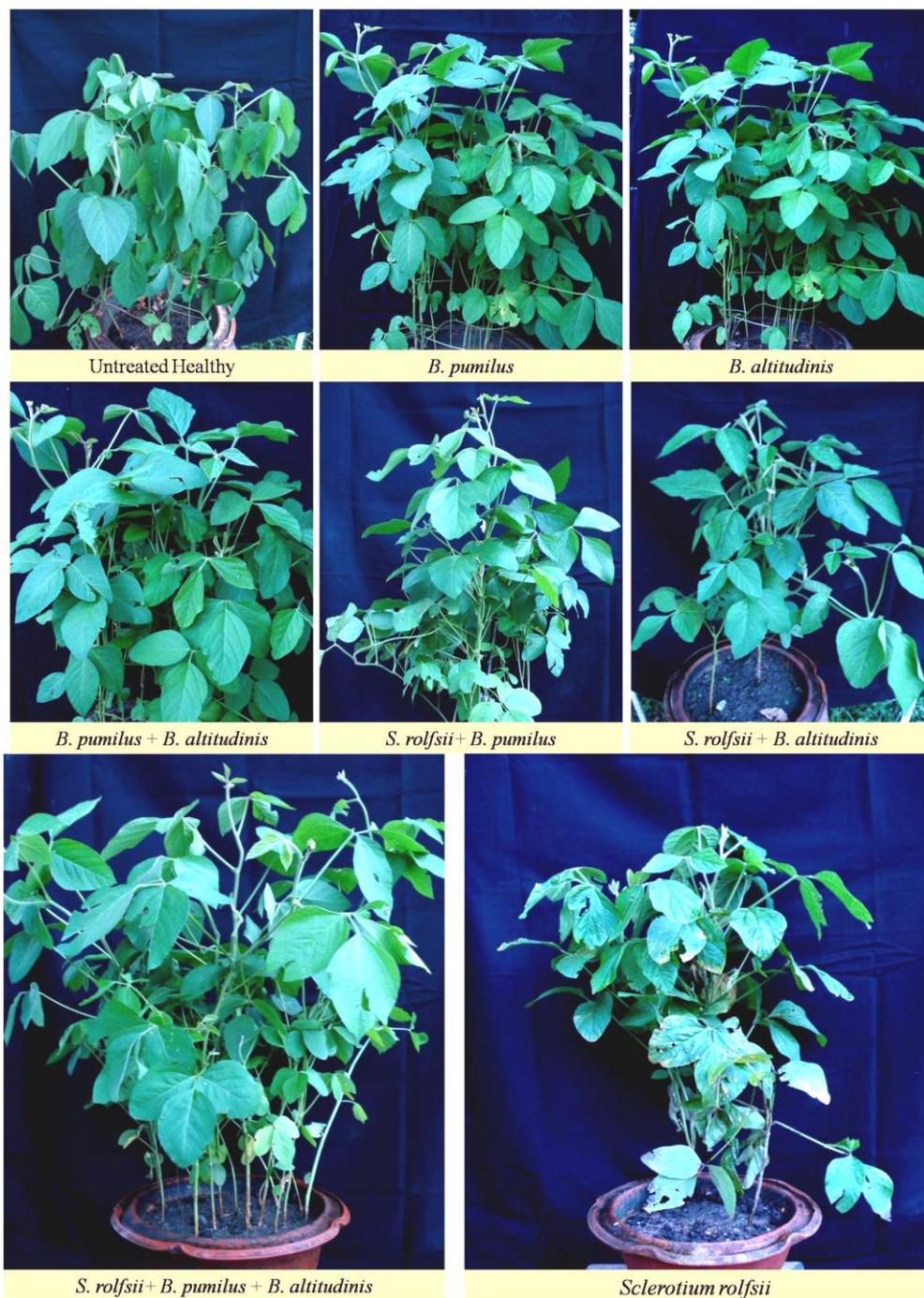


Fig. 108. Sclerotial blight development in *Glycine max* in presence and absence of PGPR isolates in pot conditions.

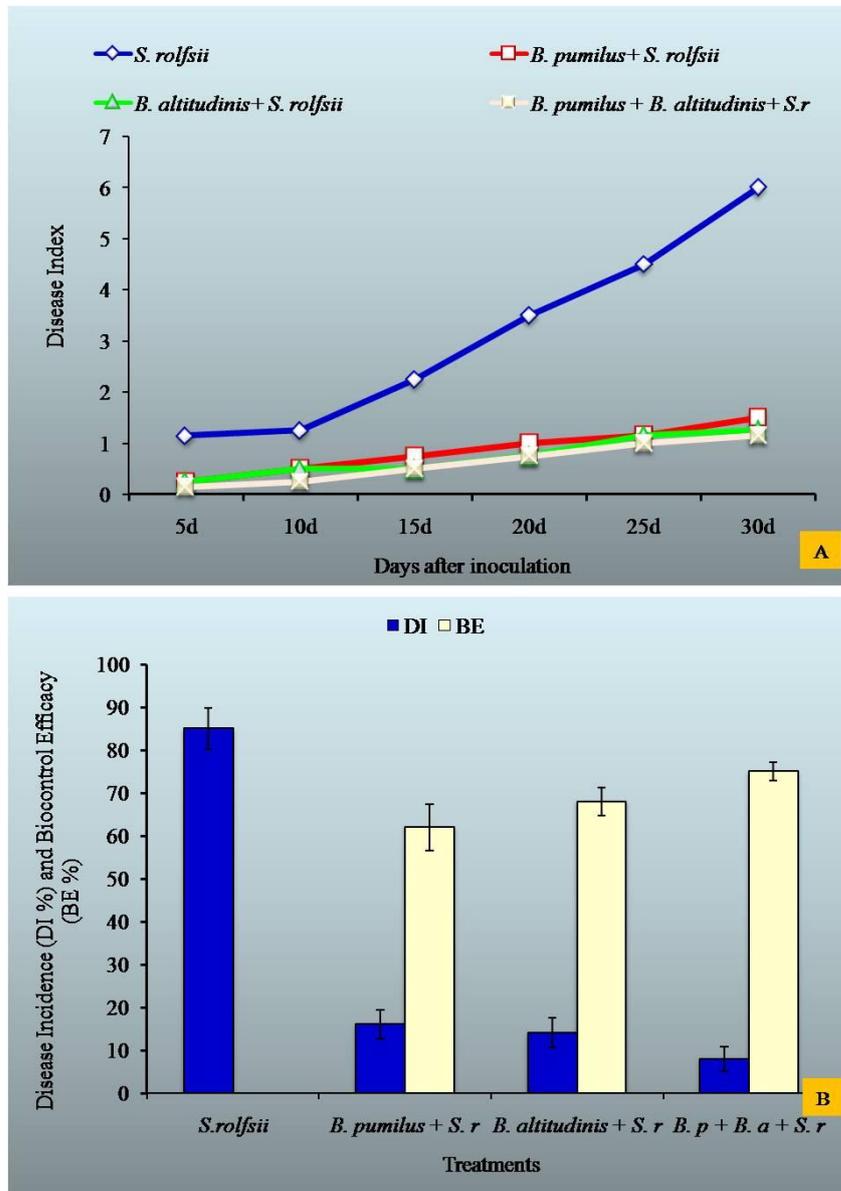


Fig. 109. Development of root rot disease of *G. max* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of *B. pumilus* and *B. altitudinis* isolates in inhibiting Sclerotial blight disease of *G. max* (B).

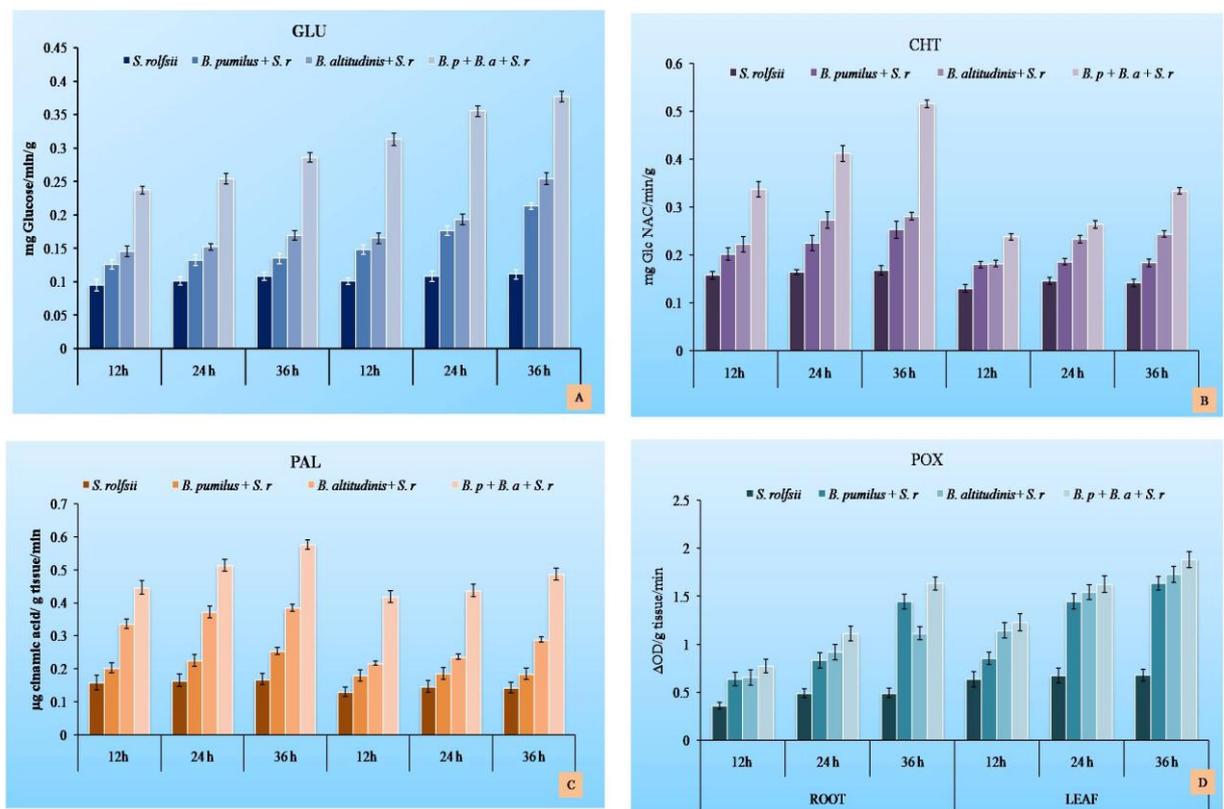


Fig. 110. Defense enzyme activities in the roots and leaves of *Glycine max* following treatment with PGPR isolates and pathogen challenge. β -1,3 Glucanase (A), Chitinase (B), Phenylalanine ammonia lyase (C) and peroxidase (D).

4.15.1.4. Tissue and cellular location of chitinase enzyme by FITC labeling

Apart from the enzymatic assessment of the defense enzymes through spectrophotometric analysis, an attempt was also made to conduct fluorescent immunocytochemical studies to locate the sites of chitinase (CHT) enzyme expression following treatment with bacterial isolate and pathogen inoculation within the tissue. *B. altitudinis* was found to elicit chitinase enzyme expression more efficiently than rest of the other isolates, therefore leave, stem and root sections of *B. altitudinis* treated plants were selected for this study. Plant sections treated with homologous antisera (PAb-CHT) and then reacted with FITC developed bright apple green fluorescence that was distributed towards the epidermis and within the vascular bundle of the plants treated with the bacterium which was evident by much brighter fluorescence than the adjoining tissues. Fluorescence of very low intensity was observed in case of control plants (Fig. 111).

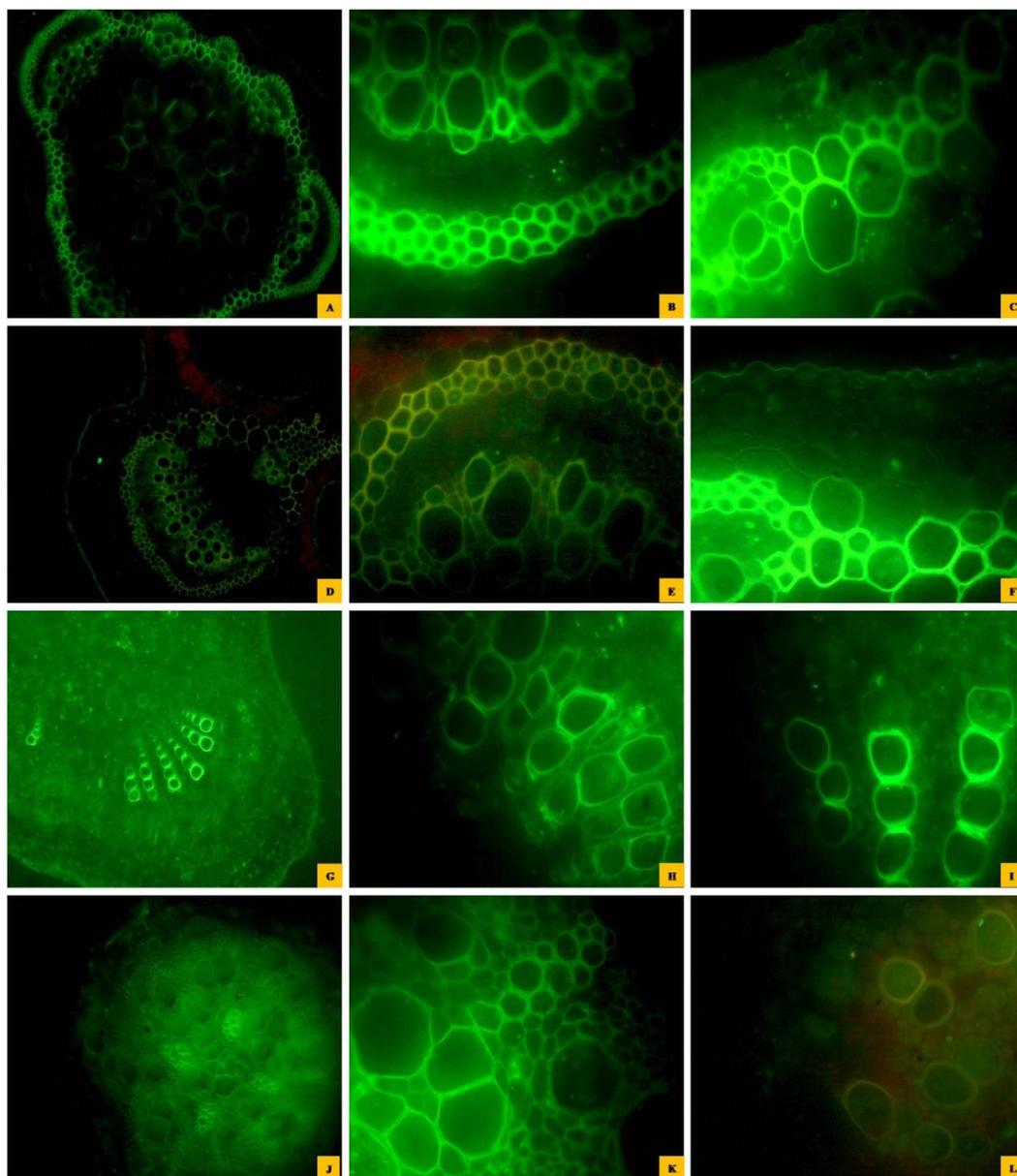


Fig. 111. FITC Labeling of stem, leaf and root tissues of *Glycine max* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73. TS of midrib (A-C); TS of leaf lamina (D-F); TS of stem showing vascular regions (G&H); TS of root showing centrally located xylem, phloem tissues and cortex (I-K); TS of stem of untreated control plant (L).

4.15.2.2. Inhibition of Sclerotial rot of *Vigna radiata* by PGPR isolates

4.15.2.2.1. Disease development

Effect of the two PGPR isolates *Bacillus pumilus*, *Paenibacillus polymyxa*, *Enterobacter cloacae*, *B. altitudinis*, *B. methylotrophicus* and *Burkholderia symbiont* in development of sclerotial blight disease of *Vigna radiata* caused by *Sclerotium rolfsii* was determined (Fig. 112). Seedlings of *Vigna radiata* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *S. rolfsii* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of all the PGPR isolates as soil drench prior to pathogen challenge were effective in reducing Sclerotial blight incidence. The disease severity in *Vigna radiata* increased with time, reaching a maximum of 6 at the end of 30 days. In contrast, when the seeds were pre treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. Among all the bacterial isolates tested, *B. altitudinis* (BRHS/S-73) followed by *B. pumilus* (BRHS/C-1) was found to be most effective. The disease development between the treated and the control untreated pots were significant however it was not significant among different treatments (Table 71, Fig. 113A).

4.15.2.1.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *V. radiata* inoculated with only *S. rolfsii* increased with time, reaching a maximum of 83.45% at the end of 30 d. In contrast, when the soil was pre treated with PGPR isolates the maximum disease severity was reduced to only 8.53 % in case of *B. altitudinis*. Biocontrol efficacy (BE%) of all the PGPR isolates were calculated and has been presented in Fig. 113 B. The results revealed that isolate *B. altitudinis* had highest biocontrol efficacy of 72.18 % followed by *B. pumilus* which showed a biocontrol efficacy of 63.88 % in controlling Sclerotial blight disease of *Vigna radiata*.

Table 71. Sclerotial blight development in the roots of *Vigna radiata* in presence and absence of PGPR isolates in pot conditions

Treatments	Days after inoculation					
	5d	10d	15d	20d	25d	30d
<i>S. rolf sii</i>	1.45	2.5	3.75	4.15	5.5	6
<i>B. pumilus</i> + <i>S. r</i>	1	1.5	1.75	2.15	2.5	2.75
<i>P. polymyxa</i> + <i>S. r</i>	1.15	1.5	2	.2.25	2.75	3
<i>E. cloacae</i> + <i>S. r</i>	1.15	1.75	2.15	2.3	2.45	2.75
<i>B. altitudinis</i> + <i>S. r</i>	1	1.25	1.5	1.75	2	2
<i>B. methylo trophicus</i> + <i>S. r</i>	1.25	1.35	1.5	2.25	2.5	2.5
<i>Burkholderia symbiont</i> + <i>S. r</i>	1.25	1.35	1.75	2	2.15	2.5

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80%of the root effected 80-85% of the shoot and leaves withered; 6-whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.15.2.2.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of *Vigna radiata* following application of biocontrol agents and challenge inoculated with the pathogen- *Sclerotium rolf sii* were determined. The reduction in Sclerotial blight incidence of *V. radiata* by pretreatment of soil by PGPR isolates were noted and the conferrence of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU both in the roots and leaves after 12, 24 and 36 h of bacterial application to the rhizosphere. The results showed that the activity of GLU and PAL were significantly higher in the leaves than the roots of the plant treated with PGPR isolates prior to pathogen challenge. Contrary to the activities of defense enzymes activities in the leaves, CHT and POX activity was found to be significantly higher in roots than the leaves even after 36 h of pathogen challenge. Over all *B. altitudinis* followed by *B. pumilus* was found to induce resistance against *S. rolf sii* more effectively than the other PGPR isolates (Fig. 114 & 115).



Fig. 112. Sclerotial blight development in *Vigna radiata* in presence and absence of PGPR isolates in pot conditions.

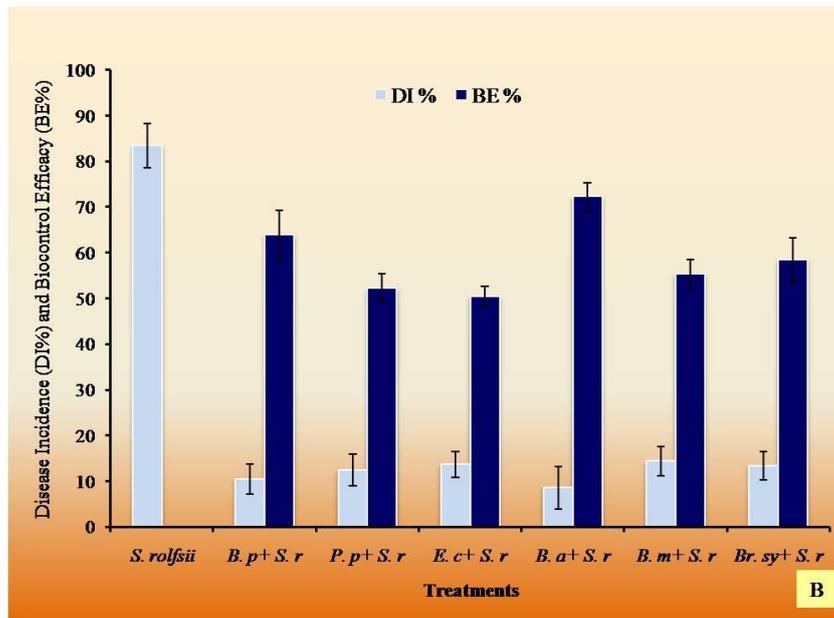
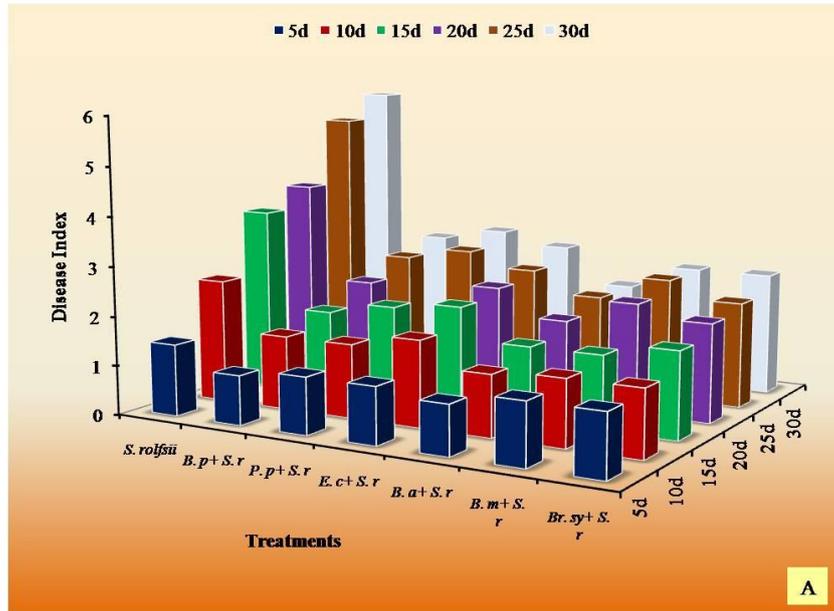


Fig. 113. Development of Sclerotial blight disease of *V. radiata* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of PGPR isolates in inhibiting Sclerotial blight disease of *V. radiata* (B).

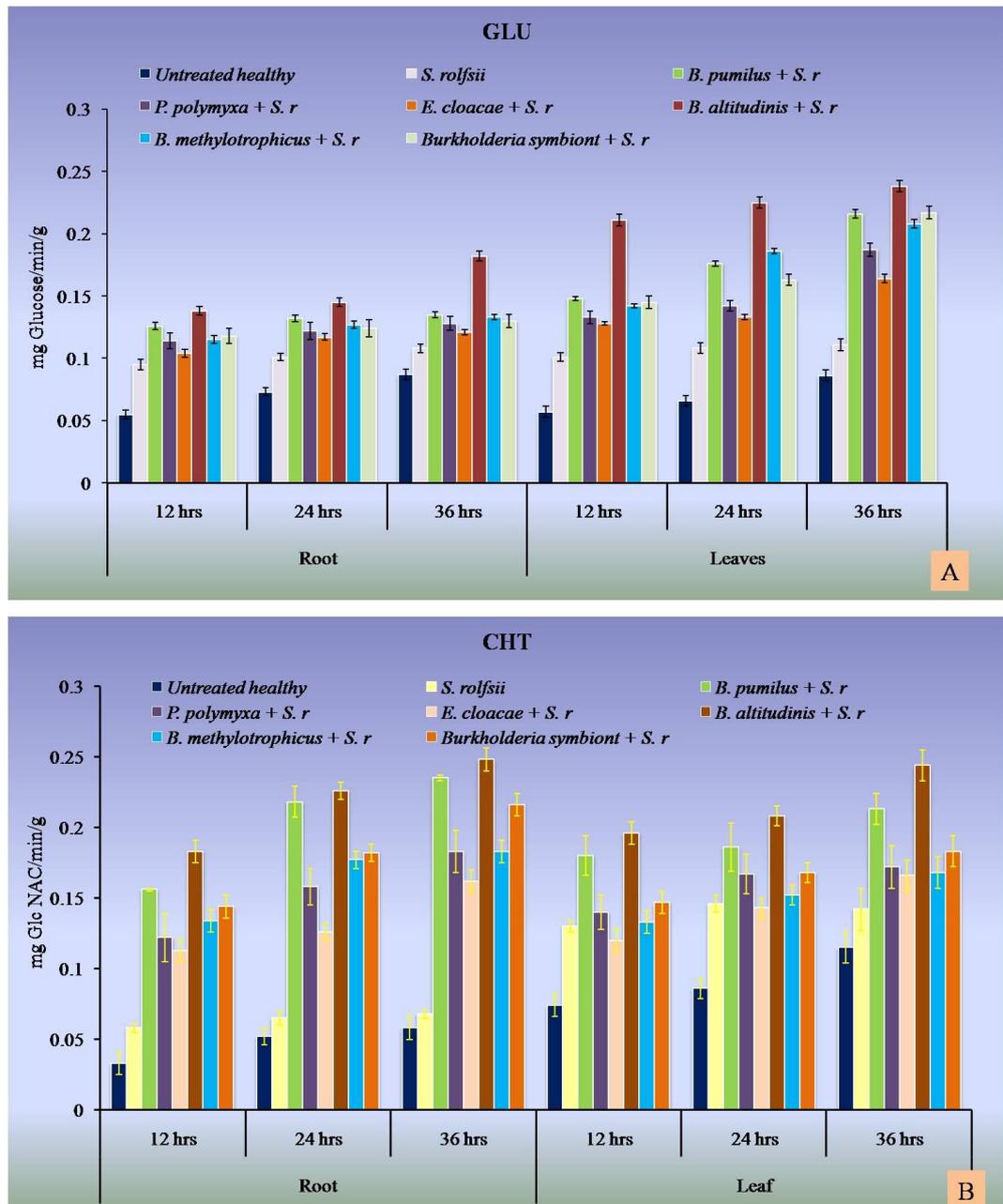


Fig. 114. Defense enzyme activities in the roots and leaves of *Vigna radiata* following treatment with PGPR isolates and pathogen challenge. β -1,3 Glucanase (A), Chitinase (B).

