Materials and Methods

Plate 4: Map of North Bengal and its Latitude and Longitudes

This is a lowland and covered with swamps and beels (small water bodies). Whereas the area south of the Kalindi is a very fertile land and is known as Diara. The plain in the south of Jalpaiguri and Cooch Behar district is also made of new alluvium deposited by numerous rivers like the Teesta, Torsa, Raidak, Jaldhaka, Sankosh, Balason, Punarbhaba, Atrai and several other small rivulets.

3.2. Soil sampling strategy

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.

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 thing that has been considered was the altitude of the sampling unit areas.

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 (1950) 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 *Trichoderma* species:

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

KH_2PO_4	1.0g
$MgSO_4.7H_2O$	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^{-1}L Oxytetracycline.

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

$MgSO_4(7H_2O)$	0.2g
KH_2PO_4	0.9g
KCl	0.14g
NH_4NO_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_2PO_4	1.0g
$(NH_4)_2HPO_4$	0.5g
$MgSO_4.7H_2O$	0.5g
$MnSO_4.6H_2O$	0.02g
Bacto yeast extract	0.3g
$FeSO_4.7H_2O$	0.1g
$CoCl_2.6H_2O$	0.02g
KCl	0.5g
Agar	18.0g

pH (before autoclaving) 6.5

After sterilization, supplemented with 300µg ml^{-1}L Oxytetracycline.

(B) 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^{-1}L 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^{-1}L Oxytetracycline.

(C) 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^{-1}L Oxytetracycline

(D) Richards agar (RA):

KNO_3	1.0g
KH_2PO_4	50g
$MgSO_4.7H_2O$	0.25g
$FeCl_3$	0.002g
Sucrose	3.0g
Agar	2.0g
Distilled H_20	100ml

(E) Carrot juice agar (CJA):

Grated carrot	20.0g
Agar	2.0g
Distilled water	100 ml

(F) Czapek dox agar (CDA):

$NaNo_3$	0.20g
$KHPO_4$	0.10g
$MgSo_4.7H20$	0.05g
KCl	0.05g
$FeSo_4.7H20$	0.05g
Sucrose	3.0g
Agar	3.0g
Distilled water	100ml

(G) Potato sucrose agar (PSA):
Peeled potato 40.0g
Sucrose 2.0g
Agar 2.0g
Distilled water 100ml

(H) Malt extract peptone agar (MPA):
Malt extract 20.0g
Peptone 1.0g
Dextrose 20.0g
Agar 20.0g
Distilled water 1L

(I) Yeast extract dextrose agar (YDA):
Yeast extract 7.50g
Dextrose 20.0g
Agar 15.0g
Distilled water 1L

(J) Flentze's soil extract agar (FSEA):
Soil extract 1L
Sucrose 1.0g
KH_2PO_4 0.20g
Dried yeast 0.10g
Agar 25.0g

3.5. Microscopic Observation

3.5.1. Bright field

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.

3.5.2. SEM studies of selected organisms

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 artefacts. The specimen dishes were then placed in vapour diffusion dehydration (VDD)

assembly, and a vacuum was drawn as described by King and Brown (1983). 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.

3.6. Biochemical tests of microorganisms

3.6.1. Gram reaction

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, heat fixed 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. Endospore stain

The bacterial smear was prepared. The fixed slide was flooded with the solution of malachite green and the slide was placed over boiling water bath for five minutes. After rinsing, the smear was counter stained with safranin.

3.6.3. Catalase

Bacterial culture (24 hour old) was flooded with 0.5 ml 10% H_2O_2 solution and gas bubbles production indicated the positive reaction.

3.6.4. 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.

3.6.5. 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.

3.6.6. Starch hydrolysis

The bacteria were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated 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.

3.6.7. Indole test

10ml 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 were 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 medium reagent interface indicating the production of indole.

3.6.8. Siderophore production

Production of siderophore was detected by standard method of Schwyn and Neiland (1987) using blue indicator chrome azurol S (CAS). The bacteria were spot inoculated at the center of the plate and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot was an indication of siderophore production.

3.6.9. Chitinase production

Production of chitinase was detected by standard method of Hsu and Lockwood (1975). Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing strains

3.6.10. Cellulase Production

Cellulose overlay agar plates were streaked with the 24h old bacterial cultures and incubated at 37°C for 7 days. The plates were observed for any clearing zone around or underneath the growth.

3.6.11. Protease production

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

3.6.12. H_2S 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 H_2S by the organisms.

3.7. Screening for phosphate solubilizing activity

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium 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.

3.8. Evaluation of phosphate solubilizing activity

Evaluation of phosphate solublizing activity of fungal 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 28^0C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate is done following amonium 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.

3.9. 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).

3.9.1. 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.

3.9.2. 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.

3.10. Screening of BCA isolates

3.10.1. Antagonistic activity

For *in vitro* evaluation of antagonistic activity of rhizobacterial and fungal isolates following fungal pathogens viz., *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* 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. 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.

3.10.2Chitinase activity

Spore suspension (1.0 x 10^6 spores per mL of culture medium of *Trichoderma* spp. 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).

3.11. Immunological studies

3.11.1. Preparation of fungal antigen

Mycelial protein was prepared following the method as outlined by Chakarborty and Saha (1994). Mycelial mats were harvested from 7-10 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 $MgCl_2$ 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.

3.11.2. Preparation of soil antigen

Soil antigens were prepared following the method of Walsh *et al.*, (1996). Soil (1 g) was crushed in 2ml of 0.05 M sodium- bicarbonate buffer (pH 9.6) in a mortar and pestle and kept overnight at 4°C. Next day, centrifugation was done at 10000 rpm for 10 min. The supernatant was collected and used as antigen for blotting purposes.

3.11.3. 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).

3.11.4. 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).

3.11.4.1. 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 ß- 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 ß- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water

3.11.4.2. 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 over layered 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 over layered 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	10% Resolving gel (ml)	5% Stacking gel (ml)
Distilled water	2.85	2.10
30% acrylamide	2.55	0.10
Tris*	1.95	0.38
10%SDS	0.075	0.030
10%APS	0.075	0.030
TEMED**	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.

3.11.4.3. 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.

3.11.4.4. 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.

3.11.4.5. 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.

3.12. Raising of polyclonal antibodies

3.12.1. 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.

3.12.2. 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.

3.12.3. 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 colt 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.

3.13. Purification of IgG

3.13.1. 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.

3.13.2. 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.

3.13.3. 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).

3.14. Immunodiffusion test

3.14.1. 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.

3.14.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the meyhod of Ouchterlony (1976). Antigen plus undiluted Antisera appropriately diluted were poured into wells with sterile 60-8- micropipette (50µl/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.

3.14.3. 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

3.15. Dot immunobinding assay

Dot blot 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 0h 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 0.45µm, 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), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Load 5µl (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.

3.16. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia and soil samples were done using FITC labeled goat antirabbit 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 antirabbit IgG conjugated with fluorescein isothiocyanate (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.

3.17. Mass multiplication of PSFs, BCAs and fungal pathogens

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 where as 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°C± 2°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.

3.18. Inoculation technique and disease assessment

Pot grown, 2-3 week old plants (*Phaseolus vulgaris*) were used for artificial inoculation with fungal pathogen. Sand maize meal media containing fungal inoculum were added carefully in the rhizosphere and ensured that inocula were attached to healthy roots. Disease assessment was done after 15 days of inoculation.

In order to determine the effects of biocontrol agents (BCA) on disease reduction, four treatments were taken in each case: i. Untreated control, ii. Inoculated with pathogen, iii. Inoculation with BCA isolates and iv. Inoculation with both BCA isolate and fungal pathogen.

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.

3.19. Assay of enzyme activities

3.19.1. β-1, 3-glucanase

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 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl 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 µg glucose released min^{-1} g^{-1} fresh tissue.

3.19.2. Chitinase

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10µl 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, 30µl of 1M K-PO_4 buffer (pH7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl 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 µg GlcNAc released /min/ g fresh wt. tissue.

3.19.3. Peroxidase

The reaction mixture contained 1 ml of 0.2M Na-phosphate buffer (pH5.4), 1.7 ml dH_2O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H_2O_2. O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.*, 1993). Specific activity expressed as the increase in Δ A 465/g tissue/min.

3.20. 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.

3.20.1. 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

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

3.20.2. Genomic DNA extraction

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia 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 60^0C 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.

3.20.3. 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.

3.20.4. Measure DNA Concentration using Spectropphotometry

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.

3.20.5. Agarose gel eletrophoresis 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.

3.20.6. 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.

3.20.7. 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.

3.21. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles 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. 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 by horizontal electrophoresis.

3.21.1. PCR primers

The following primers are used in the study.

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%

3.21.2. Amplification conditions

Temperature profile, 94°C for 4 min followed by 35cycles 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.

3.21.3. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and isolates were grouped according to their shared band patterns.

3.21.4 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.

3.21.5. Reconstruction of the phylogenetic tree

As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer.

More complex analyses involve cladistic analysis of data and reconstruction of the phylogenetic tree

3.21.6. UPGMA method

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. 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. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

3.22. ITS PCR analysis

All isolates of *Trichoderma* were taken up for ITS-PCR amplification. 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.

3.22.1. PCR primers

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
ITS-Primers pairs				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%

3.22.2. Amplification conditions

Temperature profile, 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.

3.22.3. 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.

3.23. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares.

3.23.1. Chomatogram of sequence

The chromatogram of the DNA sequence was analysed by the software Chromus.

3.23.2. Editing and alignment of sequence data

All the DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

3.24. BLAST of Sequence

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence.

3.25. Submission of rDNA gene to NCBI genbank

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

3.26. Denaturing Gradient Gel Electrophoresis (DGGE)

Materials:

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_2O	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_2O.	

0% Denaturant	
38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

Methods:

3.26.1. 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.

3.26.2. 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.

3.26.3. 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 slided 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.

3.26.4. Staining of gels and photography

Before taking DGGE units out from the tank, the run - evaporated H_2O 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 distained with running buffer. The gel was photographed under UV transilluminator.

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

Soil samples were collected from the three districts i.e. Darjeeling, Jalpaiguri and Cooch behar which includes the major surrounding arears of Terai and Dooars regions. Source of the soil samples were included from forests, river basins and agricultural crop fields. The Terai-Dooars savanna and wetlands are a mosaic of tall grasslands, savannas and evergreen and deciduous forests. The Terai and Dooars region politically constitute the plains of Darjeeling District, whole of Jalpaiguri District and upper region of Cooch Behar District in West Bengal. The slope of the land is gentle, from north to south. The general height of the land is 80 to 100 m. The entire region is made up of sand, gravel and pebbles laid down by the Himalayan rivers like the Teesta, Torsa, Mahananda, Balasan and several other small rivulets (Plate 5).

Soil samples were collected randomly from from river basins and coded accordingly. Siz river basins were selected for the collection of soil samples. These were Mahanda (RS/M), Balasan (RS/B), Panighata (RS/P), Teesta (RS/T), Dhorala (RS/D), and Torsha (RS/T). Soil samples were also collected from the different forest of Terai and Dooars regions like Sukna Forest (FS/S), Lohagarh Forest (FS/L), Cinchona Forest (FS/C), Mongpong Forest (FS/M), Terghera Forest (FS/T), Baikunthapur Forst (FS/B), Mahananda Wild Life Sanctuary (FS/M), Jaldapara Forest (FS/J), Chilapata Forest (FS/C), Gorumara Forest (FS/G). Besides, soil samples were also collected form several important crop fields like tea, rubber, lemon, paddy, bamboo, rice, wheat, soybean, cabbage and potato of terai and dooars regions (Plates 1-3).

The sampling was divided into ununiform zone and ramdom sampling patterns according to the type of vegetation they represent. For the sampling of soil samples, proper sampling tools were used. Unusual areas for sampling was avoided. Sampling area was properly divided and recorded by GPS tools (Garmin) and proper records like soil pH, texture of the samples were taken (Plate 6, Table 3). Soil samples were collected at 10-15 cm depth and mixed well in polythene bags.

The plating techniques were adopted for isolation of microorganisms (soil borne actinomycetes, bacteria and fungi) from the collected soil samples using Warcup's soil plate method..

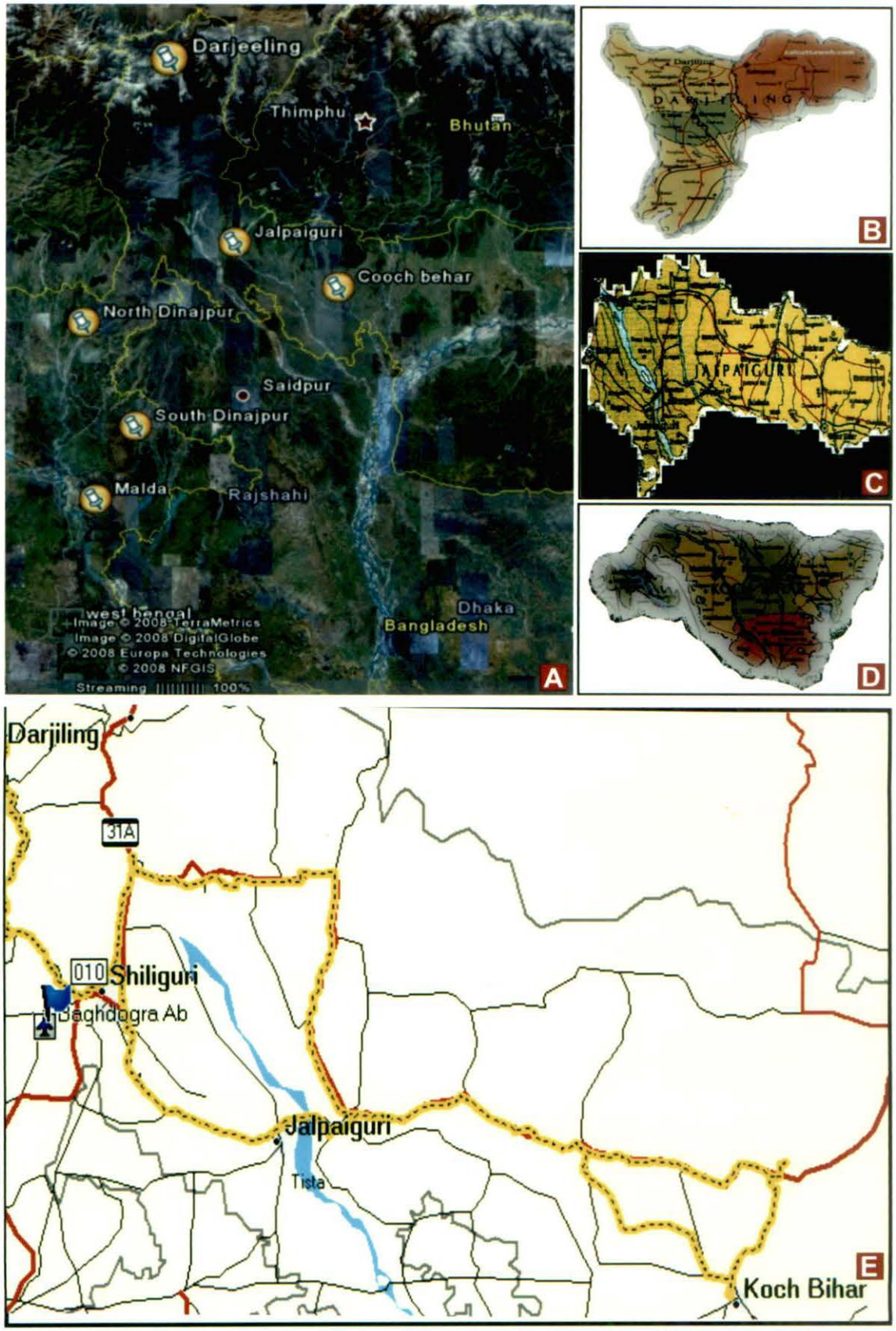


Plate 5: Satellite image of North Bengal (A), Map of Darjeeling (B) Jalpaiguri (C) Cooch behar (D) and map of study area of Terai-Dooars region by GPS tool (E)

Plate 6 : GIS locations of soil sampling areas of different regions of Terai-Dooars

Table : 3 GIS Locations of collection sites and soil characters of samples

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Soil Texture	Soil pH
Forest soil					
Sukna Forest (FS/S)	FS/SI	Siliguri	N26°47'26.81" E88°21'47.39"	Clay	4.38
	FS/SII	Siliguri	N26°47'76.11" E88°31'44.30"	Clay	4.38
	FS/SIII	Siliguri	N26°45'23.76" E88°21'67.32"	Clay	4.38
	FS/SIV	Siliguri	N26°47'20.67" E88°21'57.11"	Clay	4.38
	FS/SV	Siliguri	N26°47'21.11" E88°21'41.15"	Clay	4.38
Lohagarh Forest (FS/L)	FS/LI	Siliguri	N26°77'20.61" E88°33'31.55"	Clay	4.90
	FS/LII	Siliguri	N26°77'22.66" E88°33'30.58"	Clay	4.90
	FS/LIII	Siliguri	N26°77'25.77" E88°33'33.32"	Clay	4.90
	FS/LIV	Siliguri	N26°77'27.42" E88°33'30.32"	Clay	4.90
	FS/LV	Siliguri	N26°77'21.11" E88°33'33.25"	Clay	4.90
Cinchona Forest (FS/C)	FS/CI	Sevok	N26°58'19.90" E88°22'29.73"	Sandy	4.11
	FS/C II	Sevok	N26°58'15.77" E88°22'23.33"	Sandy	4.10
	FS/C III	Sevok	N26°58'17.89" E88°22'21.33"	Sandy	4.10
	FS/C IV	Sevok	N26°58'18.91" E88°22'26.33"	Sandy	4.10
	FS/C V	Sevok	N26°58'17.92" E88°22'28.73"	Sandy	4.11
Mongpong Forest (FS/M)	FS/M I	Chalsa	N26°52'53.53" E88°47'55.25"	Clay	3.08
	FS/M II	Chalsa	N26°52'54.33" E88°47'55.52"	Clay	3.08
	FS/M III	Chalsa	N26°52'54.35" E88°47'55.58"	Clay	3.08
	FS/M IV	Chalsa	N26°52'51.45" E88°47'54.88"	Clay	3.08
	FS/M V	Chalsa	N26°52'54.55" E88°47'55.88"	Clay	3.08
	FS/M VI	Chalsa	N26°52'54.75" E88°47'55.55"	Clay	3.08
Terghera Forest (FS/T)	FS/T I	Siliguri	N26°52'53.53" E88°47'55.25"	Clay	3.90
	FS/T II	Siliguri	N26°52'53.54" E88°47'55.26"	Clay	3.90
	FS/T III	Siliguri	N26°52'53.33" E88°47'55.30"	Clay	3.90
	FS/T IV	Siliguri	N26°52'53.43" E88°47'55.20"	Clay	3.90
	FS/T V	Siliguri	N26°52'53.93" E88°47'55.85"	Clay	3.90
Baikunthapur Forst (FS/B)	FS/B I	Siliguri	N26°45'12.73" E88°30' 29.60"	Sandy	5.5
	FS/BII	Siliguri	N26°45'12.77" E88°30' 29.80"	Sandy	5.5
	FS/B III	Siliguri	N26°45'13.63" E88°30' 27.67"	Sandy	5.5
	FS/B IV	Siliguri	N26°45'15.89" E88°30' 28.75"	Sandy	5.5
	FS/B V	Siliguri	N26°45'12.33" E88°30' 25.54"	Sandy	5.5
Mahananda Wild Life Sanctuary (FS/M)	FS/M I	Siliguri	N26°49'23.55" E88°24'40.49"	Clay	4.43
	FS/M II	Siliguri	N26°49'23.55" E88°24'40.49"	Clay	4.43
	FS/M III	Siliguri	N26°49'22.65" E88°24'41.49"	Clay	4.43
	FS/M IV	Siliguri	N26°49'25.25" E88°24'41.65"	Clay	4.43
	FS/M V	Siliguri	N26°49'20.45" E88°24'41.79"		

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Soil Texture	Soil pH
Jaldapara Forest (FS/J)	FS/J I	Jalpaiguri	N26°34'59.64" E89°22'46.50"	Clay	5.5
	FS/J II	Jalpaiguri	N26°34'58.44" E89°22'47.55"	Clay	5.5
	FS/J III	Jalpaiguri	N26°34'56.63" E89°22'44.51"	Clay	5.5
	FS/J IV	Jalpaiguri	N26°34'58.66" E89°22'44.51"	Clay	5.5
	FS/J V	Jalpaiguri	N26°34'51.60" E89°22'43.56"	Clay	5.5
Chilapata Forest (FS/C)	FS/C I	Jalpaiguri	N26°45'01.10" E89°20'60.10"	Clay	4.69
	FS/CII	Jalpaiguri	N26°45'09.12" E89°20'61.16"	Clay	4.69
	FS/C III	Jalpaiguri	N26°45'05.14" E89°20'61.23"	Clay	4.69
	FS/C IV	Jalpaiguri	N26°45'03.12" E89°20'61.34"	Clay	4.69
	FS/C V	Jalpaiguri	N26°45'03.12" E89°20'61.17"		
Gorumara Forest (FS/G)	FS/G I	Jalpaiguri	N26°44'54.08" E89°48'14.53"	Loamy	4.38
	FS/G II	Jalpaiguri	N26°44'54.88" E 89°48'14.04"	Loamy	4.38
	FS/G III	Jalpaiguri	N26°44'53.18" E 89°48'13.24"	Loamy	4.38
	FS/G IV	Jalpaiguri	N26°44'54.89" E 89°48'14.34"	Loamy	4.38
	FS/G V	Jalpaiguri	N26°44'57.23" E 89°48'15.24"	Loamy	4.38
	FS/G VI	Jalpaiguri	N26°44'55.48" E 89°48'34.12"	Loamy	4.38
Riverin soil					
Mahananda river (RS/M)	RS/M I	Darjeeling	N26°47'09.42" E88°22'06.55"	Sandy	4.11
	RS/M II	Darjeeling	N26°47'09.44" E88°22'06.53"	Sandy	4.11
	RS/M III	Darjeeling	N26°47'09.41" E88°22'06.50"	Sandy	4.11
	RS/M IV	Darjeeling	N26°47'09.47" E88°22'06.51"	Sandy	4.11
	RS/M V	Darjeeling	N26°47'09.49" E88°22'06.59"	Sandy	4.11
	RS/M VI	Darjeeling	N26°47'09.44" E88°22'06.57"	Sandy	4.11
Balasan river (RS/B)	RS/B I	Darjeeling	N26°43'11.35" E88°22'30.35"	Sandy	3.67
	RS/BII	Darjeeling	N26°43'11.39" E88°22'30.45"	Sandy	3.67
	RS/B III	Darjeeling	N26°43'11.65" E88°22'30.95"	Sandy	3.67
	RS/B IV	Darjeeling	N26°43'11.26" E88°22'30.30"	Sandy	3.67
	RS/B V	Darjeeling	N26°43'11.39" E88°22'58.45"	Sandy	3.67
Panighata (RS/P)	RS/P I	Darjeeling	N26°50'27.24" E88°26'25.85"	Sandy	3.90
	RS/P II	Darjeeling	N26°50'18.60" E88°26'28.34"	Sandy	3.90
	RS/P III	Darjeeling	N26°50'13.67" E88°26'28.03"	Sandy	3.90
	RS/P IV	Darjeeling	N26°49'51.95" E88°26'22.50"	Sandy	3.90
	RS/P V	Darjeeling	N26°49'33.35" E88°26'6.27"	Sandy	3.90
	RS/P VI	Darjeeling	N26°49'26.46" E88°26'8.82"	Sandy	3.90
Teesta (RS/T)	RS/T I	Jalpaiguri	N26°33.803' E88°45.506'	Sandy	5.10
	RS/TII	Jalpaiguri	N26°33.812' E88°45.506'	Sandy	5.10
	RS/T III	Jalpaiguri	N26°30.821' E88°42.516'	Sandy	5.10
	RS/T IV	Jalpaiguri	N26°33.834' E88°45.510'	Sandy	5.10
	RS/T V	Jalpaiguri	N26°33.834' E88°45.499'	Sandy	5.10
Dhorala (RS/D)	RS/D I	Jalpaiguri	N26°33'57.90" E88°56'11.99"	Sandy	4.43
	RS/D II	Jalpaiguri	N26°33'28.83" E88°56'27.50"	Sandy	4.43
	RS/D III	Jalpaiguri	N26°33'13.65" E88°56'41.60"	Sandy	4.43
	RS/D IV	Jalpaiguri	N26°32'39.51" E88°57'21.11"	Sandy	4.43
	RS/D V	Jalpaiguri	N26°32'17.99" E88°57'53.57"	Sandy	4.43

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Soil Texture	Soil pH
Torsha (RS/T)	RS/T I	Cooch behar	N26°16'44.40" E89°34'48.00"	Sandy	5.21
	RS/T II	Cooch behar	N26°16'44.47" E89°34'48.12"	Sandy	5.21
	RS/T III	Cooch behar	N26°16'44.67" E89°34'48.34"	Sandy	5.21
	RS/T IV	Cooch behar	N26°16'44.75" E89°34'48.85"	Sandy	5.21
	RS/T V	Cooch behar	N26°16'44.34" E89°34'48.67"	Sandy	5.21
Rhizosphere soil					
Tea (RHS/T)	RHS/T I	Darjeeling	N26°45'11.75" E88°23'28.27"	Clay	4.69
	RHS/T II	Darjeeling	N26°48'18.68" E88°21'14.61"	Sandy	3.34
	RHS/T III	Darjeeling	N26°45'11.75" E88°23'28.27"	Sandy	3.56
	RHS/T IV	Darjeeling	N26°48'18.68" E88°21'14.61"	Clay	4.69
	RHS/T V	Darjeeling	N26°48'18.56" E88°21'14.11"	Clay	4.69
	RHS/T VI	Jalpaiguri	N26°31'41.91" E89°31'33.24"	Sandy	4.49
	RHS/T VII	Jalpaiguri	N26°31'36.17" E89°31'33.8"	Sandy	4.49
	RHS/T VIII	Jalpaiguri	N26°31'43.11" E89°31'37.21"	Sandy	4.49
	RHS/T IX	Jalpaiguri	N26°31'43.09" E89°31'42.70"	Sandy	4.49
	RHS/T X	Jalpaiguri	N26°31'38.46" E 89°31'40.69"	Sandy	4.49
	RHS/T XI	Jalpaiguri	N26°31'33.15" E 89°31'37.15"	Sandy	4.49
Rubber (RHS/R)	RHS/R I	Darjeeling	N26°32.628' E88°47.980'	Sandy	3.96
	RHS/R II	Darjeeling	N26°32.610' E88°47.962'	Sandy	3.96
	RHS/R III	Darjeeling	N26°32.597' E88°47.949'	Sandy	3.96
	RHS/R IV	Darjeeling	N26°32.568' E88°47.917'	Sandy	3.96
	RHS/R V	Darjeeling	N26°32.552' E88°47.899'	Sandy	3.96
	RHS/R VI	Darjeeling	N26°32.541' E88°47.892'	Sandy	3.96
	RHS/R VII	Jalpaiguri	N26°32.521' E88°47.871'	Sandy	3.96
	RHS/RVIII	Jalpaiguri	N26°32.505' E88°47.857'	Clay	5.60
	RHS/R IX	Jalpaiguri	N26°32.486' E88°47.836'	Clay	5.60
Citrus medica (RHS/M)	RHS/M I	Darjeeling	N26°29.908' E89°31.976'	Clay	4.76
	RHS/M II	Darjeeling	N26°29.910' E89°31.926'	Clay	4.76
	RHS/M III	Darjeeling	N26°29.915' E89° 31.730'	Clay	4.76
	RHS/M IV	Darjeeling	N26°29.917' E89° 31.692'	Clay	4.76
	RHS/M V	Darjeeling	N26°29.912' E89° 31.690'	Clay	4.20
Paddy (RHS/P)	RHS/P I	Darjeeling	N26°29.889' E89° 31.641'	Clay	3.98
	RHS/P II	Darjeeling	N26°29.759' E89° 31.622'	Clay	3.98
	RHS/P III	Darjeeling	N26°29.284' E89° 31.585'	Clay	3.98
	RHS/P IV	Darjeeling	N26°29.192' E89° 31.586'	Clay	3.98
	RHS/P V	Darjeeling	N26°28.874' E89° 31.620'	Clay	3.98
	RHS/P VI	Darjeeling	N26°28.846' E89° 31.613'	Clay	3.98
	RHS/P VII	Darjeeling	N26°28.850' E89° 31.572'	Clay	3.98
	RHS/P VIII	Darjeeling	N26°28.833' E89° 31.447'	Clay	3.98
	RHS/P IX	Darjeeling	N26°28.820' E89° 31.381'	Clay	3.98

