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 $4x10^3$- $6x\ 10^4$ cfu in case of fungi and $5x10^6$cfu-$6x10^6$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. Serretiasp. Paenibacillus sp., Psedomonus sp., Enterobacter sp.* well as *Bukholderia 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 *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. One of the interesting findings of the present study was isolation of one potential fulgal 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 chtitinase activities of the isolates were determined specrtophotometrically. *T. harzianum* RHS/S-559 and RHS/S-560 obtained from the rhizosphere of *Sechium edule*, *T. viride* isolates, 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 fugal 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 solubilizinf 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 scletorial 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.