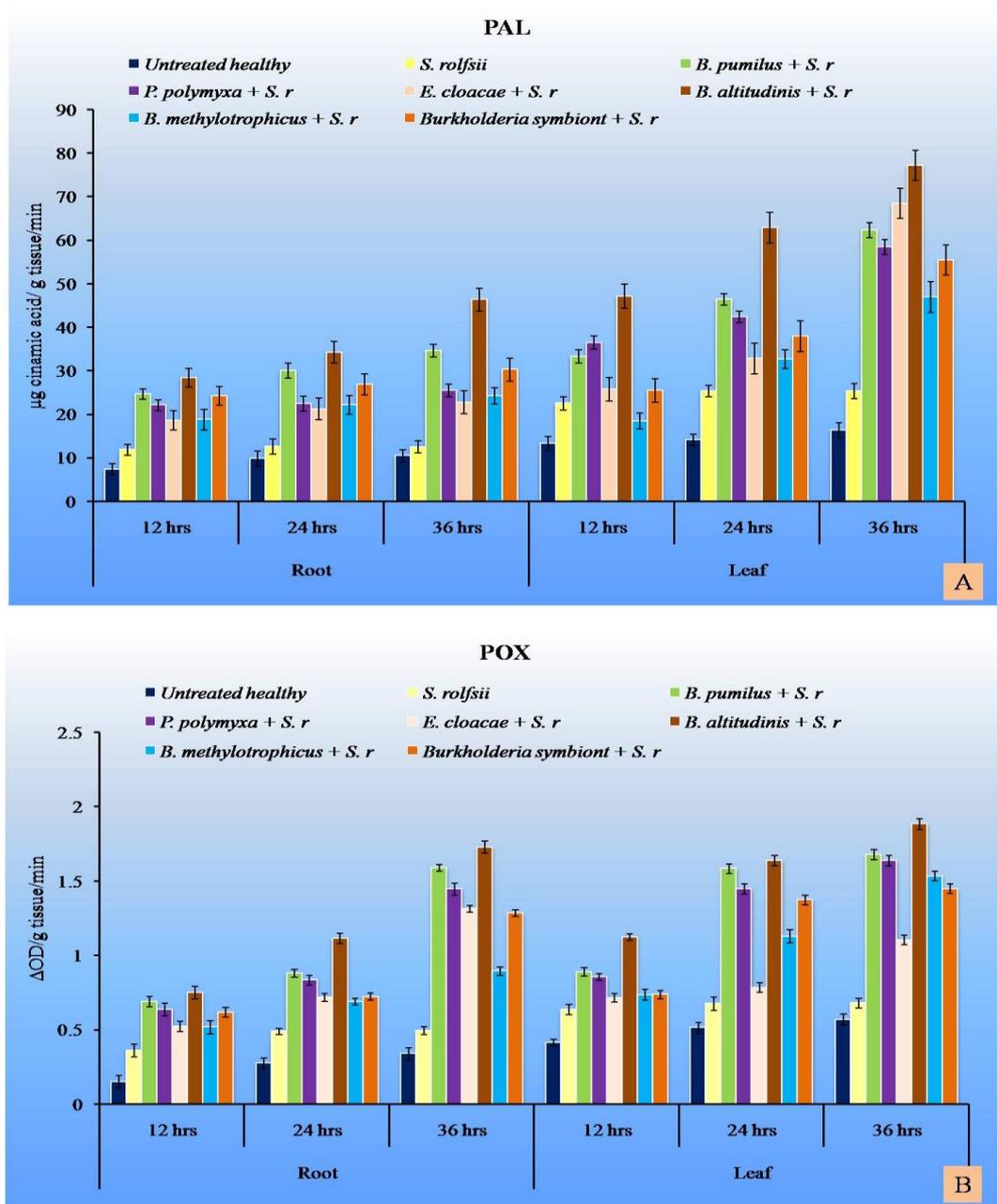


Fig. 115. Defense enzyme activities in the roots and leaves of *Vigna radiata* following treatment with PGPR isolates and pathogen challenge Phenylalanine lyase (A), Peroxidase (B).

4.15.1.4. Tissue and cellular location of chitinase enzyme by FITC labeling

Apart from the enzymatic assessment of the defense enzymes through spectrophotometric analysis, an attempt was also made to conduct fluorescent immunocytochemical studies to locate the sites of chitinase (CHT) enzyme expression following treatment with bacterial isolate and pathogen inoculation within the tissue of *V. radiata*. *B. altitudinis* was found to elicit chitinase enzyme expression more efficiently than rest of the other isolates, therefore leaf, stem and root sections of *B. altitudinis* treated plants were selected for this study. Plant sections treated with homologous antisera (PAb-CHT) and then reacted with FITC developed bright apple green fluorescence that was distributed throughout the leaf, stem and root tissues towards the epidermis and within the vascular bundle of the plants treated with the bacterium. Fluorescence of very low intensity was observed in case of control plants (Fig. 116).

4.15.2.3. Inhibition of Sclerotial rot of *Camellia sinensis* by PGPR isolates

4.15.2.3.1. Disease development

Effect of the two PGPR isolates *Bacillus pumilus* (BRHS/C-1) and *B. altitudinis* (BRHS/S-73), in development of sclerotial blight disease of four varieties of *Camellia sinensis* caused by *Sclerotium rolfsii* in nursery conditions was determined. Seedlings of *Camellia sinensis* were inoculated and disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation with *S. rolfsii* recorded in terms of disease index of the plants challenged by the pathogen. It was observed that application of both the PGPR isolates as soil drench prior to pathogen challenge were effective in reducing Sclerotial blight incidence. The disease severity in the tea seedlings increased with time, reaching a maximum of 5.75 at the end of 30 days. In contrast, when the seeds were pre treated with PGPR isolates followed by soil treatment the disease severity was significantly reduced. All the varieties responded differently towards the application of bacteria. Among all the bacterial isolates tested, *B. altitudinis* (BRHS/S-73) was found to be most effective in reducing Sclerotial blight disease however, the effect was much more greater when the isolates were applied jointly. The disease development between the treated and the control untreated plots were significant however it was not significant among different treatments (Table 72, Fig. 117A&B, 118).

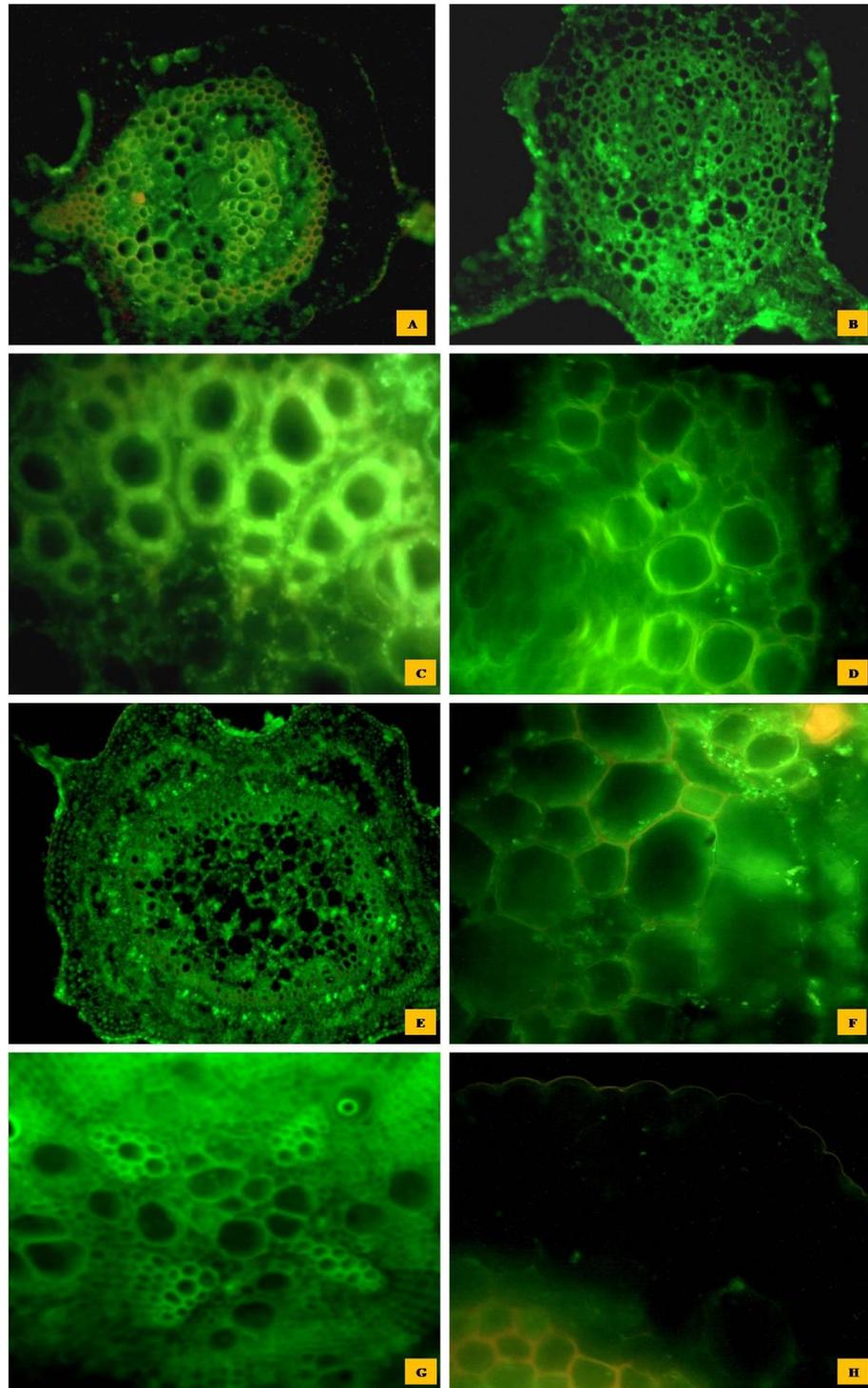


Fig. 116. FITC Labeling of stem, leaf and root tissues of *Vigna radiata* with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73 and pathogen challenge. TS of leaf (A-D); TS of stem showing vascular regions (E-F); TS of root showing centrally located xylem, phloem tissues and cortex (G); TS of leaf of untreated control plant (H).

4.15.2.3.2. Disease incidence (DE %) and biocontrol efficacy (BE %)

The disease severity in *C. sinensis* inoculated with only *S. rolfsii* increased with time, reaching a maximum of 84.46 in case of TV-9, 83.12 in case of TV-20 and an average of 76 % in case of TV-25 and TV-26 at the end of 30 d. In contrast, when the soil was pre treated with PGPR isolates the maximum disease severity was reduced to only 11.36 in case of *B. pumilus* and 14.26 in case of *B. altitudinis* treated tea seedlings. The DI % was reduced to a minimum of 4.33 % when both the isolates were applied jointly. Biocontrol efficacy (BE%) of both the PGPR isolates in reducing Sclerotial blight incidence of tea seedlings were calculated and has been presented in Fig. 117B. The results revealed that isolate *B. pumilus* had highest biocontrol efficacy of 69.26 % when applied singly and 80. 45 % when applied in combination with *B. altitudinis*.

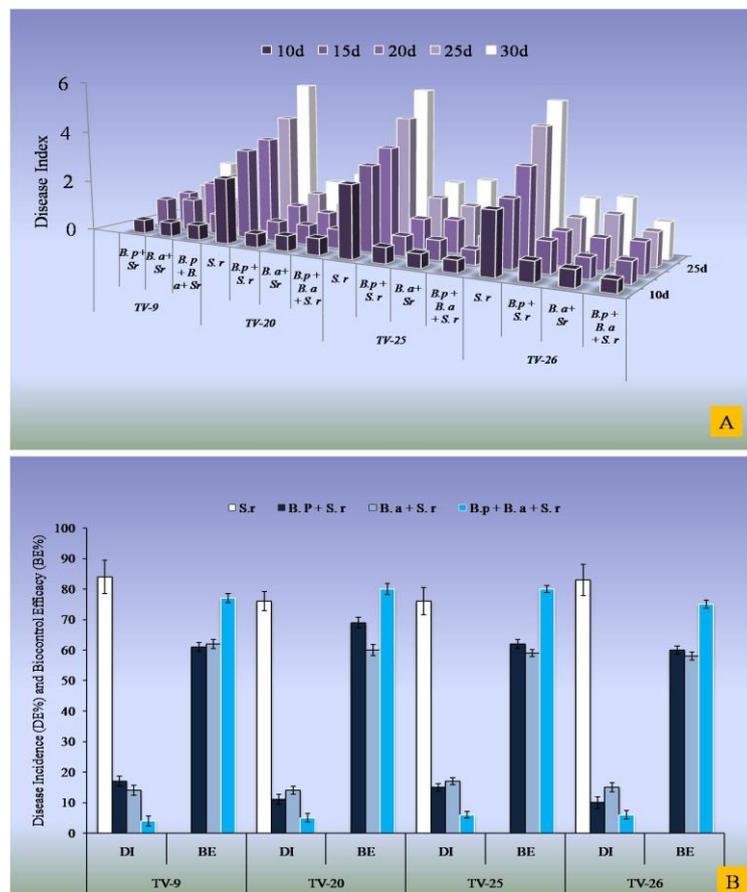


Fig. 117. Development of Sclerotial blight disease of *C. sinensis* in presence and absence of PGPR isolates in pot conditions measured in terms of disease index upto 30 days of pathogen inoculation (A); Disease incidence and biocontrol efficacy of PGPR isolates in inhibiting Sclerotial blight disease of *C. sinensis* (B).

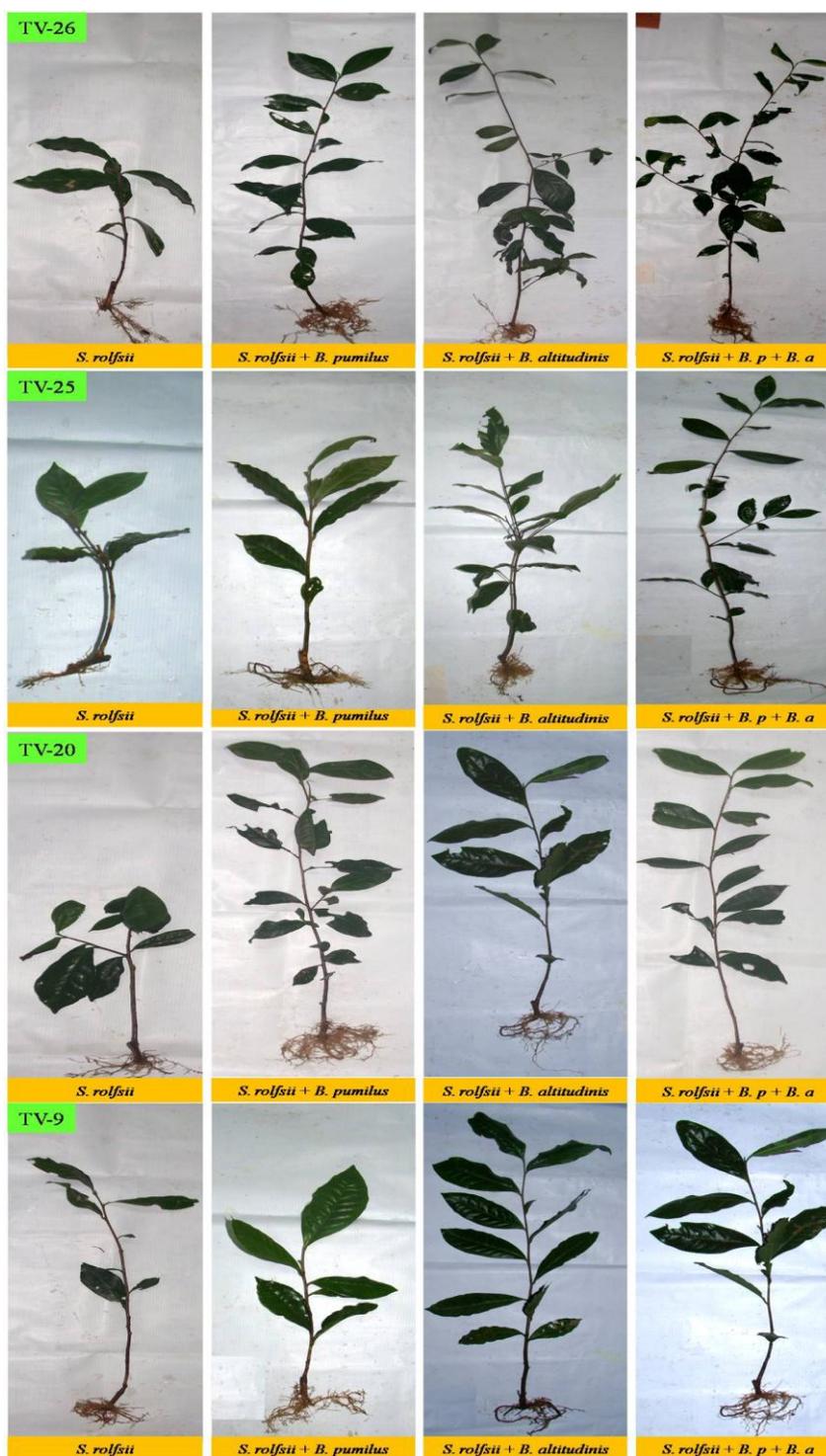


Fig. 118. Sclerotial blight development in *Camellia sinensis* in presence and absence of PGPR isolates in nursery conditions.

Table 72. Disease Index of Sclerotial blight incidence of tea seedling following bacterial treatment and pathogen challenge

Variety	Treatments	Days after inoculation					
		5d	10d	15d	20d	25d	30d
TV-9	UI (<i>S. rolfsii</i>)	1.15	2.16	2.52	3.15	4.45	5.63
		±0.06	±0.08	±0.04	±0.17	±0.23	±0.45
	<i>B. pumilus</i> + <i>Sr</i>	0.51	0.52	1.16	1.23	1.35	2.15
		±0.03	±0.02	±0.07	±0.06	±0.08	±0.05
		0.52	0.55	1.25	1.75	1.86	2.10
<i>B. altitudinis</i> + <i>Sr</i>	±0.04	±0.02	±0.03	±0.05	±0.06	±0.07	
	0.25	0.58	0.78	1.15	1.28	1.25	
TV-20	UI (<i>S. rolfsii</i>)	±0.03	±0.04	±0.07	±0.08	±0.06	±0.08
		1.13	2.53	3.45	3.75	4.50	5.75
	<i>B. pumilus</i> + <i>Sr</i>	±0.08	±0.06	±0.33	±0.13	±0.15	±0.76
		0.50	0.52	0.75	1.15	1.45	1.75
		±0.02	±0.05	±0.02	±0.07	±0.06	±0.04
<i>B. altitudinis</i> + <i>Sr</i>	0.50	0.58	0.75	1.00	1.15	2.15	
	±0.06	±0.03	±0.06	±0.05	±0.04	±0.06	
TV-25	UI (<i>S. rolfsii</i>)	0.26	0.63	0.72	0.75	0.88	1.15
		±0.01	±0.03	±0.04	±0.04	±0.03	±0.08
	<i>B. p + B. a + Sr</i>	1.16	2.75	3.25	3.75	4.75	5.75
		±0.07	±0.08	±0.16	±0.13	±0.11	±0.54
		0.54	0.60	0.75	1.15	1.75	2.15
TV-26	UI (<i>S. rolfsii</i>)	±0.02	±0.04	±0.04	±0.05	±0.07	±0.06
		0.50	0.52	0.75	1.25	1.55	2.35
	<i>B. altitudinis</i> + <i>Sr</i>	±0.01	±0.03	±0.03	±0.04	±0.08	±0.07
		0.35	0.46	0.55	0.76	1.15	1.28
		±0.03	±0.01	±0.03	±0.03	±0.07	±0.08
TV-26	UI (<i>S. rolfsii</i>)	1.23	2.33	2.50	3.46	4.75	5.56
		±0.07	±0.11	±0.13	±0.18	±0.16	±0.32
	<i>B. pumilus</i> + <i>Sr</i>	0.53	0.75	1.16	1.25	1.50	2.00
		±0.04	±0.02	±0.05	±0.06	±0.07	±0.07
		0.52	0.63	0.75	1.15	1.75	2.15
<i>B. altitudinis</i> + <i>Sr</i>	±0.03	±0.03	±0.04	±0.05	±0.07	±0.05	
	0.35	0.46	0.78	1.18	1.27	1.38	
	<i>B. p + B. a + Sr</i>	±0.01	±0.03	±0.03	±0.07	±0.06	±0.06

Disease index- 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80%of the root effected 80-85% of the shoot and leaves withered; 6-whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.15.2.2.3. Biochemical changes

Application of PGPR isolates to soil was found to affect the biochemical responses of plants, biochemical responses of *Camellia sinensis* following application of *B. pumilus* and *B. altitudinis* and challenge inoculated with the pathogen- *Sclerotium rolfsii* were determined. The reduction in Sclerotial blight incidence of *C. sinensis* by pretreatment of soil by PGPR isolates were noted and the conference of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- POX, PAL, CHT and GLU both in the roots and leaves after 5 days of bacterial application and pathogen challenge to the rhizosphere. It was observed that there was significant ($P=0.01$) enhancement of defense enzymes particularly after the pathogen challenge (Fig. 119). The defense enzyme activities were higher in the leaves than the roots. Apart from this total phenol content in the roots were also evaluated, which showed that there was a significant enhancement ($P=0.01$) in the total content of the roots and leaves were higher in those plants which were pre treated with the PGPR isolates and pathogen challenge compared to the untreated control plants (Table 73).

4.15.2.2.4. Tissue and cellular location of chitinase enzyme by FITC labeling

Apart from the enzymatic assessment of the defense enzymes through spectrophotometric analysis, an attempt was also made to conduct fluorescent immunocytochemical studies to locate the sites of chitinase (CHT) enzyme expression following treatment with bacterial isolate and pathogen inoculation within the tissue of *C. sinensis*. Leaf tissue of one of the tea varieties (TV-20) which responded to bacterial challenge better than the other varieties, was used for this study. Plant sections treated with homologous antisera (PAb-CHT) and then reacted with FITC developed bright apple green fluorescence that was distributed throughout the epidermis, mesophyll tissues as well as the vascular tissues. Of much significance was the strong reaction of PAb-CHT towards the epidermis and within the vascular bundle of the plants treated with both the bacterial isolates which were evident by much brighter fluorescence than the adjoining tissues. Fluorescence of very low intensity was observed in case of control plants (Fig. 120).

Table 73. Changes in the Total Phenol content of the roots and leaves of tea seedlings following PGPR application and pathogen challenge

Treatment	Total Phenol (Root) mg/g tissue			
	TV-9	TV- 20	TV-25	TV-26
Untreated Healthy	6.33±0.73	4.58±0.88	5.32±1.77	6.15±0.43
<i>S. rolf sii</i>	7.23±0.88	6.27±0.76	7.88±1.16	8.12±0.45
<i>B. pumilus</i>	8.26±0.46	7.45±1.16	9.16±1.43	9.35±0.33
<i>B. aaltitudinis</i>	7.88±0.73	8.43±0.93	8.83±0.95	9.76±0.37
<i>B. p + S.r</i>	10.32±0.76	10.15±0.94	10.74±0.93	10.82±0.32
<i>B. a +S .r</i>	10.43±0.75	10.26±0.75	10.3±1.43	10.21±0.39
<i>Bp+Ba+Sr</i>	12.76±0.88	13.46±0.73	11.82±1.33	12.46±0.47

Treatment	Total Phenol (Leaves) mg/g tissue			
	TV-9	TV- 20	TV-25	TV-26
Untreated Healthy	0.56±0.077	0.43±0.063	0.57±0.055	0.52±0.044
<i>S. rolf sii</i>	0.88±0.082	1.14±0.078	0.96±0.038	0.98±0.045
<i>B. pumilus</i>	1.27±0.046	1.28±0.077	1.33±0.072	1.34±0.057
<i>B. aaltitudinis</i>	1.20±0.083	1.25±0.082	1.26±0.083	1.28±0.043
<i>B. p + S.r</i>	1.54±0.055	1.46±0.074	1.33±0.074	1.38±0.043
<i>B. a +S .r</i>	1.48±0.046	1.33±0.077	1.28±0.082	1.34±0.022
<i>Bp+Ba+Sr</i>	1.73±0.058	1.83±0.062	1.82±0.062	1.76±0.036

Values are average of three replicate experiments; ±= Standard Error

Table 73a ANOVA of the data presented in Table 73, Total Phenol content(Roots)

Source of Variation	SS	df	MS	F
Rows	126.1798	6	21.02996	53.56029
Columns	2.860039	3	0.953346	2.428037
Error	7.067536	18	0.392641	
Total	136.1073	27		

Table 73b. ANOVA of the data presented in Table 73, Total Phenol content(Leaves)

Source of Variation	SS	df	MS	F
Rows	3.723386	6	0.620564	104.2965
Columns	0.002325	3	0.000775	0.130252
Error	0.1071	18	0.00595	
Total	3.832811	27		

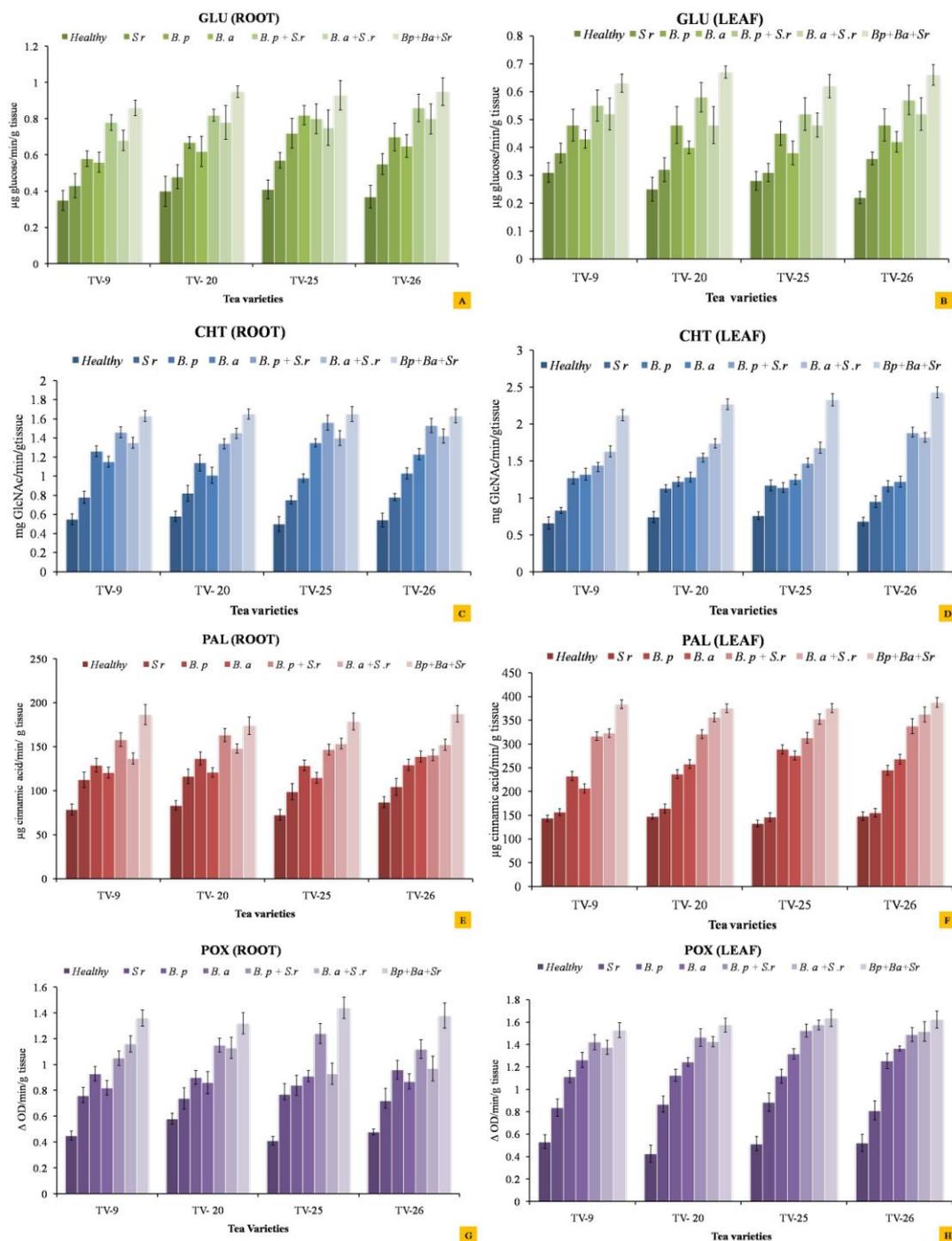


Fig. 119. Defense enzyme activities in the roots and leaves of *C. sinensis* following treatment with PGPR isolates and pathogen challenge β -1,3 Glucanase (A&B), Chitinase (B&C), Phenylalanine ammonia-lyase (D&E) and Peroxidase (F & G).