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Soil Texture	Soil pH
	RHS/P X	Darjeeling	N26°28.812' E89° 31.328'	Clay	3.98
	RHS/P XI	Darjeeling	N26°28.776' E89° 31.139'	Clay	3.98
	RHS/P XII	Darjeeling	N26°28.771' E89° 31.103'	Clay	3.98
	RHS/P XIII	Jalpaiguri	N26°28.773' E89° 31.058'	Sandy	4.89
	RHS/P XIV	Jalpaiguri	N26°28.789' E89° 31.002'	Sandy	4.89
	RHS/P XV	Jalpaiguri	N26°28.812' E89°30.938'	Sandy	4.89
	RHS/P XVI	Jalpaiguri	N26°28.837' E89° 30.860'	Sandy	4.89
	RHS/P XVII	Jalpaiguri	N26°28.872' E89° 30.760'	Sandy	4.89
	RHS/P XVIII	Cooch behar	N26°29.927' E89° 32.050'	Loamy	4.02
	RHS/P XIX	Cooch behar	N26°29.920' E89° 32.048'	Loamy	4.02
	RHS/P XX	Cooch behar	N26°29.913' E89° 32.046'	Loamy	4.02
	RHS/P XXI	Cooch behar	N26°29.907' E89° 32.038'	Loamy	4.02
	RHS/P XXII	Cooch behar	N26°29.907' E89°32.030'	Loamy	4.02
	RHS/P XXIII	Cooch behar	N26°29.908' E89°32.011'	Loamy	4.02
Bamboo (RHS/B)	RHS/B I	Darjeeling	N26°45.120' E88°26.379'	Sandy	4.11
	RHS/B II	Darjeeling	N26°45.501' E88°26.525'	Sandy	4.11
	RHS/B III	Darjeeling	N26°45.590' E88°26.557'	Sandy	4.11
	RHS/B IV	Darjeeling	N26°45.698' E88°26.599'	Sandy	4.11
	RHS/B V	Darjeeling	N26°45.829' E88°26.649'	Sandy	4.11
	RHS/B VI	Jalpaiguri	N26°43.008' E88°46.044'	Clay	4.69
	RHS/B VII	Jalpaiguri	N26°42.356' E88°46.115'	Clay	4.69
	RHS/B VIII	Jalpaiguri	N26°42.250' E88°46.104'	Clay	4.69
	RHS/B IX	Cooch behar	N26°33.196' E89°04.427'	Sandy	4.11
	RHS/B X	Cooch behar	N26°33.243' E89°04.328'	Sandy	4.11
	RHS/B XI	Cooch behar	N26°33.260' E89°04.281'	Sandy	4.11
	RHS/B XII	Cooch behar	N26°33.271' E89°04.208'	Sandy	4.11
Wheat (RHS/W)	RHS/W I	Darjeeling	N26°32.447' E88°47.797'	Clay	5.21
	RHS/W II	Darjeeling	N26°32.418' E88°47.766'	Clay	5.21
	RHS/W III	Darjeeling	N26°32.401' E88°47.748'	Clay	5.21
	RHS/W IV	Jalpaiguri	N26°42.848' E88°27.427'	Sandy	3.43
	RHS/W V	Jalpaiguri	N26°42.856' E88°27.424'	Sandy	3.43
	RHS/W VI	Jalpaiguri	N26°42.865' E88°27.421'	Sandy	3.43
	RHS/W VII	Cooch behar	N26°34.376' E89°02.028'	Loamy	3.08

Type of soil sample	Soil Sample Code	Geographical Location	GIS location of sampling	Soil Texture	Soil pH
	RHS/W VIII	Cooch behar	N26°34.346' E89°02.123'	Loamy	3.08
	RHS/W IX	Cooch behar	N26°34.376' E89°02.028'	Loamy	3.08
	RHS/W X	Cooch behar	N26°34.388' E89°02.009'	Loamy	3.08
Soybean (RHS/S)	RHS/S I	Darjeeling	N26°28.771' E89°31.103'	Sandy	4.43
	RHS/S II	Darjeeling	N26°28.766' E89°31.112'	Sandy	4.43
	RHS/S III	Darjeeling	N26°28.475' E89°31.131'	Sandy	4.43
	RHS/S IV	Darjeeling	N26°28.641' E89°31.153'	Sandy	4.43
	RHS/S V	Darjeeling	N26°28.720' E89°31.229'	Sandy	4.43
Cabbage (RHS/C)	RHS/C I	Cooch behar	N26°33.211' E89°04.170'	Clay	4.69
	RHS/C II	Cooch behar	N26°33.367' E89°03.987'	Clay	4.69
	RHS/C III	Cooch behar	N26°33.553' E89°03.311'	Clay	4.69
	RHS/C IV	Cooch behar	N26°33.543' E89°03.302'	Clay	4.69
	RHS/C V	Cooch behar	N26°33.573' E89°03.312'	Clay	4.69
Potato (RHS/P)	RHS/P I	Cooch behar	N26°33.676' E89°03.149'	Clay	5.5
	RHS/P II	Cooch behar	N26°33.752' E89°03.036'	Clay	5.5
	RHS/P III	Cooch behar	N26°34.258' E89°02.261'	Clay	5.5
	RHS/P IV	Cooch behar	N26°34.346' E89°02.123'	Clay	5.5

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 isolatation of actinomycetes and 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), Actinomycetes isolation synthetic medium, Starch nutrient agar were used for isolation of microorganism. Optimum temperature 28^0C for incubation of fungi and 37^0C for bacteria and actinomycetes were maintained. The number of fungi, bacteria and actinomycetes formed colonies on the plates were counted and the microbial populations obtained from different rhizosphere, forest soil and riverine soil were determined. Microbial population determined in soils, ranged between $5x10^3$- $15x\ 10^4$cfu in case of fungi and $10x10^6$cfu-$30x10^6$cfu in case of bacteria and actinomycetes. Finally the population of fungi, bacteria and actinomycetes as colony forming units (cfu/g of soil) were determined (Table 4, Fig 1 & 2).

4.1.1 Fungal isolates

4.1.1.1 Growth studies in solid medium

The isolated fungi were allowed to grow in Petri dishes containing sterile PDA medium for 4-6 days at 28^0C. Nature of mycelial growth, rate of growth and time of sporulation were noted. Radial growth patterns of different fungi isolated from forest soil (Plate 7), riverine soil (Plate 8) and rhizosphere soil of crop fields. (Plate 9) were studied and morphological characters of the isolated fungi have been presented in detail in Table 5.

4.1.1.2. Microscopic observation

Microscopic observations under bright field of each fungi were made and photographs were taken. On the basis of colony character, mycelia, structure of conidiophore and conidia these were identified. It was found that most of the fungal isolates belonged to the genera *Fusarium, Aspergillus, Curvularia, Penicillium, Alternaria, Scleorotia, Talaromyces, Paecilomyces, Sporotrichum, Acremonium, Drechslera, Rhizopus, Bipolaris, Rhizoctonia, Absidia, Emenicella, Noesertoria, Colletotrichum, Trichoderma* and *Macrophomina* (Plate 10).

Table 4 : Populations of fungi, bacteria and actinomycetes in soil sample from forest, riverine and rhizophere soil of crop fields

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10^4/g)	Mean	Bacterial Population (cfu x 10^6/g	Mean	Actinomycetes Population (cfu x 10^6/g)	Mean
Forest soil							
Sukna Forest (FS/S)	FS/SI	6.7	6.24	5.5	5.48	5.6	4.96
	FS/SII	6.3		5.8		5.2	
	FS/SIII	5.9		5.6		5.3	
	FS/SIV	6.1		5.2		4.1	
	FS/SV	6.2		5.3		4.6	
Lohagarh Forest (FS/L)	FS/LI	5.5	5.48	4.1	4.88	5.2	5.00
	FS/LII	5.8		4.6		5.7	
	FS/LIII	5.6		5.2		4.8	
	FS/LIV	5.2		5.7		4.9	
	FS/LV	5.3		4.8		4.4	
Cinchona Forest (FS/C)	FS/CI	4.1	4.88	4.9	4.18	4.1	3.98
	FS/C II	4.6		4.4		4.4	
	FS/C III	5.2		4.1		3.1	
	FS/C IV	5.7		4.4		4.2	
	FS/C V	4.8		3.1		4.1	
Mongpong Forest (FS/M)	FS/M I	4.9	4.18	4.2	4.62	3.9	5.4
	FS/M II	4.4		4.1		3.8	
	FS/M III	4.1		3.9		5.6	
	FS/M IV	4.4		3.8		6.1	
	FS/M V	3.1		5.6		6.4	
	FS/M VI	4.2		6.1		6.6	
Terghera Forest (FS/T)	FS/T I	4.1	4.7	6.4	6.04	7.1	6.08
	FS/T II	3.9		6.6		7.2	
	FS/T III	3.8		7.1		5.5	
	FS/T IV	5.6		5.5		5.3	
	FS/T V	6.1		4.6		5.3	
Baikunthapur Forst (FS/B)	FS/B I	6.4	6.56	5.2	5	3.4	3.9
	FS/BII	6.6		5.7		4.1	
	FS/B III	7.1		4.8		4.3	
	FS/B IV	7.2		4.9		4.2	
	FS/B V	5.5		4.4		3.5	
Mahananda Wild Life Sanctuary (FS/M)	FS/M I	5.3	4.48	4.1	3.98	4.9	5.02
	FS/M II	5.3		4.4		5.5	
	FS/M III	3.4		3.1		5.2	
	FS/M IV	4.1		4.2		5.3	
	FS/M V	4.3		4.1		4.2	

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10^4/g)	Mean	Bacterial Population (cfu x 10^6/g	Mean	Actinomycetes Population (cfu x 10^6/g)	Mean
Jaldapara Forest (FS/J)	FS/J I	4.2	3.5	3.9	5.16	4.2	4.9
	FS/J II	3.5		3.8		4.6	
	FS/J III	4.9		5.6		5.2	
	FS/J IV	5.5		6.1		5.7	
	FS/J V	5.2		6.4		4.8	
Chilapata Forest (FS/C)	FS/C I	5.3	5.76	6.6	6.075	4.9	5.72
	FS/CII	4.2		7.1		7.2	
	FS/C III	7.1		5.3		5.5	
	FS/C IV	6.1		5.3		5.3	
	FS/C V	6.1		3.4		5.3	
Gorumara Forest (FS/G)	FS/G I	6.4	6.47	4.1	4.07	3.4	4.32
	FS/G II	6.6		4.3		4.1	
	FS/G III	6.2		4.1		4.3	
	FS/G IV	6.3		4.3		4.2	
	FS/G V	6.7		4.2		3.5	
	FS/G VI	6.6		4.1		5.5	
Riverine soil							
Mahanda river (RS/M)	RS/M I	4.6	4.93	5.5	5.12	4.1	3.97
	RS/M II	5.2		4.6		4.4	
	RS/M III	5.7		5.2		3.1	
	RS/M IV	4.8		5.7		4.2	
	RS/M V	4.9		4.8		4.1	
	RS/M VI	4.4		4.9		3.9	
Balasan river (RS/B)	RS/B I	4.1	3.98	4.4	4.04	3.8	5.7
	RS/BII	4.4		4.1		5.6	
	RS/B III	3.1		4.4		6.1	
	RS/B IV	4.2		3.1		6.4	
	RS/B V	4.1		4.2		6.6	
Panighata (RS/P)	RS/P I	3.9	5.4	4.1	4.85	7.1	5.58
	RS/P II	3.8		3.9		4.6	
	RS/P III	5.6		4.1		5.2	
	RS/P IV	6.1		3.9		5.8	
	RS/P V	6.4		3.8		5.6	
	RS/P VI	6.6		5.6		5.2	
Teesta (RS/T)	RS/T I	7.1	5.48	6.1	6.68	5.3	4.98
	RS/TII	4.6		6.4		4.1	
	RS/T III	5.2		6.6		4.6	
	RS/T IV	5.7		7.1		5.2	
	RS/T V	4.8		7.2		5.7	
Dhorala (RS/D)	RS/D I	4.9	4.18	5.5	4.72	4.8	4.52
	RS/D II	4.4		5.3		4.9	
	RS/D III	4.1		5.3		4.4	
	RS/D IV	4.4		3.4		4.1	
	RS/D V	3.1		4.1		4.4	
Torsha (RS/T)	RS/T I	4.2	4.32	4.3	4.06	3.1	3.82
	RS/T II	4.1		4.2		4.2	
	RS/T III	3.9		4.1		4.1	
	RS/T IV	3.8		3.9		3.9	
	RS/T V	5.6		3.8		3.8	
Rhizosphere soil							
Tea (RHS/T)	RHS/T I	6.4	5.29	6.1	5.57	6.1	5.57
	RHS/T II	6.6		6.4		6.4	
	RHS/T III	7.1		6.6		6.6	
	RHS/T IV	4.6		7.1		7.1	
	RHS/T V	5.2		7.2		7.2	
	RHS/T VI	5.7		5.5		5.5	
	RHS/T VII	4.8		5.3		5.3	
	RHS/T VIII	4.9		5.3		5.3	
	RHS/T IX	4.4		3.4		3.4	

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10^4/g)	Mean	Bacterial Population (cfu x 10^6/g	Mean	Actinomycetes Population (cfu x 10^6/g)	Mean
	RHS/T X	4.1		4.1		4.1	
	RHS/T X1	4.4		4.3		4.3	
Rubber (RHS/R)	RHS/R I	3.1	4.87	5.5	4.84	4.2	4.57
	RHS/R II	4.2		4.6		3.5	
	RHS/R III	4.1		5.2		5.5	
	RHS/R IV	3.9		5.7		5.3	
	RHS/R V	3.8		4.8		5.3	
	RHS/R VI	5.6		4.9		3.4	
	RHS/R VII	6.1		4.4		4.1	
	RHS/RVIII	6.4		4.1		4.3	
	RHS/R IX	6.6		4.4		5.5	
Citrus medica (RHS/M)	RHS/M I	7.1	6.56	3.1	3.88	4.6	5.04
	RHS/M II	6.1		4.2		5.2	
	RHS/M III	6.4		4.1		5.7	
	RHS/M IV	6.6		3.9		4.8	
	RHS/M V	6.6		4.1		4.9	
Paddy (RHS/P)	RHS/P I	7.1	4.70	3.9	4.56	4.4	4.37
	RHS/P II	7.2		3.8		4.1	
	RHS/P III	5.5		5.6		4.4	
	RHS/P IV	5.3		5.5		3.1	
	RHS/P V	5.3		4.6		4.2	
	RHS/P VI	3.4		5.2		4.1	
	RHS/P VII	4.1		5.7		3.9	
	RHS/P VIII	4.3		4.8		4.1	
	RHS/P IX	4.2		4.9		3.9	
	RHS/P X	3.5		4.4		3.8	
	RHS/P XI	4.9		4.1		5.6	
	RHS/P XII	5.5		4.4		5.5	
	RHS/P XIII	5.2		3.1		4.6	
	RHS/P XIV	5.3		4.2		5.2	
	RHS/P XV	4.2		4.1		5.7	
	RHS/P XVI	4.1		3.9		4.8	
	RHS/P XVII	4.4		4.1		4.9	
	RHS/P XVIII	3.1		3.9		4.4	
	RHS/P XIX	4.2		3.8		4.1	
	RHS/P XX	4.1		5.6		4.4	
	RHS/P XXI	3.9		5.5		3.1	
	RHS/P XXII	3.8		4.6		4.2	
	RHS/P XXIII	5.6		5.2		4.1	

Type of soil sample	Soil Sample Code	Fungal Population (cfu x 10^4/g)	Mean	Bacterial Population (cfu x 10^6/g	Mean	Actinomycetes Population (cfu x 10^6/g)	Mean
Bamboo (RHS/B)	RHS/B I	6.1	4.85	5.7	4.30	3.9	4.70
	RHS/B II	6.4		4.8		4.1	
	RHS/B III	4.1		4.9		3.9	
	RHS/B IV	4.4		4.4		3.8	
	RHS/B V	3.1		4.1		5.6	
	RHS/B VI	4.2		4.4		5.5	
	RHS/B VII	4.1		3.1		4.6	
	RHS/B VIII	3.9		4.2		5.2	
	RHS/B IX	3.8		4.1		5.7	
	RHS/B X	5.6		3.9		4.8	
	RHS/B XI	6.1		4.1		4.9	
	RHS/B XII	6.4		3.9		4.4	
Wheat (RHS/W)	RHS/W I	6.6	5.00	3.8	4.86	4.1	4.12
	RHS/W II	7.1		5.6		4.4	
	RHS/W III	6.1		5.5		3.1	
	RHS/W IV	6.4		4.6		4.2	
	RHS/W V	4.1		5.2		4.1	
	RHS/W VI	4.4		5.7		3.9	
	RHS/W VII	3.1		4.8		4.1	
	RHS/W VIII	4.2		4.9		3.9	
	RHS/W IX	4.1		4.4		3.8	
	RHS/W X	3.9		4.1		5.6	
Soybean (RHS/S)	RHS/S I	3.8	4.64	4.4	3.94	3.1	3.82
	RHS/S II	3.9		3.1		4.2	
	RHS/S III	3.8		4.2		4.1	
	RHS/S IV	5.6		4.1		3.9	
	RHS/S V	6.1		3.9		3.8	
Cabbage (RHS/C)	RHS/C I	5.6	5.76	4.1	4.70	5.6	4.66
	RHS/C II	6.1		3.9		6.1	
	RHS/C III	4.1		3.8		4.1	
	RHS/C IV	6.4		5.6		4.4	
	RHS/C V	6.6		6.1		3.1	
Potato (RHS/P)	RHS/P I	4.4	3.95	4.1	3.98	4.2	3.90
	RHS/P II	3.1		4.4		4.1	
	RHS/P III	4.2		3.1		3.9	
	RHS/P IV	4.1		4.2		3.1	
	RHS/P V	4.0		4.1		4.2	

Table 5: **Morphology and Microscopical Characters of isolated fungi**

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	**Organisms identified (No. of Isolates)**	**Morphology and Microscopical Character**
FS/L42, FS/L41, FS/S64 RS/286, RS/M60, RS/T183, RS/T-31, RS/T58, RS/T31 RHS/H491, RHS/T190 RHS/B220, RHS/C23, RHS/P44, RHS/P202, RHS/P106, RHS/P117, RHS/P114	*Aspergillus flavus* (18)	**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, smaller heads occasionally columnar up to 300-400 µm. **Conidiophore:** Conidiophores thick-walled, hyaline, coarsely roughened, usually less than 1 µm long, 10-20 µm diam., just below the vesicle; vesicles elongated when young, becoming subglobose to globose, 25-45 µm diam.; both metulae and phialides present; metulae usually 6-10 x 5,5 µm but sometimes up to 15-16 x 8-9 µm; phialides 6,5-10 x 3-5 µm. Conidia typically globose to subglobose, conspicuously echinulate, variable, (3-) 3,5-4,5 (-6) µm diam., sometimes elliptical or pyriform at first and occasionally remaining so, and then 4.5-5.5 x 3.5- 4.5 µm.
FS/L24, FS/M259 FS/L04, FS/L-40 FS/C-140, FS/C143 FS/C-160, FS/S-165 FS/S-173, FS/S-177 FS/S-109 FS/S110, FS/S-112 FS/S-113, FS/S-262 RS/T74, RS/M60, RS/P/14, RS/D-288, RS/T-57, RS/T-58 RHS/M492, RHS/P200, RHS/S28, RHS/T72, RHS/T73, RHS/P-37, RHS/P-51, RHS/P-105, RHS/P-106, RHS/P-107, RHS/P-45, RHS/P-48, RHS/P-117, RHS/D-280, RHS/D-281 RHS/D-282, RHS/D-283, RHS/D-284, RHS/D-285, RHS/D-286, RHS/D-287	*A. niger* (42)	**Colonies:** Black on PDA mediam **Mycelia:** Hyaline, aseptate **Conidia:** Conidial heads radiate. Conidia brown, ornamented with warts and ridges, subspherical, 3.5-5.0 µm diam. **Conidiophore:** Consisting of a dense felt of conidiophores. Conidiophore stipes smooth-walled, hyaline. Vesicles subspherical, 50-100 µm diam

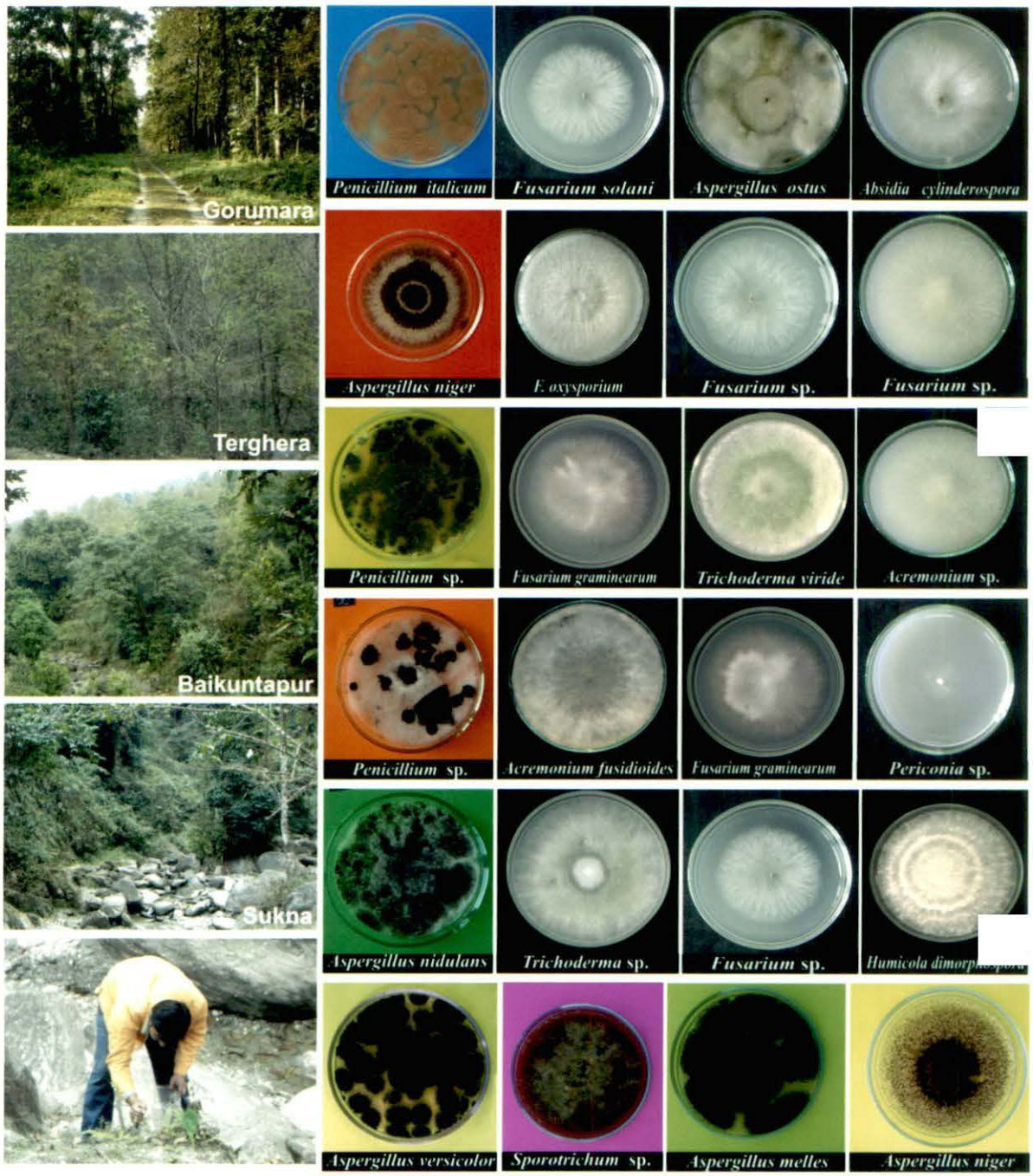


Plate 7 : Radial growth patterns of different fungi isolated from forest soil in PDA

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/L 17, FS/L-13 FS/L-18, 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 RS/R-115, RS/T-182 RS/T-183, RS/P -61 RS/P05 RHS/R-12, RHS/P-82, RHS/P-198 RHS/P-201, RHS/P-202	*A. mellus* (23)	**Colonies:** Black on PDA mediam **Mycelia:** Hyaline, aseptate **Conidia:** Conidia globose to subglobose , smooth walled or irregularly roughened, 2.8-3.5 µm diam. **Conidiophore:** Conidiophore usually 0.5-2 µm tall, thickwalled, roughened.
FS/M262 RHS/P38, RHS/P209, RHS/P205, RHS/P-38, RHS/P-114 RHS/T-99, RHS/T-190 RHS/T-191, RHS/P -50 RHS/P -54, RHS/P-43 RHS/P-47	*A. clavatus* (13)	**Colonies:** Colonies on Czapek and malt agar usually spreading, occasionally floccose, blue-green, mycelium white, inconspicuous. **Mycelia:** Hyaline, aseptate **Conidia:** Conidial heads clavate, usually splitting into several divergent columns **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.
RHS/T558 RHS/P-557 RHS/P38, RHS/P209, RHS/P205, RHS/P-205, RHS/P-209 RHS/B-220, RHS/P-114	*A. fumigatus* (9)	**Colonies:** Colony characteristics. Colonies (CzA) dark blue-green, consisting of a dense felt of 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:** Conidiophore stipes smooth-walled, often green in the upper part. Vesicles subclavate, 20-30 µm wide.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/L42 RS/T-59, RS/P -60	*A. nidulans* (3)	**Colonies:** Colonies growing rapidly, green, cream-buff or honey-yellow; reverse dark purplish. **Mycelia:** Hyaline, aseptate **Conidia:** Conidia spherical, rugulose, subhyaline, green in mass, 3-4 µm diam. 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.
RHS/M505, RHS/M499	*A. oryzae* (2)	**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. Vesicles subspherical, up to 75 µm diam. Conidiogenous cells uniseriate and biseriate. Metulae or phialides covering the entire surface or the upper three-fourths of the vesicle
RHS-544	*A. parasiticus* (1)	**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 **Mycelia:** Hyaline, aseptate **Conidia:** Conidia globose, 3.5-5.5 µm in diam, yellow-green, conspicuously rough-walled. Conidial heads green, radiate **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.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	**Organisms identified (No. of Isolates)**	**Morphology and Microscopical Character**
RHS/M506	*A. .versicolor* (1)	**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, with exudate and reverse of equally variable colour **Mycelia:** Hyaline, aseptate **Conidia:** Conidia globose, echinulate, mostly 2-3 µm diam **Conidiophore:** Conidiophores colourless or yellowish, smooth-walled, to 500-700 µm long; vesicles elongate with metulae and phialides covering most of the surface; conidial heads radiating
RHS/P196	*Curvularia lunata* var.*aeria* (1)	**Colonies:**. Colonies (PDA) expanding, black, hairy **Mycelia:** Hyaline, aseptate **Conidia:** Conidia olive brownCurved 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/P68, RHS/P66	*Fusarium graminearum* (2)	**Colonies:**. Raddish white **Mycelia:** Hyaline, aseptate **Conidia:** Conidia often formed sparsely, falcate, sickle-shaped or markedly dorsi-ventral, 3-7-septate, 25-50 x 3-4 µm, with a well developed, often pedicellate foot cell. Microconidia absent. Macroconidia produced from doliiform phialides 10-14 x 3,5-4,5 µm, formed laterally or on short multibranched conidiophores; sporodochia may form in older cultures **Conidiophore:** Chlamydospores, when present, are intercalary, single, in chains or clumps, globose, thick-walled, hyaline to pale brown with a smooth or slightly roughened outer wall, 10-12 diam. Many strains fail to develop chlamydospores on standard media

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	**Organisms identified (No. of Isolates)**	**Morphology and Microscopical Character**
RS/P2 RHS/P-388, RHS/P-137, RHS/P-137,	*Fusarium solani* (4)	**Colonies:**. Colonies growing rapidly, with white to cream-coloured aerial mycelium, usually green to bluish-brown **Mycelia:** hyaline, aseptate **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 **Conidiophore:** Conidiophores arising laterally from aerial hyphae. Monophialides mostly with a rather distinct collarette. Macroconidia produced on shorter, branched conidiophores which soon form sporodochia, usually moderately curved, with short, blunt apical and indistinctly pedicellate basal cells, mostly 3-septate, 28-42 × 4-6 µm, occasionally 5-septate
RHS-293	*Paecilomyces varioti* (1)	**Colonies:**. Colony characteristics. Colonies (MEA 2%) growing rapidly, powdery to floccose, funiculose or tufted, yellow-brown or sand colour. Odour sweet. **Mycelia:** Hyaline, aseptate **Conidia:** Conidia subspherical, ellipsoidal to fusiform, hyaline to yellow, smooth-walled, 3-5 x 2-4 µm, arising in long, divergent chains **Conidiophore:** Conidiophores bearing dense, verticillately arranged branches, each bearing 2-7 phialides, up to 150 µm in length, 3.5-6.5 µm wide. Phialides cylindrical or ellipsoidal, tapering abruptly into a long, thin, cylindrical neck.
RHS-536	*Penicillium italicum* (I)	**Colonies:**. Velutinous to fasciculate, crustose **Mycelia:** hyaline, aseptate **Conidia:**. Smooth-walled, ellipsoidal to cylindrical, 3.5-5 x 2.2-3.5 µm **Conidiophore:** Terverticillate, appressed elements, born from subsurface hyphae

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/G-173 FS/G175 FS/G-176 RHS-285	*Penicillium digitatum* (4)	**Colonies:**. Olive green **Mycelia:** Hyaline, aseptate **Conidia:**. Smooth-walled, ellipsoidal to cylindrical, 6-9 (-14) x 2.8-6 µm. **Conidiophore:** Terverticillate, appressed elements, born from subsurface or aerial hyphae
RHS/M496	*Sporotrichum pruinosum* (1)	**Colonies:**. Distinct greyish or pinkish hue; **Mycelia:** hyaline, aseptate **Conidia:** Blastoconidia from unbranched conidiophores ellipsoidal to ovoid pyriform or nearly cylindrical, 5.8 x 3.5 µm. All blastoconidia broadly attached and becoming thickwalled. Chlamydospores terminal or intercalary, hyaline, (sub)globose to broadly ellipsoidal or more rarely pyriform, 11- 60 µm diam or 11 x 7.5 µm, 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 becoming slightly thick-walled and more ellipsoidal. **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.
RHS/P129	*Acremonium fusidioides* (1)	**Colonies:**. Colonies reaching 8-10 mm diam in ten days at 20°C on MEA, ochraceous-brown, powdery; **Mycelia:** Hyaline, aseptate **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.