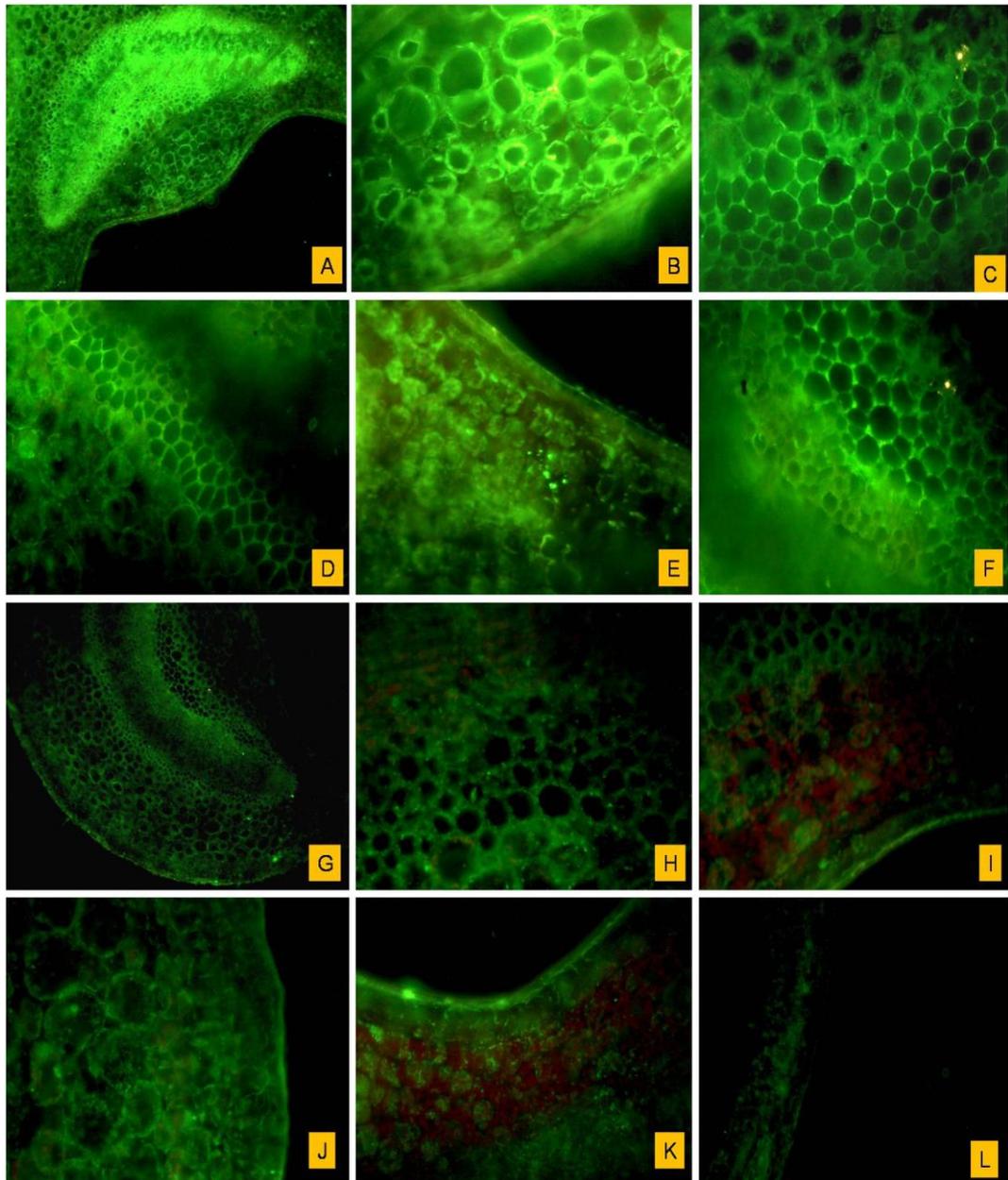


Fig. 120. FITC Labeling of leaf tissues of *Camellia sinensis* (TV-20) with PAb of Chitinase enzyme after treatment with *B. altitudinis* BRHS/S-73 and *B. pumilus* and pathogen challenge. TS of leaf treated with *B. pumilus* (A-F), TS of leaf treated with *B. altitudinis* (G-K); TS of leaf of untreated control plant (L).

4.16. Testing for survivability of pathogens *S. rolfsii* and *T. cucumeris* using Immunological formats

The survivability of the pathogen in the rhizosphere soil of test crops both in potted soils and nursery soils were determined immunologically using PABs raised against the pathogens *T. cucumeris* and *S. rolfsii*. Dot immune binding assay (DIBA) and Enzyme linked immune sorbent assay (ELISA) were conducted after 30 days of pathogen inoculation and treatment with either. Fungal biocontrol agent or PGPR isolates.

Table 74. ELISA and DIBA values of rhizosphere soil antigens inoculated with the pathogen and BCA and PGPR isolates with PAB of *Sclerotium rolfsii*

Antigens from rhizosphere of	Treatment	ELISA A ₄₀₅ values*	DIBA Colour intensity of dots **
<i>Vigna radiata</i>	<i>S. rolfsii</i>	1.733±0.042	+++
	<i>T. harzianum</i> + <i>S. rolfsii</i>	0.336±0.053	+
	<i>T. flavus</i> + <i>S. rolfsii</i>	0.383±0.066	+
	<i>T. h</i> + <i>T. f</i> + <i>S. r</i>	0.244±0.036	+
<i>Glucine max</i>	<i>S. rolfsii</i>	1.887±0.055	+++
	<i>B. pumilus</i> + <i>S. rolfsii</i>	0.316±0.038	+
	<i>B. altitudinis</i> + <i>S. rolfsii</i>	0.362±0.02	+
	<i>B. p</i> + <i>B. a</i> + <i>S. r</i>	0.211±0.027	-
	<i>Sclerotium rolfsii</i>	1.884±0.063	+++
<i>Vigna radiata</i>	<i>B. pumilus</i> + <i>S. rolfsii</i>	0.322±0.021	+
	<i>P. polymyxa</i> + <i>S. rolfsii</i>	0.277±0.033	+
	<i>E. cloacae</i> + <i>S. rolfsii</i>	0.383±0.036	+
	<i>B. altitudinis</i> + <i>S. rolfsii</i>	0.236±0.028	+
	<i>B. methylotrophicus</i> + <i>S. r</i>	0.388±0.026	+
	<i>Burkholderia symbiont</i> + <i>S. r</i>	0.317±0.033	-

*Average of three replicates; Antigen concentration- 100 µg/ml; PAB dilution 1: 100; Alkaline phosphatase dilution 1:10,000; Substrate NBT/BCIP

**+= Light purple; +++ Purple; +++ = deep purple.

Difference between ELISA values of healthy uninoculated and treated are significant at P=0.01 as determined by Student's *t* test in all cases

Table 75. ELISA and DIBA values of rhizosphere soil antigens inoculated with the pathogen and PGPR with PAb of *S. rolfsii*

Antigens from rhizosphere of	Treatment	ELISA A ₄₀₅ values*	DIBA Colour intensity of dots **
TV-9	Healthy	0.246±0.063	+
	<i>S. rolfsii</i>	1.820±0.043	+++
	<i>B. pumilus</i> + <i>S. r</i>	0.214±0.082	+
	<i>B. altitudinis</i> + <i>S. r</i>	0.242±0.064	++
	<i>B. p</i> + <i>B. a</i> + <i>S. r</i>	0.115±0.028	+
TV-20	Healthy	0.353±0.043	+
	<i>S. rolfsii</i>	1.760±0.072	+++
	<i>B. pumilus</i> + <i>S. r</i>	0.178±0.055	+
	<i>B. altitudinis</i> + <i>S. r</i>	0.165±0.073	+
	<i>B. p</i> + <i>B. a</i> + <i>S. r</i>	0.103±0.033	+
TV-25	Healthy	0.162±0.042	+
	<i>S. rolfsii</i>	1.640±0.072	+++
	<i>B. pumilus</i> + <i>S. r</i>	0.260±0.041	++
	<i>B. altitudinis</i> + <i>S. r</i>	0.276±0.083	++
	<i>B. p</i> + <i>B. a</i> + <i>S. r</i>	0.110±0.043	+
TV-26	Healthy	0.242±0.032	+
	<i>S. rolfsii</i>	1.650±0.056	+++
	<i>B. pumilus</i> + <i>S. r</i>	0.182±0.042	+
	<i>B. altitudinis</i> + <i>S. r</i>	0.216±0.060	+
	<i>B. p</i> + <i>B. a</i> + <i>S. r</i>	0.106±0.036	+

*Average of three replicates; Antigen concentration- 100 µg/ml
PAb dilution 1: 100; Alkaline phosphatase dilution 1:10,000;
Substrate NBT/BCIP

**+= Light purple; +++ Purple; +++ = deep purple.

Difference between ELISA values of healthy uninoculated and treated are significant at P=0.01 as determined by Student's *t* test in all cases

Table 76. ELISA and DIBA values of rhizosphere soil antigens inoculated with the pathogen and BCA and PGPR isolates with PAb of *Thanatephorus cucumeris*

Antigens from rhizosphere of	Treatment	ELISA A ₄₀₅ values*	DIBA Colour intensity of dots **
<i>Glycine max</i>	<i>T. cucumeris</i>	1.883±0.077	+++
	<i>T. cucumeris</i> + <i>B. pumilus</i>	0.376±0.058	+
	<i>T. cucumeris</i> + <i>P. polymyxa</i>	0.312±0.047	+
	<i>T. cucumeris</i> + <i>E. cloacae</i>	0.487±0.048	+
	<i>T. cucumeris</i> + <i>B. altitudinis</i>	0.411±0.055	+
<i>Lycopersicon esculentum</i>	<i>T. cucumeris</i>	1.766±0.083	+++
	<i>B. methylotrophicus</i> + <i>Tc</i>	0.337±0.037	+
	<i>Burkholderia symbiont</i> + <i>Tc</i>	0.382±0.042	+
	<i>Bacillus altitudinis</i> + <i>Tc</i>	0.344±0.048	+
	<i>B. m</i> + <i>B.s</i> + <i>Tc</i>	0.318±0.055	+
	<i>B.m</i> + <i>B.a</i> + <i>Tc</i>	0.326±0.037	+
	<i>B.a</i> + <i>B. s</i> + <i>Tc</i>	0.324±0.028	+
	<i>B. m</i> + <i>B. s</i> + <i>B.a</i> + <i>Tc</i>	0.310±0.022	-
<i>Brassica juncea</i>	<i>Thanatephorus cucumeris</i>	1.785 ±0.086	+++
	<i>Bacillus pumilus</i> + <i>T. c</i>	0.224±0.058	+
	<i>Paenibacillus</i> + <i>T. c polymyxa</i>	0.232±0.055	+
	<i>Enterobacter cloacae</i> + <i>T. c</i>	0.487±0.067	+
	<i>Bacillus altitudinis</i> + <i>T. c</i>	0.311±0.066	+
	<i>B. methylotrophicus</i> + <i>T. c</i>	0.353±0.054	+
	<i>Burkholderia symbiont</i> + <i>T. c</i>	0.482±0.057	+
	<i>Bacillus aerophilus</i> + <i>T. c</i>	0.316±0.048	+

*Average of three replicates; Antigen concentration- 100 µg/ml PAb dilution 1: 100; Alkaline phosphatase dilution 1:10,000; Substrate NBT/BCIP

**+= Light purple; +++ Purple; +++ = deep purple.

Difference between ELISA values of healthy uninoculated and treated are significant at P=0.01 as determined by Student's *t* test in all cases

4.16.1. Testing for survivability of *Sclerotium rolfsii* in soils treated with Biocontrol fungal isolates and PGPR.

Survivability of *Sclerotium rolfsii* in the soil pretreated with fungal biocontrol agents *T. harzianum* and *Talaromyces flavus* obtained from the rhizosphere of *Vigna radiata* was tested with the help of DIBA and ELISA formats with the PABs of *S. rolfsii*. Results presented in Table 74, shows that soils infested with pathogen without any biocontrol agent pretreatment showed maximum intensity of the dots and higher ELISA values. Though there was a significant reduction ($P=0.01$) of pathogen where *T. harzianum* and *T. flavus* were applied singly, the ELISA values of the soil pretreated by both *T. harzianum* and *T. flavus* jointly was minimum. The rhizosphere soil of *Glycine max* and *Vigna radiata* pretreated with PGPR isolates also showed reduction in pathogen population significantly (Table 74).

Similarly, the survivability of the pathogen in the rhizosphere soil of tea seedlings in untreated and treated soils was determined immunologically using PABs raised against *S. rolfsii*. DIBA and ELISA were conducted after 30d of pathogen inoculation. Results revealed that application of both the PGPRs in the rhizosphere significantly reduced the pathogen population in the rhizosphere soil of all the tested tea varieties (Table 75).

4.16.2. Testing for survivability of *Thanatephorus cucumeris* in soils treated with Biocontrol fungal isolates and PGPR.

Survivability of *Thanatephorus cucumeris* in the soil pretreated with different bacterial PGPR agents and inoculated in the rhizosphere soil of *Glycine max*, *Lycopersicon esculentum* and *Brassica juncea* was tested with the help of DIBA and ELISA formats with PABs of *T. cucumeris*. Results presented in Table 76, shows that soils infested with pathogen without any PGPR isolate pretreatment showed maximum intensity of the dots and higher ELISA values. Though there was a significant reduction ($P=0.01$) of pathogen when the PGPR isolates were applied singly, the ELISA values of the soil jointly pretreated by PGPR was minimum (Table 76).

DISCUSSION

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem and it is considered a storehouse of microbial activity. Soil microorganisms play an important role in soil processes that determine plant productivity. Diverse microorganisms are essential to a sustainable biosphere. Role of rhizosphere microbial populations for maintenance of root health, nutrient uptake and tolerance of environmental stress is now recognized. For much of the last century, microbiologists have been aware that we know the nature and identity of only a tiny fraction of the inhabitants of the microscopic landscape. While most people are very familiar with the diversity of life in the plant and animal kingdoms, few actually realize the vast amounts of variability present in the microbial populations. The current inventory of the world's biodiversity is very incomplete and that of viruses, microorganisms and invertebrates is especially deficient. Scientists have identified about 1.7 million living species on our planet. Studies indicate that the 5,000 identified species of prokaryotes represent only 1 to 10% of all microbial species and therefore we have only a small idea of our true microbial diversity (Stanley, 2002).

Soil microflora plays the most important role in the soil region of the higher plants. Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation; decomposition of organic matter; toxin removal; and the cycling of carbon, nitrogen, phosphorus, and sulphur. In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth, and changes in vegetation.

In this connection Agriculturally Important Microorganisms (AIMs) are used in a variety of agro-ecosystems both under natural conditions and artificial inoculation for diverse application such as nutrient supply, biocontrol, bioremediation and rehabilitation of degraded lands (Vessey 2003). Soil bacteria and fungi play pivotal roles in various biochemical cycles (BGC) and are responsible for the cycling of organic compounds. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition, plant health, soil structure and soil fertility.

The use of biological fertilizers in recent times, is receiving attention mainly on account of increased global preference for natural “organic” products. Isolation of microorganisms, screening for desirable characters, selection of efficient strains, production of inoculum and preparation of carrier-based formulation are important steps in the use of this microbe based environment friendly and sustainable technology (Harish 2009). A microbial inoculant containing many kinds of naturally occurring beneficial microbes called ‘Effective Microorganisms’ has been used widely in nature and organic farming (Karthick *et al.*, 2011). Biofertilizer and biopesticide containing efficient microorganisms, improve plant growth in many ways compared to synthetic fertilizers, insecticides and pesticides by way of enhancing crop growth and thus help in sustainability of environment and crop productivity (Bhattacharya and Jha, 2012). Thus the technique is environment friendly and ensures safe and healthy agricultural products. Microbial populations are instrumental to fundamental processes that drive stability and productivity of agro-ecosystems (Singh *et al.*, 2011).

Keeping in view the recent developments made so far in the field of studying soil microflora for utilization in crop improvement in eco-friendly farming practices, the present study was undertaken to explore the microbial diversity (fungi and bacteria) of Darjeeling Hill region and to characterize them for agriculturally important traits and finally workout the possibility of utilizing them for improving crop health. The initial survey of different geographical regions which includes- forest, agricultural lands and riverine soil for microbial diversity analysis showed that they varied greatly from place to place and from one soil type to another. Forest and agricultural soils were generally acidic where the pH ranged from 4.0-5.5, whereas riverine soils showed slight increase in the pH which ranged from 5.6 to 6.6 and therefore both the fungal and bacterial populations in all the soil types varied accordingly. The average fungal population in the forest soil was $5.5 \text{ cfu} \times 10^4/\text{g}$ and the average bacterial population was $4.5 \text{ cfu} \times 10^6/\text{g}$, the average fungal population in agricultural soil was $6.0 \times 10^4/\text{g}$ and average bacterial population was $5.5 \text{ cfu} \times 10^6/\text{g}$ whereas the average fungal population in the riverine soil was found to be $4.0 \text{ cfu} \times 10^6/\text{g}$ and the average bacterial population was $3.5 \text{ cfu} \times 10^6/\text{g}$. Differences in microbial populations is most probably due to the variation in biological and chemical

properties of soils of various regions. The physical, chemical and biological properties of any soil are the indicators of the quality of that soil as well as decides the nature of microbial populations it holds (Drinkwater *et al.*, 2002). The fungal population on the other hand is thought to dominate over other microbial population in certain soil types due to their wide distribution and capability to adopt in all type of environments (Zervakis *et al.*, 2002). Himalayan region represents a unique combination of plant and soil types that change drastically with altitude (Kumar *et al.*, 2011), In our study we noted that there was a considerable amount of difference among the microbial populations of the areas located in lower altitudes and higher altitudes. Fungi which are aerobic microorganisms found more in the upper layers of the soils were also found to be dominant in places like Sandakphu, Maney Dara Forest and Rimbhik Forest where the altitude is above 2000 m amsl. Diversity in microbial population is thought to be due to the quality and quantity of organic matter present in the soil as well as by other factors like soil aeration, soil density, soil structure, salinity and also the water holding capacity of the soil (Venturella *et al.*, 1997)

A total of 357 fungal isolates were obtained from soil samples collected from different forests, agricultural and riverrine soils of Darjeeling district of North Bengal. The cultural characteristics of these fungal isolates were studied. The dominant fungal isolates belonged to the genera *Absidia*, *Acremonium*, *Alternaria*, *Aspergillus*, *Byasiochlamus*, *Colletotrichum*, *Drechslera*, *Emericella*, *Fusarium*, *Curvularia*, *Gonronella*, *Macrophomina*, *Noesertoria*, *Paecilomyces*, *Penicellium*, *Pseudoeutatum*, *Rhizoctonia*, *Rhizopus*, *Sclerotianum*, *Sporotrichum*, *Syncephalastrum*, *Talaromyces*, *Thanetophorus* and *Trichoderma*. In case of bacteria, a total of 135 bacterial isolates were obtained from various regions of Darjeeling hills. Bacterial identification was performed on the basis of morphological, physiological and biochemical tests. Result revealed that out of 135 bacterial isolates, 109 were gram positive whereas 26 were gram negative. A total of 52 bacterial isolates showed phosphate solubilizing activity. Bacterial isolates were also found to produce HCN and IAA. Most of the isolates showed a positive result for catalase activity. The most common and abundant bacterial species were *Bacillus sp.*, *Micrococcus sp.*, *Coryneform sp.* *Staphylococcus sp.* *Serretiasp.* *Paenibacillus sp.*, *Pseudomonas sp.*, *Enterobacter sp.* well as *Bukholderia sp.* The bacterial population only represents the culturable

population. Soil microbial communities are often difficult to fully characterize, mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity. Bacterial populations in soil top layers can go up to more than 10^9 cells per g soil and most of these cells are generally unculturable (Torsvik et al., 1996). The fraction of the cells making up the soil microbial biomass that have been cultured and studied in any detail are negligible, often less than 5% (Torsvik *et al.*, 1990).

The next phase of this study was to characterize both fungal and bacterial isolates for their agriculturally important properties. With more and more emphasis on organic farming, efforts are on to isolate and identify beneficial microbes, and hence, plant growth promoting microorganisms (PGPMs) are finding increasing applications today as biofertilizers and bioprotectors (Vessey 2003). Several mechanisms have been postulated to explain how the PGPM stimulates plant growth which may either be direct and/or indirect (Laslo *et al.*, 2012). Direct mechanisms include production of plant growth hormones that can enhance various stages of plant growth (Zahir *et al.*, 2009), mineral phosphate solubilization (El-Tarabily *et al.*, 2008), nitrogen fixation (Bashan and de-Bashan, 2010) and stimulation of ion uptake (Mantelin *et al.*, 2004). The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds has opened the possibility of inducing microbial solubilization of phosphates in the soil (Bojinova and others 1997). The ability of rhizospheric microorganisms to promote growth by phosphate solubilization is also one of the most studied mechanisms involved in plant growth promotion (Misra and others 2012).

In this study *in vitro* screening of fungal isolates for phosphate solubilization was carried out and their phosphate solubilizing ability quantified. four isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) were found to show maximum phosphate solubilization in solid medium. These isolates were also found to solubilize rock phosphate and tricalcium phosphate more efficiently than rest of the others when tested in liquid medium. Along with phosphate solubilization, a drop in the pH of the culture medium was also noted. There have

been earlier reports that *Aspergillii* and *Penicellium* are potential phosphate solubilizers and can enhance plant growth (Pradhan and Sukla, 2005; Chakraborty *et al.*, 2008, 2010a). Several reports are available on the ability of soil fungi to solubilize inorganic phosphates (Whitelaw, 2000; Pingale *et al.* 2013). Among the bacterial isolates a total of 48 were found to solubilize phosphate when screened on solid medium. All these isolates were then subjected to three main PGPR tests conducted in liquid broth medium. For quantification of phosphate solubilization in liquid medium, all the isolates were grown in modified PKV broth medium supplemented with Rock phosphate and Tricalcium Phosphate. The results revealed that isolate *Bacillus altitudinis* BRHS/S-73 could solubilize maximum amount of rock and tricalcium phosphate followed by *B. pumilus*, BRHS/C-1, *Enterobacter cloacae*, BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* BRHS/P-92 and *B. aerophilus* BRHS/B-104. Bacterial phosphate solubilization was also associated with the lowering of the pH of the liquid medium. The results obtained in our present study is in accordance to the work conducted by Nenwani *et al.* (2010) where they have reported that soil fungi not only solubilized phosphate but also lowered the pH of the growth medium. In a previous study conducted on phosphate solubilization it was observed by Lugtenberg *et al.* (2002) that the principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings.

Many bacterial, fungal, yeast, and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied (Abd-Alla, 1994; Whitelaw, 2000). Among the bacterial genera *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Flavobacterium* and *Erwinia* are reported to be most efficient phosphate solubilizers. In this present study, *Talaromyces flavus* has been reported as a potential phosphate solubilizer for the first time (Chakraborty *et al.*, 2012). This fungal isolate obtained from Paddy rhizosphere was also found to solubilize maximum amount of rock and tricalcium

phosphate than *Aspergillus* isolates. Among the bacterial isolates, though there have been many reports of potential PGPR like *B. altitudinis*, *B. methylotrophicus*, *B. aerophilus* and *Burkholderia symbiont* from other parts of the world, their occurrence in the Darjeeling hill soil has been reported for the first time.

The present study highlights the diversity of fungal and bacterial phosphate solubilizers present in Sub-Himalayan regions of Darjeeling Hills. Though this region is known world wide for its rich floral and faunal biodiversity, a very less work has been carried out in terms of microbial diversity has been carried out. However, Pandey *et al.*, (2008) in their studies have reported that they were able to successfully characterize twenty one fungal species belonging to the genus *Penicillium*, from the Himalayan region, among them *P. oxalicum* showed maximum phosphate solubilization after day 21 of incubation. The increase in solubilization coincided with decrease in pH of the broth and that many of these species showed wide range of tolerance for temperature, pH and salt concentration. Similarly, potential bacterial isolates like *Tetrathlobacter* sp, *Bacillus* sp., as well as fluorescent pseudomonads isolated from Himalayan soils have been reported to solubilize phosphate efficiently *in vitro* (Gulati *et al.*, 2012; Singh *et al.*, 2013).

Another agriculturally important trait of fungal isolates which was studied was the chitinase and cellulase activities. In this study apart from the phosphate solubilizers, a large number of *Trichoderma* isolates were also obtained from various sources. A total of 26 isolates of *T. harzianum*, 10 of *T. viride*, 13 of *T. asperellum* and 6 of *T. erinaceium* were obtained from various sources and were tested for their ability to produce chitinase *in vitro*. The net exo and endo chitinase activities of the isolates were determined spectrophotometrically. Among the *T. harzianum* isolates, two isolates designated as RHS/S-559 and RHS/S-560 obtained from the rhizosphere of *Secchium edule* showed maximum amount of both endo and exo chitinase activities; whereas among the *T. viride* isolates, isolate RHS/G 251 showed maximum activities. Similarly among the *T. asperellum* isolates, one isolate designated as RHS/S-561 showed maximum activities and among the *T. erinaceium* isolates, isolate RHS/Rd-551 showed maximum endo and exo chitinase activities. The microorganisms produce chitinases in higher amounts than animals and plants, generally as inducible

extracellular that are of the two types, endochitinases and exochitinases. The microorganisms able to degrade chitin are widely distributed in nature. Due to its insolubility, size, molecular complexity and heterogeneous composition, chitin is not degraded inside the cell, but the microorganisms secrete enzymes with different specificity, to transform or hydrolyse chitin (Funkhouser and Aronson 2007). Filamentous fungi have many different chitinases belonging to Glycoside hydrolases (GH) family 18 (Seidl 2008). The structural scaffold of the fungal cell is composed of chitin and β -(1,3) glucan (Lalgé 2007). Fungal chitinases are therefore not only involved in exogenous chitin decomposition but also in fungal cell wall degradation and remodeling. The variability of fungal GH family 18 proteins makes them ideal candidates for the development of enzymes acting on chitinous carbohydrates used in biotechnology. Besides chitinases, GH family 18 in fungi also contains non-chitinolytic enzymes such as endo- β -N-acetylglucosaminidases which can be used for protein deglycosylation (Stals *et al.*, 2010). Most fungal chitinases that were characterized so far are involved in autolysis in various fungi (*Aspergillus nidulans* ChiB, *A. fumigatus* ChiB1, *Penicillium chrysogenum* PcChiB1, *T. atroviride* Ech42, *T. harzianum* Chit42 and *T. virens* Cht42/Tv-Ech1). However, the contribution of these different chitinases to autolysis interestingly is strongly variable among the different fungi (Shin *et al.*, 2009; Kamerewerd *et al.*, 2011). *T. atroviride* Ech42 is involved in autolysis as well as in mycoparasitism (Gruber and Seidl-Seiboth 2011). These data suggest that the same chitinases can participate in self- and non-self cell wall degradation. It was suggested that the accessibility of chitin within the fungal cell wall could be a major determinant in these processes (Hartl *et al.*, 2012). Among the fungi it is reported to be present in *Trichoderma*, *Penicillium*, *Lecanicillium*, *Neurospora*, *Mucor*, *Beauveria*, *Lycoperdon*, *Aspergillus*, *Myrothecium*, *Conidiobolus*, *Metharhizium*, *Stachybotrys* and *Agaricus*. (De la Cruz, 1992; St. Leger, 1998; Matsumoto *et al.*, 2004). Further, mycoparasitic activity of many of the reported biocontrol agents is related with the production of cell wall degrading enzymes (CWDE), such as β -1,3-glucanases, proteases and chitinases (Narayanan *et al.*, 2013). However, majority of known mycoparasite chitinase enzymes are from *Trichoderma harzianum*, *Trichoderma atroviride*, and *Trichoderma virens* and are commercially used as a source of these proteins. Additional interest in these enzymes is stimulated by the fact that chitinolytic

strains of *Trichoderma* are among the most effective agents of biological control of plant diseases (Harman *et al.*, 1993; Vinale *et al.*, 2009; Karlsson *et al.*, 2010).

The antifungal activity of some compounds produced by fungi is due to their ability to affect function or the structure of the fungal cell of the pathogens. Such compounds include enzymes, antibiotics and proteins. Cell wall-degradating enzymes such as chitinases, -1,3-glucanases, proteases and cellulases are involved in the antagonistic activity of biocontrol agents against phytopathogenic fungi (De Marco *et al.*, 2000). Therefore in this study, apart from chitinase activities of *Trichoderma* isolates, net-exo-cellulase activities of the fungal isolates were tested. Among the tested fungal isolates, isolates of *A. niger* (FS/L-04, FS/L-40, FS/C-140, RS/P/14, FS/Td-173 and RHS/T-198), *A. melleus* (FS/L-13, FS/L-17, FS/L-18, RHS/R-12 and RS/P-05), *A. fumigates* (FS/R-263), *A. clavatus* (RHS/P-38, RHS/T-99, and RHS/P-114), *P. digitatum* (RHS/T-455 and RHS/C-338), *P. italicum* (RHS/M-403 and RHS/P-414), *P. crysogenum* (RHS/T-269), *T. flavus* (RHS/P-54, RHS/P-51, RHS/P-50 and RHS/P-120), *T. harzianum* (RHS/S-559 and RHS/S-560), *T. viride* (RHS/B-245 and RHS/G-251), *T. asperellum* (RHS/S-561, RHS/Cd-601 and FS/L-188) and *T. erinacium* (RHS/T-626 and FS/Td-166) showed comparatively higher exo and endo cellulose activities. Reports on chitinases, glucanases, cellulase, ribosome-inactivating proteins, permatins and protease and the relative importance of any of these systems in the antagonistic process as antifungal agents are well documented (Duo-Chuan *et al.*, 2005; Veenekar *et al.*, 2001). High levels of cellulase produced by *Trichoderma harzianum* and *Talaromyces flavus* have been reported by Haggag *et al.*, (2006), where they have found that protease produced by *Trichoderma harzianum* and *Talaromyces flavus* grown in the presence of casein was highly effective in controlling brown spot disease caused by *Botrytis fabae* on faba bean. They have also reported that *T. flavus* exhibited high levels of extracellular protease activity compared with *T. harzianum*. Results obtained in this study also show a higher exo cellulase activity *T. flavus* than other tested isolates. The fungus species of *Talaromyces flavus* has been described as a biological control agent against several fungal pathogens including *Sclerotium rolfsii*, and *Verticillium dahliae* (Madi *et al.*, 1997) and the agents of anthracnose and powdery mildew (Nozawa *et al.*, 2004), and the action of fungal hydrolytic enzymes has been considered as the main mechanism involved in the antagonistic

process (Madi *et al.*, 1997, Duo-Chuan, *et al.*, 2005). *Talaromyces flavus*, a microorganism remarkable for its secondary metabolites with unique biological activities, enzymes applicable in the synthesis of saccharides, preparation of chiral building blocks or biotransformations, and for its application in pest biocontrol will certainly be a subject of interest for biotechnology and fungal based technology (Proska., 2010).

B. pumilus, *B. altitudinis*, *B. methylotrophicus*, *B. aerophilus*, *Enterobacter cloacae*, *Paenibacillus polymyxa* and *Burkholderia symbiont* were found to produce considerable amount of ACC deaminase *in vitro*. PGPMs have also been shown to directly stimulate plant growth through the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase that limits the plant ethylene levels and enhances growth. The enzyme is known to hydrolyze an important intermediate biosynthetic precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) to ammonia and α -ketobutyrate (Laslo and others 2012). Lowering of ethylene levels by ACC deaminase activity thus reduces the inhibitory effect of ethylene on root elongation and promotes plant growth (Glick and others 1998). ACC deaminase has been widely reported in numerous microbial species of gram negative bacteria (Babalola *et al.*, 2003) gram positive bacteria (Ghosh *et al.*, 2003) rhizobia (Uchiumi *et al.*, 2004), endophytes (Pandey *et al.*, 2005) and fungi (Jia *et al.*, 1999). There are several reports, which support the hypothesis that ACC deaminase rhizobacteria have antagonistic effects against microbial pathogens. Rasche *et al.*, (2006) reported that ACC deaminase bacteria were also capable of antagonizing at least one of the two potato pathogens *Ralstonia solanacearum* and *Rhizoctonia solani*. It is also very likely that ACC deaminase bacteria, apart from directly antagonizing pathogens, support the plant's resistance against pathogen attack.

On the other hand in the present study, isolates like *B. pumilus*, *B. altitudinis*, *B. methylotrophicus*, *B. aerophilus*, *Enterobacter cloacae*, *Paenibacillus polymyxa* and *Burkholderia symbiont* were also found to produce higher amount of IAA when grown in Tryptophan amended nutrient broth medium. Among the direct mechanisms, production of phytohormones most importantly auxins (IAA), by soil microorganisms is considered as one of the best traits for plant growth promoting (PGP) activities which directly effects root elongation and growth (Spaepen and Vanderleyden, 2011) and microorganisms

closely associated with the rhizosphere or rhizoplane of the crop plants have been found to produce higher amount of IAA than those not associated with the host plants (Spaepen *et al.*, 2007). IAA is responsible for the division, expansion and differentiation of plant cells and tissues and stimulates root elongation. The ability to synthesize IAA has been detected in many rhizobacteria as well as in pathogenic, symbiotic and free living bacterial species (Tsavkelova *et al.* 2006). At present, auxin synthesizing rhizobacteria are the most well-studied phytohormone producers (Spaepen and Vanderleyden, 2011). Among PGPR species, *Azospirillum* is one of the best studied IAA producers (Dobbelaere *et al.* 1999). Other IAA producing bacteria belonging to *Aeromonas* (Halda-Alija, 2003), *Azotobacter* (Ahmad *et al.*, 2008), *Bacillus* (Swain *et al.*, 2007; Chakraborty *et al.* 2010c, 2013), *Burkholderia* (Halda-Alija, 2003), *Enterobacter* (Shoebitz *et al.*, 2009), *Pseudomonas* (Hariprasad and Niranjana 2009), *Rhizobium* (Ghosh *et al.* 2008) and Cyanobacteria (Prasanna *et al.*, 2010; Boopathi *et al.*, 2013).

Out of a total of 135 bacterial isolates, obtained from various sources of Darjeeling hills, 48 isolates showed phosphate solubilizing abilities. Similarly all the phosphate solubilizing isolates were screened for Siderophore and HCN production. The results of this preliminary screening presented showed that out of these 48 isolates, 27 isolates showed positive tests for siderophore production and nine isolates produced HCN. Finally eight bacterial isolates (BRHS/C-1; BRHS/P-22; BRHS/R-71; BRHS/R-72; BRHS/S-7; BRHS/P-91; BRHS/P-92 and BRHS/B-104) which showed positive results for the all the PGP characters like phosphate solubilization, siderophore and HCN production were selected for further evaluation. Some rhizobacteria are capable of producing HCN (Rezzonico *et al.*, 2007). HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and that also affects negatively the growth and development of plants. HCN is a powerful inhibitor of many metal enzymes, especially copper containing cytochrome C oxidases. HCN is formed from glycine through the action of HCN synthetase enzyme, which is associated with the plasma membrane of certain rhizobacteria (Blumer and Haas 2000). To date many different bacterial genera have shown to be capable of producing HCN, including species of *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas* and *Rhizobium* (Devi *et al.*, 2007; Ahmad *et al.*, 2008; Kumar *et al.*, 2012; Kadyan *et*

al. (2013). Various studies attribute a disease protective effect to HCN, e.g. in the suppression of “root-knot” and black rot in tomato and tobacco root caused by the nematodes *Meloidogyne javanica* and *Thielaviopsis basicota*, respectively (Siddiqui *et al.*, 2006). However, there are investigations reporting harmful effects on plants, inhibition of energy metabolism of potato root cells (Bakker and Schippers 1987), and reduced root growth in lettuce (Alström and Burns 1989). Likewise, HCN produced by *Pseudomonas* in the rhizosphere inhibits the primary growth of roots in *Arabidopsis* due to the suppression of an auxin responsive gene (Rudrappa *et al.* 2008; Martínez-Viveros, 2010).

Siderophores are low molecular weight compounds that are produced and utilized by bacteria and fungi as iron (Fe) chelating agents. These compounds are produced by various types of bacteria in response to iron deficiency which normally occurs in neutral to alkaline pH soils, due to low iron solubility at elevated pH (Sharma and Johri 2003). Iron is essential for cellular growth and metabolism, such that Fe acquisition through siderophore production plays an essential role in determining the competitive fitness of bacteria to colonize plant roots and to compete for iron with other microorganisms in the rhizosphere (Crowley 2006). Siderophore producing PGPR can prevent the proliferation of pathogenic microorganisms by sequestering Fe³⁺ in the area around the root (Siddiqui 2006). Fe depletion in the rhizosphere does not affect the plant, as the low Fe concentrations occur at microsites of high microbial activity during establishment of the pathogen. Many plants can use various bacterial siderophores as iron sources, although the total concentrations are probably too low to contribute substantially to plant iron uptake. Plants also utilize their own mechanisms to acquire iron; dicots via a root membrane reductase protein that converts insoluble Fe³⁺ into the more soluble Fe²⁺ ion, or in the case of monocots by production of phytosiderophores (Crowley 2006). Various studies have isolated siderophore producing bacteria belonging to the *Pseudomonas* (Boopathi and Rao 1999), *Serratia* (Kuffner *et al.* 2008; Chakraborty *et al.* 2010c), *Streptomyces* (Kuffner *et al.* 2008) *Bacillus* sp. (Chakraborty *et al.* 2006) and *Ochrobactrum anthropi* (Chakraborty *et al.* 2009) which could promote growth of crop plants and suppress disease. Thus it is clear that all these direct mechanisms exhibited by different fungal and bacterial isolates in this study are

agriculturally important traits on the basis of which these microorganisms were selected for all other experiments.

A total of twenty six *T. harzianum* isolates, ten *T. viride* isolates, thirteen *T. asperellum* isolates and five *T. erinacium* isolates were obtained from different sources of Darjeeling hills. All these isolates were initially tested for their antagonistic effects against the fungal plant pathogens (*S. rolfsii* and *T. cucumeris*) *in vitro*. A potential phosphate solubilizer, *Talaromyces flavus* obtained from the rhizosphere of paddy was also taken up for *in vitro* antifungal analysis. Isolates of *T. harzianum* (RHS/S-559 and RHS/S-560), *T. asperellum* (RHS/S-561), *T. erinaceium* (RHS/T-439) and *T. viride* (FS/L-186) showed maximum inhibitory activities. Interactions of the antagonists and fungal pathogens were also studied with the help of scanning electron microscopy. The SEM micrographs revealed that the *Trichoderma* mycelium profusely parasitizes the pathogen mycelium and inhibits its growth. At later stage of growth, the pathogen is completely overgrown by the antagonists. On the other hand, *T. flavus* could inhibit mycelial growth and development of *S. rolfsii* in dual culture. Sclerotial germination of *S. rolfsii* with cell free culture filtrates of *T. flavus* showed 90-95 % inhibition in comparison to control. Similarly among the bacterial isolates, *Bacillus pumilus*, *Enterobacter cloacae*, *Paenibacillus polymyxa*, *B. altitudinis*, *B. methylotrophicus*, *Burkholderia symbiont* and *B. aerophilus* that showed positive result for all the tested PGP characteristics were tested for their antifungal activities against the fungal pathogens *Sclerotium rolfsii*, *Thanatophorous cucumeris*, *Rhizoctonia solani* and *Macrophomina phaseolina*. All these bacterial isolates were found to inhibit the test pathogens where the average inhibition percentage ranged from 60- 80%.

Species of the fungal genus *Trichoderma* are typically soil dwellers, existing as anamorphs belonging to the sub-division Deuteromycotina (fungi imperfecti) (Hawksworth, *et al.*, 1983) and are fast growing fungi which are commonly found in a variety of soil types, such as, agricultural, prairie, forest, salt marsh and desert soils in all climatic zones (Domsch *et al.*, 1980). *Trichoderma* species constituted up to 3% of the total fungal propagules in a wide range of forest soils (Brewer *et al.* 1971) and has been extensively studied as biological control agents against soil borne plant pathogenic fungi (Chet and Inbar, 1994). The genus *Trichoderma* has six important species; *Trichoderma*

harzianum, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii*, *T. viren* and *T. viride*. (Gajera *et al.*, 2013). Apart from these, two other species, *T. asperellum* and *T. citrinoviride* have been proposed (Kuhls *et al.*, 1999).

One of the most studied interaction between *Trichoderma* spp. and fungal pathogens is Mycoparasitism, it is a process of direct attack of one fungus on another. It is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. *Trichoderma* sp. may exert direct bio-control by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of pathogenesis related proteins, mostly chitinases, glucanases and proteases (Harman *et al.*, 2004). Mycoparasitism involves morphological changes, such as coiling and formation of appressorium like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol (McIntyre *et al.*, 2004). *Trichoderma* attaches to the pathogen with cell wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appressoria. The following step consists of the production of pathogenesis related enzymes and peptaibols (Howell *et al.*, 2003) which facilitate both the entry of *Trichoderma* hyphae into the lumen of the parasitized fungus and the assimilation of the cell wall content. These changes were evident in this study where SEM studies revealed that the pathogen cell walls were ruptured followed by extensive colonization by *Trichoderma harzianum*. Inch and Gilbert,(2011) studied the chronological events associated with the interaction between a strain of *Trichoderma harzianum* T472, with known biological control activity against perithecial production of *Gibberella zeae*, with scanning electron microscopy to investigate the mechanisms of control. They observed that the cells of the outer wall of these perithecia were abnormal in appearance and unevenly distributed across the surface grown on straw treated with *Trichoderma*. Immature perithecia were colonized by *T. harzianum* approximately 15 d after inoculation (dai) with the biocontrol agent and pathogen. Few perithecia were colonized at later stages. The affected perithecia collapsed 21 dai, compared to the perithecia in the control samples that began to collapse 28 dai. Abundant mycelium of *T. harzianum* was seen on the perithecia of treated samples.

Talaromyces flavus (RHS/P-51, NAIMCC-F-01948) was found to inhibit a number of root pathogens *in vitro*. *T. flavus* is the most common species of its genus with a worldwide distribution and is commonly isolated from soil and organic substrates in warmer regions (Domsch *et al.*, 1993). Several strains of *T. flavus* have been reported from soil and termite mounds in Trat, Sakon Nakhon, Bangkok and Mae Hong Son which were isolated using the heat and alcohol treatment methods (Manoch, 2004). This fungus is sometimes reported as *Penicillium vermiculatum*, belongs to the family Trichocomaceae of the Ascomycota and is an anamorph of *Penicillium dangeardii*. Strains of *T. flavus* can produce novel bioactive compounds, such as actofunicone, deoxy-funicone and vermistatin, which reinforce the anti *Candida albicans* activity of miconazole (Arai *et al.*, 2002). *T. flavus* has been reported as biological control agent against several plant pathogenic fungi, such as *Verticillium dahliae*, causing wilt of eggplant in Israel (Madi *et al.*, 1997), *Sclerotinia sclerotiorum*, causing white mold of dry bean in Canada (McLaren *et al.*, 1986) and *Sclerotium rolfsii*, causing stem rot of bean in Israel (Fravel, 1996) and *Vigna radiata* (Chakraborty *et al.*, 2012). The mechanisms for biocontrol included mycoparasitism (McLaren *et al.*, 1986; Fahima *et al.*, 1992; Madi *et al.*, 1997), antibiosis (Stosz *et al.*, 1996) and competition (Marois *et al.*, 1984).

Apart from fungal antagonists, bacterial isolates which showed positive results in all the tested PGP traits could also inhibit a large number of fungal pathogens *in vitro*. Some common examples of PGPR genera exhibiting plant growth promoting activity are: *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Mycobacterium*, *Mesorhizobium*, *Flavobacterium*, etc. (Singh, 2013; Chakraborty *et al.*, 2013; Bhattacharya and Jha, 2012). The potential PGPR isolates obtained in this study are *Bacillus* spp, *Paenibacillus polymyxa*, *Enterobacter cloacae* as well as *Burkholderia* spp. PGPR belonging to the genus *Bacillus* is recognized as one of the most effective biological control agent because of their properties like pathogens inhibition (Schisler *et al.*, 2004; Sid *et al.*, 2003; Abbasi *et al.*, 2011; Kadyan *et al.*, 2013), production of antibiotics such as bacitracin, polymyxin, and gramicidin, (Li *et al.*, 2009), competition to occupy an ecological niche and metabolize root exudates on pathogens affecting their growth (Doornbos *et al.*, 2012). Species of *Bacillus* and *Paenibacillus* is well documented and PGPR

members of the genus *Bacillus* can provide a solution to the formulation problem encountered during the development of BCAs to be used as commercial products, due to their ability to form heat and desiccation-resistant spores (Kloepper et al. 2004; Pathak and Keharia, 2013). On the other hand, species of *Enterobacter* are generally reported as endosymbionts but are also found to occur freely (Dutta and Podile, 2010). Fatty acid methyl ester (FAME) profiling and BOX PCR analysis of the rhizosphere soil DNA of few cereal crops have revealed that the *Enterobacteriaceae* are the most diverse growth promoting population of Gram-negative bacteria associated with rice seeds, among which *Pantoea* spp. and *Enterobacter* spp. are dominating populations (Cottyn et al., 2001). In accordance with their degree of association with the plant root cells, PGPR can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Martinez-Viveros et al. 2010). The ePGPR may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex; on the other hand, iPGPRs locates generally inside the specialized nodular structures of root cells. The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Pseudomonas* and *Serratia* belongs to ePGPR (Gray and Smith 2005). The iPGPR includes the endophytes and *Frankia* species both of which can symbiotically fix atmospheric N₂ with the higher plants (Verma et al., 2010).

It is therefore apparent from all these reports that there is an existence of diverse group of beneficial microorganisms in the soil which has also been observed in this present study.

On the basis of preliminary studies, morphological and functional diversity among both fungal and bacterial isolates were observed. This diversity was unique for certain species and locations. However in order to study the diversity among the beneficial group of fungal and bacterial isolates at molecular level the most common DNA based technique- Random Amplified Polymorphic DNA (RAPD) was utilized. RAPD is a method developed by Williams et al., (1990) involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence has been used for genetic, taxonomic and ecological studies of several fungi. Among the total collection of

phosphate solubilizers genetic relatedness among four isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromuces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) was carried out using decamer primers. Out of the 30 loci scored only 12 (40 %) were polymorphic. However, the primers differed in their capacity to detect polymorphism. Highest level of polymorphism was recorded in primer OPD-5 (75.00 %) followed by OPB-2 (62.50 %), OPD-6 (40.00 %) and AA-5 (26%). Though all these isolates were functionally similar, the degree of similarity between *T. flavus* and *Aspergillus* isolates ranged from 14.00 % to 22 % (Moderate dissimilar values). PCA of the similarity coefficient values further revealed that each group of phosphate solubilizers were grouped in separate clades. Among the Biocontrol agents, isolates *T. harzianum* (RHS/S-559, RHS/S 560, RHS/M 501, RHS/M 511) and *T. asperellum* (RHS/S 561, RHS/M 512, RHS/M 517) were found to show maximum inhibitory effect against fungal pathogens *in vitro*. Analysis of genetic relatedness on the basis of RAPD analysis revealed that out of the 17 loci scored only 10 (58.82 %) were polymorphic. However, the primers differed in their capacity to detect polymorphism. Highest level of polymorphism was recorded in primer AA-05 (62.50 %) followed by AA-11 (55.55 %) and overall the degree of similarity between *T. harzianum* and *T. asperellum* isolates ranged from 28.00 % to 71.00 % (Moderate dissimilar values). Since all the bacterial isolates were identical in their function and biochemical analysis, genetic relatedness among all the 135 bacterial isolates were carried out using decamer primers. The average number of polymorphic bands produced by the primer OPD-05 was 7 and the highest degree of polymorphism recorded was 63.63 % followed by OPD-02 (57.10 %), AA-11 (40.00%), OPD-06 (37.50 %), AA-05 (33.33%), OPA-04 (28.57 %). Similarly, Similarity co-efficient reveals that most of the bacterial isolates belonging to the same genera and species showed highest degree of similarity. Overall all the bacterial isolates were separated into four major cluster irrespective of their origin and biochemical similarities. PCA analysis of the similarity coefficient values revealed that all the bacterial isolates exhibited a wide degree of genetic diversity which has been represented with a number of dispersed points distributed in the plot area.

Random amplification of genomic DNA for analysis of genetic diversity across a wide group of organisms is a useful and rapid technique to understand the genetic relatedness among the organisms which will be further helpful for more complex analysis (Muthumeenakshi, 1994). Latha *et al.* (2002), and Venkateswarlu (2008) who studied genetic variability among the isolates of *Trichoderma* by RAPD using random primers were able to detect considerable amount of genetic variation even among isolates of same locations and RAPD primers belonging to different operon series further generated more polymorphisms among the isolates. However, the present study may be useful in linking a specific amplified RAPD fragment to its antagonistic activity, and would help in developing Sequence Characterized Amplified Region (SCAR) marker linked to a potential isolates. Chakraborty *et al.*, 2010, 2011a and 2011b have successfully shown that RAPD markers can be successfully used to study variation among fungal isolates across several groups and regions. Various phenotypic and genotypic methodologies are being used to identify and characterize bacteria. Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques include PCR (Polymerase chain reaction), RAPD (randomly amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeats) and 16S-rRNA gene sequencing. RAPD is the most reliable, rapid and practical method (Mehmood *et al.*, 2008) and is used in studying genetic variations among many organisms (Madhavan *et al.*, 2010). Shweta *et al.*, 2013 have demonstrated that *Aspergillus* isolates which were functionally different were also identical in their genetic makeup when analyzed with the help of RAPD markers.

RAPD analysis was successfully used in developing genotype fingerprints of nineteen *Trichoderma* isolates namely *T. harzianum* (five strains), *T. pseudokoningii*, (one strain), *T. koningii*, (one strain) and the other unidentified species (Magdy *et al.*, 2012).

A second level of study was conducted to analyze a specific gene sequence of selected and closely related group of microorganisms to draw variations in their genetic makeup. This was achieved with sequence data from the ITS 1 region of the ribosomal gene complex. In general, sequence data from

the ITS 1 region of the selected isolates were tested which was distinguished by Denature Gradient Gel Electrophoresis (DGGE). In the 1980s, denaturing gradient gel electrophoresis (DGGE) was developed by Fischer and Lerman, and this technology was used as a method of detecting mutations in the microbial DNA (Fischer and Lerman, 1983). Muyzer *et al.*, (1993) used DGGE to study the structure of the microbial community and proved its advantages in probing the genetic diversity of the natural microbial community and community difference. Ten years later, DGGE was widely used in various fields of microbial molecular ecology (Sanchez *et al.*, 2007; Yu *et al.*, 2010). DGGE has also been used as a powerful technique for the identification of dominant microbial species in various environmental samples (Zhao *et al.*, 2006; Mavragani *et al.*, 2011). By the analysis of the bands migrating separately on the DGGE gels, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) has succeeded in obtaining phylogenetic information about the microorganisms. Liu *et al.* (2008) suggested that the combination of soil dilution plating, DGGE and DNA sequence analysis are effective approaches to facilitate extensive examinations of the propagule numbers of *Trichoderma*, to reliably identify *Trichoderma* species in soils with different management practices. Vendan *et al.*, 2012, while conducting their studies on the shifts in the PCR-DGGE profiles of bacterial communities associated with the rhizosphere soil of ginseng at varying age levels have suggested that DGGE profiles can be used to detect low levels of similarity values even among the members of the same genus. In a similar study, Rytkonen *et al.*, (2011) have demonstrated that PCR-DGGE method can be used to detect multiple species of *Phytophthora* directly in infected plant tissues by using *Phytophthora* specific primers and have suggested that very closely related species can be detected using this technique. On the other hand Sun *et al.*, (2013) have suggested that PCR-DGGE technique is not only helpful in knowing un-cultured communities but also helps to track the populations of known organisms with the help of reference sequences. They further suggested that microbial communities of ecological samples other than soils can also be effectively monitored. All these studies support the findings of this study where PCR-DGGE was successfully used to distinguish between different groups of functionally similar microorganisms. The technique which depends on slight variation of the similar

gene sequences was useful enough to determine genetic diversity of even closely related fungal and bacterial isolates.