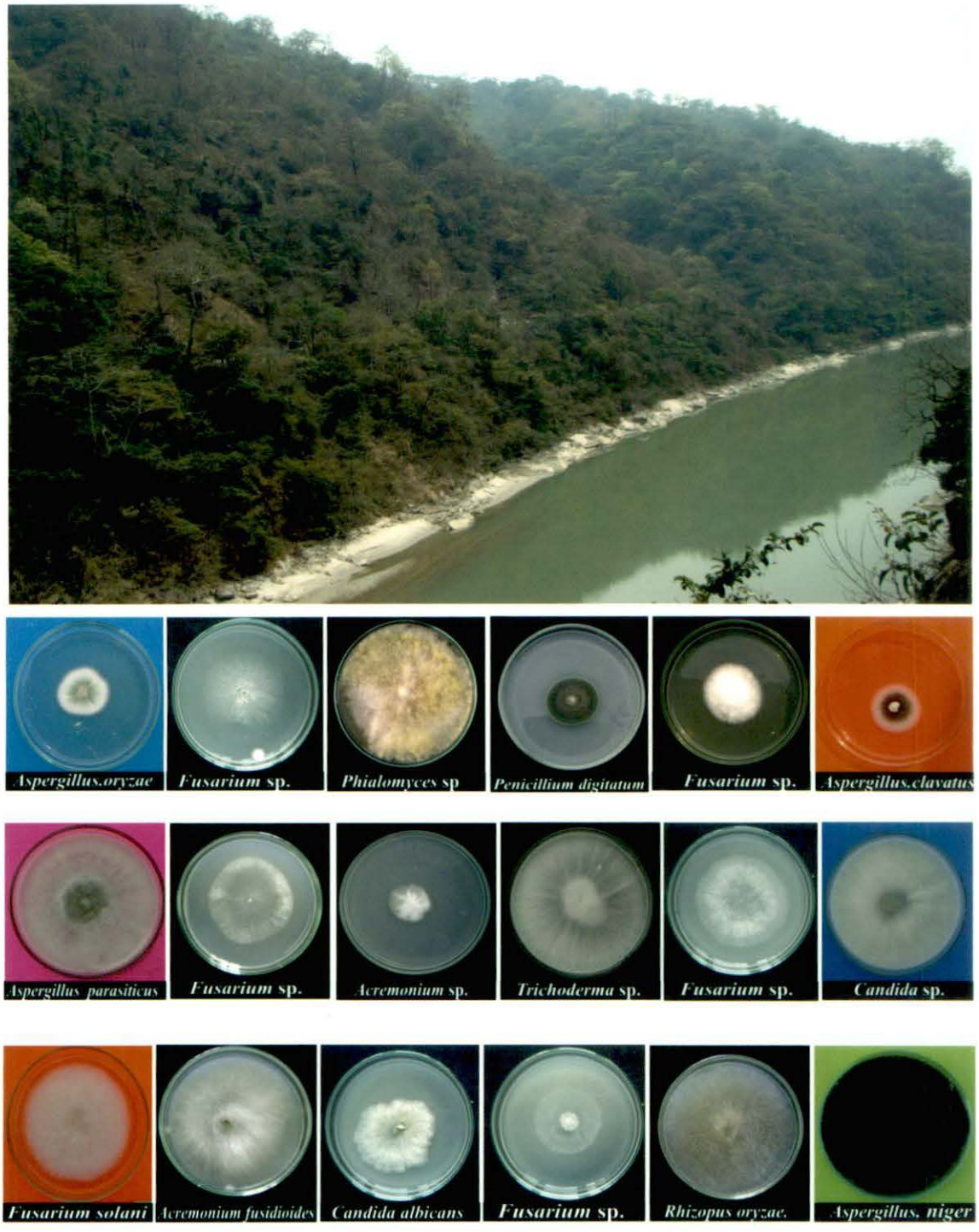


Plate 8 : Radial growth patterns of different fungi isolated from riverine soil in PDA

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/T71	*Drechslera sp.* (1)	**Colonies:**. Colonies effuse, grey, brown or blackish brown, often hairy, sometimes velvety **Mycelia:** Hyaline, aseptate Mycelium mostly immersed. Stroma present in some species. Sclerotia or protothecia often formed in culture. Setae and hyphopodia absent **Conidia:**. Conidia solitary, in certain species also sometimes catenate or forming secondary conidiophores which bear conidia, acropleurogenous, simple, straight or curved, clavate, cylindrical rounded at the ends, ellipsoidal, fusiform or obclavate, straw-coloured or pale to dark brown or olivaceous brown, sometimes with cells unequally coloured, the end cells then being paler than intermediate ones, mostly smooth, rarely verruculose, pseudoseptate. **Conidiophore:** Conidiophores macronematous, mononematous, sometimes caespitose, straight or flexuous, often geniculate, unbranched or in a few species loosely branched, brown, smooth in most species. Conidiogenous cells polytretic, integrated, terminal, frequently becoming intercalary, sympodial, cylindrical, cicatrized
RHS/P222	*Rhizopus oryzae* (1)	**Colonies:**. Colonies (MEA, 30°C) expanding, up to 1 cm high, whitish to greyish-brown. **Mycelia:** Hyaline, aseptate **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; columella comprising 50-70% of sporangium, spherical; apophysis short, 3-12 µm high. Sporangiospores greyish-green, angular, subspherical to ellipsoidal, longitudinally striate, 6-8 x 4.5-5.0 µm **Chlamydospore:** Single or in chains, spherical to ovoidal, 10-35 µm diam, hyaline, smooth-walled. Zygospores. Zygospores red to brown, spherical or laterally flattened, 60-140 µm, with flat projections. Suspensors unequal, spherical and conical. Heterothallic

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/T387	*Bipolaris sorokiniana* (1)	**Colonies:**. Dark brown to black **Mycelia:** Hyaline, aseptate **Conidia:**. Asci cylindrical to clavate, 1-8-spored, bitunicate, 110-230 x 30-45 µm. Ascospores hyaline to light brown, 6-13-septate, filiform, coiled in a helix in the ascus, often surrounded by a thin mucilaginous sheath, 160-360 x 6-9 µm. Pseudoparaphyses hyaline, filiform, branched. **Conidiophore:** Conidiophores solitary or in small groups, straight to flexuous, sometimes geniculate, pale to mid-dark brown, up to 220 µm long, 6-10 µm wide. Conidiogenous cells polytretic, integrated, terminal, sympodial, cylindrical, cicatrized. Conidia curved to straight, fusiform to broadly ellipsoidal, 3-12-distoseptate, 40-120 x 17-28 µm
RHS/T-382	*Sclerotium rolfsii* (1)	**Colonies:**. Whitish **Mycelia:** Mycelium septate with clamp connections at hyphal septa **Sclerotia:** Spherical, slightly ellipsoidal, quite uniform in size with a smooth surface. The surface color of the sclerotia was initially white, turned to pinkish buff, then to olive-brown, and eventually to clove brown as sclerotia matured
RHS/T-383	*Colletotrichum gloeosporioides* (1)	**Colonies:**. Colonies (OA) extremely variable, effuse, grey to brown, with pinkish patches; reverse dark brown with vinaceous stains. **Mycelia:** hyaline, aseptate **Conidia:**. Conidia straight, cylindrical, obtuse at the apex, 9-24 x3.0-4.5 µm. **Appressoria**: 6-20 x4-12 µm, clavate or irregular
RHS/S450	*Macrophomina phaseolina* (1)	**Colonies:**. Pycnidia dark brown, solitary or gregarious on leaves and stems, immersed, becoming erumpent, 100-200 µm diam **Mycelia:** hyaline, aseptate **Conidia:**. Conidia hyaline, ellipsoid to obovoid, 14-30 x 5-10 µm **Conidiophore:** Conidiophores (phialides) hyaline, short obpyriform to cylindrical, 5-13 x 4-6 µm..

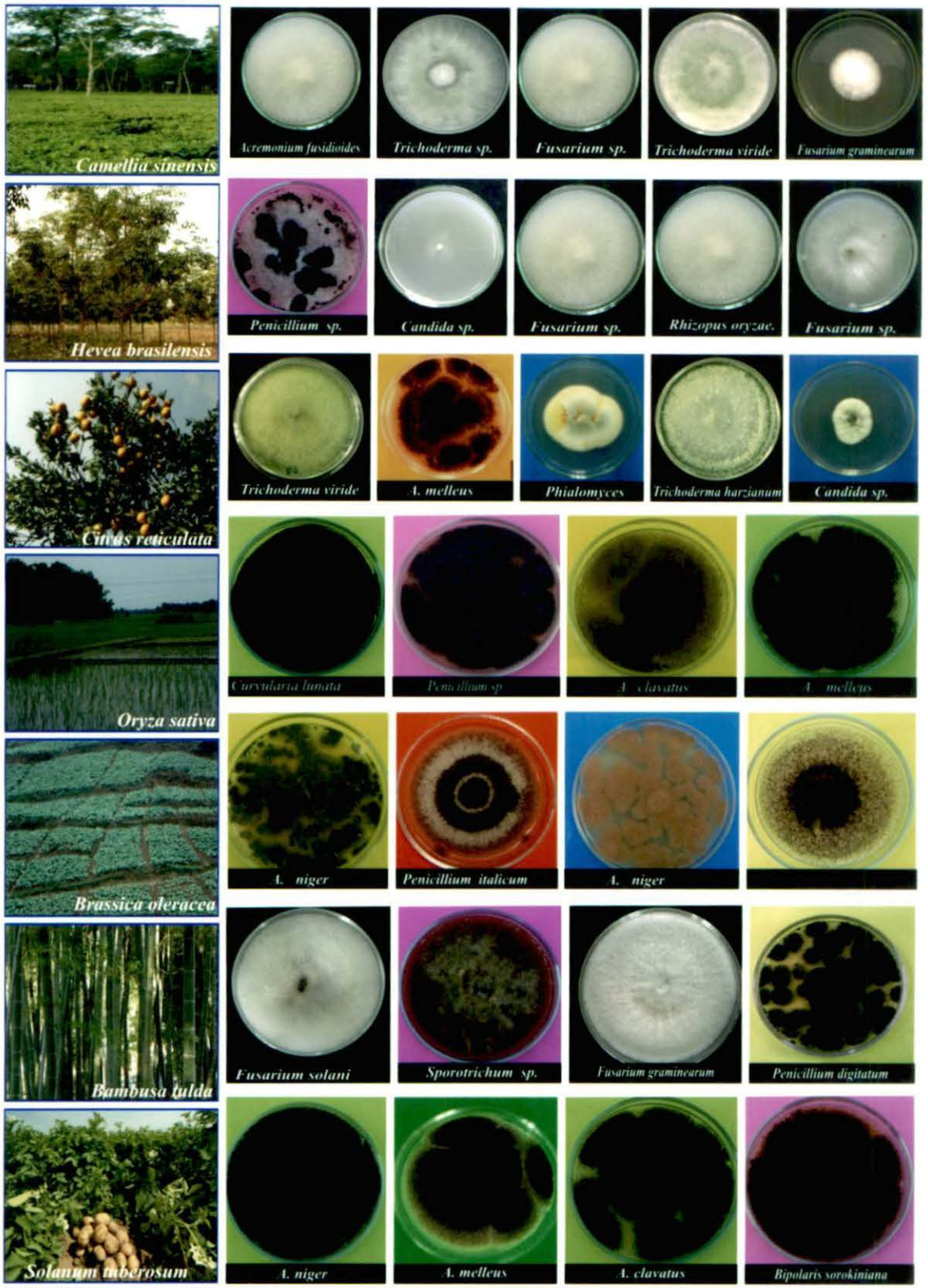


Plate 9 : Radial growth patterns of different fungi isolated from rhizosphere soil of different crop fields in PDA

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/P577	*Rhizoctonia solani* (1)	**Colonies:**. Fruitbody loosely adnate, hypochnoid to pellicular, usually whitish or cream-coloured. **Mycelia:** Hyaline, aseptate **Conidia:**. Basidia short-cylindrical, 20 x 12 µm, normally with four, rather stout and 8-10 µm long sterigmata **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
RHS/P578	*Absidia cylinderospora* (1)	**Colonies:**. The rapid growing, flat, woolly to cottony, and olive gray colonies mature within 4 day. **Mycelia:** Hyaline, aseptate **Rhizoid:** Rhizoids are rarely observed. When present, the sporangiophores arise on stolons from points between the rhizoids, but not opposite the rhizoids **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
RHS/T-384	*Alternaria alternate* (1)	**Colonies:**. *Alternaria* spp. grow rapidly and the colony size reaches a diameter of 3 to 9 cm following incubation at 25°C for 7 days on potato glucose agar. The colony is flat, downy to woolly and is covered by grayish, short, aerial hyphae **Mycelia:** Hyaline, septate, brown hyphae **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 **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.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RS/T-182	*Aspergillus ostus* (1)	**Colonies:**. Colonies on potato dextrose agar at 25°C are white to yellow to drab gray to brown, but never green **Mycelia:** Hyphae are septate and hyaline **Conidia:**. Conidial heads are radiate to loosely columnar and biseriate **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
RHS/H509	*Emenicella nidulans* (1)	**Colonies:**. Colonies (PDA) growing rapidly, green, cream-buff or honey-yellow; reverse dark purplish **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
FS/S108	*Noesertoria fischeri* (1)	**Colonies:**. Colony white to cream coloured cleistothecia, exudate clear texture velutinous **Mycelia:** Hyaline, aseptate **Conidia:**. conidia sparse and grey green **Ascospore:** Ascospores with two longtitudinal thin Ascospores are spherical, conidial heads spherical to ellipsoidal .
RHS/P-221, RHS/P-223	*Candida albicans* (2)	**Colonies:**. Colonies on Glucose Peptone Agar incubated at 25°C: after 3 days cream-coloured, smooth, dull, dome-shaped, often becoming wrinkled with a mycelial border on prolonged incubation. **Mycelia:** Hyaline, true mycelium and pseudomycelium **Conidia:**. Yeast-like cells generally globose to short-ovoid, thin-walled, hyaline with substantial size variation between cultures of different isolates and also of a single isolate, 2.0-7.0 x 3.0-8.5 µm, usually single or budding, occasionally forming short chains. **Chlamydospore:** Spherical, smooth, thick-walled spores (often referred to as chlamydospores) are produced terminally both on the main hyphae and on short, acute lateral branches. .

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RS/D-376, RS/D-377	*Cladosporium cladosporioidies* (2)	**Colonies:**. Colonies expanding, velvety to powdery, olivaceous green to olivaceous brown; reverse olivaceous black. **Conidia:** Conidia ellipsoidal to limoni-form, smooth-walled or slightly verrucose, olivaceous brown, 1-celled, with dark scars, easily liberated. **Conidiospore:** Conidiophores of variable length, up to 350 µm long, 2-6 µm wide, without swellings, with terminal and lateral ramifications, bearing branched conidial chains, pale olivaceous brown.
RHS/P-229	*Doratomyces sp.* (1)	**Colonies:**. Colonies effuse, grey, brown, blackish brown or black, velvety, floccose or powdery. Hyphae superficial and immersed. Stroma none. Setae and hyphopodia absent **Conidia:** Conidia catenate, dry, acrogenous, simple, ellipsoidal, ovoid, obovoid, spherical **Conidiospore:** Conidiogenous cells monoblastic, sometimes integrated terminal on branches but mostly diserete, penicillately arranged, percurrent, ampulliform or lageniform Conidiophores typically macronematous, synnematous, dark brown to black, threads straight or flexuous, individually pale brown to brown, mostly smooth, branched towards the apex with the branches splaying out to form a head.
RHS/B-247, RHS/B-290, RHS/B-291	*Humicola dimorphospora* (3)	**Colonies:**. Colonies on PDA 25-44 mm diam after 21 d at 25 C, flat, smooth, umbonate, yellowish white (4A2). Vegetative hyphae subhyaline to pale olive, smooth- and thin-walled, 1-3 µm wide, commonly forming mycelial strands **Mycelia:** Aseptate, Hyaline **Conidia:**. Sympodial conidia mostly guttuliform, 3-8(4.3 ± 0.8) x 1.5-3(2.2 ± 0.3) µm, hyaline to subhyaline, smooth- and thin-walled. Lateral blastoconidia from undifferentiated hyphae mostly subglobose to obovoid, 3-5(4 ± 0.4) x 3-5(3.8 ± 0.4) µm, smooth- and thick-walled, brown, always becoming dark gradually, sessile or on subhyaline to weakly pigmented pedicels **Conidiospore:** Conidiophores more or less differentiated, mostly unbranched, up to 90 µm long. Conidiogenous cells terminal or intercalary, sympodial, denticulate, 22-43 long, 1-1.5 µm wide toward the center, often inflated at the apex up to 5 µm wide.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/B-252, RHS/B-253, RHS/B-254	*Periconia sp.* (3)	**Colonies:**. Colonies effuse or, in a few species, small and compact, grey, brown, olivaceous brown or black, hairy. **Mycelia:** Mycelium mostly immersed but sometimes partly superficial. Stroma frequently present, mid to dark brown, pseudoparenehymatous. Separate setae absent but in a few species the apex of the conidiophore is sterile and setiform **Conidia:**. Conidia catenate, chains often branched, arising at one or more points on the curved surface of the conidiogenous cell, simple, usually spherical or subspherical, occasionally ellipsoidal, oblong or broadly cylindrical, pale to dark brown, **Conidiophore:** Conidiophores macronematous and sometimes also micronematous. Macronematous conidiophores mostly with a stipe and spherical head, looking like round-headed pins, branches present or absent, stipe straight or flexuous, in one species torsive, pale to dark brown, often appearing black and shining by reflected light, smooth or rarely verrucose; sometimes the apex is sterile and setiform. Conidiogenous cells monoblastic or polyblastic, diserete on stipe and branches, determinate, ellipsoidal, spherical or subspherical.
RHS/C-309, RHS/C-310, RHS/C-333	*Phialomyces sp.* (3)	**Colonies:**. Colonies effuse, at first yellow, later greyish olive. **Mycelia:** Mycelium partly superficial, partly immersed. Stroma none **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 cach end, 0-septate **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.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
RHS/C-340, RHS/C-341	*Scytalidium sp.* (2)	**Colonies:**. Colonies effuse, dark blackish brown. Mycelium immersed and superficial. Hyphae smooth, some narrow, cylindrical, colourless, others thicker, pale to mid brown with oceasional darker swollen cells and often thick, very dark brown septa. **Mycelia:** The hyphae often lie parallel to one another and may be closely adpressed forming bundles. Stroma none. **Conidia:**. Catenate, separating, dry, schizogenous, simple, smooth, 0-septate, with septa sometimes thick and very dark, of two kinds: (1) colourless, thin-walled, cylindrical or oblong, truncate at each end, (2) broader, mid or dark brown, thick-walled, oblong, doliif0rm or broadly ellipsoidal. Type species: Scytalidium lignicola Pesante **Conidiophores:** Micronematous, mononematous or sometimes synnematous, branched or unbranched, straight or flexuous, colouiless or brown, smooth. Conidiogenous cells fragmenting and forming arthroconidia, integrated, intercalary, determinate, Cylindrical, ellipsoidal.
RHS/P-134, RHS/P-135	*Sporobolomyces sp.* (2)	**Colonies:**. Colonies salmon pink, orange-red, red, cream, or yellow-brown; butyrous or rather tough; smooth or irregular, warty, venose or reticulate, sometimes somewhat pruinose. **Conidiophore:** Cells ellipsoidal, globose, fusiform or cylindrical, monokaryotic; conidiogenesis usually polar, rarely lateral or multilateral; conidia enteroblastically formed, sessile, on denticles or on distinct stalks, which are sometimes branched; scars distinct or indistinct, sympodially or percurrently proliferating; pseudohyphae and/or septate hyphae present or absent; chlamydospores present or absent; sterigmata laterally or terminally on yeast cells and hyphae, frequently sympodially branched. Ballistoconidia bilaterally symmetrical, ellipsoidal, falcate, allantoid, lacrymoid or amygdaliform, germinating with ballistoconidia, hyphae or yeast cells. Clamp connections absent.

Soil type [Forest soil- FS, Riverine soil- FS Rhizosphere soil- RHS] Isolate code	Organisms identified (No. of Isolates)	Morphology and Microscopical Character
FS/C-90 FS/S-455 FS/S-458 RHS/T- 477 Ag/S476 Ag/S471 Ag/S479 RHS/AC480 RHS/AC481 RHS/AC482 RHS/AC483	*Trichoderma harzianum* (11)	**Colonies:**. Dark green **Conidia:**. subglobose to ovoidal, 3.5 to 4.0 µm long, smooth, green **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. **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; Terminal phialides in a whorl or solitary, typically cylindrical or at least not conspicuously swollen in the middle and longer than the subterminal phialides.
FS/L-20 FS/S-473 FS/S-474 FS/S-475 FS/S-478 RHS/T- 460 RHS/T- 463 RHS/T- 472	*Trichoderma viride* (8)	**Colonies:**. Geen **Conidia:** Confluent to discrete, 0.5-1.0 mm diam, **Conidiophore**: Typically 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 ot 2-3 at the tips of lateral branches or at the tip of the conidiophore. The central axis 2.2-3.2µm wide. **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, arisingfrom a cell 2.2-3.2 µm wide.

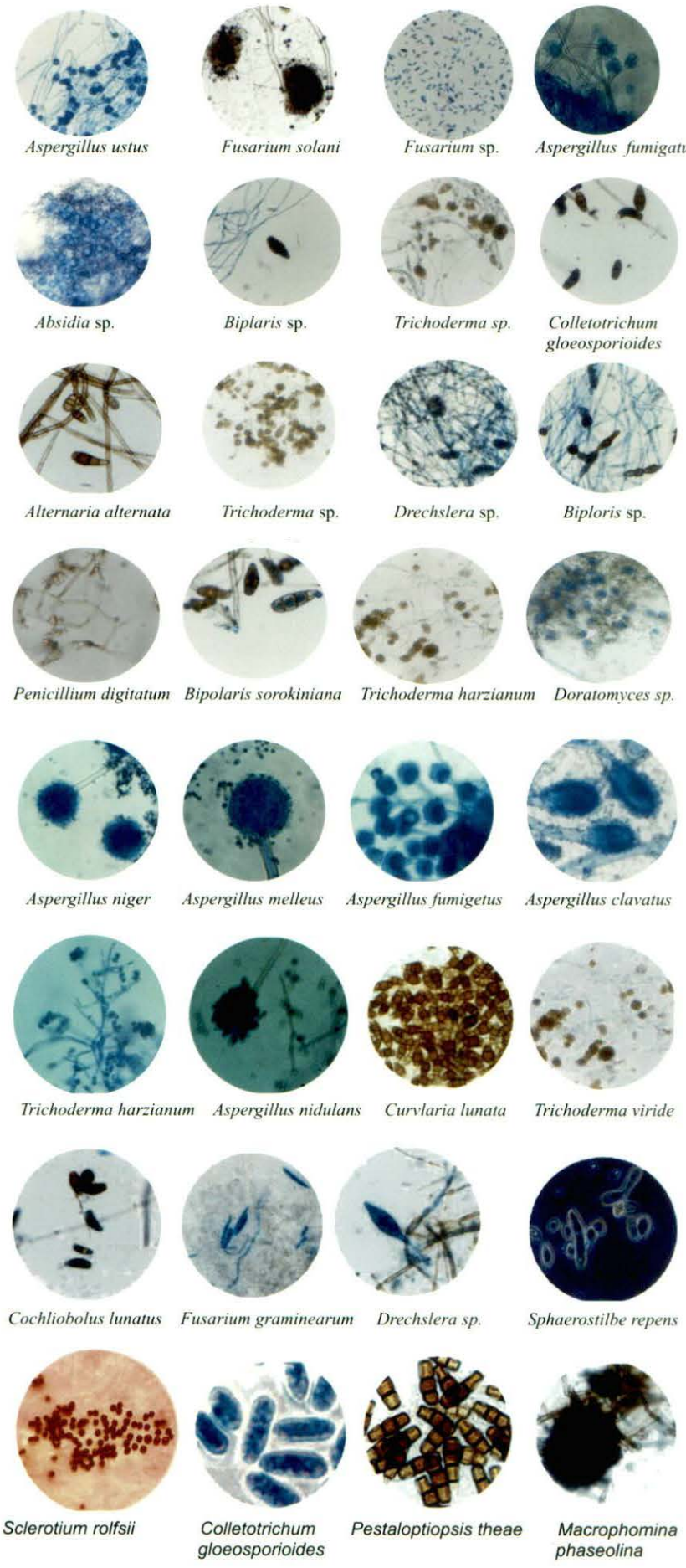


Plate 10 : Light microscopic views of different fungi isolated from forest, riverine and rhizosphere soil

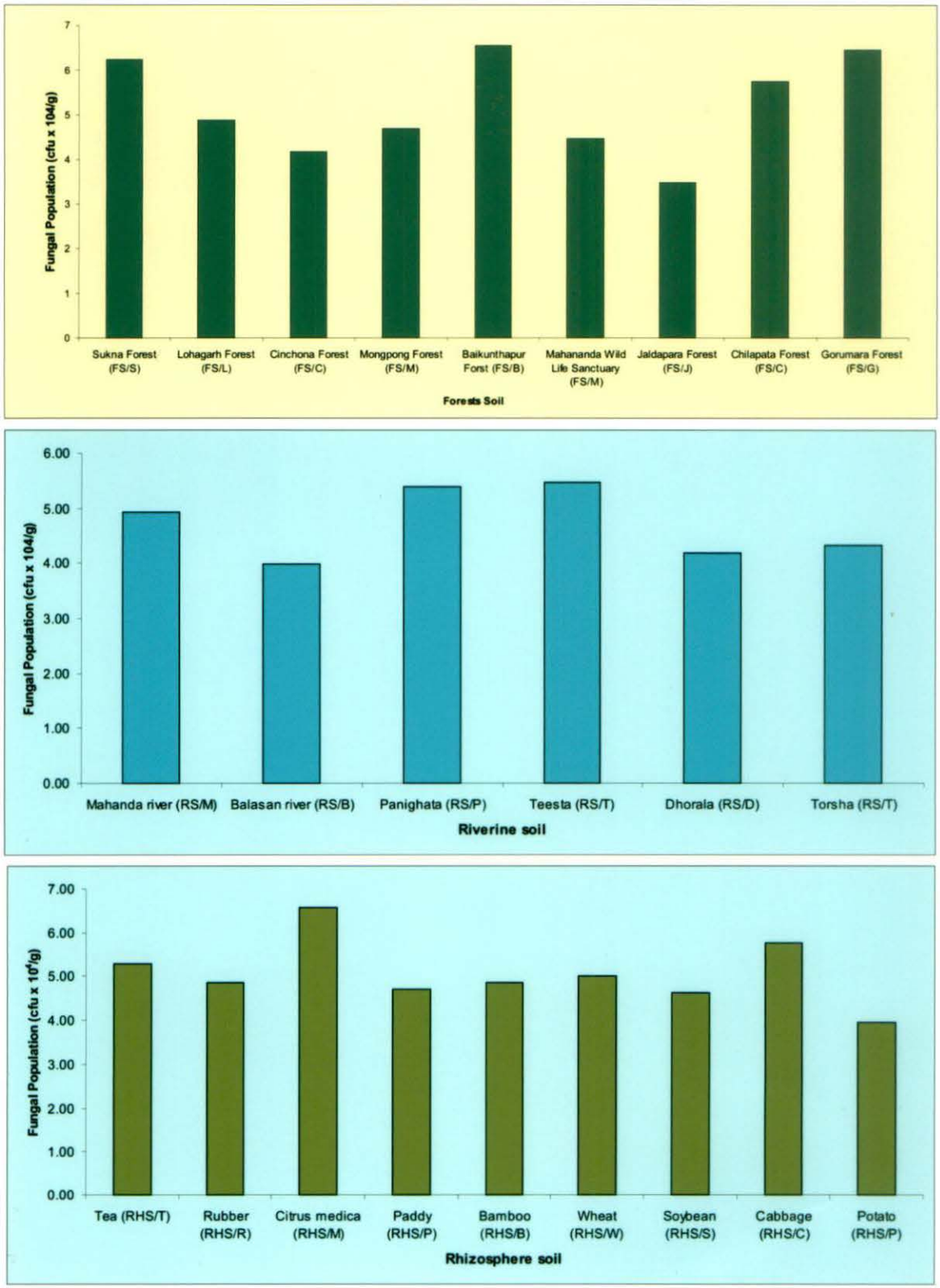


Figure 1: Fungal population of forest soil, riverine soil and rhizosphere soil

4.1.2. Bacterial isolates

A list of bacteria isolated from the forest, riverine and rhizosphere soil of crops fields and their GIS locations along with their codes have been presented in Table 6.