The next phase of investigation was the analysis of rDNA sequences of selected, pathogen, PSF, BCA and PGPR isolates for identification and phylogenetic placements. For *in vitro* antagonistic studies and for *in vivo* disease suppression studies, one very notorious fungal pathogen, *Thanatephorus cucumeris* was isolated from naturally infected *Vigna radiata* seedlings. The pathogenicity of the obtained fungal pathogen was confirmed through Koch's postulate and used for further studies; the pathogen was coded as RHS/V-566. The identity of this pathogen was confirmed with the help of 18S rDNA sequences obtained by ITS-PCR amplification of genomic DNA with the help of Universal primers. After direct sequencing of the ITS-PCR amplicons, the sequences were queried against the ex-type sequences of NCBI-Genbank database through BLAST algorithm. The analysis revealed isolate RHS/V-566/NAIMCC-F-02903 to have 99% homology with *Thanatephorus cucumeris*. The sequences were approved as 18S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,400). The accession number for isolate *T. cucumeris* provided by NCBI is JN248540. Multiple sequence alignment of the sequences showing maximum identity score (96-99 %) along with the sequence of *R. solani* was conducted to determine sequence homology using CLUSTAL-W software. Similarly, among all the phosphate solubilizing fungal isolates, isolate RHS-P-51/NAIMCC-F-01948, which was initially identified on the basis of morphological characters as *Talaromyces flavus*, was found to be the most potential PSF and its identity was also confirmed on the basis of 18S rDNA sequences. The obtained sequence (8000 bp) was further used to query against NCBI Genbank sequences through BLAST. The analysis revealed isolate RHS/P-51 to have 100% homology with *Talaromyces flavus*. The sequences were approved as 18S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,230). The accession number for isolate *T. flavus* provided by NCBI is GU324073. Multiple sequence alignments and phylogenetic analysis of *T. flavus* was conducted based on the 18S rDNA sequences of some commonly occurring phosphate solubilizers (*A. niger*, *A. melleus* and *A. clavatus*) obtained from NCBI Genbank database. The phylogenetic tree shows that *T. flavus* is closely related to *A. niger* group whereas distantly related to *A. clavatus* group. In

case of *Trichoderma* isolates, isolates, FS/L-20, SF/S-474, FS/S-475, FS/S-478, RHS/T-460, RHS/S-559, RHS/S-560 and RHS/S-561 showed highest amount of antagonistic activities against the fungal pathogens. The identity of all these BCA isolate were also confirmed with help of 18S rDNA sequences amplified with the help of *Trichoderma* specific universal primers. The BLAST analysis of the individual gene sequences confirmed the identities of these isolates as, *Trichoderma erinaceum* (FS/L-20 (NAIMCC-01949), FS/S-474 (NAIMCC-F-01960), FS/S-475 (NAIMCC-F-01953), FS/S-478(NAIMCC-F-01954) *Trichoderma harzianum* (RHS/S-559 (NAIMCC-F-01968), RHS/S-560(NAIMCC-F-01966) and *Trichoderma asperellum* (RHS/S-561(NAIMCC-F-01967). The accession number for all these isolates, provided by NCBI are HM107419, GU187915, GU191829, HM117841, HQ334995, HQ334997, HQ334995 and HQ334996 for *T. erinaceum*, *T. harzianum* and *T. asperellum* isolates respectively. The 18S DNA sequence based phylogenetic analysis of *T. harzianum*, *T. asperellum* and *T.erinaceum* isolate was conducted with other ex-type strains btained from NCBI-Genbank database which showed maximum homology (98-100 %) with the respective query sequences. The phylogenetic tree showed that there is a geographical variation among the isolates. The isolates belonging to the same geographical zone were clustered in the same clade. Over all 18S rDNA sequences show that isolates of *T. harzianum* (Telomorph-*Hypocrea lixii*) is closely related to *T. erinaceum* which is indicated by their position in the phylogenetic tree, whereas *T. asperellum* isolates showed comparatively lesser affinity with *T. harzianum* isolates. Though *T. viride* was used as an out group for this analysis they remained dispersed showing more or less affinities with all the three groups of *Trichoderma spp.* Among the PGPR isolates, from the total collection of 135 bacteria, at least 48 isolates were found to solubilize phosphate however only eight bacterial isolates viz BRHS/C-1, BRHS/P-22, BRHS/R-71, BRHS/R-72, BRHS/S-73, BRHS/P-91, BRHS/P-92 and BRHS/B-104 were found to be the best PGPR with promising PGP traits *in vitro*. In case of bacterial isolates, 16S rDNA specific primers were used. The ITS region of the bacterial isolates were amplified with the help of universal primer pair. After direct sequencing of the PCR product 16S rRNA gene sequence of approximately 800 to 1,400 base pairs were obtained. The obtained sequence was further used to query against NCBI

Genbank sequences through BLAST. The analysis revealed isolate BRHS/C-1 had 99 % similarity with *Bacillus pumilus*, BRHS/P-22 showed 98% with *Bacillus altitudinis*, BRHS/R-71 showed 98 % similarity with *Enterobacter cloacae*, BRHS/R-72 had 99 % similarity with *Paenibacillus polymyxa*, BRHS/S-73 had 99% homology with *Bacillus altitudinis*, BRHS/P-91 had 99 % similarity with *Bacillus methylotrophicus*, BRHS/P-92 had 99 % similarity with *Burkholderia sp.* and BRHS/B-104 had 99 % similarity with *Bacillus aerophylus*. The sequences were approved as 16S rRNA gene sequence by NCBI after complete annotation. The NCBI Accession numbers for each isolate is JF836847, HQ849482, KC703974, KC703775, JF899300, JQ765577, JQ765578 and KC603894 respectively.

Methods commonly used in taxonomy can be utilized to differentiate between organisms from such communities, but they require cultivation of purified isolates from environmental samples (Mahaffee and Kloepper, 1997). Therefore, approaches detecting the diversity of directly extracted signature molecules of microorganisms, such as fatty acids (Frostegård, et al., 1996) or DNA (Zhou *et al.*, 1997), have been developed. DNA-based characterization techniques have the advantage that specific genes can be amplified from a community mixture or pure culture by PCR and that products of such amplifications can be further characterized, e.g., by subcloning and DNA sequencing. Such data can be directly compared to DNA sequence databases and thus provide information about similarity to already-known genes (Ueda *et al.*, 1995). Molecular methods have recently been introduced into fungal taxonomy for distinction between teleomorphs (Samuels *et al.*, 2002). These techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera (Sherriff *et al.*, 2007). Taylor, *et al.*, (1999), demonstrated that molecular techniques indicating interrelations among species combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relationships. Internal transcribed spacer sequences of ribosomal DNA (rDNA) analysis and universally primed polymerase chain reaction have been used to categorize the isolates of biocontrol agents (Rameshkumar *et al.*, 2012). Jeewon *et al.*, 2013, in their studies related to the identification and phylogenetic characterization of endophytic and saprobic fungi from a medicinal plant- *Antidesma*

madagascariense have reported the presence of *Aspergillus*, *Guignardia*, *Fusarium*, *Penicillium*, *Pestalotiopsis*, and *Trichoderma* for the first time in the leaves of *Antidesma madagascariense*, the identities of the fungal isolates were confirmed with the help of 18S rDNA sequences. Phylogenetic analyses revealed that fungi recovered belong to 5 different fungal lineages (Hypocreaceae, Trichocomaceae, Nectriaceae, Xylariaceae, and Botryosphaeriaceae). DNA data from the ITS regions were reliable in classification of all recovered isolates up to genus level. They were able to successfully show the reliability of ITS sequence data for possible identification and discovering of evolutionary scenarios among isolates that do not sporulate under cultural conditions.

rDNA sequences were further analyzed to compare nucleotide frequencies, DNA molecular weights, and number of open reading frames (ORF) of individual sequences. Number of ORFs varied in different groups and individual organisms. The nucleotide combinations like-G, A, T, C, GG, GA, GT, GC, AG, AA, AT, AC, TG, TA, TT, TC, CG, CA, CT, CC [G,C] and [A,T] of all the individual PSF, BCA and PGPR isolates were determined. Chakraborty *et al.*, 2011a, have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. This analysis could have other applications in DNA barcoding besides cluster analysis. The determination of frequencies of DNA strings would enable easy identification of taxon-specific strings that can be used as taxon specific probes in DNA chip for species identification.

Series of *in vivo* experiments were carried out next with the selected phosphate solubilizing fungi, Biocontrol agents and PGPR isolates to determine their plant growth promoting activity in the field and potted conditions. On the basis of initial screening of fungal isolates for phosphate solubilization, *A. niger* FS/L-04, *A. melleus* FS/L-17, *A. clavatus* RHS/P-38 and *T. flavus* RHS/P-51 were found to be most efficient phosphate solubilizers. Evaluation of these isolates for enhancement of growth of six different crop plants viz. *Phaseolus vulgaris*, *Glycine max*, *Cicer arietinum*, *Vigna radiata*, *Pisum sativum* and *Oryza*

sativa in green house condition was carried out. These PFS isolates were applied separately to the soils after multiplying them in farm yard manure. Seeds were then sown in PSF amended soils which resulted in significant increase in growth, measured in terms of height, leaf number and dry biomass over similar increase in control. Effect of *T. flavus* amendment was found to be significantly higher in all the tested crops in comparison to the other *Aspergillus* isolates. Enhancement of growth by these phosphate solubilizing fungal isolates was directly associated with the soil phosphate mobilization. All the PSF isolates could solubilize phosphate *in vitro* as evident by the appearance of halo zone around the colony in PVK medium. The potential of these isolates were further tested in the pot conditions where they were amended in the soil. The total residual phosphate in un-inoculated soil was found to be much higher than the soil amended with PSF isolates while root and leaf phosphate contents significantly increased in plants grown in PSF amended soil comparison to control. The total soil phosphate content was reduced by almost 50% by *T. flavus* compared to other PSF isolates. Similarly, the total phosphate content of the roots and leaves was also higher in all the crops treated with *T. flavus*. Among the test crops, soil phosphate content of *Glycine max* was reduced to a greater extent, however root and leaf phosphate content was found to be maximum in case of *Pisum sativum*. In all the cases the difference between the treated and control was found to be significant at the level $P=0.5$ when tested with students' *t* test. As phosphate solubilization is a prime process for plant growth, the importance of phosphate solubilizing microorganisms is well recognized (Velazquez and Rodriguez-Barrueco 2007). Earlier studies with other isolates have shown them to possess plant growth promoting and or biocontrol activities. Though records of phosphate solubilizing isolates from the Indian Himalayan soil are very few, the available literatures in recent times have suggested that *Aspergillus* and *Penicillium* in particular, survive and dominate in acidic and low-temperature areas of the Indian Himalayan region (Pandey *et al.*, 2008). Rinu and Pandey (2010) in their study have isolated pH and cold tolerant species of *Aspergillus* from the Himalayan soil and have studied their growth requirements and tricalcium phosphate solubilization at different temperatures and have found that *Aspergillii* from different locations could efficiently solubilize phosphate at different temperatures. The results obtained in our present study is in accordance to the

work conducted by Nenwani *et al.*, (2010) where they have reported that soil fungi not only solubilized phosphate but also enhanced phosphate uptake in plants grown in nursery condition. There have been earlier reports that *Aspergillii* are potential phosphate solubilizers and can enhance plant growth (Chakraborty *et al.*, 2008). Apart from *Aspergillus* and *Penicillium* isolates which are the most common phosphate solubilizers in the soil, *Talaromyces flavus* has also been reported as a potential phosphate solubilizer by (Chakraborty *et al.*, 2012) for the first time. *Talaromyces flavus* (Klocker) Stolk and Samson is one of the most important species of antagonistic fungi. This ascomycete is frequently isolated from soil, although it may also occur on organic materials undergoing decomposition (Domsch *et al.*, 1980). The organic soluble metabolites of this fungus include D-glucono-1,4-lactone; 5-hydroxymethylfurfural; 4,6-dihydroxy-5-methylphthalimide; methyl 4-carboxy-5-hydroxyphthalaldehyde; hexaketide; 7-hydroxy-2,5-dimethylchromone; 3-hydroxymethyl-6,8-dimethoxycoumarin; altenusin, desmethyldehydroaltenusin, talaroflavone, deoxytalaroflavone, 2-methylsorbic acid, sorbic acid, bromomethylsorbic acid, and bromosorbic acid (Ayer and Racok 1990; Wakelin and others 2004). Some of above-mentioned metabolites (2-methylsorbic acid, sorbic acid, bromomethylsorbic acid, and bromosorbic acid) play a fundamental role in the biogeochemical cycling of phosphorus (P) in natural and agricultural ecosystems (Wakelin *et al.*, 2004). Pandya and Saraf (2010) have found that *T. flavus* after inoculation to the soil in the cotton and potato fields of Iran could mobilize nutrients, help mineralization and facilitate nutrients and water. Naraghi *et al.*, (2012) have shown that antagonistic fungus *Talaromyces flavus* enhances growth of cotton and potato when a combined method of seed treatment and direct application to the soil was followed in their split-plot trial experiments. Measured parameters were root length, crown length, plant height, plant fresh weight, and plant dry weight. The type of growth promotion may be similar to that produced by the addition of *Trichoderma* spp. which has been found to enhance the growth of various plants (Contreras-Cornejo *et al.*, 2009; Hajieghrari *et al.*, 2010; Masunaka *et al.*, 2011). There is overwhelming evidence in the literature indicating that plant growth-promoting fungi (PGPF) can be a true success story in sustainable agriculture (Sudha *et al.*, 2011). In fact, through their numerous direct and indirect modes of

action, PGPF can allow a significant reduction in the use of pesticides and chemical fertilizers.

Among the several isolates of PGPR obtained from different regions of Darjeeling hills, seven PGPR isolates, *Bacillus pumilus* BRHS/C-1, *Enterobacter cloacae* BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. altitudinis* BRHS/S-73, *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* BRHS/P-92 and *B. aerophilus* BRHS/B-104 were selected for *in vivo* evaluation of their effects on growth differsnt crop plants. In the first set of field trials, effect of PGPR on growth of *Vigna radiata*, *Cicer arietinum*, *Glycine max* and *Triticum aestivum* was evaluated. All the crops were grown at different intervals according to their growing season in complete randomized block design. Evaluation of their effect on overall growth and development of the test crops were computed in terms of shoot length, root length and shoot and root fresh weight. Results revealed that seed bacterization followed by application of the bacterial isolates as soil drench to the natural environment could enhance growth of all the tested crop plants. However, *B. altitudinis* BRHS/S-73 followed by *B. pumilus* BRHS/C-1 could enhance growth of all the tested crops more efficiently. In the second set of trials, *B. altitudinis* BRHS/S-71 and *B. pumilus* BRHS/C-1 were further tested for their effect in enhancing the growth of four different varieties of tea seedling (TV-9, TV-20, TV-25 and TV-26) in nursery conditions where they were applied to the rhizosphere of six month old tea plants in nursery conditions at a regular interval of 15 days for one month. Growth promotion of different varieties by individual bacterium was noted as compared to untreated control in terms of increase in height, leaf fresh and dry mass. Results revealed an increase in all the parameters by single as well as dual application of bacteria. The overall result reveals that the growth of tea seedlings grown under same environmental and physical conditions was enhanced to a greater extent when both the bacterial isolates were applied jointly. In both the cases the growth promotion was found to be correlated with total phosphate content and phosphatase activities of the soil. Different strains of *Bacillus* have been reported to be potential PGPR. The recognition of plant growth-promoting rhizobacteria (PGPR) as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years to where today researchers are able to repeatedly use them successfully in field experiments (Saharan and Nehra, 2011). Enhancement of

plant growth by root-colonizing species of *Bacillus* and *Paenibacillus* is well documented and PGPR members of the genus *Bacillus* can provide a solution to the formulation problem encountered during the development of BCAs to be used as commercial products, due to their ability to form heat and desiccation-resistant spores (Kloepper *et al.*, 2004).

B. altitudinis was first isolated by Shivaji and his co-workers while analyzing air samples for microbial populations from high altitude (Shivaji *et al.*, 2006) PGPR activity of *B. altitudinis* has been recently reported by other authors where the bacterium not only enhanced growth but also suppressed root pathogens (Gopalakrishnan *et al.*, 2010; Jin *et al.*, 2012) Reports of bacterial inoculants being able to increase plant growth, speed up seed germination, improve seedling emergence, responses to external stress factors, protect plants from disease and root growth pattern have also been reported in earlier studies by Rinu and Pandey (2009). Plant growth promotion in tea, *Camellia sinensis* by *Bacillus megaterium*, *B. pumilus*, *Ochrobactrum anthropi* and *Serratia marcescens* has been reported by Chakraborty *et al.* (2004, 2006, 2009, 2013). Ranjan *et al.*, (2013) in their study conducted to isolate potential PSB from Indian soil have reported *Pseudomonas aeruginosa*, *Micrococcus* sp., *Enterobacter* sp. and *Bacillus pumilus* to be most efficient phosphate solubilizers. These isolates proved to be successful inoculum in promoting growth of different rice cultivars grown in water stress conditions. Similarly, Souza *et al.*, (2013) have demonstrated that inoculation with isolates AC32 (*Herbaspirillum* sp.), AG15 (*Burkholderia* sp.), CA21 (*Pseudacidovorax* sp.), and UR51 (*Azospirillum* sp.) alone without ant fertilization could achieve similar growth of rice crops achieved by full fertilization and these strains were used for formulation new bioinoculants.

Interestingly, the total phosphate content of the PSF and PSB treated soils were significantly reduced while root and leaf phosphate contents of all the test crops showed an increase in comparison to control untreated plots. The results are in conformity with those of other workers who have reported the ability of rhizospheric microorganisms to promote growth and have suggested phosphate solubilization to be one of the mechanisms involved in plant growth promotion (Misra *et al.*, 2012). Some PGPR biofertilizers also influence the availability of phosphate by secreting phosphatases for mineralization of organic phosphates

(Bünemann, 2008). Phosphate is often the limiting nutrient for microbial and plant growth in soil, phosphatases remove the phosphate from organic compounds and convert it in soluble form available to the plants. Increase in both acid and alkaline phosphatase activities in bacterial treated rhizosphere soil in our study indicated that microbial phosphatases were involved in solubilization of insoluble phosphate in the soil. To this end the results clearly confirms that all the fungal and bacterial isolates tested in this study have the ability of efficiently mobilize phosphate in the soil.

Apart from phosphate solubilizers, a large number of Biocontrol agents (BCA) were also obtained from various sources which included *Trichoderma harzianum*, *T. asperellum* and *T. erinaceum*. Another phosphate solubilizing fungus *T. flavus* was also found to inhibit a number of phytopathogens *in vitro*. In the present investigation, *T. harzianum*, *T. asperellum* were found to efficiently reduce sclerotial blight incidence of *Vigna radiata* caused by *Sclerotium rolfsii* and root rot disease of *Cicer arietinum* caused by *T. cucumeris* when applied in the soil either singly or in combination with another efficient biocontrol fungus *T. flavus*. Reduction of disease incidence was found to be correlated with the enhancement of defense enzyme activities tested after application of biocontrol agents and pathogen challenge. The direct effects of *Trichoderma* spp. on plants are remarkable and are found to be efficient inducers of systemic and localized resistance in plants Woo *et al.*, 2006. Another mechanism proposed to explain biocontrol activity by *Trichoderma* species is that of induction of resistance in the host plant. This concept is supported by the work of Yedidia *et al.* (1999), who demonstrated that inoculating roots of 7-day-old cucumber seedlings in an aseptic hydroponic system with *T. harzianum* (T-203) spores initiated plant defense responses in both the roots and leaves of treated plants. Increased enzyme activities were observed in both roots and leaves. Later, Harman (2006) showed that inoculation of plants with *T. harzianum* induced an array of pathogenesis-related proteins, including a number of hydrolytic enzymes. Successful biocontrol agents like *Trichoderma* use several of them mechanisms for disease control. Both indirect and direct mechanisms may act coordinately and their importance in the bio-control process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions including nutrient availability, pH, temperature and iron concentration. Activation of each

mechanism implies the production of specific compounds and metabolites such as plant growth factors, pathogenesis related lytic enzymes, siderophores, antibiotics, and carbon and nitrogen permeases. These metabolites can be either overproduced or combined with appropriate bio-control strains in order to obtain new formulations for use in more efficient control of plant diseases (Gajera *et al.*, 2013). On the other hand *Talaromyces flavus* is one of the most important species of antagonistic fungi. This ascomycete is frequently isolated from soil, although it may also occur on organic materials undergoing decomposition (Domsch *et al.*, 1980). *T. flavus* is a biological control agent that has been used in biological control of important soil-borne pathogens such as *Verticillium dahliae*, *V. albo-atrum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Marois *et al.*, 1984; Naraghi *et al.*, 2010, 2012). In our earlier investigation we have shown that *T. flavus*, apart from efficiently solubilizing phosphate could successfully reduce sclerotial blight incidence of Mung bean seedlings. (Chakraborty *et al.*, 2012). However, utilization of *T. flavus* and *T. harzianum* jointly in reduction of disease has been demonstrated for the first time.

Similarly, the PGPR under investigation was also effective in suppressing sclerotial blight of *Glycine max* caused by *S. rolfsii* grown in pot conditions. Its efficiency was also found to be correlated with the enhancement of key defence enzymes- chitinase (CHT), β -1, 3-glucanase (GLU), Phenyl alanine ammonia lyase (PAL) and Peroxidase (POX) which increased significantly specially in the presence of the pathogen. Increase in these key defense enzymes during plant growth promotion and disease suppression of tea was reported in the earlier studies by Chakraborty *et al.* (2004 a, b, 2006, 2009, 2013) where PGPR like *Bacillus megaterium*, *B. pumilus*, *Ochrobactrum anthropi* and *Serratia marcescens* were successfully utilized to overcome several root diseases of tea. Our results are in agreement with the earlier reports where enhancement of defense enzymes like has been shown to be key mechanisms in suppressing root diseases and induction of resistance by bacterial isolates (Chen *et al.*, 2010; Liang *et al.*, 2011; Attia *et al.*, 2011; George *et al.* 2013). Induction of systemic resistance by both BCA and PGPR is suggestive of the fact that the plant becomes more resistant to a future pathogen attack. This long lasting, broad spectrum resistance, called induced systemic resistance (ISR) (Van Peer *et al.*, 1991), is phenotypically similar to SAR, but molecular events leading to its

induction are different. ISR has been shown to operate by activating gene encoding defense enzymes -peroxidase, chitinase, phenylalanine ammonia-lyase, β -1,3-glucanase and others, involved in synthesis of phytoalexin (M'Piga *et al.*, 1997).

The overall result of the present study has shown that there is there is a huge microbial diversity in the soils of sub Himalayan soils of Darjeeling Hills. The occurrence of functionally diverse groups of phosphate solubilizers, chitin degraders, biocontrol agents, plant growth promoting rhizobacteria in all the tested soil types suggests presence abundant Beneficial Microorganisms in the region. RAPD and DGGE based genetic relatedness analysis of these beneficial microorganisms suggested that they were not only functionally diverse but also showed significant variation in their genetic make up. ITS sequences employed in identification of important and selected microorganisms proved to be a reliable tool and can be utilized in future for identifying closely related organisms. Multiple sequence alignment of the conserved sequences was useful for drawing strain specific differences which is helpful for designing strain or group specific primers. *In vivo* applications of specific fungi like *T. flavus*, *A. niger*, *A. melleus* and *A. clavatus* to enhance plant growth have shown good potential to develop efficient bioinoculants. Potential PGPR like *B. pumilus*, *Enterobacter cloacae*, *P. polymyxa*, *B. altitudinis*, *B. methylotrophicus*, *Burkholderis spp.* and *B. aerophilus* were found to be efficient plant growth promoters in field as well as in nursery seedlings. Both the PSF and PSB were found to efficiently mobilize soil phosphate when applied to soil. The enhanced soil phosphatase activities of treated soils further suggested that microbial phosphatases were involved in solubilizing insoluble phosphate in the soil. Apart from this, potential biocontrol agents like *Trichoderma harzianum*, *T. asperellum*, and *Talaromyces flavus* showed high degree of biocontrol efficacy in controlling root diseases of potted legumes. Their effect was found to be enhanced when applied jointly. Similarly potential PHPR as listed could also suppress root diseases of a wide range of vegetable crops, cereals, legumes as well as plantation crops in pot and nursery conditions. Biopriming of the seeds and seedlings prior to sowing and after germination proved to be effective in growth enhancement and to induce resistance against fungal root pathogens. Reduction of root diseases by both BCA

and PGPR was associated with all the elements commonly known to be involved in the induced systemic resistance which were found to have been enhanced. Regarding the mechanism of action of the beneficial microorganisms, it seems probable that these organisms act through a combination of methods. Though it is difficult to predict the actual happening in the soil environment, it is assumed that on one hand these microorganisms secrete metabolites into the soil which in turn elicit responses in the host which was evident by differential expression of enzymes both in the roots and leaves of treated plants and on the other hand suppress pathogen population by antibiotics, HCN and Siderophore secretion. Among the PSF isolates *Talaromyces flavus* RHS/P-51 has been shown to possess dual attributes of phosphate solubilization and biocontrol efficacy for the first time in this study. Although PGPRs like *B. altitudinis*, *B. aerophilus*, *Enterobacter cloacae*, *B. methylotrophicus* and *Burkholderia spp.* have been reported from other parts of India and abroad, in this study they have been reported for the first time to be isolated from high altitude regions of Darjeeling Hills. Beneficial microorganisms investigated in this study could be used in suitable formulations commercially which would enhance the use of biological products to replace or supplement chemical use is the need of the hour.

CONCLUSION

Soil microflora plays the most important role in the soil region of the higher plants. The variable microfloras changes the soil fertility conditions of a specific plant and are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation; decomposition of organic matter; toxin removal; and the cycling of carbon, nitrogen, phosphorus, and sulphur. In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth, and changes in vegetation. To this end the present study was carried out to analyze soil microbial diversity of Darjeeling Hills which included forests, agricultural rhizosphere soil and riverine soils followed by evaluation of potential plant growth promoting fungus (PGPF), Biocontrol agents (BCA) and Plant growth promoting Rhizobacteria (PGPR) for utilization for improvement of different legumes, cereals, vegetable crops as well as chief plantation crop-tea grown in this region. The overall findings of this study have been concluded as under:

- ❖ Microbial population in soils ranged between 4×10^3 - 6×10^4 cfu in case of fungi and 5×10^6 cfu- 6×10^6 cfu in case of bacteria. A total of 637 fungal isolates were obtained from the major forest, agricultural fields and river basins of Darjeeling hills. Out of the total collection, 205 isolates were obtained from forest, 373 from agricultural and 59 isolates were obtained from river basins. Similarly, a total of 135 bacterial isolates were obtained from various sources. Among them 39 were obtained from forest soil, 73 from rhizosphere of agricultural crops and 23 from riverine soil.
- ❖ Dominant fungal isolates belonged to the genera *Absidia*, *Acremonium*, *Alternaria*, *Aspergillus*, *Byasiochlamus*, *Colletotrichum*, *Drechslera*, *Emericella*, *Fusarium*, *Curvularia*, *Gonronella*, *Macrophomina*, *Noesertoria*, *Paecilomyces*, *Penicellium*, *Pseudoeutatum*, *Rhizoctonia*, *Rhizopus*, *Sclerotianum*, *Sporotrichum*, *Syncephalastrum*, *Talaromyces*, *Thanetophorus* and *Trichoderma*.

- ❖ The most common and abundant bacterial species were *Bacillus sp.*, *Micrococcus sp.*, *Coryneform sp.*, *Staphylococcus sp.*, *Serratia sp.*, *Paenibacillus sp.*, *Pseudomonas sp.*, *Enterobacter sp.* well as *Burkholderia sp.*
- ❖ Isolates of *Aspergillus niger* (FS/L-04, RS/P-14, FS/L-40, FS/C-140), four isolates of *A. melleus* (RHS/R 12, FS/L 13, FS/L 17, FS/L 18), three isolates of *A. clavatus* (RHS/P 38, RHS/P-114, RHS/T-99) and four isolates of *Talaromyces flavus* (RHS/P 50, RHS/P 51, RHS/P 54, RHS/P 120) were found to solubilize rock phosphate and tricalcium phosphate more efficiently than rest of the others and were designated as potential plant growth promoting fungus (PGPF)
- ❖ One of the interesting findings of the present study was isolation of one potential fungal isolate *Talaromyces flavus* RHS/P-51/ NAIMCC-F-01948, which is reported as a potential phosphate solubilizers for the first time in this study. This fungal isolate not only solubilized phosphate efficiently *in vitro* but also inhibited a number of root pathogens to a greater extent.
- ❖ Isolate *B. pumilus*, BRHS/C-1, *Bacillus altitudinis* BRHS/S-73 & BRHS/P-22, *Enterobacter cloacae*, BRHS/R-71, *Paenibacillus polymyxa* BRHS/R-72, *B. methylotrophicus* BRHS/P-91, *Burkholderia symbiont* BRHS/P-92 and *B. aerophilus* BRHS/B-104 were found to possess all the tested plant growth promoting traits like phosphate solubilization, IAA, siderophore, HCN and ACC deaminase production. Apart from this these potential bacterial isolates could inhibit root pathogens upto 80%. They were designated as potential plant growth promoting rhizobacteria (PGPR) isolates.
- ❖ Among the PGPR isolates *B. altitudinis*, *B. aerophilus*, *Burkholderia* spp. and *Enterobacter cloacae* though there are reports from the other parts of the world to be potential PGPRs, they have been isolated and characterized for the first time from soils of Sub-Himalayan regions of Darjeeling hills.
- ❖ A total of 26 isolates of *Trichoderma harzianum* 10 isolates of *T. viride*, 13 isolates of *T. asperellum* and 6 isolates of *T. erinaceum* were obtained from various sources and were tested for their ability to produce Chitinase *in vitro*. *T. harzianum* RHS/S-559/ /NAIMCC-F-01968 and RHS/S-560//NAIMCC-F-01967, *T. viride* isolates, isolate RHS/G 251, *T. asperellum* and *T. erinaceum* RHS/Rd-551 showed maximum endo and exo chitinase activities. All these potential *Trichoderma* isolates were designated as BCA isolates. Potential PSF

and BCA as well as commonly occurring fungal isolates have been deposited to National Agriculturally Important Culture Collection (NAIMCC) of NBAIM.