4.1.2.1 Biochemical tests

Isolated bacteria studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of systematic Bacteriology. Bacterial identification was performed on the basis of morphological, physiological and biochemical tests. Isolates were characterized for H_2S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, protese production, urase production, cellulase production and indolae production. Detail result have been presented in Table 7. Result revealed that out of 87 bacterial isolates, 75 bacteria showed gram positive reaction and rest negative where as 30 bacterial isolates showed phosphate solubilizing activity and 45 isolates were cellulase and siderophore producers. All isolates showed positive result in catalase activities. Overall, *Bacillus sp., Micrococcus sp., Coryneform sp. Staphylococcus sp.* as well as *Psedomonus sp.* were found to be more abundant.

4.1.3. Actinomycetes isolates

A list of actinomycetes isolated from the forest, reverine and rhizosphere soil of various crops fields and their locations as recorded by GPS tool have been presented in Table 8 .

4.1.3.1 Biochemical tests

The actinomycetes isolates studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of systematic Bacteriology. Identification actinomycetes was performed on the basis of morphological and biochemical tests. Isolates were characterized for H_2S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, urase production, cellulase production and indolae production. Detail result has been presented in Table 9. Result revealed that 10 actinomycetes isolates showed phosphate solubilizing activity. 24 isolates showed chitin degrading activity. 24 isolates showed cellulase production. Eight isolates produced indole. Overall, *Streptomyces griseorubens* and *Streptomyces griseus* occurred profusely in the soil.

Total number of fungi, bactera and actinomycetes which were isolated from forest, riverine and rhizosphere soil of crop fields of terai-dooars have been presented in Fig 3.

Table 6 : GIS location of isolated bacteria

Source	Code	*GPS location*	
		Latitude	*Longitude*
Sukna Forest (FS/S)	B/FS/S1	N 26°47'26.81"	E 88°21'47.39"
	B/FS/S11	N 26°47'76.11"	E 88°31'44.30"
	B/FS/S12	N 26°45'23.76"	E 88°21'67.32"
	B/FS/S13	N 26°47'20.67"	E 88°21'57.11"
	B/FS/S16	N 26°47'21.11"	E 88°21'41.15"
	B/FS/S18	N 26°47'76.11"	E 88°31'44.30"
Terghera Forest (FS/T)	B/FS/S19	N 26°52'53.53"	E 88°47'55.25"
	B/FS/T4	N 26°52'53.54"	E 88°47'55.26"
	B/FS/T5	N 26°52'53.33"	E 88°47'55.30"
	B/FS/T6	N 26°52'53.43"	E 88°47'55.20"
	B/FS/T7	N 26°52'53.93"	E 88°47'55.85"
	B/FS/T8	N 26°52'53.54"	E 88°47'55.26"
	B/FS/T9	N 26°52'53.33"	E 88°47'55.30"
	B/FS/T10	N 26°52'53.23"	E 88°47'55.45"
Baikunthapur Forst (FS/B)	B/FS/B14	N 26°45' 12.73"	E 88°30' 29.60"
	B/FS/B15	N 26°45' 12.73"	E 88°30' 29.60"
	B/FS/B17	N 26°45' 12.77"	E 88°30' 29.80"
	B/FS/B3	N 26°45' 13.63"	E 88°30' 27.67"
	B/FS/B5	N 26°45' 15.89"	E 88°30' 28.75"
	B/FS/B6	N 26°45' 12.33"	E 88°30' 25.54"
Mahananda Wild Life Sanctuary (FS/M)	B/FS/B21	N 26°49' 23.55"	E 80°24' 40.49"
	B/FS/M23	N 26°49' 23.55"	E 80°24' 40.49"
	B/FS/M24	N 26°49' 22.65"	E 80°24'41.49"
	B/FS/M25	N 26°49'25.25"	E 80°24'41.65"
	B/FS/M26	N 26°49'25.12"	E 80°24' 41.95"
	B/FS/M27	N 26°49'25.55"	E 80°24'41.45"
Jaldapara Forest (FS/J)	B/FS/J28	N 26°34'59.64"	E 89°22'46.50"
	B/FS/J29	N 26°34'59.64"	E 89°22'46.50"
	B/FS/J30	N 26°34'58.44"	E 89°22'47.55"
	B/FS/J31	N 26°34'56.63"	E 89°22'44.51"
	B/FS/J32	N 26°34'58.66"	E 89°22'44.51"
	B/FS/J33	N 26°34'51.60"	E 89°22'43.56"
Chilapata Forest (FS/C)	B/FS/C33	N 26°45'00.10"	E 89°20'60.05"
	B/FS/C34	N 26°45'09.12"	E 89°20'61.16"
	B/FS/C35	N 26°45'05.14"	E 89°20'61.23"
	B/FS/C36	N 26°45'03.12"	E 89°20'61.34"
	B/FS/C37	N 26°45'03.12"	E 89°20'61.17"
	B/FS/C38	N 26°45'09.12"	E 89°20'61.16"
Gorumara Forest (FS/G)	B/FS/G39	N 26°44'54.08"	E 88°48'14.53"
	B/FS/G40	N 26°44'54.88"	E 88°48'14.04"
	B/FS/G42	N 26°44'53.18"	E 88°48'13.24"
	B/FS/G43	N 26°44'54.89"	E 88°48'14.34"
	B/FS/G44	N 26°44'57.23"	E 88°48'15.24"
Mahanda river (RS/M)	B/RS/M 51	N26°47'09.44"	N88°22'06.53"
	B/RS/M 54	N26°47'09.41"	N88°22'06.50"

Source	**Code**	***GPS location***	
		Latitude	***Longitude***
	B/RS/M 55	N26°47'09.47"	N88°22'06.51"
	B/RS/M 56	N26°47'09.49"	N88°22'06.59"
	B/RS/M 57	N26°47'09.44"	N88°22'06.53"
Balasan river (RS/B)	B/RS/B 52	N26°43'11.39"	E88°22'30.45"
	B/RS/B 53	N26°43'11.65"	E88°22'30.95"
	B/RS/B 58	N26°43'11.26"	E88°22'30.30"
	B/RS/B 61	N26°43'11.39"	E88°22'58.45"
	B/RS/B 62	N26°43'11.39"	E88°22'30.45"
	B/RS/B 64	N26°43'11.40"	E88°22'31.65"
Teesta (RS/T E)	B/RS/TE 66	N 26°42'48.92"	E 88°37'44.26"
	B/RS/TE 67	N 26°42'48.90"	E 88°37'44.28"
	B/RS/TE 68	N 26°42'48.89"	E 88°37'44.11"
	B/RS/TE 65	N 26°42'48.76"	E 88°37'44.65"
Torsha (RS/TO)	B/RS/TE 66	N 26°15'50.33"	E 89°33'16.90"
	B/RS/T69	N 26°16'44.08"	E 89°34'36.75"
	B/RS/T 71	N 26°16'17.46"	E 89°34'48.32"
	B/RS/T 72	N 26°15'38.01"	E 89°35'21.63"
Tea	B/RHS/T 73	N 26°46'52.34"	E 88°23'43.08"
	B/RHS/T74	N 26°47'0.39"	E 88°23'52.34"
	B/RHS/T75	N 26°46'52.76"	E 88°23'40.70"
	B/RHS/T76	N 26°46'48.31"	E 88°23'53.76"
Rubber	B/RHS/R77	N26° 32.628'	E88° 47.980'
	B/RHS/R78	N26° 32.610'	E88° 47.962'
	B/RHS/R79	N26° 32.597'	E88° 47.949'
	B/RHS/R80	N26° 32.568'	E88° 47.917'
Paddy	B/RHS/P81	N26° 29.192'	E89° 31.586'
	B/RHS/P22	N26° 28.874'	E89° 31.620'
	B/RHS/P82	N26° 28.846'	E89° 31.613'
	B/RHS/P83	N26° 28.850'	E89° 31.572'
Lemon	B/RHS/C86	N26° 29.908'	E89° 31.976'
	B/RHS/C87	N26° 29.910'	E89° 31.926'
Potato	B/RHS/P88	N26° 33.676'	E89° 03.149'
	B/RHS/P89	N26° 33.752'	E89° 03.036'
	B/RHS/P91	N26° 34.258'	E89° 02.261'
	B/RHS/P92	N26° 34.346'	E89° 02.123'
	B/RHS/P93	N26° 33.752'	E89° 03.036'
	B/RHS/P94	N26° 33.676'	E89° 03.149'
Wheat	B/RHS/W95	N26° 32.418'	E88° 47.766'
	B/RHS/W96	N26° 32.408'	E88° 47.74'
	B/RHS/W97	N26° 42.848'	E88° 27.427'
	B/RHS/W98	N26° 42.856'	E88° 27.424'
	B/RHS/W99	N26° 42.865'	E88° 27.421'

Table : 7 Morphology and biochemical tests of isolated bacteria

Code	Shape	Pigment	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
B/FS/S1	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/S11	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/S12	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/FS/S13	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/S16	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/FS/S18	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/S19	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/FS/T4	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/T5	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/T6	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Staphylococcus sp.*
B/FS/T7	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Streptococcus sp.*
B/FS/T8	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/T9	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/T10	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/B14	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/B15	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/B17	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/B3	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/B5	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/FS/B6	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/FS/B21	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/FS/M23	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/M24	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/M25	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Aerococcus sp.*
B/FS/M26	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/M27	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/J28	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/J29	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/J30	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/FS/J31	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/FS/J32	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Staphylococcus sp.*
B/FS/J33	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/FS/C33	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/FS/C34	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/FS/C35	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/C36	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/C37	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/C38	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/G39	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/G40	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/G42	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/FS/G43	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/FS/G44	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RS/M 51	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*

Code	Shape	Pigment	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
B/RS/M 54	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RS/M 55	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RS/M 56	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RS/M 57	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RS/B 52	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Staphylococcus sp.*
B/RS/B 53	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Staphylococcus sp.*
B/RS/B 58	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/RS/B 61	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/RS/B 62	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/RS/B 64	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RS/TE 66	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus luteus*
B/RS/TE 67	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RS/TE 68	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RS/TE 65	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RS/TE 66	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sphaericus*
B/RS/T69	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RS/T 71	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RS/T 72	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/T 73	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/T74	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/RHS/T75	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/RHS/T76	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Coryneform sp.*
B/RHS/R77	Rod	W	+	+	-	-	+	+	+	+	+	+	-	+	-	*Bacillus sp.*
B/RHS/R78	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sphaericus*
B/RHS/R79	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/R80	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/P81	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/P22	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus pumilus*
B/RHS/P82	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/P83	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/M86	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/M87	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/P88	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/P89	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/P91	Rod	W	+	-	-	+	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/P92	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/P93	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/P94	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/W95	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/W96	Spherical	Y	-	+	-	-	+	+	-	-	+	+	-	-	-	*Micrococcus sp.*
B/RHS/W97	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/W98	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/W99	Rod	W	+	+	-	-	+	+	+	+	+	+	_	+	-	*Bacillus sp.*

Table 8 : GIS location of isolated bacteria

Source	Code	*GPS location*	
		Latitude	*Longitude*
Sukna Forest (FS/S)	A/FS/S1	N 26°47'26.81"	E 88°21'47.39"
	A/FS/S11	N 26°47'76.11"	E 88°31'44.30"
	A/FS/S12	N 26°45'23.76"	E 88°21'67.32"
	A/FS/S13	N 26°47'20.67"	E 88°21'57.11"
	A/FS/S16	N 26°47'21.11"	E 88°21'41.15"
	A/FS/S18	N 26°47'76.11"	E 88°31'44.30"
Baikunthapur Forst (FS/B)	A/FS/B14	N 26°45' 12.73"	E 88°30' 29.60"
	A/FS/B15	N 26°45' 12.73"	E 88°30' 29.60"
	A/FS/B17	N 26°45' 12.77"	E 88°30' 29.80"
	A/FS/B3	N 26°45' 13.63"	E 88°30' 27.67"
	A/FS/B5	N 26°45' 15.89"	E 88°30' 28.75"
	A/FS/B6	N 26°45' 12.33"	E 88°30' 25.54"
Mahananda Wild Life Sanctuary (FS/M)	A/FS/B21	N 26°49' 23.55"	E 80°24'40.49"
	A/FS/M23	N 26°49' 23.55"	E 80°24'40.49"
	A/FS/M24	N 26°49' 22.65"	E 80°24'41.49"
	A/FS/M25	N 26°49' 25.25"	E 80°24'41.65"
	A/FS/M22	N 26°49' 25.12"	E 80°24'41.95"
	A/FS/M27	N 26°49' 25.55"	E 80°24'41.45"
Jaldapara Forest (FS/J)	A/FS/J28	N 26°34' 59.65"	E 89°22'46.51"
	A/FS/J29	N 26°34' 59.64"	E 89°22'46.50"
	A/FS/J30	N 26°34'58.44"	E 89°22'47.55"
	A/FS/J31	N 26°34'56.63"	E 89°22'44.51"
	A/FS/J32	N 26°34'58.66"	E 89°22'44.51"
	A/FS/J33	N 26°34'51.60"	E 89°22'43.56"
Gorumara Forest (FS/G)	A/FS/G39	N 26°44'54.08"	E 88°48'14.53"
	A/FS/G40	N 26°44'54.88"	E 88°48'14.04"
	A/FS/G42	N 26°44'53.18"	E 88°48'13.24"
	A /FS/G43	N 26°44'54.89"	E 88°48'14.34"
	A/FS/G44	N 26°44'54.08"	E 88°48'14.53"
	A /FS/G 72	N 26°44'54.89"	E 88°48'14.34"
Mahanada river	A /RS/M 101	N26°47'09.42"	E88°22'06.55"
	A /RS/M 102	N26°47'09.42"	E88°22'06.55"
	A /RS/M 103	N26°47'09.42"	E88°22'06.55"
	A /RS/M 104	N26°47'09.42"	E88°22'06.55"
	A /RS/M 105	N26°47'09.42"	E88°22'06.55"
Balasan river	A /RS/M 106	N26°43'11.35"	E88°22'30.35"
	A /RS/M 107	N26°43'11.35"	E88°22'30.35"
	A /RS/M 108	N26°43'11.35"	E88°22'30.35"
	A /RS/M 109	N26°43'11.35"	E88°22'30.35"
	A /RS/M 110	N26°43'11.35"	E88°22'30.35"
Tea	A /RHS/T 73	N 26°32'29.58"	E 89°31'21.48"
	A /RHS/T74	N 26°32'14.42"	E 89°31'30.99"
	A /RHS/T75	N 26°32'6.75"	E 89°31'32.63"
	A /RHS/T76	N 26°31'56.91"	E 89°31'35.92"
Rubber	A /RHS/R77	N 26°42'41.19"	E 88°20'55.17"
	A /RHS/R78	N 26°42'39.61"	E 88°20'55.92"
	A /RHS/R79	N 26°42'38.95"	E 88°20'57.11"
	A /RHS/R80	N 26°42'39.22"	E 88°20'55.96"
Paddy	A /RHS/P81	N26°29.192'	E89°31'.586'
	A /RHS/P22	N26° 28.874'	E89 31.620'
	A /RHS/P82	N26° 28.846'	E89° 31.613'
	A /RHS/P83	N26° 28.850'	E89° 31.572'
Lemon	A /RHS/M86	N26° 29.908'	E89° 31.976'
	A /RHS/M87	N26° 29.910'	E89 °31.926'
Potato	A /RHS/PO26	N26° 33. 676'	E89° 03.149'
	A /RHS/PO89	N26° 33.752'	E89° 03.036'
	A /RHS/PO91	N26° 34.258'	E89° 02.261'
	A /RHS/PO92	N26° 34.346'	E89° 02.123'
	A /RHS/PO93	N26° 33.752'	E89° 03.036'
	A /RHS/P9O4	N26° 33.676'	E89° 03.149'

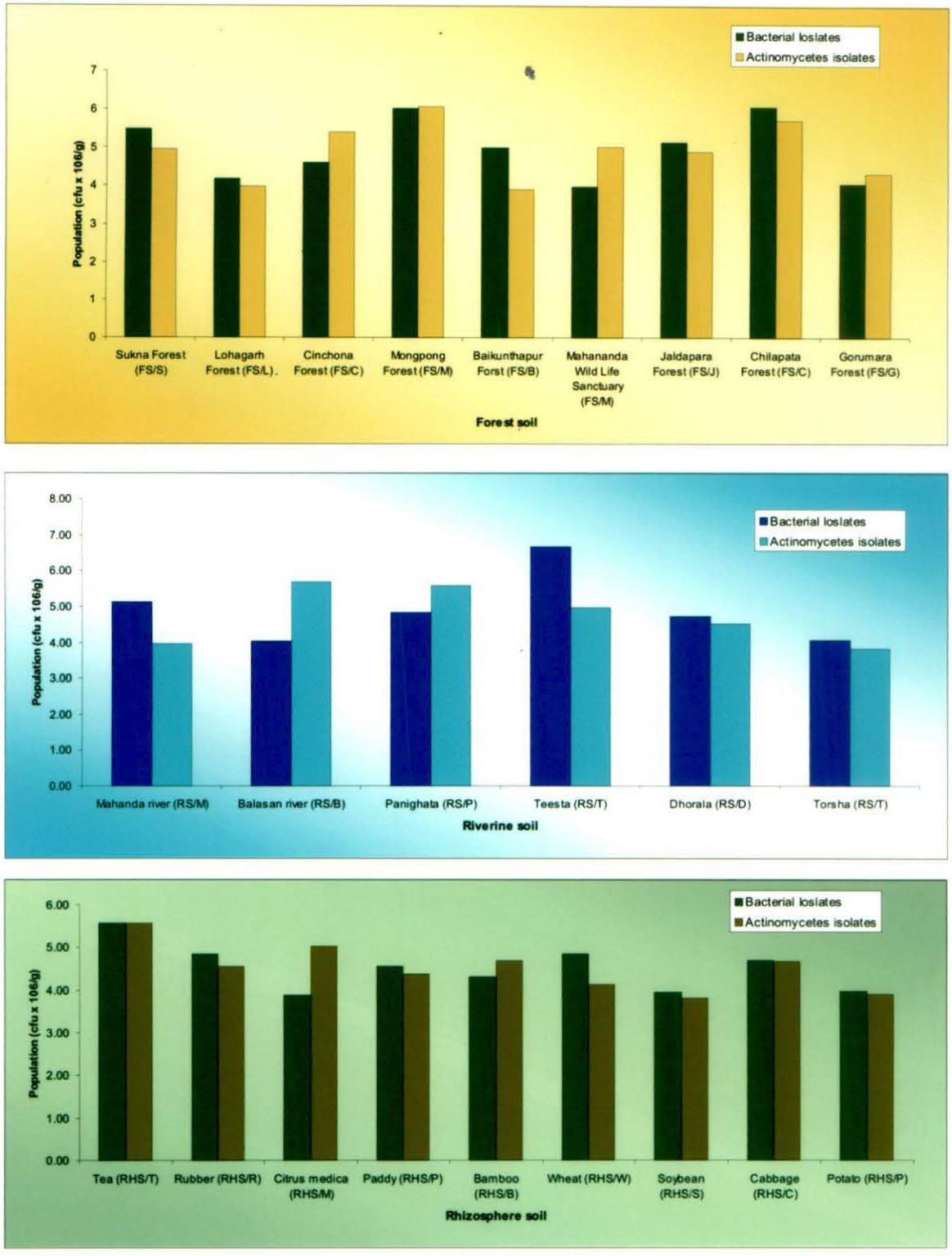


Figure 2: Microbial population of forest soil, riverine soil and rhizosphere soil

Table 9: Morphology and biochemical tests of isolated actinomycetes

Code	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Urase production	Cellulase Production	Indolae Production	Identification
A/FS/S1	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/S11	+	+	-	-	+	+	+	+	+		+	-	*Streptomyces sp.*
A/FS/S12	+	+	-	+	+	+	+	+	+	_	+	-	*Streptomyces viridis.*
A/FS/S13	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces griseorubens*
A/FS/S16	-	+	-	-	+	+	-	-	+	-	-	-	
A/FS/S18	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces griseolus*
A/FS/B14	+	+	-	+	+	+	+	+	+	_	+	-	*Streptomyces griseus*
A/FS/B15	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/B17	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/B3	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/B5	+	+	-	-	+	+	+	+	+		+	-	*Streptomyces sp.*
A/FS/B6	+	+	-	-	+	+	+	+	+		+	-	*Streptomyces sp.*
A/FS/B21	+	+	-	+	+	+	+	+	+	_	+	-	*Streptomyces griseus*
A/FS/M23	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/M24	+	+	-	+	+	+	+	+	+		+	-	*Streptomyces sp.*
A/FS/M25	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces globisporus*
A/FS/M22	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces globisporus*
A/FS/M27	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/J28	+	+	-	+	+	+	+	+	+		+	-	*Streptomyces sp.*
A/FS/J29	+	+	-	+	+	+	+	+	+	_	+	-	*Streptomyces viridis.*
A/FS/J30	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces viridis.*
A/FS/J31	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/J32	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/J33	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/G39	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/G40	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A/FS/G42	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /FS/G43	+	+	-	-	+	+	+	+	+		+	-	*Streptomyces sp.*

Code	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Urase production	Cellulase Production	Indolae Production	Identification
A/FS/G44	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /FS/G72	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RHS/T 73	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RHS/T74	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces viridis.*
A /RHS/T75	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces viridis.*
A /RHS/T76	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RHS/R77	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RHS/R78	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RHS/R79	+	+	-	+	+	+	+	+	+	_	+	-	*Streptomyces griseus*
A /RHS/R80	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RHS/P81	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces griseorubens*
A /RHS/P22	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces griseorubens*
A /RHS/P82	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces griseorubens*
A /RHS/P83	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RHS/M86	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RHS/M87	+	+	-	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RHS/PO26	+	+	+	+	+	+	+	+	+	_	+	+	*Streptomyces griseus*
A /RHS/P89	+	+	+	+	+	+	+	+	+	_	+	+	*Streptomyces sp.*
A /RHS/P91	+	+	+	+	+	+	+	+	+	_	+	+	*Streptomyces sp.*
A /RHS/P92	-	+	+	-	+	+	-	-	+	-	-	+	*Streptomyces sp.*
A /RHS/P93	-	+	-	+	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RHS/P94	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RS/M 101	+	+	-	-	+	+	+	+	+	_	+	+	*Streptomyces sp.*
A /RS/M 102	+	+	-	-	+	+	+	+	+	_	+	+	*Streptomyces sp.*
A /RS/M 103	-	+	-	-	+	+	-	-	+	-	-	+	*Streptomyces sp.*
A /RS/M 104	-	+	-	-	+	+	-	-	+	-	-	+	*Streptomyces sp.*
A /RS/M 105	-	+	+	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RS/M 106	+	+	+	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RS/M 107	+	+	+	-	+	+	+	+	+	_	+	-	*Streptomyces sp.*
A /RS/M 108	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RS/M 109	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*
A /RS/M 110	-	+	-	-	+	+	-	-	+	-	-	-	*Streptomyces sp.*

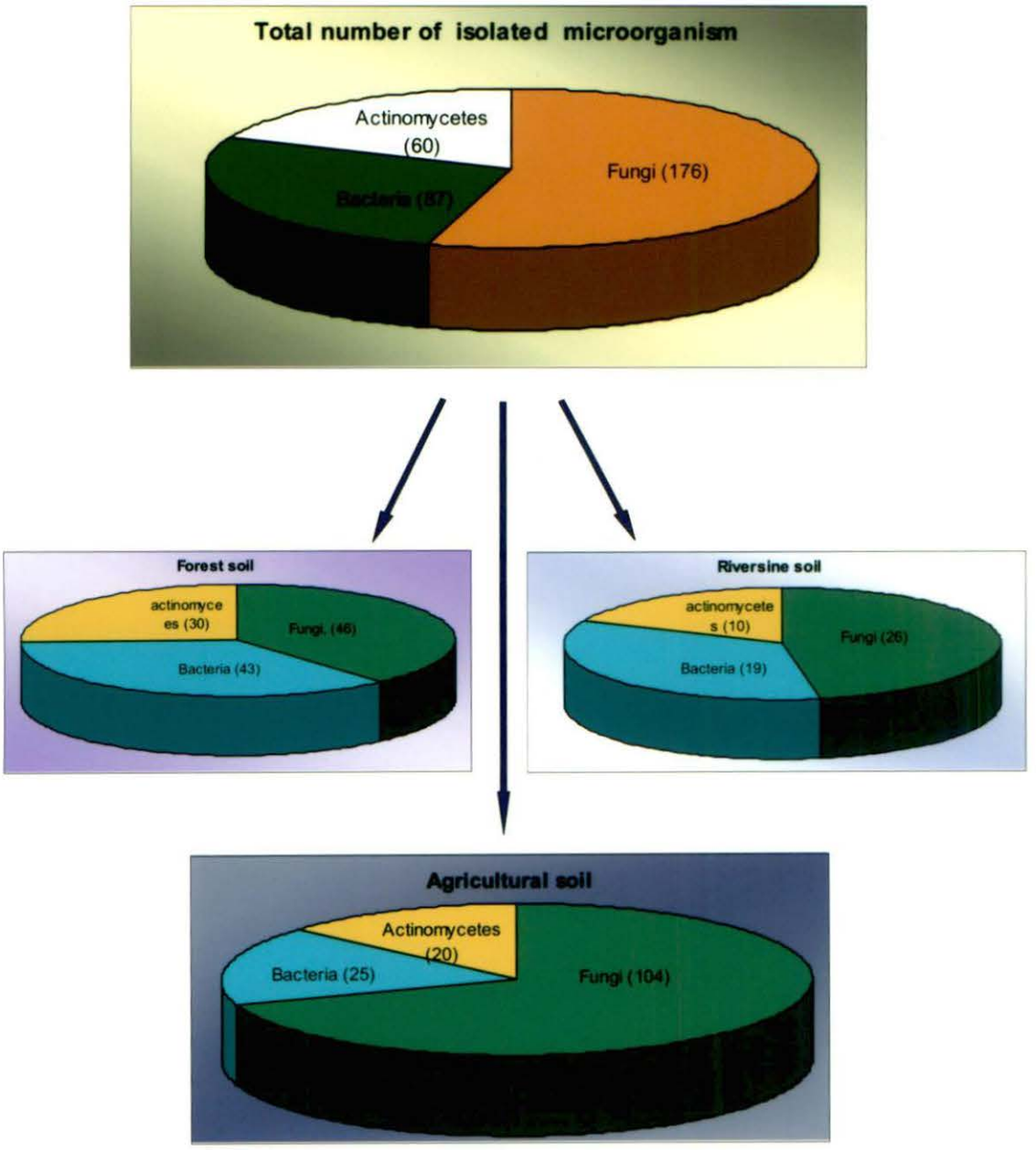


Figure 3: Number of microorganisms isolated from Terai-Dooars regions

4.2. *In vitro* screening of fungal isolates for phosphate solubilizing activities

4.2.1 Screening in solid medium

Soil samples from forest, riverine and agricultural land (rhizosphere of plantation and agricultural crops) yielded 46, 26 and 104 fungal isolates respectively. These were screened for phosphate solubilizing activity in Pikovskaya's agar medium. Formation of halo zones around the fungal colony showed positive result for characterization of phosphate solubilization. Each of the isolates were allowed to grow in PKV medium separately and incubated for 96 hr. After screening a total number of 70 fungal isolates showed phosphate solubilizing activity (Table 10). Among them *Aspergillus niger* showed maximum phosphate solubilization on PVK medium while *A. melleus* and *A. clavatus* showed minimum activity (Plate 11).