- ❖ Diversity among all the PGPF, PGPR and BCA isolates were carried out using RAPD markers and DGGE formats. The analysis of genetic relatedness using random decamer primers revealed a significant amount of genetic variation among the tested organisms. Similarly DGGE analysis of the conserved sequences could detect genetic variation even among closely related isolates. This technique was useful in detecting similarities among the functionally similar group of isolates as well as to find out the similarities between unidentified organisms with reference to a known one.
- ❖ ITS-PCR technique was successfully used to confirm the identities of all the potential PGPF, PBCA and PGPR isolates using universal primers. The sequences have been deposited to NCBI Genbank database and accession number for each isolate has been provided. The potential isolates and their NCBI Acc. numbers are: *T. flavus* RHS/P-51 (GU324073), *Trichoderma erinaceum* (HM107419, GU187915, GU191829, HM117841), *T. harzianum* (HQ334995, HQ334997), *T. asperellum* (HQ334996), *B. pumilus* BRHS/C-1 (JF836847), *B. altitudinis* BRHS/P-22 & BRHS/S-73 (HQ849482 & JF899300) *Enterobacter cloacae* BRHS/R-71 (KC703974), *Paenibacillus polymyxa* BRHS/R-72 (KC703775), *Bacillus methylotrophicus*-BRHS/P-91 (JQ765577), *Burkholderia* spp. BRHS/P-92 (JQ765578) and *B. aerophilus* BRHS/B-104 (KC603894)
- ❖ All the PGPF, BCA and PGPR isolates were tested for their effect on enhancement of growth and resistance against root diseases of few important legumes (*Vigna radiata*, *Cicer arietinum*, *Glycine max*, *Pisum sativum* and *Phaseolus vulgaris*) Cereals (*Triticum aestivum* and *Oriza sativum*) vegetable crops (*Lycopersicon esculentum* and *Brassica juncea*) and plantation crop (*Camellia sinensis*). Among the PGPF isolates *T. flavus* was found to enhance the growth of tested crops more efficiently than the others and was accompanied by high soil phosphatase activities. Among the PGPR isolates *B. pumilus* and *B. altitudinis* were most effective in enhancing growth in the field conditions. Both BCA and PGPR isolates successfully reduced Sclerotial blight and root rot incidence of the test crops. which was accompanied with enhanced activities of key defense enzymes like β -1,3 Glucanase, Chitinase, Phenylalanine ammonia lyase and Peroxidase and enhanced levels of phenolics. Bio-priming of the

seeds and seedlings prior to sowing and after germination proved to be effective in growth enhancement and to induce resistance against fungal root pathogens. Reduction of root diseases by both BCA and PGPR was associated with all the elements commonly known to be involved in the induced systemic resistance which were found to have been enhanced.

BIBLIOGRAPHY

- Abbasi MK, Sharif S, Kazmi M, Sultan T and Aslam M.** Isolation of plant growth promoting rhizobacteria from wheat rhizosphere and their effect on improving growth, yield and nutrient uptake of plants. *Plant. Biosys.* **145:** 159-168, 2011.
- Abd-Alla MH.** Use of organic phosphorus by *Rhizobium leguminosarum* bv. *viciae* phosphatases. *Biol. Fertil. Soils.* **8:** 216–218, 1994.
- Afzal A and Bano A.** *Rhizobium* and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum* L.). *Int. J. Agri. Biol.* **10:**85-88, 2008.
- Agrawal PK, Agrawal S, Verma SK, Singh SK and Shukla KP.** Characterization of plant growth promoting bacteria from soil of central and upper Himalaya region. *Int J. Appl Biol and Pharma. Technol.* **2:** 363-369, 2011.
- Ahmad F, Ahmad I and Khan MS.** Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* **163:** 173–181, 2008.
- Ahmad F, Ahmad I, Aqil F, Wani AA, and Sousche YS.** Plant growth promoting potential of free living diazotrophs and other rhizobacteria isolated from northern Indian soil. *Biotechnol. J.* **1:** 1112–1123, 2006.
- Ahmad F, Husain FM and Ahmad I. (2011)** Rhizosphere and Root Colonization by Bacterial Inoculants and Their Monitoring Methods: A Critical Area in PGPR Research. In: Ahmad *et al.* (eds.). *Microbes and Microbial Technology: Agricultural and Environmental Applications* Springer Science+Business Media, LLC. pp.5-14, 2011.
- Alam S, Khalil S, Ayub N and Rashid M.** 2002. *In vitro* solubilization of inorganic phosphate by phosphate solubilizing microorganism (PSM) from maize rhizosphere. *Intl. J. Agric. Biol.* **4:** 454-458, 2002.
- Alba APC and Devay HE.** Detection of cross-reactive antigens between *Phytophthora infestans* (Mont.) de Bary and *Solanum* species by indirect

- Enzyme-Linked Immunosorbent Assay. *Phytopathology*. **112**: 97-104, 1985.
- Alpei J, Bonkowski M and Scheu S.** Protozoa, Nematoda and Lumbricidae in the rhizosphere of *Hordelymus europaeus* (Poaceae): Faunal interactions, response of microorganisms and effects on plant growth. *Oecologia*. **106**:111–126, 1996.
- Alström S, Burns RG.** Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biol. Fertil. Soils* **7**: 232–238, 1989.
- Altomare C, Norvell WA, Björkman T and Harman GE.** Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* **65**: 2926–2933, 1999.
- Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ.** Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410, 1990.
- Anderson LM, Stockwell VO and Loper JE.** An extracellular protease of *Pseudomonas fluorescens* inactivates antibiotics of *Pantoea agglomerans*. *Phytopathology* **94**: 1228-1234, 2004.
- Arai M, Tomoda H, Okuda T, Wang H, Tabata N, Masuma R, Yamaguchi Y and Omura S.** Funicone- related compounds, potentiators of antifungal miconazole activity, produced by *Talaromyces flavus* FKI-0076. *J. Antibiotic.* **55**:172-180, 2002.
- Attia M, Nemat M, Awad A and Turky S.** Induction of defense responses in soybean plants against *Macrophomina phaseolina* by some strains of plant growth promoting Rhizobacteria. *J. Appl. Sci. Res.* **7**: 1507-1517, 2011.
- Audenaert K, Pattery T and Cornelis P.** Induction of systemic resistance to Botrytis cinerea in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin and pyocyanin. *Mol. Plant-Microbe. Interact.* **15**: 1147-1156, 2002.
- Ayer WA, Racok JS.** The metabolites of *Talaromyces flavus*: Part 1. Metabolites of the organic extracts. *Can. J. Chem.* **68**: 2085–2094, 1990.
- Babalola O, Osir EO, Sanni AI, Odhaimbo GD, Bulimo WD.** Amplification of 1-aminocyclopropane-1-carboxylic (ACC) deaminase from plant

- growth promoting rhizobacteria in *Striga*-infested soils. *Afr. J. Biotechnol.* **2**:157–160, 2003.
- Baki A and Anderson JD.** Vigor determination in Soybean seed by multiple criteria. *Crop Sci.* **13**: 630-633, 1973.
- Bakker AW, Schippers B.** Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. *Soil Biol. Biochem.* **19**: 249–256, 1987.
- Bakker PAHM, Pieterse CMJ and Van Loon LC.** Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology.* **97**: 239-243, 2007.
- Bakonyi T, Derakhshifar I, Grabensteiner L and Nowotny N.** Development and evaluation of PCR assays for the detection of *Paenibacillus* larvae in honey samples: comparison with isolation and biochemical characterization. *Appl. Environ. Microbiol.* **69**: 1504-1510, 2003.
- Banik S. and Dey BK.** Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate solubilizing microorganisms. *Plant Soil.* **69**:353-364, 1982.
- Bartholdy BA, Berreck M and Haselwandter K.** Hydroxamate siderophore synthesis by *Phialocephala fortinii*, a typical dark septate fungal root endophyte. *Bio. Metals.* **14**:33-42, 2001.
- Bashan Y, de-Bashan LE.** How the plant growth promoting bacterium *Azospirillum* promotes plant growth –a critical assessment. *Adv. Agron.* **108**: 77–136, 2010.
- Be' langer RR andAvis TJ** Ecological processes and interactions occurring in leaf surface fungi. In: SE Lindow, EI Hecht-Poinar, and VJ Elliot (eds.). *Phyllosphere Microbiology.*, APS Press, St. Paul, MN, pp. 193–207, 2002.
- Benhamou N.** Potential of the mycoparasite, *Verticillium lecanii*, to protect citrus fruit-against *Penicillium digitatum*, the causal agent of green mold: A comparison with-the effect of chitosan. *Phytopathol.* **94**: 693-705, 2004.
- Bhatacharyya PN and Jha DK.** Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *J. Microbiol. Biotechnol.* **28**: 1327-1350, 2012.
- Blumer C, Haas D.** Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* **173**: 170–177, 2000.

- Bojinova D, Velkova R, Grancharov I, Zhelev S.** The bioconversion of Tunisian hosporphite using *Aspergillus niger*. *Nutr. Cyc. Agroecosyst.* **47:** 227-232, 1997.
- Boller T and Mauch F.** Colorimetric assay for chitinase. *Meth. Enzymol.* **161:** 430-435, 1988.
- Boopathi E, Rao KS.** A siderophore from *Pseudomonas putida* type A1: structural and biological characterization. *BBA-Protein Struct. M.* **1435:** 30–40, 1999.
- Boopathi T, Balamurugan V, Gopinath S and Sundararaman M.** Characterization of IAA Production by the Mangrove Cyanobacterium Phormidium sp. MI405019 and Its Influence on Tobacco Seed Germination and Organogenesis. *J. Plant Growth Regul.* DOI 10.1007/s00344-013-9342-8, 2013.
- Bowen GD and Rovira AD.** The rhizosphere and its management to improve plant growth. *Adv. Agron.* **66:** 1–102, 1999.
- Brewer D, Calder FW, MacIntyre TM and Taylor A.** Ovine ill-thrift in Nova Scotia: The possible regulation of the rumen flora in sheep by the fungal flora of permanent pasture. *The J. Agri.Science.* **76:** 465-477, 1971.
- Brimecombe MJ, De Leij FA and Lynch JM.** The Rhizosphere- The Effect of Root Exudates on Rhizosphere Microbil Populations. In: R Pinton, Z Varanini and P Nannipieri (eds.). *The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface.* Marcel Dekker, New York, pp. 95- 140, 2001.
- Broggini GAL, Duffy B, Hollinger E, Scharer HJ, Gessler C and Patocchi A.** Detection of fire blight biocontrol agent *Bacillus subtilis* BD170 in a Swiss apple orchard. *Eur.J. Plant Pathol.* **111:** 93–100, 2005.
- Buchanan RE, Gibbons NE. (Ed.)** Bergey's Manual of Determinative Bacteriology, 8th ed. Williams & Wilkins Co. Baltimore, Md. 21202, 1974.
- Buhariwalla HK, Srilakshmi P, Kannan S, Kanchi RS, Chandra S, Satyaprasad K, Waliyar F, Thakur RP and Crouch JH.** AFLP analysis of *Trichoderma* spp. from India compared with sequence and morphological-based diagnostics. *J. Phytopathol.* **153:** 389–400, 2005.

- Bull CT, Shetty KG and Subbarao KV.** Interactions between Myxobacteria, plant pathogenic fungi and biocontrol agents. *Plant Dis.* **86:** 889-896, 2002.
- Bünemann EK.** Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. *Soil Biol. Biochem.*, **40:** 2116-2129, 2008.
- Cabello M, Irrazabal G, Bucsinzky AM, Saparrat M and Schalamuck S.** Effect of an arbuscular mycorrhizal fungus, *G. mosseae* and a rock-phosphate-solubilizing fungus, *P.thomii* in *Mentha piperita* growth in a soilless medium. *J. Basic Microbiol.* **45:**182-189, 2005.
- Chakraborty BN.** Microbial resources and their importance in agricultural biotechnology. In: Eco-Conservation and Sustainable living (Eds. Gurung, C and Bhandari, J.B.), Narosa Publishing House, New Delhi, pp.11-30, 2013.
- Chakraborty BN and Saha A.** Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. *Physiol. Mol. Plant Pathol.* **44:** 403 – 416, 1994.
- Chakraborty BN and Chakraborty U.** Microbial resources for crop improvement. Satish Publishing House, Delhi. 275pp, 2013.
- Chakraborty BN, Chakraborty U, Sunar K and Dey PL.** Evaluation of plant growth promoting and antifungal activities of *Talaromyces flavus* (NAIMCC-F-01948) against *Sclerotium rolfsii*. *Indian Phytopath.* **65:** 258-263, 2012.
- Chakraborty BN, Chakraborty U, Sunar K and Dey PL.** RAPD profile and rDNA sequence analysis of *Talaromyces flavus* and *Trichoderma* species. *Ind. J. Biotechnol.* **10:** 487-495, 2011a.
- Chakraborty BN, Chakraborty U, Saha A, Dey PL and Sunar K.** Morphological and Molecular Characterization of *Trichoderma* Isolates of North Bengal. *J. Mycol.Plant Pathol.* **41:** 207-214, 2011b.
- Chakraborty BN, Chakraborty U, Saha A, Dey PL and Sunar, K.** Evaluation of phosphate solubilizer from soil of North Bengal and their diversity analysis. *World J. Agri. Sci.* **6:** 195-200, 2010a.
- Chakraborty BN, Chakraborty U, Dey PL and Sunar K.** Phylogenetic relationship of *Trichoderma* isolates of North Bengal based on sequence

analysis of ITS region of rDNA *Journal of Applied Science and Research* **6**:1477-1482, 2010b.

Chakraborty BN, Chakraborty U, Saha A, Dey PL and Sunar K. Screening of phosphate solubilizing Aspergilli isolates from soils of North Bengal and their effects on soybean. *J. Mycol. Plant Pathol.* **38**: 227-233, 2008.

Chakraborty U, Chakraborty BN, Chakraborty AP, Sunar K and Dey PL. Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. *Indian J. Biotechnol.* **12**: 20-31, 2013.

Chakraborty U, Chakraborty BN and Chakraborty AP. Influence of *Serratia marcescens* TRS-1 on growth promotion and induction of resistance in *Camellia sinensis* against *Fomes lamaoensis*. *J. Plant. Interact.* **5**: 261-272, 2010c.

Chakraborty U, Chakraborty BN, Basnet M, Chakraborty AP. Evaluation of *Ochrobactrum anthropi* TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. *J. Appl. Microbiol.* **107**: 625–634, 2009.

Chakraborty U, Basnet M and Chakraborty BN. Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. *J. Basic Microbiol.* **46**: 186-195, 2006.

Chakraborty U, Chakraborty BN, Tongden C, Roychowdhury P. et al., Assessment of Tea rhizosphere microorganisms as plant growth promoters. *J. Tea Res.* **25**: 38-47, 2004.

Chakraborty U, Chakraborty BN and Kapoor M. Changes in levels of peroxidase and phenylalanine ammonia lyase in Brassica napus cultivars showing variable resistance to *Leptosphaeria maculans*; *Folia Microbiol.* **38**: 491-499, 1993.

Chapon A, Boutin M, Rime D, Delalande L, Guillerm AY and Sarniguet A. Direct and specific assessment of colonization of wheat rhizoplane by *Pseudomonas fluorescens* Pf29A. *Eur. J. Plant Pathol.* **109**: 61–70, 2003.

Chen F, Wang M, Zheng Y and Luo J. Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber fusarium wilt by *Bacillus subtilis* B579. *World J. Microbiol. Biotechnol.* **26**: 675-684, 2010.

- Chen YP, Rekha D, Arunshen AB, Lai WA and Young CC.** Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Appl. Soil Ecol.* **34**: 33-41, 2006.
- Chet I and Inbar J.** Biological Control of fungal pathogens. *Appl. Biochem. Biotechnol.* **48**: 37-43, 1994.
- Cipollini D, Purrington CB and Bergelson J.** Costs of induced responses in plants. *Basic Appl. Ecol.* **4**: 79–85, 2003.
- Clausen J.** Immunochemical techniques for the identification of macromolecules. In: *Laboratory techniques in biochemistry and molecular biology*. Vol -1 Part III. (Eds. R. H. Burden and P.H. Van Knippenberg). pp 64-65, 1988
- Compant S, Duffy B, Nowak J, Clément C and Barka EA.** Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **71**:4951–4959, 2005.
- Conn VM, Walker AR and Franco CMM.** Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **21**: 208-218, 2008.
- Contreras-Cornejo HA, Macias-Rodriguez L, Cortes-Penagos C, Lopez-Bucio J.** *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiol.* **149**:1579–1592, 2009.
- Cook RJ.** Making greater use of microbial inoculants in agriculture. *Annu. Rev. Phytopathol.* **31**: 53-80, 1993.
- Cottyn B, Regalado E, Lanoot B, De Cleene M.** 2001. Bacterial populations associated with the rice seed in the tropical environment. *Phytopathol.* **91**: 282–292, 2001.
- Crowley DE.** Microbial siderophores in the plant rhizosphere. In: LL Barton and J Abadía (eds). *Iron Nutrition in Plants and Rhizospheric Microorganisms*. Springer, Netherlands, pp. 169–198, 2006.
- Curl EA and Harper JD.** Fauna-microflora interactions. In: JM Lynch (ed.). *The Rhizosphere*, John Wiley & Sons, Chichester, pp.369–388, 1990.
- Dakora FD and Philipps DA.** Root exudates as mediators of mineral acquisition in low nutrient environments. *Plant Soil.* **245**: 35–47, 2002.

- de Boer W, Folman LB, Summerbell RC and Boddy L.** Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol. Rev.* **29**:795–811, 2005.
- De la Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pintor-Toro JA. and Llobel A.** *Eur. J. Biochem.* **206**: 859-867, 1992.
- De Marco, J, Lima L, Sousa M and Felix C.** A *Trichoderma harzianum* chitinase destroys the cell wall of the phytopathogen *Crinipellis pernicioso*, the causal agent of witches broom disease of cocoa. *World J. Microbiol. Biotechnol.* **16**: 383- 386, 2000.
- De Meyer G and Hofte M.** Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinera* on bean. *Phytopathology.* **87**: 588-593, 1997.
- De Weert S and Bloemberg G.** Rhizosphere competence and the role of root colonization in biocontrol. In: SS Gnanamanickam (ed.). *Plant-Associated Bacteria*, Springer: The Netherlands, pp. 317–333, 2006.
- Deubel A, Gransee H and Merbach W.** Transformation of organic rhizodeposits by rhizoplane bacteria and its influence on the availability of tertiary calcium phosphate. *J. Plant Nutr. Soil Sci.* **163**: 387-392, 2000.
- Devi KK, Seth N, Kothamasi S, Kothamasi D.** Hydrogen cyanide producing rhizobacteria kill subterranean termite *Odontotermes obesus* (Rambur) by cyanide poisoning under *in Vitro* Conditions. *Curr. Microbiol.* **54**: 74–78, 2007.
- Dobbelaere S, Croonenberghs A, Thys A, Van De Broek A, et al.** Photostimulatory effects of *Azospirillum brasilense* wild type and mutant strain altered in IAA production in wheat. *Plant Soil*, **212**: 155–164, 1999.
- Dodor DE and Tabatabai AM.** Effect of cropping systems on phosphatases in soils. *J. Plant Nutr. Soil Sci.* **166**: 7–13, 2003.
- Domsch KH, Gams W, Anderson TH.** *Compendium of soil fungi*, Vol 1. Academic Press, London, pp 752-759, 1980.
- Doornbos RF, Loon LC, Bakker PAHM.** Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. *Agron. Sustain. Dev.* **32**: 227-243, 2012.
- Drinkwater LE.** Improving fertilizer nitrogen use efficiency through an ecosystem-based approach. In: A Mosie, JK Syers and J Freney (eds)

Agriculture and the Nitrogen Cycle: Assessing the Impacts of Fertilizer Use on Food Production and the Environment, Vol 65. Island Press, Washington, DC, pp 93–102, 2002.

- Droog F.** Plant glutathione S-transferases, a tale of theta and tau. *J. Plant Growth Regul.* **16**:95–107, 1997.
- Dunn C, Crowley JJ, Moenne-Loccoz Y, Dowling DN, de Bruijn FJ and O’Gara F.** Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W18 is mediated by an extracellular proteolytic activity. *Microbiol.* **143**:3921–3931, 1997.
- Duo-Chuan LI, Chen S and Jing L.** Purification and partial characterization of two chitinases from the mycoparasitic fungus *Tararomyces flavus*. *Mycopathologia* **159**: 223-229, 2005.
- Duponnois R., Kisa M and Plenchette C.** Phosphate solubilizing potential of the nematofungus *Arthrobotrys oligospora*. *J. Plant Nutr. Soil Sci.* **169**: 280–282, 2006.
- Dutta S, Podile AR.** Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. *Crit. Rev. Microbiol.* **36**: 232–244, 2010.
- Elad Y and Baker R.** Influence of trace amounts of cations and siderophore-producing pseudomonads on chlamydiospore germination of *Fusarium oxysporum*. *Phytopathology.* **75**: 1047-1052, 1985.
- Elad Y, Chet I and Karan J.** *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rotsii* and *Rhizoctonia solani*. *Phytopathol.* **70**: 119-121, 1980.
- El-Tarabily KA, Nassar AH and Sivasithamparam K.** Promotion of growth of bean (*Phaseolus vulgaris* L.) in a calcareous soil by a phosphate-solubilizing, rhizospherecompetent isolate of *Micromonospora endolithica*. *Appl. Soil Ecol.* **39**: 161–171, 2008.
- Fahima T, Madi L and Henis Y.** Ultrastructure and germinability of *Verticillium dahliae* microsclerotia parasitized by *Talaromyces flavus* on agar medium and in treated soil. *Biocont. Sci. Technol.* **2**: 69-78, 1992.
- Fallah A.** Abundance and distribution of phosphate solubilizing bacteria and fungi in some soil samples from north of Iran. 18th World Congress of Soil Science, July 9-15, 2006, Philadelphia, Pennsylvania, USA, 2006.

- Fankem H, Nwaga D, Deubel A, Dieng L, Merbach W and Etoa FT.** Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *African J. Biotech.* **5**: 2450-2460, 2006.
- Fox R, Comerford NB, and W. W. Mcfee.** Phosphorus and aluminium release from a spodic horizon mediated by organic acids. *Soil Sci. Soc. Am. J.* **54**: 1763-1767, 1990.
- Fravel DR.** Interaction of biological fungi with sublethal rates of metham sodium for control of *Verticillium dahliae*. *Crop Protection.* **15**: 115-119, 1996.
- Frostegård Å, Tunlid A and Bååth E.** Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biol. Biochem.* **28**:55–63, 1996.
- Gajera H, Domadiya R, Patel S, Kapopara M, Golakiya B.** Molecular mechanism of Trichoderma as bio-control agents against phytopathogen system – a review. *Curr. Res. Microbiol. Biotechnol.* **4**: 133-142, 2013.
- Garbaye J.** Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol.* **128**: 197–210, 1994.
- George E, Marochner, H, Jakobson I.** Role of Arbuscular Mycorrhizal fungi in uptake of phosphorous and nitrogen from soil. *Crit. Rev. Biotechnol.* **15**: 257-270, 1995.
- George P, Gupta A, Gopal M, Thomas L, Thomas GV.** Multifarious beneficial traits and plant growth promoting potential of *Serratia marcescens* KiSII and *Enterobacter* sp. RNF 267 isolated from the rhizosphere of coconut palms (*Cocos nucifera* L.). *World. J. Microbiol. Biotechnol.* **29**:109–117, 2013.
- Gerretsen FC.** The influence of microorganisms on the phosphate intake by the plant. *Plant Soil.* **1**: 51-81, 1948.
- Ghosh S, Penterman JN, Little RD, Chavez R, Glick BR.** Three newly isolated plant growth-promoting bacilli facilitate the seedling growth of canola, *Brassica campestris*. *Plant Physiol. Biochem.* **41**: 277–281, 2003.
- Ghosh S, Sengupta C, Maiti TK, Basu PS.** Production of 3-indolylacetic acid in root nodules and culture by a *Rhizobium* species isolated from root nodules of the leguminous pulse *Phaseolus mungo*. *Folia Microbiol.* **53**: 351–355, 2008.

- Glazebrook J.** Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**:205-227, 2005.
- Glick BR, Penrose DM and Li J.** A model for the lowering of plant ethylene concentration by plant growth promoting bacteria. *J. Theor. Biol.* **190**: 63–68, 1998.
- Glick BR.** The enhancement of plant growth by free living bacteria. *Can J Microbiol* 41:109–117, 1995.
- Goenadi D, Siswanto Sugiarto Y.** Bio-activation of poorly soluble Phosphate rocks with a Phosphorus-Solubilizing Fungus. *Soil Sci. Soc. Am. J.* **64**: 927-932, 2000.
- Goldstein AH.** Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. *Am. J. Altern. Agric.* **1**: 51-57, 1986.
- Goldstein AH.** Bioprocessing of rock phosphate ore: essential technical considerations for the development of a successful commercial technology. Proc. 4th Int. Fert. Assoc. Tech. Conf.. IFA, Paris. p. 220, 2000.
- Goldstein AH.** Involvement of the quinoprotein glucose dehydrogenases in the solubilization of exogenous phosphates by gram-negative bacteria. In: A T Gorini, E Yagil and S Silver (eds.). Phosphate in Microorganisms: Cellular and Molecular Biology, ASM Press, Washington, D. C. pp. 197-203, 1994.
- Gopalakrishnan S, Pagidi H, Bandru KK, Iyer GKK.** Evaluation of bacteria isolated from rice rhizosphere for biological control of charcoal rot of sorghum caused by *Macrophomina phaseolina* (Tassi) Goid. *World J. Microbiol. Biotechnol.* **27**: 1313-1321, 2010.
- Gruber S, Seidl-Seiboth V.** Self vs. non-self: fungal cell wall degradation in *Trichoderma*. *Microbiology*. doi:10.1099/ mic.0.052613-0, 2011.
- Gulati A, Rahi P, Vyas P:** Characterization of phosphate-solubilizing fluorescent pseudomonads from the rhizosphere of seabuckthorn growing in the cold deserts of Himalayas. *Curr. Microbiol.* **56**: 73-79, 2012.
- Gyaneshwar P, Parekh LJ, Archana, Podle PS, Collins MD, Hutson RA and Naresh KG.** Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. *FEMS Microbiol. Lett.* **171**: 223-229, 1999.

- Haas D and Defago G.** Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* **3**: 307-319, 2005.
- Haggag A, Kansoh L and Amal Aly AM.** Proteases from *Talaromyces flavus* and *Trichoderma harzianum*: purification, characterization and antifungal activity against brown spot disease on Faba Bean. *Plant Pathol. Bulletin.* **15**: 231-239, 2006.
- Halda-Alija L.** Identification of indole-3- acetic acid producing freshwater wetland rhizosphere bacteria associated with *Juncus effusus* L. *Can. J. Microbiol.* **49**: 781–787, 2003.
- Hamill JD.** Alterations in auxin and cytokinin metabolism of higher plants due to expression of specific genes from pathogenic bacteria: a review. *Aust. J. Plant Physiol.* **20**:405–423, 1993.
- Hao X, Cho CM, Racz GJ and Chang C.** Chemical retardation of phosphate diffusion in an acid soil as affected by liming. *Nutr. Cycl. Agroecosys.* **64**: 213-224, 2002.
- Hariprasad P, Niranjana SR.** Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. *Plant Soil.* **316**: 13–24, 2009.
- Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Tronsmo A.** Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathol.* **83**: 313–318, 1993.
- Harman GE, Howell CR, Viterbo A et al.** *Trichoderma* species- opportunistic, avirulent plant symbionts. *Nature Reviews* **2**: 43-56, 2004.
- Harrison MJ.** Signaling in the arbuscular mycorrhizal symbiosis. *Annu. Rev. Microbiol.* **59**:19-42, 2005.
- Hartl L, Zach S, Seidl-Seiboth V.** Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl. Microbiol. Biotechnol.* **93**: 533–543, 2012.
- Hawksworth DL, Sutton BC and Ainsworth GC.** Ainsworth and Bisby's Dictionary of fungi. Seventh Edition. Commonwealth Mycological Institute, Kew, Surry, UK, 1983.
- He ZL, Bian W and Zhu J.** Screening and identification of microorganisms capable of utilizing phosphate adsorbed by goethite. *Comm. Soil Sci. Plant Anal.* **33**: 647-663, 2002.

- Heid CA, Stevens J, Livak KJ and Williams PM.** Real time quantitative PCR. *Genome Res.* **6**: 986–994, 1996.
- Henri F, Laurette NN, D. Annette D John Q, Wolfgang M, François-Xavier E and Dieudonné N.** Solubilization of inorganic phosphates and plant growth promotion bystrains of *Pseudomonas fluorescens* isolated from acidic soils of Cameroon. *African J. Microbiol. Res.* **2**: 171-178, 2008.
- Herbert RA.** Methods for enumerating microorganisms and determining biomass in natural environments. *Methods Microbiol.* **129**: 207–212, 1990.
- Heydari A and Misaghi IJ.** Biocontrol activity of *Burkholderia cepacia* against *Rhizoctonia solani* in herbicide-treated soils. *Plant Soil.* **202**: 109-116, 1998.
- Heydari A and Misaghi IJ.** The role of rhizosphere bacteria in herbicide-mediated increase in *Rhizoctonia solani*-induced cotton seedling damping-off. *Plant Soil.* **257**: 391-396, 2003.
- Heydari A and Pessarakli M.** A Review on Biological Control of Fungal Plant Pathogens Using Microbial Antagonists. *J Biological Sciencies* **10**: 270-290, 2010.
- Heydari A, Fattahi H, Zamanizadeh HR, Hassanzadeh N and Naraghi L.** Investigation on the possibility of using bacterial antagonists for biological control of cotton seedling damping-off in green house. *Appl. Entomol. Phytopathol.* **72**: 51-68, 2004.
- Hilda R. and Fraga R.** Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotech. Adv.* **17**: 319-359, 2000.
- Hinsinger P.** Bioavailability of soil inorganic P in the rhizosphere as affected by rootinduced chemical changes: a review. *Plant Soil.* **237**: 173-195, 2001.
- Hodge A, Stewart J, Robinson D, Griffiths BS and Fitter AH.** Plant, soil fauna and microbial responses to N rich organic patches of contrasting temporal availability. *Soil Biol. Biochem.* **31**:1517–1530, 1999.
- Howell CR.** Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* **87**: 4-10, 2003.
- Hryniewicz K and Baum C.** The Potential of Rhizosphere Microorganisms to Promote the Plant Growth in Disturbed Soils. In: A Malik and E Grohmann (eds.). Environmental Protection Strategies for Sustainable

Development, Strategies for Sustainability. Springer Science Business Media B.V. pp. 35-64, 2011.

Hrynkiewicz K, Baum C and Leinweber P. Density, metabolic activity and identity of cultivable rhizosphere bacteria on *Salix viminalis* in disturbed arable and landfill soils. *J. Plant Nutr. Soil Sci.* **173**:747–756, 2010a.

Hrynkiewicz K, Ciesielska A, Haug I and Baum C. Conditionality of ectomycorrhiza formation and willow growth promotion by associated bacteria: role of microbial metabolites and use of C sources. *Biol. Fertil. Soils.* **46**:139–150, 2010b.

Huston MA. Biological Diversity, Cambridge University Press, Cambridge, UK, 1994.

Igual J, Valverde MA, Cervantes E and Velázquez E. Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. *Agronomie.* **21**: 561-568, 2001.

Inch S and Gilbert J. Scanning electron microscopy observations of the interaction between *Trichoderma harzianum* and perithecia of *Gibberella zeae*. *Mycologia.* **103**: 1–9, 2011.

Isherword KF. Fertilizer use and environment. In: N. Ahmed and A. Hamid (eds.). Proc. Symp. Plant Nutrition Management for Sustainable Agricultural Growth. NFDC, Islamabad. pp. 57-76, 1998.

Islam MT, Yasuyuki H, Abhinandan D, Toshiaki I and Satoshi T. Suppression of damping-off-disease in host plants by the rhizoplane bacterium *Lysobacter sp.* strain SB-K88 is-linked to plant colonization and antibiosis against soilborne peronosporomycetes. *Appl. Environ. Microbiol.* **71**: 3786-3796, 2005.

Janisiewicz WJ and Peterson DL. Susceptibility of the stem pull area of mechanically harvested apples to blue mold decay and its control with a biocontrol agent. *Plant Dis.* **88**: 662-666, 2004.

Jeewon, R., Jayesh Ittoo,Devendra Mahadeb,Yasmina Jaufeerally-Fakim,Hong-Kai Wang,and Ai-Rong Liu. DNA Based Identification and Phylogenetic Characterisation of Endophytic and Saprobic Fungi from *Antidesma madagascariense*, a Medicinal Plant in Mauritius. *J. Mycol.* **10**: 55-67, 2013. doi.org/10.1155/2013/781914.

- Jentschke G, Bonkowski M, Godbold DL, and Scheu S.** Soil protozoa and forest tree growth: Non-nutritional effects and interaction with mycorrhizae. *Biol. Fertil. Soils.* **20**:263–269, 1995.
- Jilani G, Akram A, Ali RM, Hafeez FY, Shamsi IH, Chaudhry AN and Chaudhry AG.** Enhancing crop growth, nutrients availability, economics and beneficial rhizosphere microflora through organic and biofertilizers. *Ann. Microbiol.* **57**:177-183, 2007.
- Jin X, Sun R, Zhu J, Xu Z.** Isolation and Identification of *Bacillus altitudinis* ZJ 186 from Marine Soil Samples and its antifungal activity against *Magnaporthe oryzae*. *Curr. Res. Bacteriol.* **5**: 13-23, 2012.
- Jing Y, He Z and Yang X.** Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. *J Zhejiang Univ. Sci. B.* **8**:192–207, 2007.
- Jones DL.** Organic acids in the rhizosphere - a critical review. *Plant Soil.* **205**: 25-44, 1998.
- Joseph CM and Phillips DA.** Metabolites from soil bacteria affect plant water relations. *Pl. Physiol. Biochem.* **41**:189–192, 2003.
- Kadyan S, Panghal M, Kumar S, Singh K and Yadav JP.** Assessment of functional and genetic diversity of aerobic endospore forming Bacilli from rhizospheric soil of *Phyllanthus amarus* L. *World. J. Microbiol. Biotechnol.* DOI 10.1007/s11274-013-1323-3, 2013.
- Kageyama K and Nelson EB.** Differential inactivation of seed exudates stimulation of *Pythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. *Appl. Environ. Microbiol.* **69**: 1114-1120, 2003.
- Kamerewerd J, Zadra I, Kurnsteiner H, Kuck U.** PchI-BI, encoding a class V chitinase, is affected by PcVelA and PcLaeA and responsible for cell wall integrity in *Penicillium hrysogenum*. *Microbiol.* doi:10.1099/mic.0.051896-0, 2011.
- Kamil Z, Rizk M, Saleh M and Moustafa S.** Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. *Global J. Mol. Sci.* **2**: 57–66, 2007.

- Karlsson M, Ihrmark K, Asmail N, Ubhayasekera W, Melin P, Stenlid J.** Comparative molecular evolution of *Trichoderma* chitinases in response to mycoparasitic Interactions. *Evol. Bioinform.* **6**: 1–26, 2010.
- Karthick S, Namsivayam Raja, Narendrakumar G, and Arvind Kumar J.** Evaluation of Effective Microorganism (EM) for treatment of domestic sewage. *J. Exp. Sci.* (2)**7**: 30-32, 2011.
- Kaymak HC.** Potential of PGPR in Agricultural Innovations. In: DK Maheshwari (ed) Plant growth and health promoting bacteria. Microbiol monographs. Springer, Berlin **18**:45–79, 2011.
- Keel C, Voisard C, Berling CH Kahir G and Defago G.** Iron sufficiency is a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA, under gnotobiotic conditions. *Phytopathology.* **79**: 584-589, 1989.
- Khan MS, Zaidi A and Wani PA.** Role of phosphate solubilizing microorganisms in sustainable agriculture: a review. *Agron. Sustain. Dev.* **27**: 29–43, 2007.
- Khiari L and Parent LE.** Phosphorus transformations in acid light-textured soils treated with dry swine manure. *Can. J. Soil Sci.* **85**:75-87, 2005.
- Kim KY, Jordan D and McDonald GA.** Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. *Biol. Fert. Soils* **26**: 79-87, 1998.
- King EJ, Brown MF.** A technique for preserving aerial fungal structure for scanning electron microscopy. *Can. J. Microbiol.* **29**: 653–658, 1983.
- Klingstrom E and Johansson SM.** Antagonism of *Scytalidium* isolates against decay fungi. *Phytopathol.* **63**: 473-479, 1973.
- Kloepper JW, Ryu CM and Zhang S.** Induce systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology.* **94**:1259-1266, 2004.
- Knoester M, Pieterse CMJ, Bol JF and Van Loon LC.** Systemic resistance in Arabidopsis induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Mol. Plant Microbe Interact.* **12**:720-727, 1999.
- Knudsen D, Beegle D.** Recommended phosphorous tests. In: Dahnke, W.C. (Ed.), Recommended Chemical Soil Tests Procedures for the North

Central Region. Bull North Dakota Agric. Exp. Stn., North Dakota, USA, pp. 122-115, 1988.

- Kpombrekou K and Tabatabai MA.** Effect of organic acids on release of phosphorus from phosphate rocks. *Soil Sci.* **158**: 442-453, 1994.
- Kucey RMN, Janzen H and Legget ME.** Microbial mediated increases in plant available phosphorus. *Adv. Agron.* **42**:199 – 228, 1989.
- Kuffner M, Puschenreiter M, Wieshammer G, Gorfer M, Sessitsch A.** Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. *Plant Soil.* **304**: 35–44, 2008.
- Kuhls K, Lieckfeldt E, Borner T et al.** Molecular reidentification of human pathogenic *Trichoderma* isolates as *Trichoderma longibrachiatum* and *Trichoderma citrinoviride*. *Med. Mycol.* **37**: 25-33, 1999.
- Kullnig CM and Kubicek CP.** Evolution and phylogeny of the genus *Trichoderma* based on a multisequence approach. *Institut fuer Biochemische Technologieand Mikrobiologie*, Austria, 2000.
- Kumar A, Kumar A, Devi S, Patil S, Payal C and Negi S.** Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent. Res. Sci. Technol.* **4**: 01-05, 2012.
- Kumar A, Prakash A and Johri BN.** *Bacillus* as PGPR in crop ecosystem. In: DK Maheshwari (ed.) *Bacteria in agrobiolgy: Crop ecosystem*, Springer-Verlang, Berlin, pp.37-59, 2011.
- Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature.* **227**: 680-685, 1970.
- Lafontaine PJ and Benhamou N.** Chitosan treatment: An emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. *Biocont.Sci.Technol.* **6**:111-124, 1996.
- Lange L, Heide M, Hobolth L and Olsol WL.** Serological detection of *Plasmodiophora brassicae* by dot immuno-binding and visualization of the serological reactions by scanning electron microscopy. *Phytopathl.* **79**: 1066-1075, 1989.

- Laslo E, Gyorgy E, Mara G, Tamas E.** Screening of plant growth promoting rhizobacteria as potential microbial inoculants. *Crop protection*. **40**: 43-48, 2012.
- Latha J, Verma A and Mukherjee KP.** PCR fingerprinting of some *Trichoderma* isolates from two Indian type culture collations—need for reidentification of these economically important fungi. *Curr.Sci.* **83**: 372-374, 2002.
- Lemanceau P, Alabouvette C and Meyer JM.** Production of fusarinine and iron assimilation by pathogenic and non-pathogenic *Fusarium*. In: TR Swinburne (ed.). Iron, siderophores and plant diseases. Plenum, London, pp. 251–259, 1985.
- Li J, Yang Q, Zhao L, Zhang S, Wang Y, Zhao X (2009)** Purification and characterization of a novel antifungal protein from *Bacillus subtilis* strain B29. *J Zhejiang. Univ. Sci. B.* **10**: 264–272, 2009.
- Liang J, Tao R, Hao Z and Wang L.** Induction of resistance in cucumber against seedling damping-off by plant growth-promoting rhizobacteria (PGPR) *Bacillus megaterium* strain L8. *Af. J. Biotechnol.* **10**: 6920-6927, 2011.
- Lima G, De Curtis F, Castoria R and De Cicco V.** Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. *Eur. J. Plant Pathol.* **109**: 341–349, 2003.
- Liu B, Glenn D and Buckley K.** *Trichoderma* communities in soils from organic, sustainable, and conventional farms, and their relation with Southern blight of tomato. *Soil Biol. Biochem.* **40**: 1124–1136, 2008.
- Loper JE and Buyer JS.** Siderophores in microbial interactions of plant surfaces. *Mol. Plant-Microbe Interact.* **4**: 5-13, 1991.
- Lowder M, Unge A, Maraha N, Jansson JK, Swigget J and Oliver JD.** Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. *Appl. Environ. Microbiol.* **66**: 3160–3165, 2000.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ.** Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**: 265-275, 1951.
- Lynch JM and Whipps JM.** Substrate flow in the rhizosphere. *Pl. Soil.* **129**:1–10, 1990.

- M'Piga P, Belanger RR, Paulitz TC and Benhamou N.** Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. *Physiol. Mol. Plant Pathol.* **50**: 301–320, 1997.
- Madhavan S, Paranidharan V, Velazhahan R.** RAPD and virulence analyses of *Colletotrichum capsici* isolates from chilli (*Capsicum annuum*). *J. Plant Dis. Protect.* **117**: 253–257, 2010.
- Madi L, Katan J and Henis Y.** Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. *Phytopathol.* **87**: 1054-1060, 1997.
- Magdy H, Abd El-Twabl and Momein HA El-Katatny1.** Genome characterization and biochemical analysis of some *Trichoderma* strains by RAPD, Ac Transposable Elements, 5S rDNA, CMC-ase and PGase Egypt. *J. Bot.* 2nd International conference, 29-30 April, Minia Univ., pp. 125 – 140, 2012.
- Mahadevan A and Sridhar R.** Methods in physiological plant pathology. II. Ed. Sivakami Publ. pp. 157-159, 1982.
- Mahaffee WF and JW Kloepper.** Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field grown cucumber (*Cucumis sativus* L.). *Microb. Ecol.* **34**: 201–223, 1997.
- Mahaffee WF, Bauske EM, van Vuurde JW, van der Wolf JM, van den Brink M and Kloepper JW.** Comparative analysis of antibiotic resistance, immunofluorescent colony staining, and a transgenic marker (bioluminescence) for monitoring the environmental fate of a rhizobacterium. *Appl. Environ. Microbiol.* **63**: 1617–1622, 1997.
- Manoch L, Jeamjitt O, Dethoup T, Kokaew J and Poochinya P.** SEM study on ascospore ornamentation *Aspergillus* and *Penicillium* teleomorphs from soil at termite mounds. *Journal of Microscopy Society of Thailand.* **18**: 98-103, 2004.
- Mantelin S and Touraine B.** Plant growth-promoting bacteria and nitrate availability: impact on root development and nitrate uptake. *J. Exp. Bot.* **55**: 27–34, 2004.

- Margulis L.** Symbiosis in cell evolution: microbial communities in the archea and proterozoic eons, 2nd edition. W.M. Freeman and Co., New York, 1993.
- Marois JJ, Fravel DR and Papavizas GC.** Ability of *Talaromyces flavus* to occupy the rhizosphere. *Soil Biol. Biochem.* **16**: 387–390, 1984.
- Martínez-Viveros O, Jorquera MA, Crowley DE, Gajardo G and Mora ML.** Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *J. Soil Sci. Plant Nutr.* **10**: 293 – 319, 2010.
- Masunaka A, Hyakumachi M, Takenaka S.** Plant growthpromoting fungus, *Trichoderma koningi* suppresses isoflavonoid phytoalexin vestitol production for colonization on/in the roots of *Lotus japonicus*. *Microb. Environ.* **26**: 128–134, 2011.
- Mathesius U, Mulders S, Gao MS, Teplitski M, Caetano-Anolles G, Rolfe BG and Bauer WD.** Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sci.* **100**:1444–1449, 2003.
- Mathew KA. and Gupta SK.** Studies on web blight of French bean caused by *Rhizoctonia solani* and its management. *J. Mycol. Pl. Pathol.* **26**: 171-177, 1996.
- Mathivanan N, Kabilan V and Murugesan K.** Production of chitinase by *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*. *Indian J. Exp. Biol.* **35**: 890–893, 1997.
- Maurhofer M, Hase C, Meuwly P, Metraux JP and Defago G.** Induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonization *Pseudomonas fluorescens* strain CHAO: Influence of the gac A gene and of pyoverdine production. *Phytopathology.* **84**: 139-146, 1994.
- McCaig AE, Glover LA and Prosser JI.** Numerical analysis of grassland bacterial community structure under different land management regimes using 16S rDNA sequence data and DGGE banding patterns. *Appl. Env. Microbiol.* **67**: 4554–4559, 2001.
- McCaig AE, Phillips CJ, Stephen JR, Kowalchuk GA, Harvey SM, Herbert RA, Embley TM and Prosser JI.** Nitrogen cycling and community

structure of β -subgroup ammonia oxidizing bacteria within polluted, marine fish-farm sediments. *Appl. Env. Microbiol.* **65**:213–220, 1999b.

McIntyre M, Nielsen J and Arnau J. Proceedings of the 7th European Conference on Fungal Genetics. Copenhagen, Denmark, 2004.

Mclaren DL, Huang HC and Rimmer SR (1986). Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. *C. J Plant Pathol.* **8**: 43-48, 1986.

Mehmood S, Bashir A, Ahmad A, Akram Z, Jabeen N and Gulfraz M. Molecular characterization of regional *Sorghum bicolor* varieties from Pakistan. *Pak. J. Bot.* **40**: 2015-2021, 2008.

Mehrvarz S, Chaichi MR and Alikhani HA. Effects of phosphate solubilizing microorganisms and phosphorus chemical fertilizer on yield and yield components of Barely (*Hordeum vulgare* L.). *Am-Euras. J. Agric. & Environ. Sci.* **3**: 822-828, 2008.

Meliani A, Bensoltane A and Mederbel H. Microbial Diversity and Abundance in Soil: Related to plant and soil type. *American J. Plant Nutrition and Fertilization Technol.* **2**: 10-18, 2012.

Meziane H, Van der Sluis I, Van Loon LC, Hofte M and Bakker PAHM. Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Mol. Plant Pathol.* **6**:177-185, 2005.

Milgroom MG and Cortesi P. Biological control of chestnut blight with hypovirulence: a critical analysis. *Annu. Rev. Phytopathol.* **42**: 311-338, 2004.

Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Biotechnol. Bioeng. Symp.* **5**: 193 – 219, 1972.

Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry.* **31**: 426-428, 1956.

Misra N, Gupta G, Jha PN. Assessment of mineral phosphate-solubilizing properties and molecular characterization of zinc-tolerant bacteria. *J. Basic Microbiol.* **52**: 549-558, 2012.

Molano J. et al. A rapid and sensitive assay for chitinase using tritiated chitin. *Analytical Biochemistry* **83**: 648-656, 1977.

Morris CE, Bardin M, Berge O, Frey-Klett P, Fromin N, Girardin H, Guinebretiere MH, Lebaron P, ThieryJM and Troussellier M. Microbial biodiversity: approaches to experimental design and hypothesis

testing in primary scientific literature from 1975 and 1999. *Microbiol. Mol. Biol. Rev.* **66**: 592–616, 2002.

- Moyne AL, Shelby R, Clevel TE and Tuzun S.** Bacillomycin D: aniturin with antifungal activity against *Aspergillus flavus*. *J. Appl. Microbiol.* **90**: 622-629, 2000.
- Muthumeenakshi S, Mills PR, Brown AE and Seaby DA.** Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiol.* **140**: 769-777, 1994.
- Nahas E.** Factors determining rock phosphate solubilization by microorganism isolated from soil. *World J. Microb. Biotechnol.* **12**:18-23, 1996.
- Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara, G and Renella G.** Microbial diversity and soil functions. *Eur. J. Soil. Sci.* **54**: 655-670, 2003.
- Naraghi L. Heydari A, Rezaee S, Razavi M (2012).** Biocontrol agent *Talaromyces flavus* stimulates the growth of Cotton and Potato. *J. Plant. Growth Regul.* **31**: 471–477, 2012.
- Naraghi L., Heydari A, Rezaee S, Razavi M. and Afshari-Azad H.** Biological control of greenhouse cucumber *Verticillium* wilt disease by *Talaromyces flavus*. *Phytopathol. Mediterr.* **49**: 321–329, 2010.
- Nenwani V, Doshi P, Saha T and Rajkumar S.** Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity. *J. Yeast and Fungal Research.* **1**: 009-014, 2010.
- Nozawa H, Ishikawa S, Nakayama K, Ogawa S and Izu S.** Establishment of a procedure to control strawberry anthracnose and powdery mildew with Biotrust WP (*Talaromyces flavus*). *Plant Protection Society* **51**: 37-42, 2004.
- Nurnberger T, Brunner F, Kemmerling B and Piater L.** Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* **198**:249-266, 2004.
- Omar SA.** The role of rock-phosphate-solubilizing fungi and vesicular–arbuscular mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. *World J. Microbiol. Biotechnol.* **14**: 211-218, 1998.

- Otani T, Ae N and Tanaka H.** Phosphorus (P) uptake mechanisms of crops grown in soils with low P status. II. Significance of organic acids in root exudates of pigeonpea. *Soil Sci Plant Nutr.* **42:** 553-560, 1996.
- Ouchterlony O.** *Immunodiffusion and immunoelectrophoresis.* In: Handbook of Experimental Immunology (Ed. D.M. Weir). Blackwell Sci. Publ. Oxford and Edinburgh, 1967.
- Ovreas L and Torsvik V.** Microbial diversity and community structure in two different agricultural soil communities. *Microbial. Ecol.* **36:** 303–315, 1998.
- Pace NA, Stahl DA, Lane DJ, Olsen G.** The analysis of natural microbial communities by ribosomal RNA sequences. *Adv. Microbial. Ecol.* **9:** 1-55, 1986.
- Pan SQ, Ye XS. and Kuc J.** A technique for detection of chitinase, β -1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectric focussing. *Phytopathol.* **81:** 970-974, 1991.
- Pandey A, Das N, Kumar B, Rinu K, Trivedi P.** Phosphate solubilization by *Penicillium* spp. isolated from soil samples of Indian Himalayan region. *World J. Microbiol. Biotechnol.* **24:** 97–102, 2008.
- Pandey P, Kang SC, Maheshwari DK.** Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. *Curr. Sci.* **89:** 170–180, 2005.
- Pandya U and Saraf M** (2010) Application of fungi as a biocontrol agent and their biofertilizer potential in agriculture. *J. Adv. Dev. Res.* **1:** 90–99, 2010.
- Pathak KV and Keharia H.** Characterization of fungal antagonistic bacilli isolated from aerial roots of banyan (*Ficus benghalensis*) using intact-cell MALDI-TOF mass spectrometry (ICMS). *J. Appl. Microbiol.* **114:** 1300—1310, 2013.
- Phillips AD, Fox TC, King MD, Bhuvaneshwari TV and Teuber LR.** Microbial products trigger amino acid exudation from plant roots. *Plant Physiol.* **136:** 2887-2894, 2004.
- Pickup RW.** Development of molecular methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137:** 1009–1019, 1991.

- Pieterse CMJ and Van Loon LC.** NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant. Biol.* **7**: 456-464, 2004.
- Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ and Van Loon LC.** A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell.* **10**:1571-1580, 1998.
- Pikovskaya RE.** Solubilization of phosphorous in soil in connection with vital activity of some microbial species. *Microbiologia.* **17**: 362-370, 1948.
- Pingale SS and Popat SV.** Study of influence of phosphate dissolving microorganisms on yield and phosphate uptake by crops. *Eur. J. Exp. Biol.* **3**: 191-193, 2013.
- Ponmurugan P and Gopi C.** Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. *J. Agron.* **5**: 600-604, 2006.
- Pozo MJ and Azcon-Aguilar C.** Unraveling mycorrhiza-induced resistance. *Curr. Opin. Plant Biol.* **10**: 393-398, 2007.
- Prabhu V, Biolchini PF and Boyer GL.** Detection and identification of ferricrocin produced by ectendomycorrhizal fungi in the genus *Wilcoxina*. *Bio. Metals.* **9**: 229–234, 1996.
- Pradhan N and Sukla B.** Solubilization of inorganic phosphates by fungi isolated from agriculture soil. *Af. J. Biotech.* **5**: 850-854, 2005.
- Prasanna R, Joshi M, Rana A, Nain L.** Modulation of IAA production in cyanobacteria by tryptophan and light. *Polish J. Microbiol.* **59**: 99–105, 2010.
- Proksa B.** *Talaromyces flavus* and its metabolites. *Chemical Papers* **64**: 696–714, 2010.
- Rameshkumar N, Ayyadurai N, Kayalvizhi N and Gunasekaran P.** Genotypic and phenotypic Dpdiversity of PGPR fluorescent Pseudomonads isolated from the rhizosphere of sugarcane (*Saccharum officinarum* L.) *J. Microbiol. Biotechnol.* **22**: 13–24, 2012.
- Ramette A, Moe-Loccoz Y and Dgo G.** Prevalence of fluorescent-pseudomonads producing antifungal phloroglucinols and or hydrogen cyanide in soils naturally suppressive or conducive to tobacco root rot. *FEMS Microb. Ecol.* **44**: 35-43, 2003.

- Ranjan A, Mahalakshmi MA and Muruhan S.** Isolation and characterization of phosphate-solubilizing bacterial species from different crop fields of Salem, Tamilnadu, India. *International J of Nutrition, Pharmacology, Neurological Diseases*: 29-33, 2013. DOI-10.4103/2231-0738.106982
- Rasche F, Marco-Noales E, Velvis H, Overbeek LS, López MM, Elsas JD, Sessitsch A.** Structural characteristics and plant beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes. *Plant Soil* **298**: 123–140, 2006.
- Reyes I, Valery A and Valduz Z.** Phosphate solubilizing microorganisms isolated from rhizospheric and bulk soils of colonizer plants at an abandoned rock phosphate mine. In: First International Meeting on Microbial Phosphate Solubilization. E.Velázquez and C. Rodríguez-Barrueco (eds.), pp. 69-75, 2007.
- Richardson AE.** Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Aust. J. Plant Physiol.* 28:897-906, 2001.
- Rinu K and Pandey A.** *Bacillus subtilis* NRRL B-30408 inoculation enhances the symbiotic efficiency of *Lens esculenta* Moench at a Himalayan location. *J. Plant Nutr. Soil Sci.*, **172**, 134–139, 2009.
- Rinu K and Pandey A.** Temperature-dependent phosphate solubilization by cold- and pH-tolerant species of *Aspergillus* isolated from Himalayan soil. *Mycoscience*. **51**: 263–271, 2010.
- Rovira AD.** Rhizosphere research- 85 years of progress and frustration. In: DL Kleister and PB Cregan (eds.). *The Rhizosphere and Plant Growth*. Kluwer Academic Publishers, The Netherlands. pp.3–13, 1991.
- Rudrappa T, Splaine RE, Biedrzycki ML and Bais HP.** Cyanogenic pseudomonads influence multitrophic interactions in the rhizosphere. *PLoS ONE* **3**: 33-41, 2008.
- Ruppel S, Ruhlmann J and Merbach W.** Quantification and localization of bacteria in plant tissues using quantitative real-time PCR and online emission fingerprinting. *Plant Soil*. **286**: 21–35, 2006.