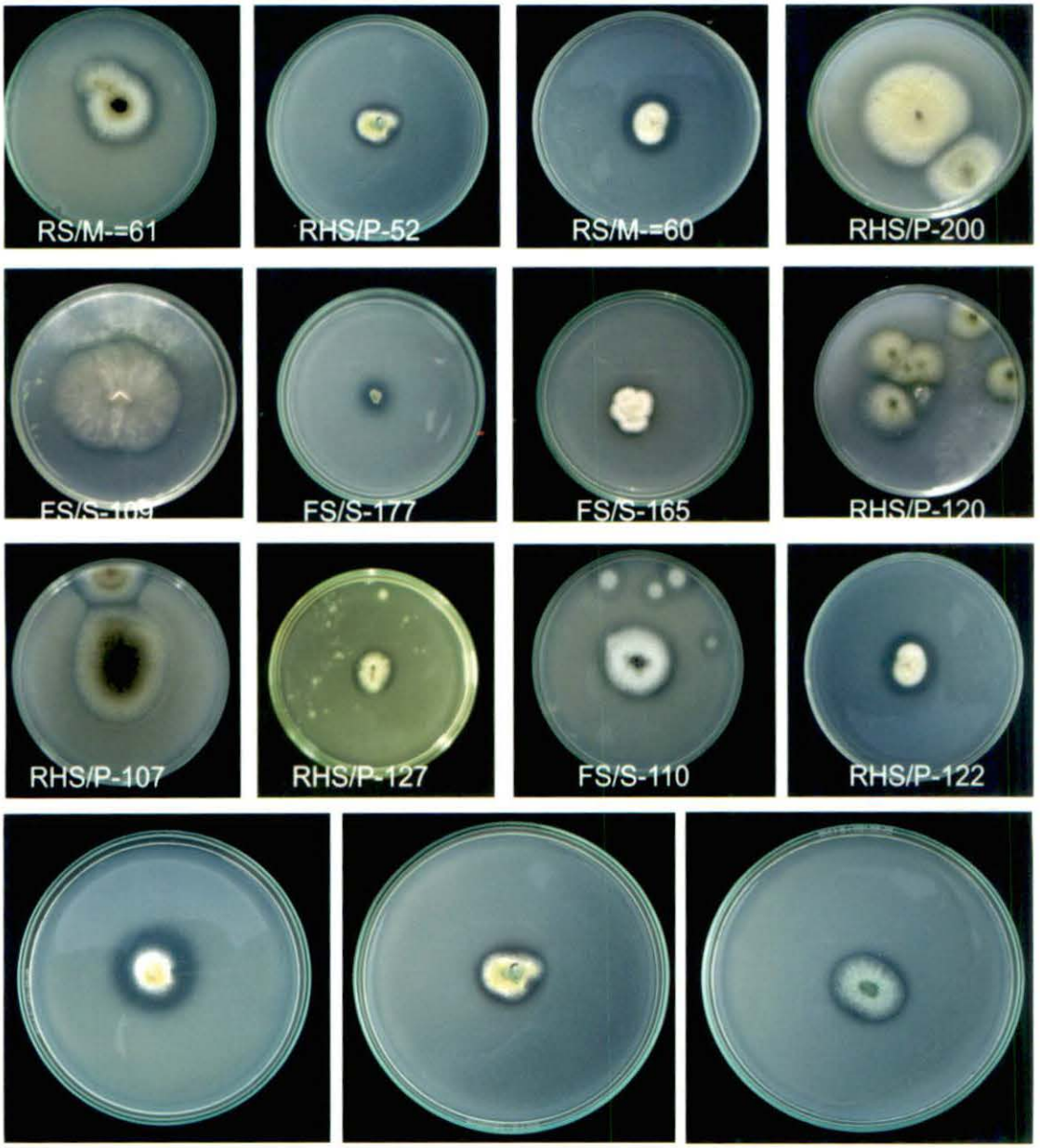
4.2.2. Evaluation in liquid medium

These isolates were further evaluated for their phosphate solubilizing ability using two types of inorganic phosphates-tricalcium and rock phosphate in liquid medium. Isolate *A. niger* (FS/L 14) showed maximum solubilization of phosphorous (852 mg/L) while isolate *A. melleus* (FS/S 262) showed minimum of 795 mg/L of phosphorous solubilization when medium was supplemented with tricalcium phosphate .When the medium was supplemented with rock phosphate, isolate *A.melleus* (FS/L 13) showed maximum of 381 mg /L phosphorous solubilization and isolate *A. niger* (FS/S 64) showed minimum of 211 mg/L phosphorous solubilization when the media was supplemented with rock phosphate (Table 11, Fig 4). The isolates of *Aspergillus* isolated from agricultural soil showed maximum level of phosphate solubilization activity *in vitro.* When liquid medium was supplemented with both tricalcium phosphate and rock phosphate separately, isolates of *Aspergillus* obtained from riverine soil showed minimum level of phosphate solubilization. In the present study, in both cases the average drop in the pH was from 7 to 3.5; however no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium.

Table 10 : *In vitro* screening of fungal isolates for phosphate solubilizing activity

PSF	Isolates	Clear zone (cm)*		PSF	Isolates	Clear zone (cm)*	
		48 h	96h			48 h	96h
Forest Soil		0.4	0.7	**Rhizosphere soil**			
Aspergillus niger	FS/L04						
	FS/L-40	0.4	0.7		RHS/P-37	0.4	0.7
	FS/C-140	0.4	0.7	*Aspergillus niger*	RHS/P-51	0.4	0.7
	FS/C143	0.4	0.7		RHS/P-105	0.4	0.7
	FS/C-160	0.4	0.7		RHS/P-106	0.4	0.7
	FS/S-165	0.4	0.7		RHS/P-107	0.4	0.7
	FS/S-173	0.4	0.7		RHS/P-45	0.4	0.7
	FS/S-177	0.4	0.7		RHS/P-48	0.4	0.7
	FS/S-140	0.4	0.7		RHS/P-117	0.4	0.7
	FS/S-109	0.4	0.7		RHS/D-280	0.4	0.7
	FS/S110	0.4	0.7		RHS/D-281	0.4	0.7
	FS/S-112	0.4	0.7		RHS/D-282	0.4	0.7
	FS/S-113	0.4	0.7		RHS/D-283	0.4	0.7
	FS/S-262	0.2	0.6		RHS/D-284	0.4	0.7
Aspergillus melleus	FS/L-42	0.3	0.5		RHS/D-285	0.2	0.6
	FS/L-13	0.3	0.5		RHS/D-286	0.4	0.7
	FS/L-17	0.3	0.5		RHS/D-287	0.4	0.7
	FS/L-18	0.3	0.5		RHS/P-200	0.4	0.7
	FS/G-226	0.3	0.5		RHS/P-82	0.4	0.7
	FS/S-64	0.3	0.5	*Aspergillus melleus*	RHS/P-198	0.3	0.5
	FS/L-41	0.3	0.5		RHS/R-12	0.3	0.5
	FS/S -63	0.3	0.5		RHS/P-201	0.3	0.5
	FS/S-24	0.3	0.5		RHS/P-202	0.3	0.5
	FS/S-278	0.3	0.5		RHS/P-205	0.3	0.5
Riverine Soil	RS/P/14	0.4	0.7	*Aspergillus fumigatus*	RHS/P-209	0.2	0.6
Aspergillus niger	RS/D-288	0.3	0.5		RHS/B-220	0.2	0.6
	RS/T-57	0.3	0.5		RHS/P-114	0.2	0.6
	RS/T-58	0.3	0.5		RHS/P-43	0.3	0.6
Aspergillus nidulans	RS/T-59	0.2	0.6	*Aspergillus clavatus*	RHS/P-114	0.3	0.6
	RS/P -60	0.2	0.6		RHS/T-99	0.3	0.6
Aspergillus melleus	RS/R-115	0.3	0.5		RHS/T-190	0.3	0.6
	RS/T-182	0.3	0.5		RHS/T-191	0.3	0.6
	RS/T-183	0.3	0.5		RHS/P -50	0.3	0.6
	RS/P -61	0.5	0.3		RHS/P -54	0.3	0.6
	RS/P05	0.5	0.3		RHS/P-38	0.2	0.6
					RHS/P-47	0.3	0.6

*Average of three replicates



Aspregillus niger *Aspergillus melleus* *Aspergillus clavatus*

Plate 11 : Screening of fungal isolates for phosphate solubilizing activity showing characteristic halo zone formation in Pikovskaya's agar

Table 11 Evaluation of phophphorus solubilization by fungal isolates obtained from forest, riverrine and rhizosphere soil in liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP).

PSF	Isolates	TCP	RP	PSF	Isolates	TCP	RP
Forest Soil				**Rhizosphere soil**			
Aspergillus niger	FS/L04	850±2.58	366±3.76				
	FS/L-40	852±4.41	370±3.22		RHS/P-37	807±1.15	345±2.92
	FS/C-140	824±3.06	344±3.25		RHS/P-51	849±2.92	374±2.76
	FS/C143	821±303	345±1.15	*Aspergillus niger*	RHS/P-105	807±2.76	349±2.85
	FS/C-160	824±2.86	346±2.92		RHS/P-106	813±2.85	344±1.31
	FS/S-165	830±2.75	352±2.76		RHS/P-107	807±1.31	355±1.99
	FS/S-173	802±2.91	343±2.85		RHS/P-45	842±1.99	287±1.57
	FS/S-177	843±2.03	341±1.31		RHS/P-48	841±1.57	342±1.38
	FS/S-108	808±3.55	350±1.99		RHS/P-117	837±1.38	360±1.09
	FS/S-109	802±2.55	355±1.57		RHS/D-280	806±1.09	336±0.92
	FS/S110	842±2.92	367±1.38		RHS/D-281	807±0.92	362±3.55
	FS/S-112	842±2.76	354±1.09		RHS/D-282	817±1.30	355±2.55
	FS/S-113	848±2.85	360±0.92		RHS/D-283	816±1.38	334±2.92
	FS/S-262	795±1.31	360±1.30		RHS/D-284	840±1.09	375±2.76
Aspergillus melleus	FS/L-42	830±1.98	360±2.91		RHS/D-285	839±0.92	370±2.85
	FS/L-13	817±1.82	381±2.03		RHS/D-286	804±1.09	336±1.31
	FS/L-17	820±1.82	379±3.55±		RHS/D-287	807±1.32	340±1.99
	FS/L-18	821±3.07	376±2.55		RHS/P-200	838±1.09	345±1.57
	FS/G-226	847±1.70	352±2.92		RHS/P-82	838±0.92	350±1.38
	FS/S-64	842±1.86	211±2.76	*Aspergillus melleus*	RHS/P-198	841±1.38	346±2.92
	FS/L-41	843±2.36	214±2.85		RHS/R-12	810±1.09	385±2.76
	FS/S -63	839±1.54	332±1.31		RHS/P-201	836±0.92	342±2.85
	FS/S-24	810±2.51	338±1.99		RHS/P-202	829±1.30	350±1.31
	FS/S-278	829±3.17	339±1.57		RHS/P-205	842±0.92	340±1.38
Riverine Soil	RS/P/14	852±1.99	360±1.38	*Aspergillus fumigatus*	RHS/P-209	827±2.51	331±2.85
Aspergillus niger	RS/D-288	830±1.57	350±1.09		RHS/B-220	837±2.54	344±1.31
	RS/T-57	809±1.38	352±0.92		RHS/P-114	838±3.17	335±1.99
	RS/T-58	802±1.09	354±1.30		RHS/P-38	799±3.14	288±1.57
Aspergillus nidulans	RS/T-59	830±2.86	350±2.92	*Aspergillus clavatus*	RHS/P-114	829±2.15	340±1.38
	RS/P -60	840±2.36	340±2.76		RHS/T-99	832±3.91	341±1.99
Aspergillus melleus	RS/R-115	836±4.24	338±1.38		RHS/T-190	825±3.03	350±1.57
	RS/T-182	810±3.25	309±2.91		RHS/T-191	827±1.55	351±1.38
	RS/T-183	850±3.91	317±2.03		RHS/P -50	850±1.55	342±1.09
	RS/P -61	847±3.2	343±3.55		RHS/P -54	839±2.12	350±0.92
	RS/P05	854±3.25	370±2.55		RHS/P-43	812±2.76	350±3.55
					RHS/P-47	811±2.85	348±2.55

PSF= Phosphate solubilizing fungi; TCP=Tricalcium phosphate (P=997 mg/L); RP= Rock phosphate (P=500 mg/L). Values are mean of 3 replicates.

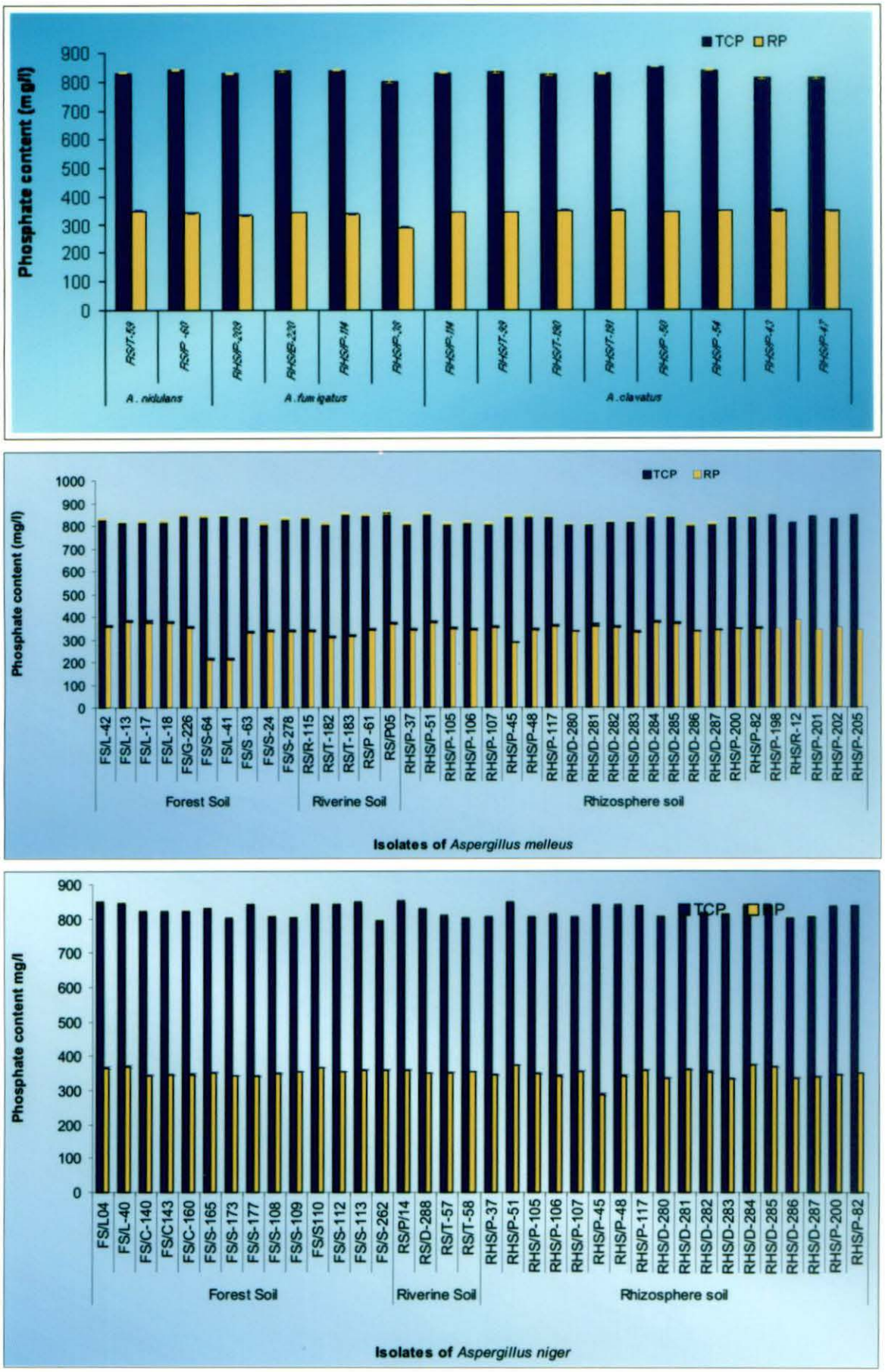


Figure 4: Estimation of phosphate solubilized by fungal isolates *in vitro*

4.3. *In vivo* application of selected phosphate solubilizing fungi and their effect on plant growth

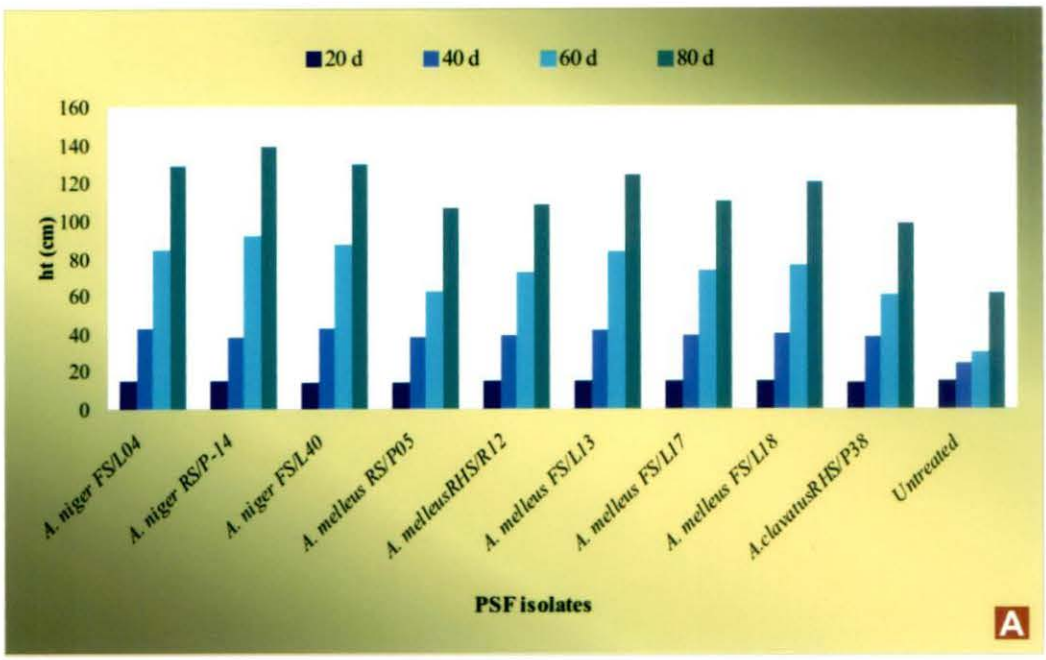
Among seventy phosphate solubilizer identified, nine PSF of which three isolates of *A.niger*, five *A. melleus* and one isolates of *A. clavatus* were selected on the basis of their greater phosphate solubilizing activity *in vitro* for their further evaluation on plant growth. PSF isolates were grown separately in the PDA medium for sporulation over a period of 4-5 days after which harvested spore mass was suspended in sterile distilled water. Well decomposed FYM heaps (5kg) were mixed with spore suspension (100 ml) containing 10^6 spores / ml, moistened slightly to optimize the PSF growth and kept in polythene bags in shade for 10 days.

Glycine max was selected for this study using nine PSF isolates as mentioned above. Surface sterilized soybean seeds were sown in pots filled with the amended PSF. For each treatment 15 replicate plants were taken and FYM without any amendments served as control for all the treatments. After an interval of every 20 days plant height, leaf area, nodulation, phosphate content in the roots as well as soil was measured to monitor the effect of amendments. Plants treated with *A. niger* (FS/L-04, RS/P-14, FS/L-40) showed a max increase in leaf area in comparison to the control plants (Plate 12). However, isolates of *A. melleus* also showed an increase in leaf area. Differences in both leaf area and nodulation in all treatments were significantly greater than that in control. All the tested PSF isolates increased growth in relation to control of which three isolates of *A. niger* were most effective (Table 12 Fig 5 A). Phosphate level in the roots were also found to be more in those plants grown in soil amended with PSF isolates (Fig 5 B).

Table 12 : Effect of amendment of soil with PSF isolates on growth and nodulation of soybean plant

PSF	Isolates	Leaf area (cm^2)	No. of nodules/plant
Soil amended with *A.niger*	FS/L 04	38.0 ± 1.42	21±1.42
	RS/P-14	41.4 ±2.96	26±2.96
	FS/L 40	36.1±1.52	23±4.58
A.melleus	RS/P 05	24.8 ± 1.41	17±1.04
	RHS/R-12	28.1 ±1.56	16±1.56
	FS/L-13	33.1±0.59	17±0.59
	FS/L 17	30.0±0.63	17±0.62
	FS/L-18	31.0± 1.99	19±1.99
A.clavatus	RHS/P-38	25.0±1.64	16±1.64
Unamended soil (Control)		11.1±0.53	06±0.53

Plate 12: (A-D) Control and treated 20 day old soybean plants in green house condition grown on *A. niger*, *A. melleus* and *A. clavatus* amended with FYM respectivly



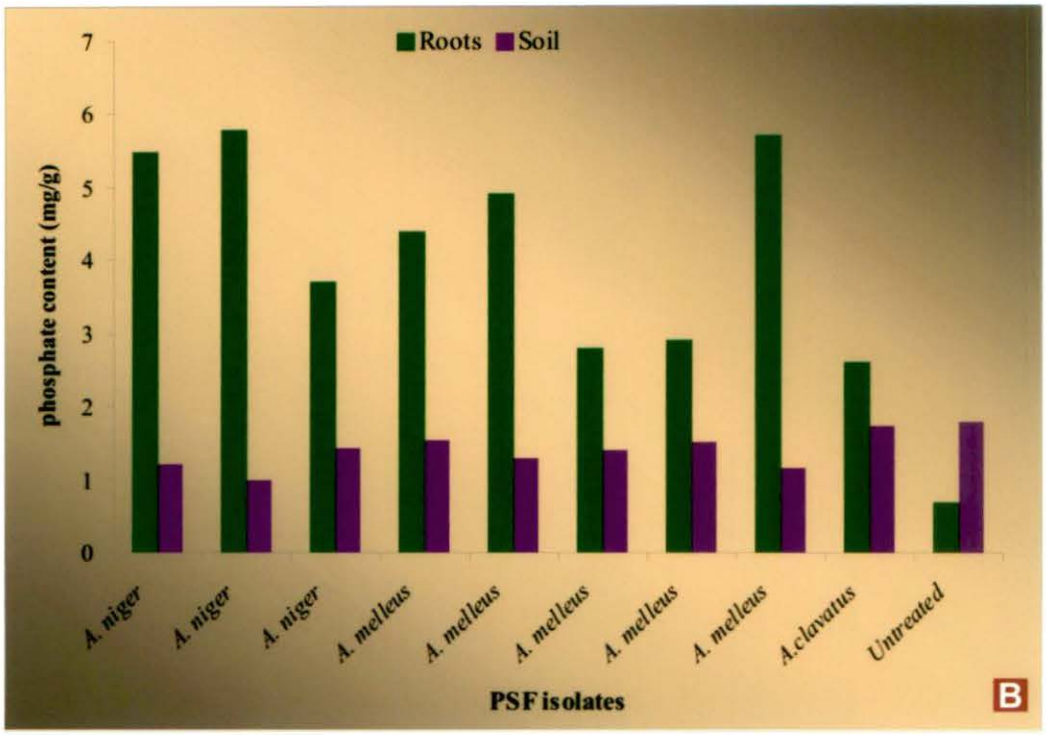


Figure 5:) Height of soybean plants after amendment of PSF isolates in the soil (A) Phosphate content of root and soil (B)

4.4. Evaluation of selected bacterial isolate on plant growth promotion *in vitro* and *in vivo*

In preliminary screening experiments one of the baterial isolates B/RHS/P22 obtained from paddy rhizoshrere showed phosphate solubilization, starch hydrolysis, protease and chitinase activities *in vitro* conditions (Plate 13 A-D). This isolate was also found to inhibit fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani in vitro*), which was identified as *Bacillus pumilus*. The identification of this isolate was further conformed by Microbial Type Culture Collection, Chandigarh as *Bacillus pumillus* (MTCC 10653). Evalualation of *B. pumillus* (MTCC 10653) on the growth of *Vigna radiata* and *Cicer arietinum* was conducted both *in vitro* and *in vivo* conditions. Bacterization of seed enhanced germination percentage in both *C. arietinum* and *V. radiata* in comparison to the control sets (Table 13) as well as increased seedling growth *in vitro* conditions (Table 14).

Table 13. Effect of seed bacterization with *B. pumillus* (MTCC 1065) on seedling germination.