- Ryle GJA, Powell CE, and Gordon AJ.** Respiratory costs of nitrogen-fixation in soybean, cowpea, and white clover and Nitrogen-fixation and the respiration of the nodulated root. *J. Exp. Bot.* **30**:135–144, 1979.
- Rytönen A, Lilja A and Hantula J.** PCR-DGGE method for *in planta* detection of *Phytophthora* species. *Forest Pathology*. DOI: 10.1111/j-149-0329.2011.00716.x, 2011.
- Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW and Pare PW.** Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* **134**: 1017-1026, 2004.
- Sagoe CI, Ando T, Kouno K and Nagaoka T.** Relative importance of protons and solution calcium concentration in phosphate rock dissolution by organic acids. *Soil Sci. Plant Nutr.* **44**: 617-625, 1998.
- Saharan BS and Nehra V.** Plant Growth Promoting Rhizobacteria: A Critical Review, *Life Sciences and Medicine Research*. 21: 43-50, 2011.
- Samuels GJ, Dodd SL, Gams W, Castlebury LA and Petrini O.** *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*, *Mycologia*. **94**: 146-170.
- Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E and Kubicek CP.** The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Stud. Mycol.* **41**: 1-54, 1998.
- Schisler DA, Slininger PJ, Behle RW, Jackson MA.** Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathol.* **94**: 1267-1271, 2004.
- Schwyn B, Neilands JB.** Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**: 47–56, 1987.
- Shahraki M, Heydari A and Hassanzadeh N.** Investigation of antibiotic, siderophore and volatile metabolites production by *Bacillus* and *Pseudomonas* bacteria. *Iran. J. Biol.* **22**: 71-85, 2009.
- Sharma A, Johri BN.** Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. *Microbiol. Res.* **158**: 243–248, 2003.
- Sharma K, Dak G, Agrawal A, Bhatnagar M and Sharma R.** Effect of phosphate solubilizing bacteria on the germination of *Cicer arietinum* seeds and seedling growth. *J. Herb. Med. Toxicol.* **1**: 61-63, 2007.

- Sherriff C, Whelan MJ, Arnold GM, Lafai JF, Brygoo Y and Bailey JA.** Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Exp. Mycology*. **18**: 121–138, 2007.
- Shivaji S, Chaturvedi P, Suresh K, Reddy GSN, et al.** *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int. J. Syst. and Evol. Microbiol.* **56**: 1465-1473, 2006.
- Shoebitz M, Ribaudó CM, Pardo MA, Cantore ML, Ciampi L, Curá JA.** Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* rhizosphere. *Soil Biol. Biochem.* **41**: 1768–1774, 2009.
- Shweta SK, Kannan S, Madhavan R, Adhithya V, Paranidharan and Velazhahan R.** RAPD analysis of genetic diversity among the isolates of *Aspergillus flavus* from different hosts and locations. *Archives Phytol. Plant Protection*. doi.org/10.1080/03235408.2013.787701, 2013.
- Sid AA, Ezziyyani M, Pérez-Sanchez C, Candela ME.** Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *Eu J Plant Pathol* 109:633-637, 2003.
- Siddiqui ZA.** PGPR: Prospective biocontrol agents of plant pathogens. In: ZA Siddiqui (ed). *PGPR: Biocontrol and Biocontrol*. Springer, Netherlands, pp: 112–142, 2006.
- Silva HAS, Romeiro RDS, Macagnan D, Halfeld-Vieira BDA, Pereira MCB and Munteer A.** Rhizobacterial induction of systemic resistance in tomato plants: Non-specific protection and increase in enzyme activities. *Biol. Control.* **29**: 288-295, 2004.
- Singh JS, Pandey VC, Singh DP.** Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development, *Agriculture Ecosystems and Environment.* **140**: 339–353, 2011.
- Singh JS.** Plant Growth Promoting Rhizobacteria Potential Microbes for Sustainable Agriculture. *Resonance* **3**: 275-281, 2013.
- Smith KP and Goodman RM.** Host variation for interaction with beneficial plant associated microbes *Ann. Rev. Phytopathol.* **37**: 473-491, 1999.

- Smith SE and Read DJ.** Mycorrhizal Symbiosis. Academic Press, London, 1997.
- Somers E and Vanderleyden J.** Rhizosphere bacterial signaling: a love parade beneath our feet. *Crit. Rev. Microbiol.* **30**: 205–240, 2004.
- Son TN, Diep CN and Giang TTM. 2006.** Effect of bradyrhizobia and phosphate solubilizing bacteria application on Soybean in rotational system in the Mekong delta. *Omonrice.* **14**:48-57, 2006.
- Souza RD, Beneduzi A, Ambrosini A, Costa PB, Meyer J, Vargas LK, Schoenfeld R and Passaglia LMP.** The effect of plant growth-promoting rhizobacteria on the growth of rice (*Oryza sativa* L.) cropped in Southern Brazilian fields. *Pl. Soil.* **366**:585–603, 2013.
- Spaepen S, Vanderleyden J,** Auxin and plantmicrobe interaction. *Cold Spring Harb Perspect Biol.*, **3**: 14-21, 2011.
- Spaepen S, Vanderleyden J, Remans R.** Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**: 425–448, 2007.
- Spaink HP.** Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol.* **54**: 257-288, 2000.
- St. Leger R, Joshi L. Roberts D.** *Appl. Environ. Microbiol.* 64: 709-713, 1998.
- Stanley J.** Biodiversity of Microbial life. Wiley-Liss, New York, NY. **2002**.
- Staunton S and Leprince F.** Effect of pH and some organic anions on the solubility of soil phosphate: implications for P bioavailability. *Eur. J. Soil Sci.* **47**: 231-239, 1996.
- Stephen J and Jisha MS.** Buffering reduces phosphate solubilizing ability of selected strains of bacteria. *World J. Agric. Sci.* **5**:135-137, 2009.
- Stevenson FJ. 2005.** Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients. John Wiley and Sons, New York, 2005.
- Sudha LJ, Kuberan T, Anbaraj J, Sundaravadivelan C, Kumar P and Dhanaseeli M.** Effect of plant growth promoting fungal inoculant on the growth of *Arachis hypogea* (L.) and its role on the induction of systemic resistance against *Rhizoctonia solani*. *Int. J. Appl Biol. Pharm. Tech.* **2**: 222–232, 2011.

- Sun S, Guo Z, Yang R, Sheng Z, and Cao P.** Analysis of microbial diversity in tomato paste wastewater through PCR-DGGE. *Biotechnol. Bioprocess Eng.* **18**: 111-118, 2013.
- Sundara B, Natarajan V and Hari K.** Influence of phosphorus solubilizing bacteria on the changes in soil available phosphorus and sugarcane yields. *Field Crops Res.* **77**: 43-49, 2002.
- Swain MR, Naskar SK and Ray RC.** Indole-3-acetic acid production and effect on sprouting of Yam (*Dioscorea rotundata* L.) minisetts by *Bacillus subtilis* isolated from culturable cowdung microflora. *Pol. J. Microbiol.* **56**: 103–110, 2007.
- Taylor JW, Jacobson DJ and Fisher M.** The evolution of asexual fungi: speciation and classification, *Annual Review Phytopathol.* **37**: 197-246, 1999.
- Thomas A Nicholas DP and Parkinson D.** Modification of the agar flim technique for assaying length of mycelium in soil. *Nature (London).* **205**:105, 1965.
- Thomashow LS and Weller DM.** Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *Tritici*. *J. Bacteriol.* **170**: 3499-3508, 1988.
- Tominaga N, Takeshi M.** A sulfite dependent acid phosphatase of *Thiobacillus thiooxidans*. *J. Biochem.*, **76**: 419-428, 1974.
- Torsvik V, Goksoyr J and Daae FD.** High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* **56**: 782–87, 1990.
- Torsvik V, Øvreås L.** Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* **5**:240–245, 2002.
- Torsvik VL, Sorheim R and Goksoyr, J.** Total bacterial diversity in the soil and sediment communities – a review. *J. Industrial Microbiol.* **17**: 170-178, 1996.
- Trevors JT.** Bacterial diversity in soil with an emphasis on chemically contaminated soils. *Water air soil Pollut.* **101**: 45-67, 1998.
- Troxler J, Zala M, Natsch A, Moënné-Loccoz Y and Défago G.** Autecology of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the rhizosphere and inside roots at later stages of plant development. *FEMS Microbiol. Ecol.* **23**: 119–130, 1997.

- Uchiumi T, Oowada T, Itakura M, Mitsui H, Nukui N, Dawadi P, Kaneko T, Tabata S, Yokoyama T, Tejima T, Saeki K, Oomori H, Hayashi M, Maekawa T, Sriprang R, Murooka Y, Tajima S, Simomura K, Nomura M, Suzuki A, Shimoda S, Sioya K, Abe M and Minamisawa K.** Expression islands clustered on symbiosis island of *Mesorhizobium loti* genome. *J. Bacteriol.* **186**: 2439–2448, 2004.
- Ueda T, Suga Y and Matsuguchi T.** Molecular phylogenetic analysis of a soil microbial community in a soybean field. *Eur. J. Soil Sci.* **46**: 415–421, 1995.
- Ulhoa CJ and Peberdy JF.** Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. *Enzyme Microb. Technol.* **14**: 236-240, 1992.
- Unge A, Tombolini R, Molbak L and Jansson JK.** Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with dual *gfp-luxAB* marker system. *Appl. Environ. Microbiol.* **65**: 813–821, 1999.
- Vallad GE and Goodman R.** Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Sci.* **44**: 1920-1934, 2004.
- Van der Ent S, Verhagen BWM, Van Doorn R, Bakker D, Verlaan MG, Pel MJC, Joosten RG, Proveniers MCG, Van Loon LC and Ton J.** MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiol.* **146**:1293-1304, 2008.
- Van Elsas J D, Trevors JT and Starodub ME.** Bacterial conjugation between pseudomonads in the rhizosphere of wheat. *FEMS Microbiol. Lett.* **53**: 299–306, 1998.
- Van Elsas JD and Boersma FGH.** A review of molecular methods to study the microbiota of soil and the mycosphere. *European J. Soil Biology.* **47**:77-87, 2011.
- Van Loon LC, Bakker PAHM and Pieterse CMJ.** Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopathol.* **36**: 453-483, 1998.
- Van Oosten VR, Bodenhausen N, Reymond P, Van Pelt JA, Van Loon LC, Dicke M and Pieterse CMJ.** Differential effectiveness of microbially induced resistance against herbivorous insects in *Arabidopsis*. *Mo. Plant Microbe Interact.* **21**:919-930, 2008.

- Van Peer R and Schippers B.** Lipopolysaccharides of plant growth-promoting *Pseudomonas* spp. strain WCS 417r induce resistance in carnation to *Fusarium wilt*. *Neth. J. Plant Pathol.* **98**: 129-139, 1992.
- Van Peer R, Nieman GJ and Schippers B.** Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathol.* **81**: 728-734, 1991.
- Van Wees SC, Pieterse CM, Trijssenaar A, van Westende YA, Hartog F and van Loon LC.** Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* **10**: 716-724, 1997.
- Van Wees, Saskia CM, Sjoerd Van der Ent and Pieterse CMJ.** Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant. Biol.* **11**: 443–448, 2008.
- Vassilev N, Vassileva M and Nikolaeva I.** Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. *Appl. Microbiol. Biotechnol.* **71**: 137–144, 2006.
- Vassileva M, Serrano M, Bravo V, Jurado E, Nikolaeva I, Martos V and Vassilev N.** Multifunctional properties of phosphate-solubilizing microorganisms grown on agro-industrial wastes in fermentation and soil conditions. *Appl. Microbiol. Biotechnol.* **85**:1287–1299, 2010.
- Veenekar JV, Tanksale A, Ghatge M and Deshpande V.** Novel biofunctional alkaline protease inhibitor: protease inhibitory activity as the biochemical basis of antifungal activity. *Biochem. Biophys. Res. Commun.* **285**: 1018-1024, 2001.
- Vendan RT, Sun Hee Lee, Young Joon Yu and Young H R.** Analysis of bacterial community in the Ginseng soil using Denaturing Gradient Gel Electrophoresis (DGGE). *Indian J. Microbiol.* **52**: 286–288, 2010.
- Venkateswarlu, R., K.M. Reddi, E.N.P. Reddy, Sudhakar P., (2008).** Molecular characterization of *Trichoderma* spp. Used against *Fusarium wilt* with PCR based RAPD and ITS-PCR. *J. Mycol. Plant Pathol.*, 38(3):569-563, 2008.
- Venturella G., Perini C, Barluzzi C, Pacioni G, Bernicchia A, Padovan F, Quadraccia L and Onofri S.** Towards a red data list of fungi for Italy. – *Bocconeia.* **5**: 867-872, 1997.

- Verhagen BWM, Glazebrook J, Zhu T, Chang H-S, Van Loon LC and Pieterse CMJ.** The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.* **17**:895-908, 2008.
- Verma JP et al.** Impact of plant growth promoting rhizobacteria on crop production. *Int. J. Agric. Res.* **5**: 954–983, 2010.
- Vessey JK.** Plant growth promoting rhizobacteria as biofertilizers. *Plant Soils.* **255**: 571-586, 2003.
- Vinale F, Ghisalberti EL, Sivasithamparam K, Marra R, Ritieni A, Ferracane R, Woo S, Lorito M (2009)** Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens. *Lett. App. Microbiol.* **48**: 705–711, 2009.
- Viterbo A, Ramot O, Chemin L et al.** (2002). Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Ant van Leeuw.* **81**: 549-556, 2002.
- Wakeham AJ and White JG.** Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiol. Mol. Plant Pathol.* **48**:289-303, 1996.
- Wakelin SA, Warren RA, Harvey PR and Ryder MH.** Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. *Biol. Fertil. Soils.* **40**: 36–43, 2004.
- Walker TS, Bais P, Grotewold E and Vivanco JM.** Root exudation and rhizosphere biology. *Plant Physiol.* **132**: 44–5, 2003.
- Waller F Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C and von Wettstein D.** The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci. USA.* **102**:13386-13391, 2005.
- Walsh JA, Merz U and Harrison JG.** Serological detection of spore balls of *Spongospora subterranean* and quantification in soil. *Plant Pathol.* **45**: 884-895, 1996.
- Wamberg C, Christensen S and Jakobsen I.** Interactions between foliar-feeding insects, mycorrhizal fungi, and rhizosphere protozoa on pea plants. *Pedobiologia.* **47**: 281–287, 2003.
- Warcup JH** The origin of the colonies of fungi on soil dilution plates. *Trans.Br.Mycol.Soc.***38**: 298, 1955.

- Wardle, DA.** Communities and Ecosystems, Linking aboveground and belowground components. Monographs in Population Ecology 34, Princeton University Press, 2002.
- Warmink JA, Van Elsas JD.** Selection of bacterial populations in the mycosphere of *Laccaria proxima*: is type III secretion involved? *ISME J.* 2: 887-900, 2008.
- Watson RD.** Soil washing improves the value of the soil dilution and plate count method of estimating populations of soil fungi. *Phytopathol.* **50**: 792-794, 1960.
- Whitelaw MA.** Growth promotion of plants inoculated with phosphate solubilizing fungi. *Adv. Agron.* **69**: 99-151, 2000.
- Winkelmann G.** Surface polymers and hydroxyl acids. A model of iron supply in sideramine-free fungi. *Arch. Microbiol.* **121**:43–51, 1979.
- Woo SL, Scala F, Ruocco M et al.** (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathol.* **96**: 181-185, 2006.
- Woodward AW and Bartel B.** Auxin: regulation, action and interaction. *Ann. Bot.* **95**:707–73, 2005.
- Wright SF, Upadhyaya A.** A survey of soil aggregate stability and glomalin, a glycoprotein produced by hyphae of mycorrhizal fungi. *Plant soil.* **198**: 97-107, 1998.
- Xavier LJC and Germida JJ.** Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biol. Biochem.* **35**: 471–478, 2003.
- Xue L, Xue Q, Chen Q, Lin C, Shen G and Zaho J.** Isolation and evaluation of rhizosphere actinomycetes with potential application for biocontrol of *Verticillium* wilt of cotton. *Crop Protection.* **43**: 231-240, 2013.
- Yadaf RS and Tarafdar JC.** Influence of organic and inorganic phosphorus supply on the maximum secretion of acid phosphatase by plants. *Biol. Fert. Soils.* **34**:140-143, 2001.
- Yazdani M, Bahmanyar MA, Pirdashti H and Esmaili MA.** Effect of Phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of Corn (*Zea mays L.*). *Proc. World Acad. Science, Eng. Technol.* **37**: 90-92, 2009.

- Yu HT, Shen ZB, Shen DR, Jin J and Wang GH.** Inspection of soil bacterial diversity along soybean rhizosphere with different distances by PCR-DGGE method. *Bull. Agr. Sci. Technol.* **6**: 65–68, 2010.
- Zahir ZA, Ghani U, Naveed M, Nadeem SM.** Comparative effectiveness of *Pseudomonas* and *Serratia sp.* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch. Microbiol.* **191**: 415–424, 2009.
- Zaidi A. and Khan MS.** Co-inoculation effects of phosphate solubilizing microorganisms and *Glomus fasciculatum* on green gram - *Bradyrhizobium* symbiosis. *Turk. J. Agric.* **30**: 223-230, 2006.
- Zake DR, Pregitzer KS, Burton AJ and Edwards HK.** Microbial responses to a changing environment : implications for the future functioning of terrestrial ecosystems. *Fungal Ecology.* **4**: 386-395. 2011.
- Zervakis G, Polemis E and Dimou, D.** Mycodiversity studies in selected ecosystems of Greece: III. Macromycetes recorded in *Quercus* forests in southern Peloponnisos. *Mycotaxon.* **84**: 141-162, 2002.
- Zhao G and Wang HY.** Soil microorganism bio-diversity molecule ecology research approach. *For. By-Prod. Spec. China,* **80**: 54–56, 2006.
- Zhou J, Davey ME, Figueras JB, Rivkina E, Gilichinsky D and Tiedje JM.** Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. *Microbiology.* **143**: 3913–3919, 1997.
- Zimmer D, Baum C, Leinweber P, Hrynkiewicz K and Meissner R.** Associated bacteria increase the phytoextraction of cadmium and zinc from a metal-contaminated soil by mycorrhizal willows. *Int. J. Phytorem.* **11**: 200–213, 2009.
- Zwart KB, Kuikman PJ, and van Veen JA.** Rhizosphere protozoa: Their significance in nutrient dynamics. In: Soil Protozoa. Darbyshire, J.F. (ed.), CAB International, Wallingford, 93–122, 1994.

APPENDIX-A: List thesis related publications

In Journals

1. **Sunar K**, Dey PL, Chakraborty U and Chakraborty BN. Biocontrol efficacy and plant growth promoting activity of *Bacillus altitudinis* isolated from Darjeeling hills, India. *J Basic Microbiology*, 2013 (In Press)
2. Chakraborty BN, Chakraborty U, **Sunar K** and Dey PL. Evaluation of plant growth promoting and antifungal activities of *Talaromyces flavus* (NAIMCC-F-01948) against *Sclerotium rolfsii*. *Indian Phytopathol.* **65**: 258-263, 2012.
3. Chakraborty BN, Chakraborty U, **Sunar K** and Dey PL. RAPD profile and rDNA sequence analysis of *Talaromyces flavus* and *Trichoderma* species. *Indian Journal of Biotechnology* 10:487-495, 2011.
4. Chakraborty BN, Chakraborty U, Saha A, **Sunar K** and Dey PL. Evaluation of phosphate solubilizer from soil of North Bengal and their diversity nalysis. *World journal of Agricultural Science*, **6**: 195-200.2010.

In proceeding volume

1. **Sunar K**, Dey PL, Chakraborty U and Chakraborty BN. Evaluation of *Talaromyces flavus* for phosphate solubilization and biocontrol activity and its molecular analysis. In: *Microbial Resources for crop improvement* (eds. BN Chakraborty and U Chakraborty). Satish Publishing House, Delhi, pp.129-144, 2012.
2. Chakraborty BN, Chakraborty U, Dey PL and **Sunar K**. (2012). Exploitation of agriculturally important microorganisms from soil and their evaluation for improvement of crop health status. In: *Biodiversity Conservation: Fundamental and Applications* (eds. H Saha, ML Ghosh, G Gangopadhyay, D Saha, PK Singh, S Sarkar and SC Das) Sarat Book Distributors, Kolkata, pp. 41-56.

APPENDIX- B: List of Abbreviations

1. ACC- 1-amino-cyclopropane-1-carboxylic acid hydrochloride
2. AIMS- Agriculturally Important Microorganisms
3. APS- Ammonium per sulphate
4. *A. clavatus*- *Aspergillus clavatus*
5. *A. melleus*- *Aspergillus melleus*
6. *A. niger*- *Aspergillus niger*
7. *B. altitudinis*- *Bacillus altitudinis*
8. *B. aerophilus*- *Bacillus aerophilus*
9. *B. methylotrophicus*- *Bacillus methylotrophicus*
10. *B. symbiont*- *Burkholderia symbiont*
11. BCA-Biocontrol Agent
12. BE %- Biocontrol Efficacy %
13. BLAST- Basic local alignment search tool
14. BSA- Bovine serum albumin
15. $\text{Ca}_3(\text{PO}_4)_2$. Tri-calcium phosphate
16. CaHPO_4 - Calcium phosphate
17. CAS- Chrome azurol S
18. CAT- Catalase
19. CDA- Chitinase detection agar
20. cfu- Colony forming unit
21. CHT- Chitinase
22. DAC-ELISA- Direct antigen coating- Enzyme linked immune-sorbent assay
23. DGGE- Denaturing Gradient Gel Electrophoresis
24. DI%- Disease Incidence %
25. DNA- Deoxyribonucleic acid
26. dNTPs- Deoxy nucleotide tri-phosphates
27. *E. cloacae*- *Enterobacter cloacae*
28. EDTA- Ethylene diamine tetra acetic acid
29. ELISA- Enzyme linked immune- sorbent assay
30. ER- Root endosphere
31. FITC- Fluorescein isothiocyanate
32. g- gram
33. GlcNAc- N-acetyl glucosamine
34. HCl- Hydrochloric acid
35. HCN- Hydrocyanic acid
36. IAA- Indole acetic acid
37. ISR- Induced systemic resistance
38. KCl- Potassium chloride
39. MEGA 4- Molecular Evolutionary Genetics Analysis 4
40. μl - Micro litre
41. mg- Mili gram
42. ml- Mili litre
43. NB- Nutrient Broth
44. NBT/BCIP substrate- Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
45. NCBI- National Center for Biotechnology Information
46. NCM- Nitrocellulose membrane

47. *P. polymyxa*- *Paenibacillus polymyxa*
48. PAb-Polyclonal antibody;
49. PAL- Phenylalanine ammonia lyase
50. PAGE- Poly-acrylamide gel electrophoresis
51. PBS-Tween- Phosphate buffer saline- Tween
52. PCA- Phenazine-1-carboxylic acid
53. PCI- Water saturated phenol: Chloroform: Isoamyl alcohol
54. PCR- Polymerase chain reaction
55. PCR-RAPD- Polymerase chain reaction- Random Amplified Polymorphic DNA
56. PSF-Phosphate solubilizing Fungus
57. PGPR- Plant Growth Promoting Rhizobacteria
58. PGPTs- Plant growth promoting traipNPP- p- nitrophenyl phosphate
59. POX- Peroxidase
60. PR proteins- Pathogenesis related proteins
61. PSB- Phosphate solubilising bacteria
62. PVK- Pikovskaya s agar
63. PVP- Poly vinyl- pyrrolidone
64. RAPD- Random amplified polymorphic DNA
65. RNA- Ribonucleic acid
66. Rock phosphate
67. SAR- Systemic acquired resistance
68. SDS- Sodium dodecyl sulphate
69. SDS-PAGE- Sodium dodecyl sulphate- Poly-acrylamide gel electrophoresis
70. SKM- Skim milk agar
71. *S. rolfsii*- *Sclerotium rolfsii*
72. TV- Toklai varietyTAE buffer- Tris Acetic Acid and EDTA buffer
73. TE buffer- Tris-EDTA buffer
74. TEMED- N,N,N',N'-Tetramethylethylenediamine
75. Tris Hcl- Tris hydrochloric acid
76. *T. cucumeris*- *Thanatephorus cucumeris*
77. *T. asperellum*- *Trichoderma asperellum*
78. *T. erinaceum*- *Trichoderma erinaceum*
79. *T. harzianum*- *Trichoderma harzianum*
80. *T. viride*- *Trichoderma viride*
81. *T. flaus*- *Talaromyces flaus*
82. UPGMA- Unweighted Pair Group Method with Arithmetic Mean
83. yr- Year
84. β -1,3-GLU- β -1,3 glucanase

APPENDIX- C: List of Chemicals

1. Ammonium chloride
2. Ammonium per sulphate
3. Bovine serum albumin
4. Calcium phosphate
5. Carboxy methyl cellulose
6. Chrome azurol S
7. Colloidal chitin
8. Copper sulphate
9. Deoxy nucleotide tri-phosphates
10. Di methyl amino benzaldehyde
11. Di sodium hydrogen phosphate
12. Diethyl aminoethyl cellulose
13. Dinitro salicylic acid
14. Ethylene diamine tetra acetic acid
15. Ferric chloride
16. Fluorescein isothiocyanate
17. Helicase (3%)
18. Hexa-decytrimethyl ammonium bromide
19. Hydrochloric acid
20. Hydrocyanic acid
21. Hydrogen peroxide
22. Indole acetic acid
23. Magnesium chloride
24. N,N,N',N'-Tetramethylethylenediamine
25. N-acetyl glucosamine
26. Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
27. O-dianisidine (5 mg/ml methanol)
28. p- nitrophenyl phosphate
29. Phosphate buffer saline- Tween
30. Poly vinyl- pyrrolidone
31. Potassium chloride
32. Potassium dihydrogen phosphate
33. Sodium azide
34. Sodium carbonate
35. Sodium chloride
36. Sodium dodecyl sulphate
37. Sodium Hydroxide
38. Sodium molybdate
39. Sodium nitrite
40. Sulphuric acid
41. Tri-calcium phosphate
42. Tris Acetic Acid and EDTA buffer
43. Tris hydrochloric acid
44. Tris-EDTA buffer
45. Water saturated phenol: Chloroform: Isoamyl alcohol
46. 0.05(M) sodium phosphate buffer (pH 6.8)
47. 0.1(M) sodium acetate buffer (pH 5.0)

48. 0.2M Na-phosphate buffer (pH 5.4)
49. 0.3mM borate buffer (pH 8.0)
50. 1 M K-PO₄ buffer (pH 7.1)
51. 1-amino-cyclopropane-1-carboxylic acid hydrochloride
52. 1M Na-acetate buffer (pH 4)
53. Sodium borate buffer (pH 8.8)
54. 2 mM β- mercaptoethanol
55. 1 M Na-borate buffer (pH 9.8)
56. 2% L-phenylalanine
57. 2,4-Diacetylphloroglucinol
58. 4 mM H₂O₂.
59. 4% laminarin