Sl. No	Organism	*Cicer arietinum (%)*	*Vigna radiate (%)*
1	CONTROL	70	72
2	*B.pumilus* (B/RHS/P22)	84	83

Table: 14 Effect of seed bacterization with *B. pumillus* (MTCC 1065) on seedling growth *in vitro* conditions

Tratment	*Vigna radiata*				*Cicer arietinum*			
	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length(cm)	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length(cm)
CONTROL	12.5±0.58	2±0.57	2.4±0.58	4.5±1.78	12.5±1.21	5±3.46	6.0±0.17	5.5±0.6
B. pumilus (B/RHS/P22)	17.5±1.27	3±1.73	2.5±0.80	5.4±0.05	25.5±1.21	9±1.154	2.0±0.17	13.0±1.96

Values are average of 10 replicate plants

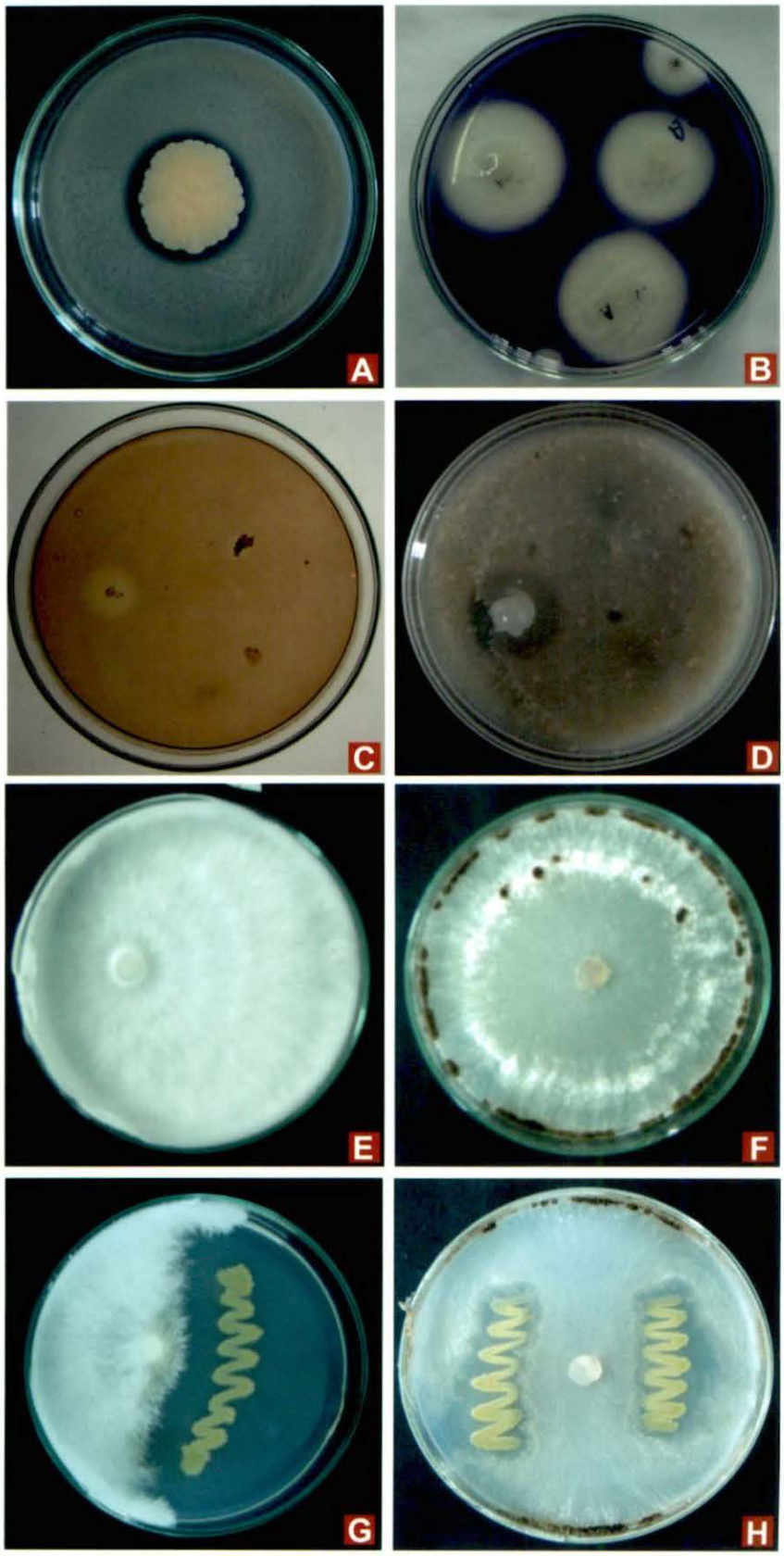


Plate13 : Screening for phosphate solubilizing activity (A)starch hydrolysis (B), chiniase activity (C), protease activity (D) and antifungal activity of *Bacillus pumilus* B/RHS/P 22 (MTCC 10653) against *Sclerotium rolfsii* (G) and *Rhizoctonia solani* (H). Control plates (E & F) respectively for (G &H)

Marked increase in growth of *V. radiata* and *C. arietinum* was noticed when *B.pumilus* was applied in the rhizosphere of these plants. Increase in the growth was observed in terms of increase in height of seedlings, number of shoots and number of leaves and roots. It was observed that treatment with the bacteria increased the rate of growth of the plants in relation to untreated control. Increase in growth was recorded from 7 days onwards. Bacterial inoculation led to as much as 125% increase in growth, as against 15-25% in control. (Table 15). Percentage increase in height of the plants, no. of leaves and leaf length, and root length have been recorded at 7day interval upto 20 day following application. Effective growth pattern of *Vigna radiata* following application of *B.pumillus* under field condition in relation to untreated control has been presented in Plate 14.

Table. 15 Effect of foliar application of *B. pumillus* (MTCC 1065) on the growth of *Vigna radiata* and *Cicer arietinum*

Treatmet	Days	*Vigna radiata*				*Cicer arietinum*			
		Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length(cm)	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length (cm)
CONTROL	7	10±1.73	4	6.78±1.21	3.0±0.58	16.5±0.58	7	1.46±1.21	10.0±0.58
	14	18±0.23	8	7.86±0.24	3.5±0.56	21.5±0.64	18	2.15±0.23	15.0±0.56
	20	20±1.64	16	9.72±1.21	4.21±0.68	23.2±0.56	23	3.56±1.21	19.0±0.58
B. pumilus (B/RHS/P22)	7	18±0.57	4	8.55±0.69	7.0±0.63	26.5±0.57	9	2.52±1.22	15.5±0.57
	14	24±0.32	12	10.22±0.89	9.0±0.63	31.5±0.52	26	3.25±0.27	18.5±0.45
	20	25±0.48	26	13.45±0.69	11.0±0.53	36.6±0.47	30	4.52±1.22	25.5±0.23

Values are average of 10 replicate plants

Since plant growth promotion could also be due to induction of biochemical responses within the host, experiments were conducted to assess the effect of *B. pumilus* on the accumulation of defense enzymes in 7 day old seedlings of *V. radiata* and *C. arietinum*. Grown in *in vitro* condition as well as in the field conditions where both seed bacterization and foliar applications of *B. pumilus* were done. In this case samplings were taken two weeks following applications.Three specific defense enzyme like peroxidase, ß-1, 3- glucanase and chitinase activities were determined and compared with health control. Results (Tables 16 & 17) revealed enhanced production of peroxidase, chitinase and ß-1, 3- glucanase in the leaf of both the plants following application of *B. pumilus*.

Plate 14 : Control (A) and *Bacillus pumilus* B/RHS/P 22 (MTCC 10653) treated *Vigna radiata* in field condition (B)

Table : 16 Changes in defense enzymes of *Vigna radiata* and *Cicer arietinum* seedlings following seed bacterization with *B. pumilus* under *in vitro* condition

Plants	Tests	CONTROL	*B. pumilus* (MTCC 1065)
Vigna radiata	Peroxidase (ΔOD/gm/min)	111.7±1.73	145.0±1.73
	Chitinase (mg Glc NAC/g/hr)	33.5±1.21	52.5±1.15
	Glucanase (µg/g/min)	0.12±0.057	0.22±0.058
Cicer arietinum	Peroxidase (ΔOD/gm/min)	115±2.30	220±1.73
	Chitinase (mg Glc NAC/g/hr)	5.8±0.69	13.6±0.86
	Glucanase (µg/g/min)	0.48±0.057	0.65±0.173

Table 17 Changes in defense enzymes of *Vigna radiata* and *Cicer arietinum* seedlings following foliar application of *B. pumilus* in field condition

Plants	Tests	CONTROL	*B. pumilus* (B/RHS/P22)
Vigna radiata	Peroxidase (ΔOD/gm/min)	71.0±1.15	86.5±0.577
	Chitinase (mg Glc NAC/g/hr)	07.8±0.63	30.0±1.15
	Glucanase (µg/g/min)	0.260±0.0017	0.575±0.0005
Cicer arietinum	Peroxidase (ΔOD/gm/min)	80.0±2.30	124.5±0.577
	Chitinase (mg Glc NAC/g/hr)	06.5±0.635	15.0±1.154
	Glucanase (µg/g/min)	0.285±0.00057	0.300±0.0011

Following application of *B. pumilus* , phosphate content both in soil and root tissues were also determined. Results (Table 18) shows that the phosphate content of roots of treated plants were higher in relation to control whereas soil phosphate content decreased with the application of *B. pumilus.*

Table 18. Total P- content of Soil and roots of *Vigna radiata* and *Cicer arietinum*

Treatments	µg P/g soil		µg P/g root tissue	
	Vigna radiata	*Cicer arietinum*	*Vigna radiata*	*Cicer arietinum*
CONTROL	35	44	9	7
B. pumilus (B/RHS/P22)	26	28	12	12

Values are replicate of three samples

4.5. Evaluation of selected actinomycetes on plant growth promotion *in vitro* and *in vivo*

In preliminary screening experiments one of the actinomycetes isolates A /RHS/PO26 obtained from potato rhizoshrere showed phosphate solubilization, starch hydrolysis, protease and chitinase activities *in vitro* conditions (Plate 15 A-F). This isolate was also found to inhibit fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani in vitro*), which was identified as *Streptomyces griseus.* The identification of this isolate was further conformed by National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08). Evalualation of *S. griseus* (NCFT 2578.08) on the growth of *Vigna radiata* and *Cicer arietinum* was conducted both *in vitro* and *in vivo* conditions. Bacterization of seed enhanced germination percentage in both *C. arietinum* and *V. radiata* in comparison to the control sets (Table 19) as well as increased seedling growth *in vitro* conditions (Table 20).

Table 19. Effect of seed bacterization with *S. griseus* (NCFT 2578.08) on seedling germination.

Sl. No	Organism	*Cicer arietinum (%)*	*Vigna radiata (%)*
1	Control	70	72
2	*Streptomyces griseus* (A /RHS/PO26)	80	71

Table: 20 Effect of seed bacterization with *S. griseus* (NCFT 2578.08) on seedling growth *in vitro* conditions

	Vigna radiata				*Cicer arietinum*			
	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length(cm)	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length(cm)
CONTROL	12.5±0.58	2±0.57	2.4±0.58	4.5±1.78	12.5±1.21	5±3.46	6.0±0.17	5.5±0.6
Streptomyces griseus (A /RHS/PO26)	15.5±1.73	7±1.15	1.8±0.58	5.5±2.36	16.5±0.69	7±0.577	3.0±0.57	10.5±1.32

Values are average of 10 replicate plants

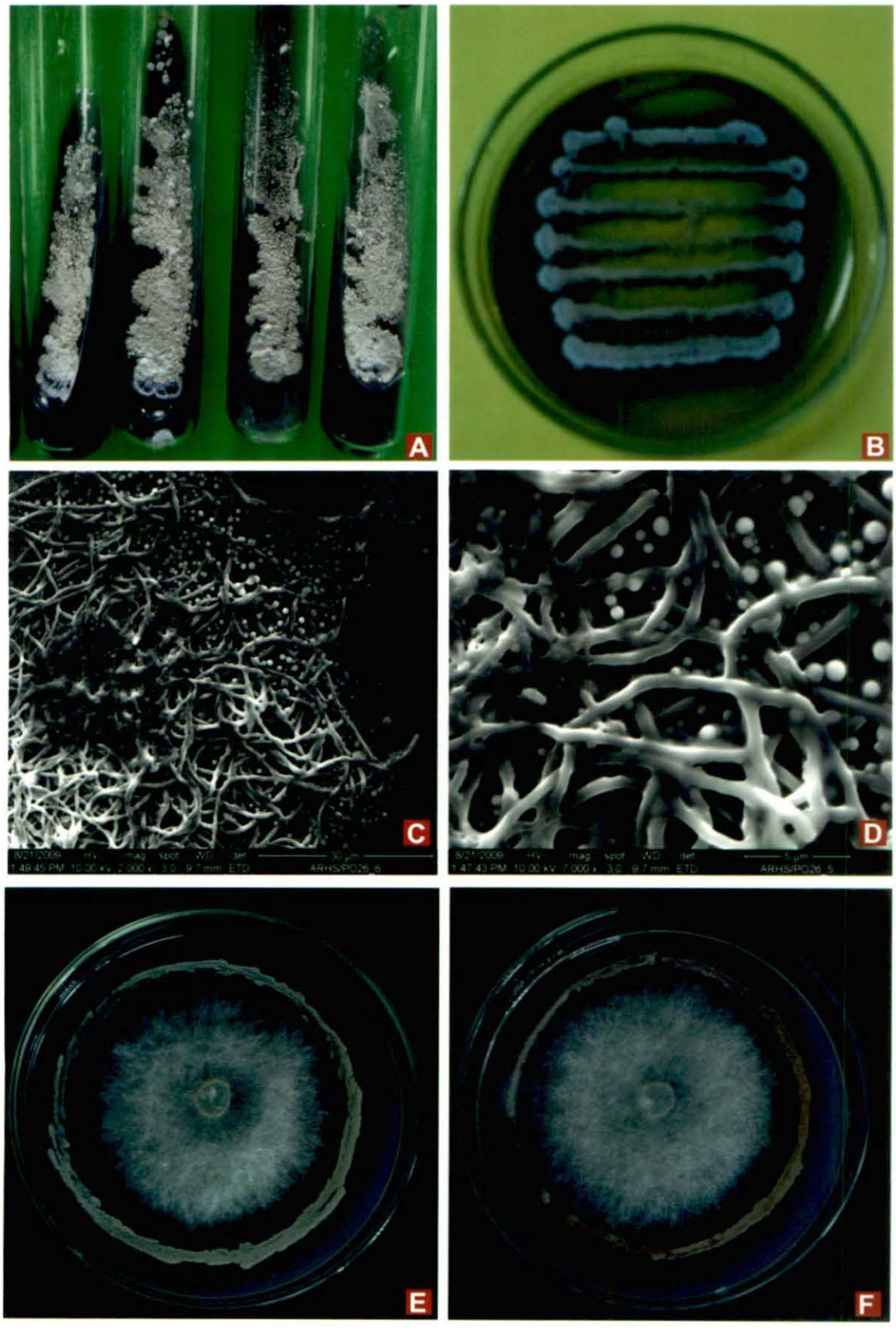


Plate 15 : *Streptomyces griseus* ARHS/PO 26 (NCFT 2578.08) in SCN medium (A&B) SEM view (C&D) and its antifungal activity against *Sclerotium rolfsii* (E) and *Rhizoctonia solani* (F)

Marked increase in growth of *V. radiata* and *C. arietinum* was noticed when *S. griseus* was applied in the rhizosphere of these plants. Increase in the growth was observed in terms of increase in height of seedlings, number of shoots and number of leaves and roots. It was observed that treatment with the bacteria increased the rate of growth of the plants in relation to untreated control. Increase in growth was recorded from 7 days onwards. Actinomycetes inoculation led to as much as 125% increase in growth, as against 15-25% in control. (Table 21). Percentage increase in height of the plants, no. of leaves and leaf length, and root length have been recorded at 7day interval upto 20 day following application. Effective growth pattern of *Vigna radiata* following application of *S. griseus* under field condition in relation to untreated control has been presented in Plate 16.

Table. 21 Effect of foliar application of *S. griseus* (NCFT 2578.08) on the growth of *Vigna radiata* and *Cicer arietinum*

	Days	*Vigna radiata*				*Cicer arietinum*			
		Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length (cm)	Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length (cm)
CONTROL	7	10±1.73	2	6.78±1.21	3.0±0.58	16.5±0.58	7	1.46±1.21	3.0±0.58
	14	16±1.37	6	7.66±1.21	5.0±0.44	21±0.58	13	2.23±1.11	14.0±0.59
	20	19±1.72	12	8.68±1.31	6.9±0.54	19.5±0.22	18	2.96±1.41	16.0±0.34
S. griseus (A /RHS/PO26)	7	20±1.15	2	9.27±1.15	5.5±3.46	21.0±1.15	8	2.79±0.63	5.5±3.46
	14	26±1.51	11	10.47±1.10	9.5±1.43	27±1.05	19	3.23±0.33	21.0±1.12
	20	.29±1.22	18	11.27±1.35	12.5±3.34	29.0±1.21	23	3.79±0.43	24.0±1.23

Values are average of 10 replicate plants

Since plant growth promotion could also be due to induction of biochemical responses within the host, experiments were conducted to assess the effect of *S. griseus* on the accumulation of defense enzymes in 7 day old seedlings of *V. radiata* and *C. arietinum*. Grown in *in vitro* condition as well as in the field conditions where both seed bacterization and foliar applications of *S. griseus* were done. In this case samplings were taken two weeks following applications.Three specific defense enzyme like peroxidase, ß-1, 3- glucanase and chitinase activities were determined and compared with health control. Results (Tables 22& 23) revealed enhanced production of peroxidase, chitinase and ß-1, 3- glucanase in the leaf of both the plants following application of *S. griseus*.

Plate16 : Control (A) and *Streptomyces griseus* ARHS/PO 26 (NCFT 2578.08) treated *Cicer aritenum* in field condition (B)

Table : 22 Changes in defense enzymes of *Vigna radiata* and *Cicer arietinum* seedlings following seed bacterization with *S. griseus* under *in vitro* condition

Plants	Tests	CONTROL	*Streptomyces griseus* (A /RHS/PO26)
Vigna radiata	Peroxidase (ΔOD/gm/min)	111.7±1.73	198.0±1.15
	Chitinase (mg Glc NAC/g/hr)	33.5±1.21	60.0±0.69
	Glucanase (µg/g/min)	0.12±0.057	0.35±0.115
Cicer arietinum	Peroxidase (ΔOD/gm/min)	115±2.30	330±2.30
	Chitinase (mg Glc NAC/g/hr)	5.8±0.69	16.2±0.69
	Glucanase (µg/g/min)	0.48±0.057	0.74±0.058

Table 23 Changes in defense enzymes of *Vigna radiata* and *Cicer arietinum* seedlings following foliar application of *S. griseus* in field condition

Plants	Tests	CONTROL	*Streptomyces griseus* (A /RHS/PO26)
Vigna radiata	Peroxidase (ΔOD/gm/min)	71.0±1.15	80.0±2.30
	Chitinase (mg Glc NAC/g/hr)	07.8±0.63	06.5±0.635
	Glucanase (µg/g/min)	0.260±0.0017	0.285±0.00057
Cicer arietinum	Peroxidase (ΔOD/gm/min)	91.0±1.73	180.0±0.57
	Chitinase (mg Glc NAC/g/hr)	17.3±0.57	22.5±1.21
	Glucanase (µg/g/min)	0.510±0.00057	0.375±0.0011

Following application of *S. griseus*, phosphate content both in soil and root tissues were also determined. Results (Table 24) shows that the phosphate content of roots of treated plants were higher in relation to control whereas soil phosphate content decreased with the application of *S. griseus.*

Table 18. Total P- content of Soil and roots of *Vigna radiata* and *Cicer arietinum*

Treatments	µg P/g soil		µg P/g root tissue	
	Vigna radiata	*Cicer arietinum*	*Vigna radiata*	*Cicer arietinum*
CONTROL	35	44	9	7
Streptomyces griseus (A /RHS/PO26)	20	35	13	12

Values are replicate of three samples

4.6 Screening of the fungal isolates showing antagonistic activities against selected phytopathogens.

Among the isolated fungi initially *Trichoderma* sp. which were identified morphologically were grown in PDA (Plate 17). All the isolates of *T. harzianum*, *T. viride* as well as isolates of *Aspergillus niger*, *A. melleus*, *A. clavatus*, and *A. fumigatus* were tested for their antagonistic activity against three selected phytopathogens – *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium oxysporium* by dual paring tests (Plate 18). 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 plates. Then same size of another agar disc of selected phytopathogens were similarly placed at the periphery but on the opposing end of the same Perti dish. The percent inhibition in the radial colony growth was calculated by the following formula: Per cent inhibition = C – T/ C ×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 25. *Aspergillus* group of fungi showed antagonistic activity moderately. *Aspergillus niger* had overgrown against phytopathogen. In case of *Trichoderma viride* isolates were successfully inhibited the pathogens. The group of *T. harzianum* isolates like RHS/AC 480, AG/S476 showed highly antagonistic activity against *Rhizoctinia solani*, *Sclerotium rolfsii* and *Fusarium oxysporium* .

Table 25 Antagonistic activities against phytopathogens

Antagonists	*Rhizoctinia solani* Colony diam.(mm)			*Fusarium oxysporium* diam.(mm)			*Sclerotium rolfsii* Colony diam.(mm)		
	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)
T. viride (FS/L-20)	67	22	75.6	67	22	75.6	67	21	76.7
T. viride (FS/S-475)	66	20	77.8	76	13	85.6	67	22	75.6
T. viride (FS/S-478)	67	22	75.6	66	20	77.8	68	22	75.6
T. viride (FS/S-474)	68	22	75.6	67	22	75.6	73	17	81.1
T. viride (RHS/T- 460)	61	23	74.4	73	17	81.1	76	14	84.4
T. harzianum (FS/S-458)	76	13	85.6	75	13	85.6	76	14	84.4
T. harzianum (FS/S-455)	61	22	75.6	73	17	81.1	76	14	84.4
T. viride (FS/S-473)	66	20	77.8	66	20	77.8	66	20	77.8

Antagonists	*Rhizoctinia solani* Colony diam.(mm)			*Fusarium oxysporium* diam.(mm)			*Sclerotium rolfsii* Colony diam.(mm)		
	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)
T. viride (RHS/T-472)	67	22	75.6	67	22	75.6	67	22	75.6
T. viride (RHS/T-463)	68	22	75.6	68	22	75.6	68	22	75.6
T. harzianum (Ag/S476)	77	12	86.7	75	13	85.6	78	11	87.8
T. harzianum (RHS/AC480)	77	12	86.7	76	14	84.4	79	12	86.7
T. harzianum (RHS/AC481)	76	14	84.4	71	19	78.9	73	17	81.1
T. harzianum (RHS/AC482)	75	13	85.6	61	23	74.4	76	14	84.4
T. harzianum (RHS/AC483)	75	13	85.6	76	14	84.4	71	19	78.9
T. harzianum (FS/C-90)	71	19	78.9	79	12	86.7	73	17	81.1
T. harzianum (RHS/T-477)	79	19	78.9	78	14	84.4	79	13	85.6
T. harzianum (Ag/S471)	76	14	84.4	76	14	84.4	76	14	84.4
T. harzianum (Ag/S479)	79	12	86.7	79	12	86.7	79	12	86.7
A. niger (FS/L04)	59	29	67.8	26	31	65.6	28	36	60.0
A. niger (FS/L-40)	42	39	56.7	27	36	60.0	29	36	60.0
A. niger (FS/C-140)	42	30	66.7	58	29	67.8	27	34	62.2
A. niger (FS/S-112)	42	38	57.8	28	36	60.0	58	29	67.8
A. niger (FS/S-113)	44	39	56.7	29	36	60.0	27	36	60.0
A. niger (FS/S-262)	41	30	66.7	27	34	62.2	58	29	67.8
A melleus (FS/L-42)	58	29	67.8	58	29	67.8	28	36	60.0
A melleus (FS/L-13)	28	36	60.0	27	36	60.0	29	36	60.0
A melleus (FS/L-17)	29	36	60.0	58	29	67.8	42	30	66.7
A melleus (FS/L-18)	27	34	62.2	28	36	60.0	42	38	57.8
A. clavatus (RHS/T-99)	29	36	60.0	42	38	57.8	58	29	67.8
A. clavatus (RHS/T-190)	27	34	62.2	44	39	56.7	59	29	67.8
A. clavatus (RHS/T-191)	58	29	67.8	41	30	66.7	42	39	56.7
A. clavatus (RHS/P -50)	27	36	60.0	58	29	67.8	42	30	66.7
A. clavatus (RHS/P -54)	58	29	67.8	28	36	60.0	42	38	57.8
A. clavatus (RHS/P-43)	28	36	60.0	42	30	66.7	44	39	56.7
A. fumigatus (RHS/P-209)	29	36	60.0	42	38	57.8	41	30	66.7
A. fumigatus (RHS/B-220)	58	29	67.8	44	39	56.7	58	29	67.8
A. fumigatus (RHS/P-114)	27	36	60.0	41	30	66.7	28	36	60.0

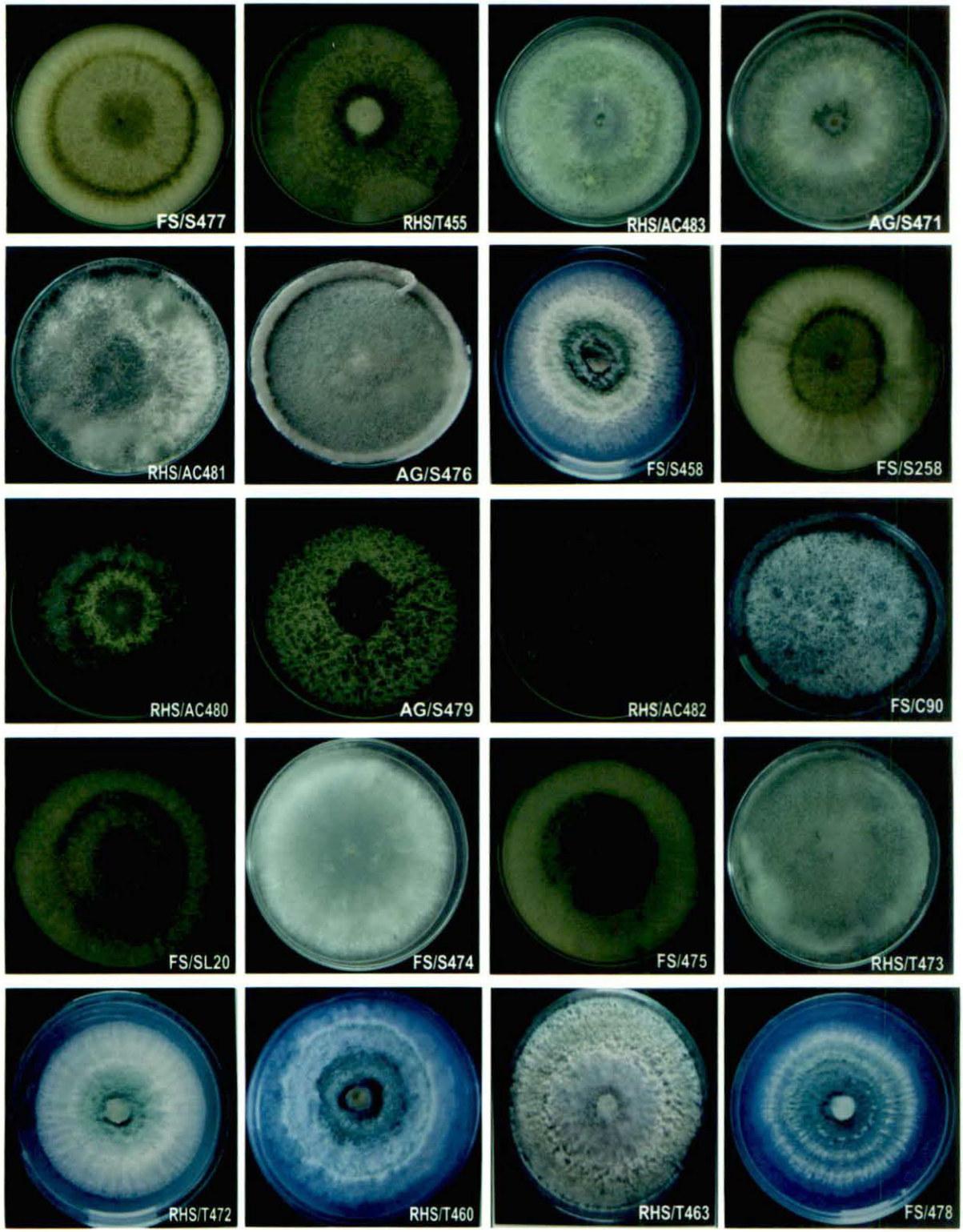


Plate 17: Radial growth of different isolates of *Trichoderma* obtained from forest, riverine and agricultural soil.

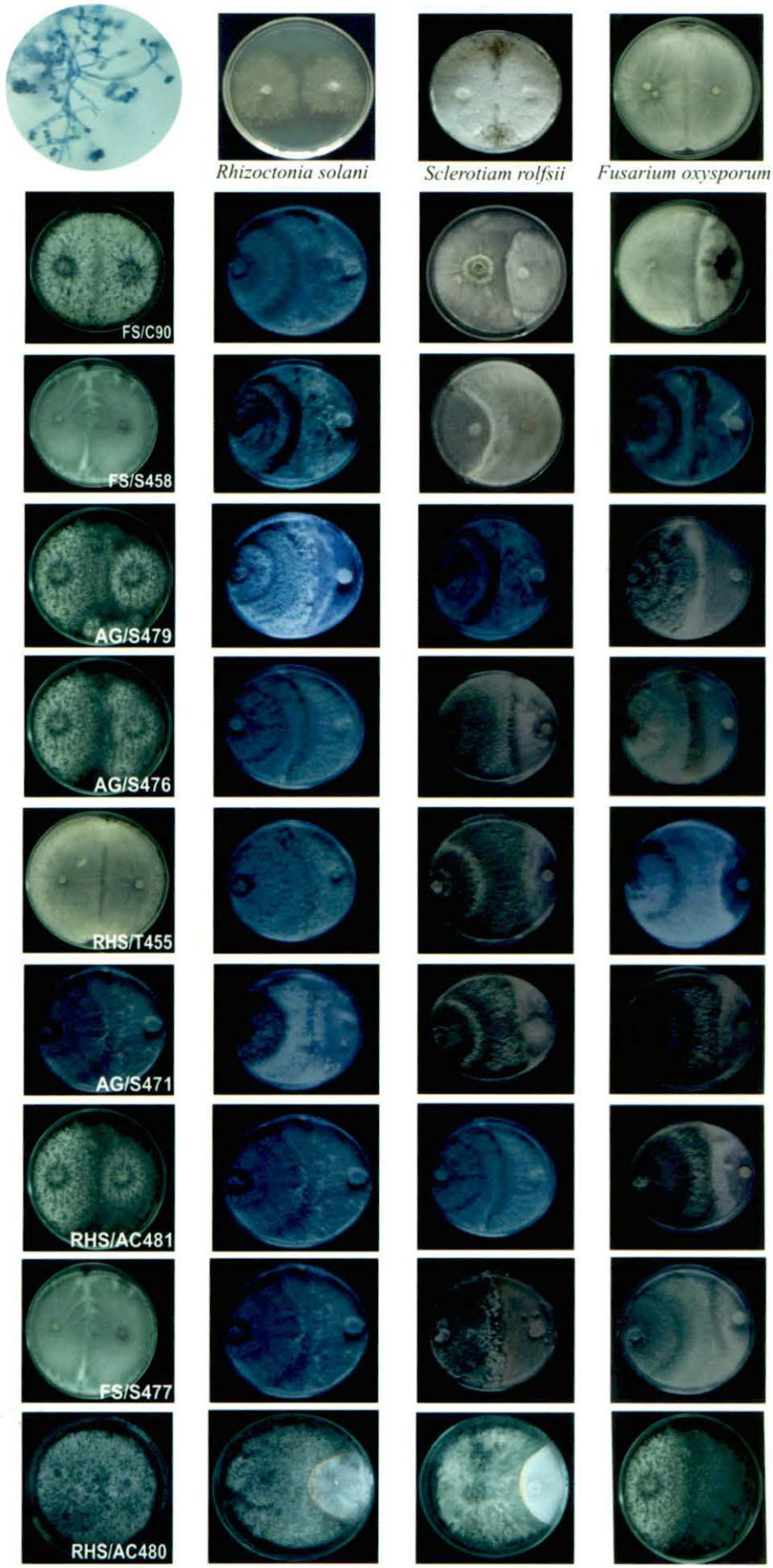


Plate 18 : Antagonistic activity of isolates of *Trichoderma harzianum* against *Rhizoctonia solani*, *Scleoratium rolfsii* and *Fusarium oxysporum*

4.7 Determination of cellulase activity of selected fungal isolates showing antifungal activities

Cellulase producing fungi have the capacity for breaking down forest litter and other agricultural wastes into easily utilizable carbon sources. Hence, such fungi are potentially useful in agriculture. In order to select such fungi, the fungal isolates were grown in medium containing only cellulose as carbon source and those which could grow in such media were selected. Exo and endocellulase activities of such fungi were further assayed.

The amount of glucose released by endocellulase activity of fungal hyphae during their growth using cellulose as C source was measured, where the culture filtrate was collected from the fermentation media by centrifugation. In the present study , 11 isolates of *T. harzianum*, 8 isolates of *T. viride*, 6 isolates of *A. niger*, 7 isolates of *A. melleus*, 4 isolates of *A. clavatus* and 3 isolates of *A. flavus* were selected for determination of their cellulase activities. The isolates of *A. melleus*,, *A. clavatus* and *A. fumigatus* showed moderate cellulase activities while isolates of *A. niger* and *Trichoderma* showed good celluase activities (Table 26)

Table 26 : Net exocellulase activity of selected fungal isolates

Antagonists	Exo and endo cellulase activity (µg reducing sugar produced/ml/hr.)	Amount reducing sugar due to Endocellulase activity (µg/ml)	Net exo-cellulase activity (µg/ml/hr.)
T. harzianum (FS/S-458)	12.5	5.0	17.6 ±1.61
T. harzianum (Ag/S476)	23.5	6.7	16.8±1.61
T. harzianum (FS/C-90)	21.5	7.6	13.9±1.61
T. harzianum(FS/S-455)	21.7	5.4	16.3±1.61
T. harzianum (RHS/AC480)	19.9	3.7	16.2±1.23
T. harzianum (RHS/AC481)	18.7	2.8	15.9±1.23
T. harzianum (RHS/AC482)	17.4	1.9	15.5±1.23
T. harzianum (RHS/AC483)	25.9	9.8	16.1±1.23
T. harzianum (RHS/T- 460)	22	5.8	16.2±1.23
T. harzianum (RHS/T- 477)	21.5	6.7	14.8±1.23
T. harzianum(Ag/S471)	21.9	7.1	14.8±1.23
T. harzianum(Ag/S479)	21.8	9.3	12.5±1.55
T. viride (FS/L-20)	22	10.5	11.5±1.55
T. viride (FS/S-475)	17.5	10	7.5±1.55
T. viride (FS/S-478)	12.5	5	7.5±1.55
T. viride(FS/S-474)	25	17.5	7.5±1.55
T. viride (FS/S-258)	18.9	6.2	12.7±1.55
T. viride (FS/S-473)	13.2	6.3	6.9±1.55
T. viride (RHS/T- 472)	13.3	5.6	7.7±1.55

Antagonists	Exo and endo cellulase activity (µg reducing sugar produced/ml/hr.)	Amount reducing sugar due to Endocellulase activity (µg/ml)	Net exo-cellulase activity (µg/ml/hr.)
T. viride (RHS/T- 463)	12.3	2.4	9.9±1.61
A. niger (FS/L04)	21.2	11.5	9.7±1.55
A. niger(FS/L-40)	21.2	11.5	9.7±1.96
A. niger (FS/C-140)	22.8	10	12.8±1.75
A. niger(FS/S-112)	21.5	5	16.5±2.06
A. niger (FS/S-113)	23.3	6.7	16.6±2.43
A. niger (FS/S-262)	19.9	5.2	14.7±1.12
A melleus (FS/L-42)	18.7	11.1	7.6±1.55
A melleus (FS/L-13)	19	6.7	12.3±2.98
A melleus (FS/L-17)	20.8	12.4	8.4±2.43
A melleus (FS/L-18)	20.8	13.1	7.7±1.55
A melleus (FS/S -63)	17.6	11.1	6.5±2.43
A melleus (FS/S-24)	21.4	14.2	7.2±1.63
A melleus(FS/S-278)	13.5	8.4	5.1±3.40
A. clavatus (RHS/T-99)	12.3	8.1	4.2±1.55
A. clavatus (RHS/P -50)	13.2	4.9	8.3±1.55
A. clavatus (RHS/P -54)	13.2	7.3	5.9±1.63
A. clavatus (RHS/P-43)	16.3	10.9	5.4±1.46
A. fumigatus (RHS/P-209)	13.5	7.8	5.7±0.93
A. fumigatus (RHS/B-220)	17.3	13.1	4.2±1.04
A. fumigatus (RHS/P-114)	9.9	5.1	4.8±1.46

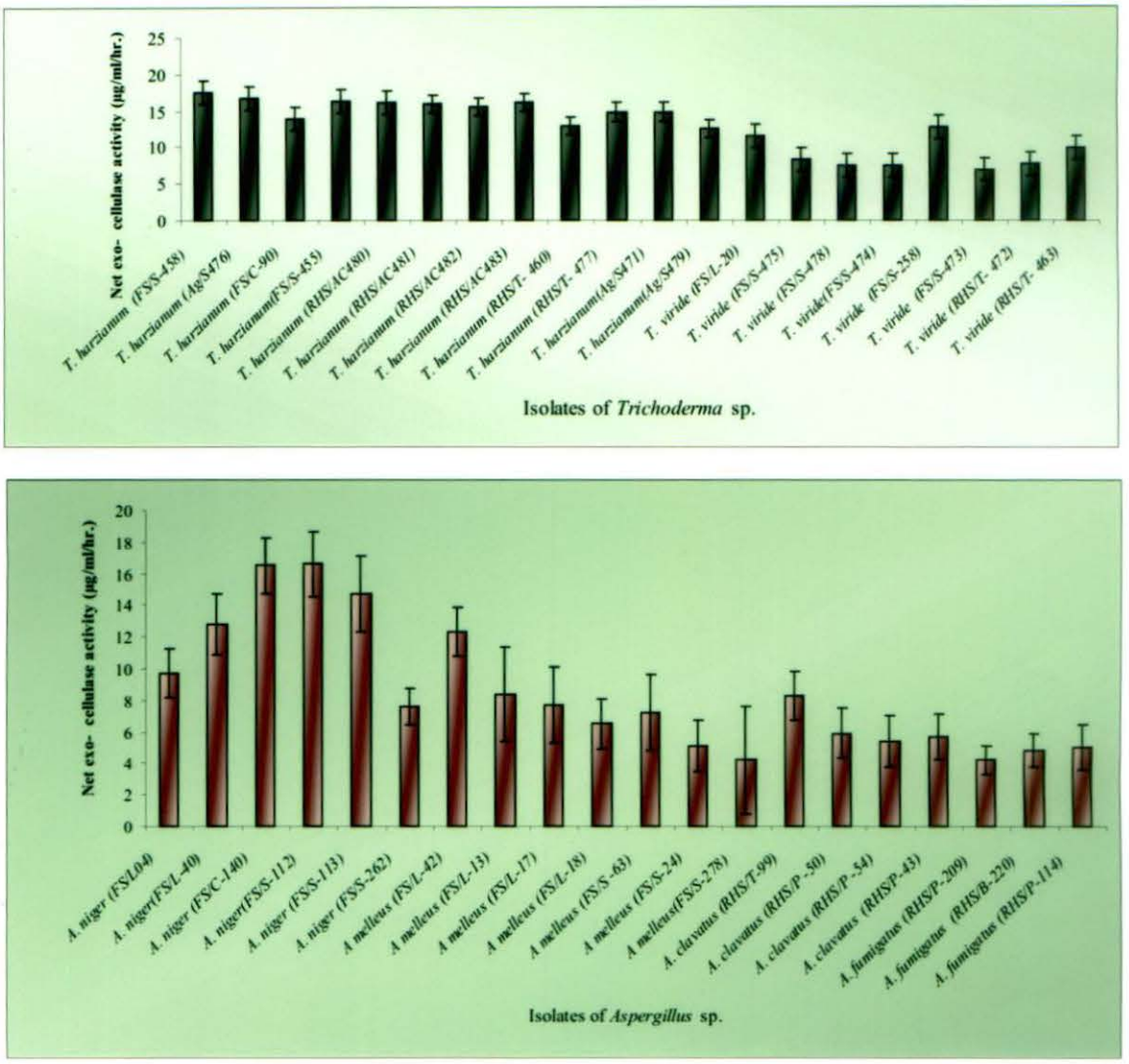


Figure 6: Cellulase activity of fungal isolates

4.8. Determination of chitinase activities of *Trichoderma* isolates showing antifungal activities

Nineteen *Trichoderma* isolates which showed antagonistic activity were selected further for determining the chitinase activities for comparison among the isolates. These isolates were also taken for determining their spore, conidia , phialide structures. These isolates were deposited to National Agriculturally Important Microbial Culture Collection (NAIMCC), Maunath Bhnjan and their accession numbers have been provided in Tables 27 and 28. Spore suspensions were prepared from individual culture. Drops of spore suspension were placed on clean grease free glass- slides, mounted with lacto phenol cotton blue, covered with cover slip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spore measured . Microscopic observations under bright field of all these isolates have been presented in Plate 19. Detailed informations on conidiophore, conidia and phialides of these isolates have been presented in Table 27 and 28 .

Scanning electron microscopic observations of the conidia of isolates of *T. viride* and *T. harzianum* were also made. Photographic presentations of *T. harzianum* and *T. viride* have been presented in Plates 20 and 21. Results revealed that isolates had smooth conidial surfaces. The conidia were an irregular pyramidal shape with a diameter within the 100 to 200 nm size range. The conidia of different isolates varied in shape from globose to sub globose and in size, with diameters ranging from 3.0 to 3.5 μm. Fragments of what appeared to be a thin layer of tissue were observed on and around the conidia in most conidial preparations.

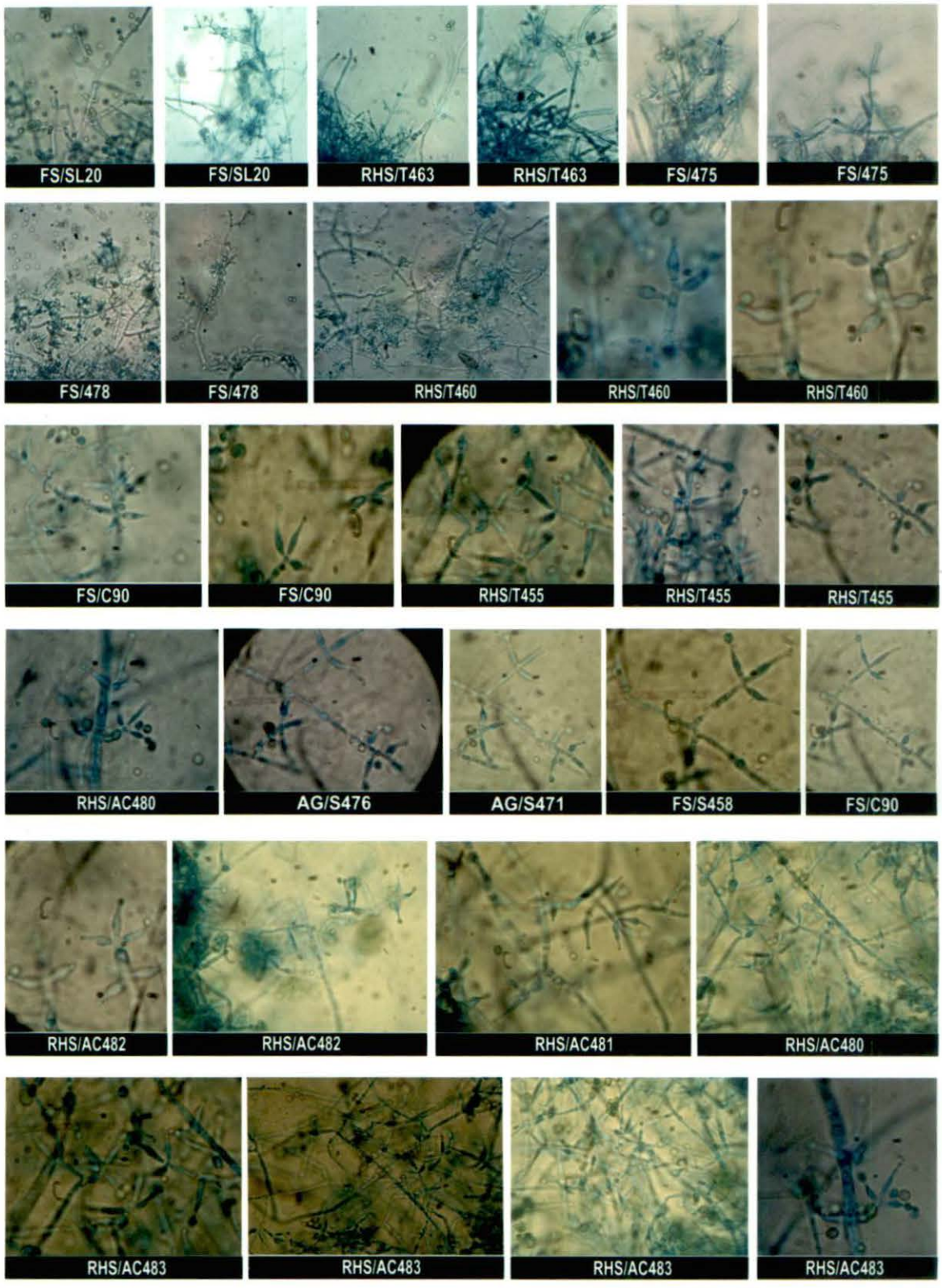


Plate 19: Microscpical views of the isolates of *Trichoderma harzianum and T. viride*

Table 27 . Morphological characteristics of different isolates of *Trichoderma viride*

Isolate Code	Conidiophores Central axis (µm)	Phialide dimensions (µm) a	b	c	d	Conidia (µm)	NAIMCC acc.no.*
FS/L-20	1.7 X 3.2	11.5	4.0	3.3	4.5	2.0x 1.5	NAIMCC-F-01949
FS/S-473	1.9 X 4.2	9.8	3.8	4.1	4.1	2.2x 1.2	NAIMCC-F-0157
FS/S-474	1.6 X 4.1	11.1	4.1	3.1	4.1	2.3x 1.7	NAIMCC-F-01958
FS/S-475	1.9 X 4.2	10.5	3.0	3.1	4.1	2.1x 1.5	NAIMCC-F-01959
FS/S-478	1.5 X 3.2	11.6	3.3	3.4	4.4	2.9x 1.6	NAIMCC-F-01960
RHS/T- 460	1.8 X 3.9	10.5	4.6	3.7	4.9	2.7x 1.8	NAIMCC-F-01953
RHS/T- 463	1.7 X 3.2	11.7	4.1	3.1	4.9	2.5x 1.9	NAIMCC-F-01954
RHS/T- 472	1.9 X 4.0	10.5	3.9	3.9	4.9	2.0x 1.2	NAIMCC-F-01956

a- length; b- width at widest point; c- width at base; d- width where arising from a cell
*National Agriculturally Important Microbial Culture Collection (NAIMCC)

Table 28 Morphological characteristics of different isolates of *T. harzianum*

Isolate Code	Length of terminal branches of Conidiophores (µm)	Phialide dimensions (µm) a	b	c	d	Conidia (µm)	NAIMCC acc.no.*
FS/C-90	146	8.9	4.6	2.6	3.8	3.0x 2.1	NAIMCC-F-01950
FS/S-455	149	10.7	4.9	3.7	3.9	2.1x 1.7	NAIMCC-F-01955
FS/S-458	144	11.8	4.6	4.3	5.5	2.9x 1.4	NAIMCC-F-01952
RHS/T- 477	150	6.5	3.4	2.5	5.1	3.9x 2.8	NAIMCC-F-01962
RHS/AC480	143	6.7	3.5	2.5	4.9	4.2x 3.9	NAIMCC-F-01961
RHS/AC481	148	6.5	3.3	2.6	5.2	4.3x 3.8	NAIMCC-F-01963
RHS/AC482	145	6.4	4.2	2.5	4.2	3.3x 2.8	NAIMCC-F-01964
RHS/AC483	147	6.5	3.3	2.6	5.1	3.1x 2.4	NAIMCC-F-01965
Ag/S476	. 149	6.2	3.3	2.7	5.4	2.7x 1.8	NAIMCC-F-01966
Ag/S471	151	6.6	3.5	2.7	5.5	2.6x 1.9	NAIMCC-F-01967
Ag/S479	152	6.7	3.0	2.9	5.5	2.5x 1.6	NAIMCC-F-01968

a- length; b- width at widest point; c- width at base; d- width where arising from a cell
*National Agriculturally Important Microbial Culture Collection (NAIMCC)

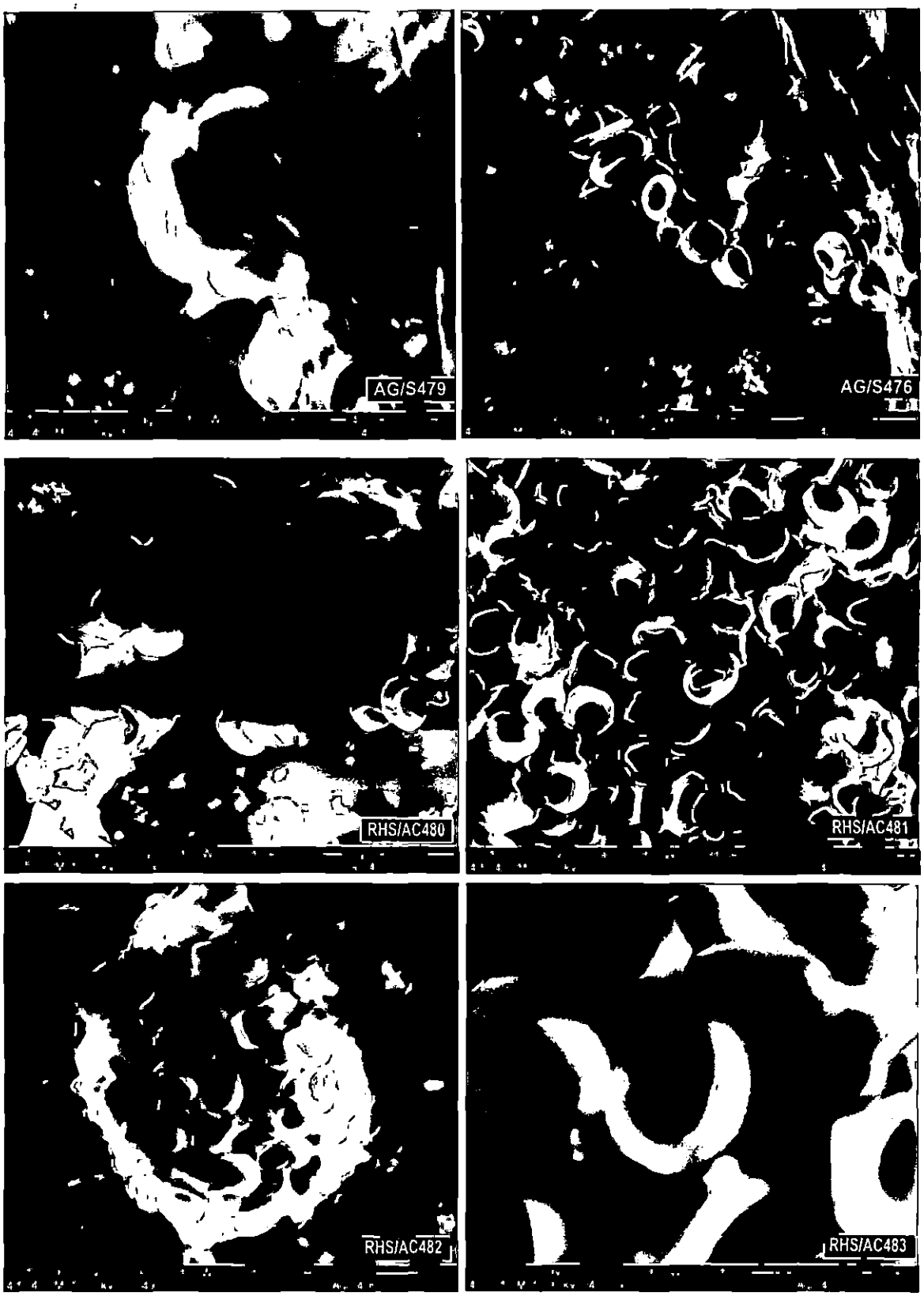


Plate 20: SEM of spores of *Trichoderma harzianum* isolates

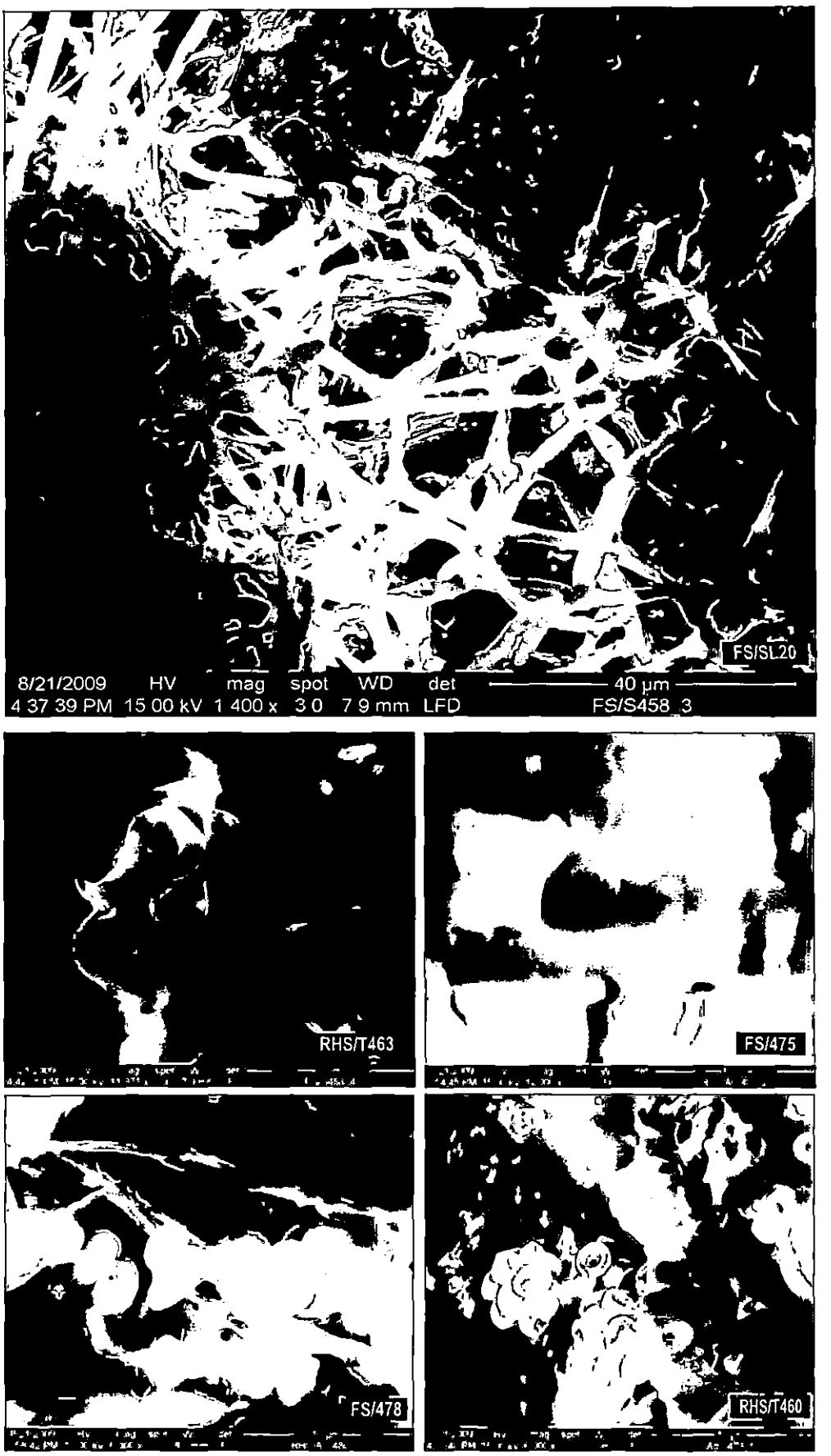


Plate 21: SEM hyaphe and spores of *Trichoderma viride* isolates

Chitinase activities of these nineteen isolates of *Trichoderma* species were also determined. Spore suspension (1.0 x 10^6 spores per mL of culture medium) were used and inoculated into duplicate 250 mL flasks containing 30 ml of unbuffered mineral synthetic medium . Following two weeks, culture filtrates were centrifuged at 4 °C for 10 min at 5000 x g and the clear supernatants were immediately tested for enzyme activity. The assay mixture contained 1 mL of 0.5 % pure chitin 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 reagent. Activity was expressed as µg N-acetyl glucosamine produced. Assay of both endo and exo chitinase activities of the different isolates of *T. harzianum* and *T. viride* revealed that isolate RHS/AC481 of *T. harzianum* had maximum while isolates Ag/s 476 of *T. harzianum* had minimum chitinase activity (Table 29, Fig 7).

Table 29. Chitinase activities of different isolates of *Trichoderma harzianum* and *Trichoderma viride*

Isolates	Code	Chitinase activity	
		Exo*	Endo*
T. viride	FS/L-20	4.12±0.63	26.12±0.63
	FS/S-473	4.47±0.40	25.41±0.24
	FS/S-474	4.35±0.55	26.59±0.46
	FS/S-475	4.29±0.55	25.65±0.51
	FS/S-478	4.24±0.63	24.24±0.23
	RHS/T- 460	4.24±0.46	25.88±0.63
	RHS/T- 463	4.47±0.93	25.65±0.46
	RHS/T- 472	4.18±0.04	24.47±0.63
T. harzianum	FS/C-90	4.18±0.46	24.47±0.04
	FS/S-455	4.34±0.46	23.82±0.46
	FS/S-458	4.53±0.55	26.59±0.51
	RHS/T- 477	4.41±0.55	24.24±0.52
	RHS/AC480	4.65±0.63	27.8±0.69
	RHS/AC481	5.00±0.46	25.18±0.46
	RHS/AC482	4.71±0.63	28.94±0.63
	RHS/AC483	4.76±0.04	28.71±0.24
	Ag/S476	3.94±0.46	24.47±0.46
	Ag/S471	4.53±0.44	23.76±0.26
	Ag/S479	4.18±0.61	26.12±0.24

* Chitinase activity expressed as µg N-Acetyl glucosamine released/ ml culture filtrate/h (Endo) and µg N-Acetyl glucosamine released/ g mycelium /h (Exo)

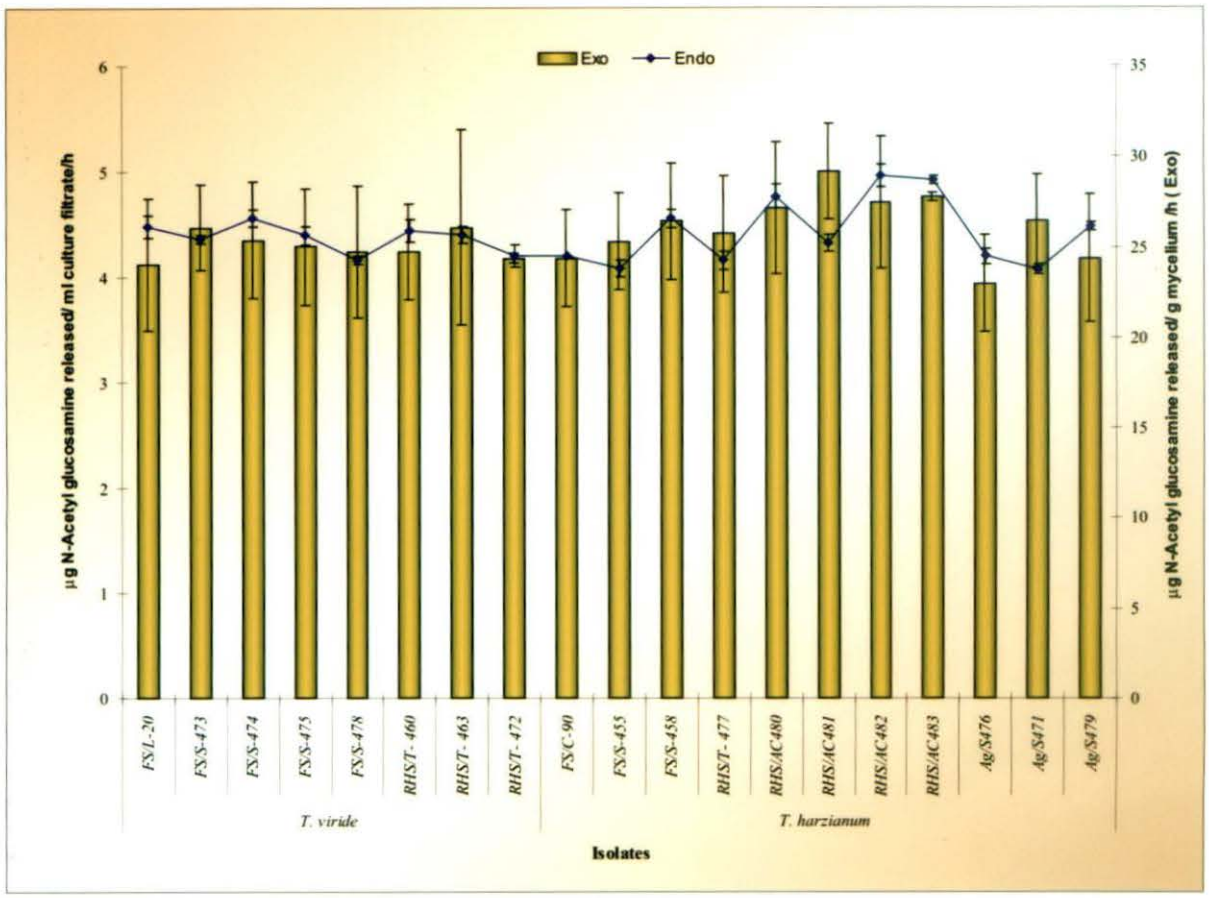


Figure 7: Chitinase assay of *Trichoderma* isolates

4.9 Immunological characterization of *Trichoderma* isolates

4.9.1 Soluble protein

Soluble protein prepared from mycelia of eight isolates of *T. viride* and eleven isolates of *T. hrazianum* were analysed. Initially, soluble proein content was estimated. Mycelial protein content of *T. viride* isolates were ranging between 1.21 – 2.25 mg/gm fresh wt. whereas protein content of *T. harzianum* isolates were ranging between 2.03 – 2.68 mg/gm fresh wt. tissue (Table 30)

Soluble proteins extracted from *Trichoderma* isolates were further analysed by SDS-PAGE (Plate 22 B). The molecular weight of protein bands visualized after staining with coomassie blue were determined by comaring with known molecular weight marker. Mycelial protein of *T. viride* exhibited 19-24 bands in SDS-PAGE ranging in molecular weight (Ca.200 kDa to 12 kDa) whereas mycelial protein of *T. harzianum* exhibited 20-28 bands in SDS-PAGE ranging in molecular weight (Ca.205 kDa to 10 kDa) of varying intensities and more proteins of lower molecular weight were present.

4.9.2. Serology

Polyclonal antibodies (PAbs) were raised in rabbit against mycelial proteins as described previously and these were used in various immunological formats. For antigen sourse normal sera were collected before immunization. The effectiveness of the purified antigen of *T. harzianum* (RHS/AC 480) in raising PAbs were checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction was also done with PAb raised against 60-80% fractionated protein and results shows four separated, sharp bands of the isolates of *T. harzianum* (RHS/AC 480). IgG fractions were purified and experiments were done with purified IgG fraction of this PAb.

Dot immunobinding assay was performed using soluble antigens prepared from eight isolates of *T. viride* and eleven isolates of *T. hrazianum.* Purified IgG of 1st, 2nd, 3rd and 4th bleedings were taken into consideration. In this case IgG prepared from *T. harzianum* gave positive colour reactions, though dots were of low intensity in four isolates of *T. viride* or the reactions were very weak (Table 31; Plate 22 A). Identification of *Trichoderma* directly from soil taken root rhizosphere was also carried out using dot immunobinding assay,western blot analyses reaction as well as indirect immunofluoresce.

Table 30 : Soluble Protein content and Protein pattern of *Trichoderma* isolates

Isolates	Code	Protein content (mg/gm)	Molecular weight (kDa)
T. viride	FS/L-20	2.20	12, 26, 29, 31, 38, 41, 42, 43, 50,55 66,70, 75, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
	FS/S-473	1.82	12, 20, 29, 31, 35, 41, 50, 60,70, 75, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
	FS/S-474	1.88	12, 20, 29, 31, 35, 41, 50, 60,70, 75, 88, 95, 120,150, 160, 180, 185, 190, 200
	FS/S-475	2.42	12, 20, 29, 31, 35, 41, 50, 60,70, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
	FS/S-478	1.21	12, 20, 29, 31, 35, 41, 50, 60, 75, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
	RHS/T- 460	2.25	12, 20, 29, 31, 35, 41, 50, 60,70, 75, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
	RHS/T- 463	2.25	12, 20, 29, 31, 35, 41, 50, 65,70, 75, 80, 95, 120,150, 160, 180, 185, 190, 200
	RHS/T- 472	2.07	12, 20, 29, 31, 35, 41, 50, 60,70, 75, 88, 95, 120,135,150, 160, 170, 180, 185, 190, 200
T. harzianum	FS/C-90	2.57	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 110,130,140,150,160, 170, 180,190, 200,205
	FS/S-455	2.24	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 110,130,140,160, 170, 180,190, 205
	FS/S-458	2.03	10, 21, 22, 24, 30, 35, 40, 42, 43, 45, 66,75, 110, 130, 140, 160, 170, 180, 200, 205
	RHS/T- 477	2.32	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 110,130,140,160, 170, 180,190, 205
	Ag/S476	2.64	10, 21, 22, 24, 30, 35, 40, 42, 43, 45, 66,75, 110, 130, 140, 160, 170, 180, 200, 205
	Ag/S471	2.24	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 110,130,140,160, 170, 180,190, 205
	Ag/S479	2.62	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 50,55 66,70, 75, 88, 95, 110,130,140,160, 170, 180,190, 205
	RHS/AC480	2.68	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 110,130,140,150,160, 170, 180,190, 200,205
	RHS/AC481	2.51	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 100,130,140,150,155, 170, 180,195, 200,205
	RHS/AC482	2.21	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 105,130,140,150,161, 170, 180,190, 200,205
	RHS/AC483	2.21	10, 21, 22, 24, 29, 30, 35, 40, 42, 43, 45, 50,55 66,70, 75, 88, 95, 120,130,140,150,160, 175, 180,195, 200,205

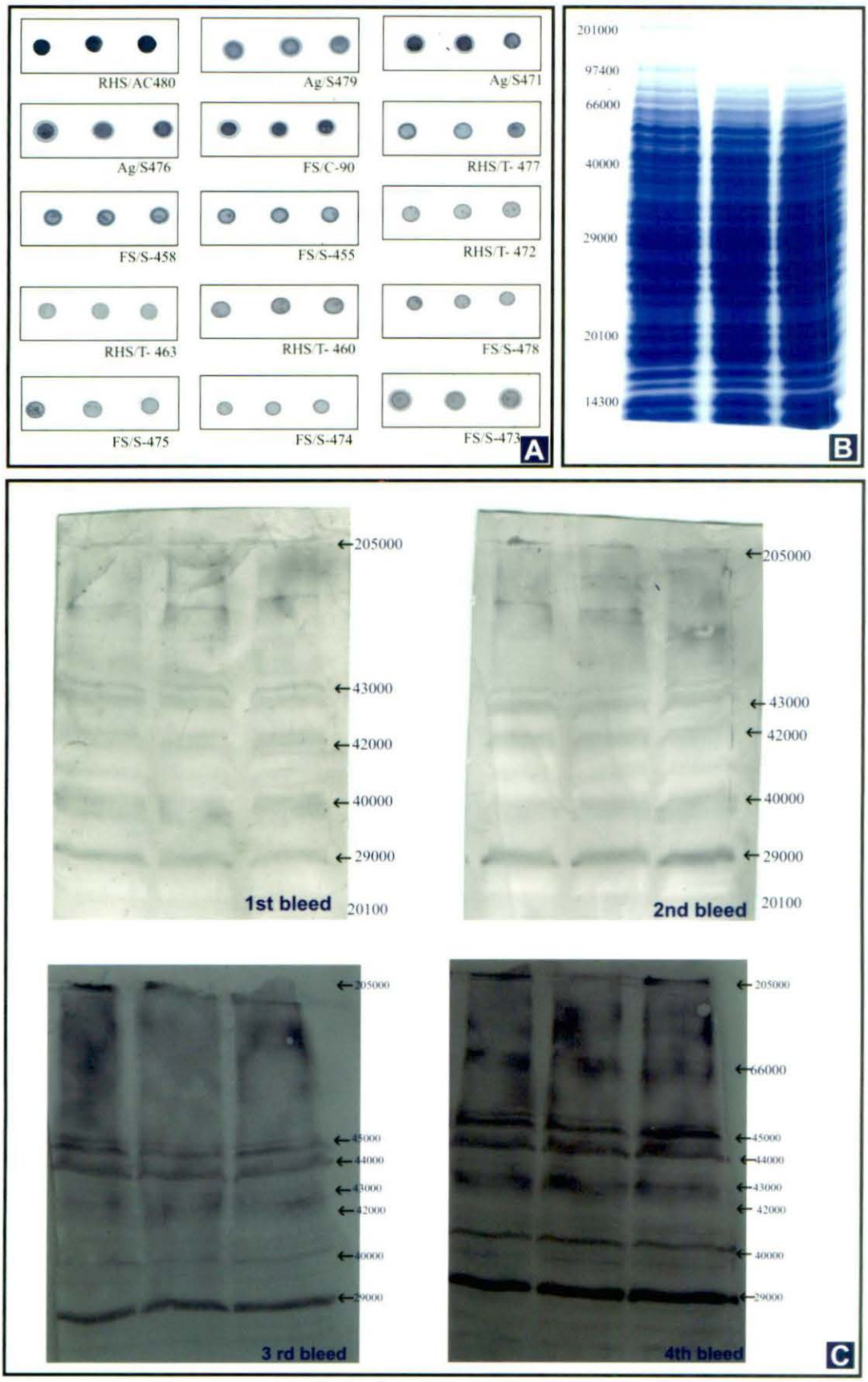


Plate 22: Dot immunobinding assay (A) on nitrocellulose paper with PAb of *Trichoderma harzianum* (RHS/AC480) against mycelial antigen of *T.harzianum* and *T. viride*. SDS-PAGE (B) and Western blot analysis (C)of *T. harzianum* (RHS/AC480)

Isolates of *T. viride*	Colour intensity[a] Using mycelial PAb (4^{th} bleed)	Isolates of *T. harzianum*	Colour intensity[a] Using mycelial PAb (4^{th} bleed)
FS/L-20	±	FS/C-90	+++
FS/S-473	±	FS/S-455	+++
FS/S-474	±	FS/S-458	+++
FS/S-475	±	RHS/T- 477	+++
FS/S-478	++	Ag/S476	+++
RHS/T- 460	++	Ag/S471	+++
RHS/T- 463	++	Ag/S479	+++
RHS/T- 472	++	RHS/AC480	++++
		RHS/AC481	++++
		RHS/AC482	++++
		RHS/AC483	++++

a [a] Fast red colour intensity : Pinkish red ; ++++ Bright high , +++ Medium, ++ Low, ± Faint, - no reaction ; IgG concentration 40µg/ml.

Western blot analyses using IgG of *T. harzianum* RHS/AC480 revealed that the homologous antigens showed maximum 10 bands ranging from 20 to 205kDa hybridized with IgG of 4^{th} bleed, 5 bands ranging from 20-45 kDa in 3^{rd} bleed, 4 bands ranging from 29-43 kDa in 2^{nd} bleed and 1^{st} bleed (Table 32 Plate 22 C).

Table 32 : Western blot analysis of *Trichoderma harzianum* (RHS/AC 480).

1^{st} bleed Molecular weight (kDa)	2^{nd} bleed Molecular weight (kDa)	3^{rd} bleed Molecular weight (kDa)	4^{th} bleed Molecular weight (kDa)
29, 40,42, 43	29, 40,42, 43	20, 29, 40, 43,45	20, 24, 29, 40, 42,43,44,45,66, 205

Indirect immunofluorescence of hyphae and phialides of *Trichoderma harzianum* (RHS/AC 480) were studied. Mycelia of *T. harzianum* (4-day-old) treated with PAb and reacted with FITC labeled antibodies of goat specific for rabbit globulin showed strong apple green fluorescence. Young hyphae and phialides confirmed the bright fluorescence which were used for serological detection (Plate 23) .

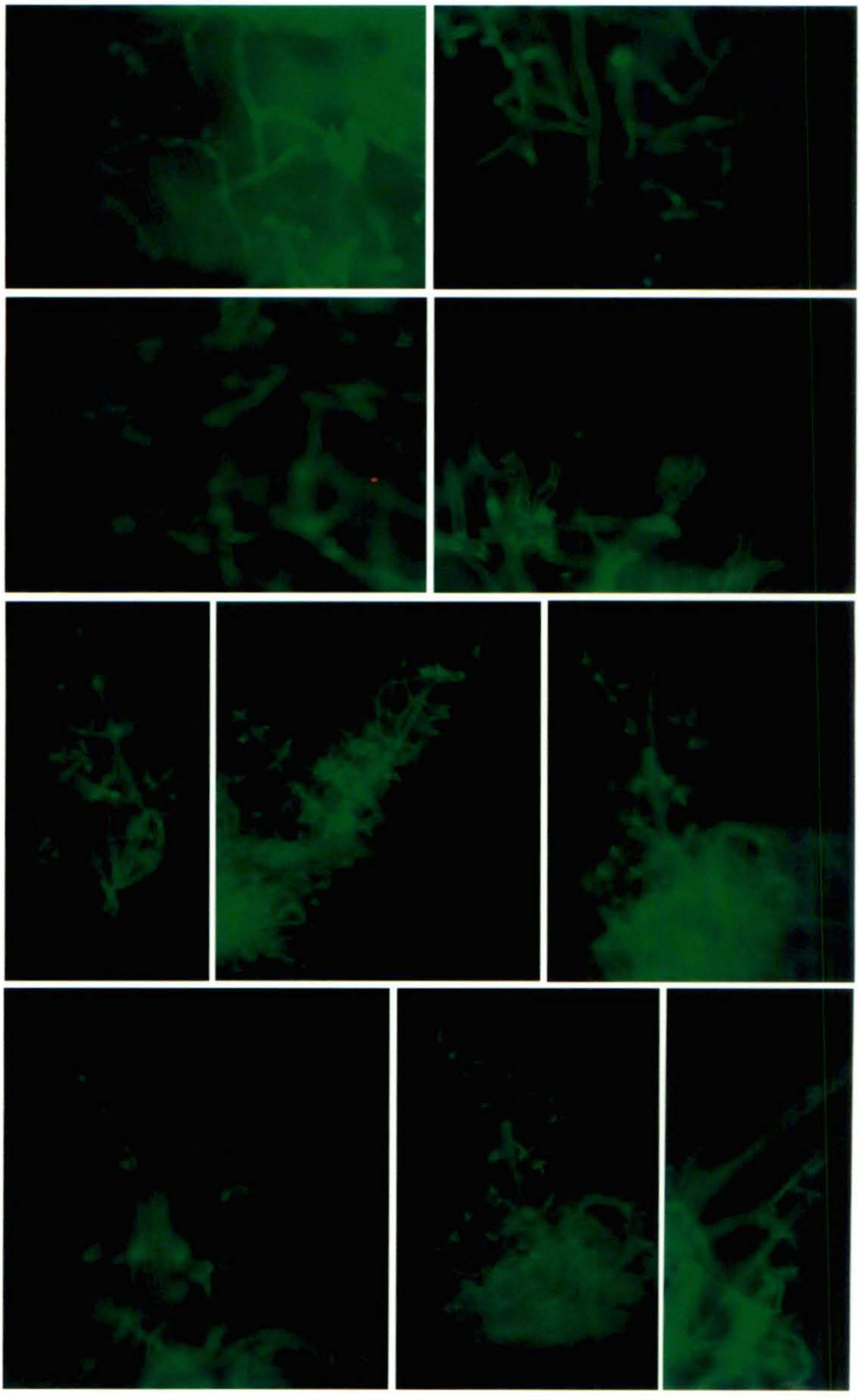


Plate 23: Indirect immunofluorescence of hyphae and phialides of *Trichoderma harzianum* (RHS/AC480) (treated with PAb and reacted with FITC labelled antibodies of goat specific for rabbit globulin)

4.10. *In vivo* test of BCA isolates

In order to assess the biocontrol potential of *Trichoderma harzianum*, eight isolates of *T. harzianum* were selected on the basis of their sporulation behaviour as well as serological identity. In this experiment, *Phaseolous vulgaris* was considered as a host material and *Sclerotium rolfsii* , one of the pathogens showed anatagonistic activity when tested against *T. harzianum in vitro.* The experimental set up was carried out with following treatments : (a) pathogen (*S. rofsii*), (b) biocontrol agent (*T. harzianum*), (c) BCA (*T. harzianum*) and pathogen (*S. rofsii*) and (d) Untreated healthy control. Soil was amended with mass multiplied inocula of the BCA isolates separately prior to sowing the seeds. Ten day old plants were inoculated with *S. rolfsii* and disease development was recorded after 10 day of inoculation . Results (Table 33) revealed that among the isolates

Table 33: Evaluation of eight isolates of *Trichoderma harzianum* on the development of scleotial blight incidence of *Phaseolus vulgaris*

	Disease Index [a]
Treatments	***Phaseolus vulgaris*[b]**
S. rofsii	5.58
S. rofsii + *T.harzianum* (Ag/S476)	0.97
S. rofsii + *T.harzianum* (RHS/AC480)	0.86
S. rofsii + *T.harzianum* (FS/C-90)	1.85
S. rofsii + *T.harzianum* (FS/S-455)	1.28
S. rofsii + *T.harzianum* (FS/S-458)	1.34
S. rofsii + *T.harzianum* (RHS/T- 477)	1.05
S. rofsii + *T.harzianum* (Ag/S479)	1.65
S. rofsii + *T.harzianum* (Ag/S471)	1.73

No disease was observed in uninoculated control, or those inoculated with either *T. harzianum* alone.

[a] 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

[b] Age of plants 3 week

Average of 3 separate inoculated plants (10 days after inoculation)

tested, sclerotial blight disease development was markedly reduced with prior applications of two isolates of *T. harzianum* (Ag/S476 and RHS/AC480). However all other isolates reduced the disease to some extent. Disease development, scleriotia formation in advanced stages as well as disease reduction due to application of BCA isolates have been presented in Plate 24.

Two isolates of *T. harzianum* (Ag/S476 and RHS/AC480) which showed significant disease reduction, were futher tested *in vivo* and accumulation of defense enzymes in host plants (*Phaseolus vulgaris*) against *S. rolfsii* following application of *T. harzianum* were determined. Accumulation of three defense enzymes (peroxidase, ß-1, 3- glucanase and chitinase) were compared with untreated healthy control. Enzyme activities were higher in pretreated soil with *T. harzianum* as well as treated and inoculated plants than untreated healthy control (Table 34).

Table 34 : Induction of defense enzymes in *Phaseolus vulgaris* against *Sclerotium rofsii* following application of *Trichoderma harzianum*

Treatments	Enzyme activity		
	Glucanase (µg/g/min)	Chitinase mg GlcNAC/g /min	Peroxidase ΔOD/g/min
Untreated Healthy	19.7	13.9	63
Treated			
T. harzianum (Ag/S476)	33.2	21.7	85
T. harzianum (RHS/AC480)	34.7	24.0	86
Inoculated			
S. rofsii	13.5	16.6	61
Teated and inoculated			
T. harzianum(Ag/S476) +*S. rofsii*	29.3	20.6	79
T. harzianum (RHS/AC480) +*S. rofsii*	31.1	23.3	83

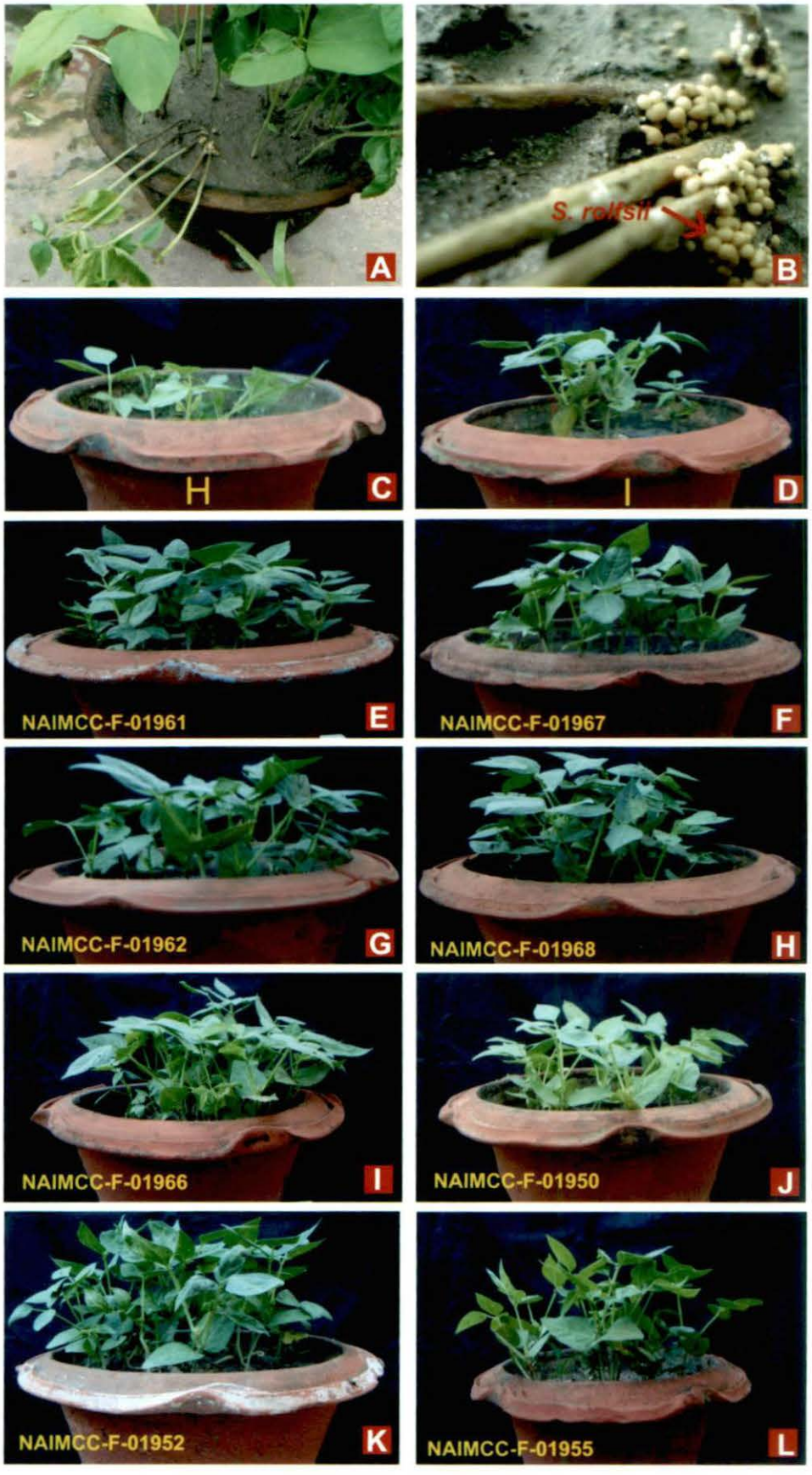


Plate 24: Evaluation of eight isolates of *Trichoderma harzianum* on the development of sclerotial blight incidence of *Phaseolus vulgaris*.
(A&B) Sclerotial blight, (C) Untreated control, (D) Inocualted with *S. rolfsii,* (E-L) treated with *T. harzianum* and inoculated with *S. rolfsii*

4.11. Diversity analysis of PSF isolates

4.11.1 Isolation and purification of genomic DNA from PSF isolates

Ten phosphate solubilizing fungi (*A. niger* (FS/L-04), *A. niger* (RS/P-14) *A. niger* (FS/L-40), *A. niger* (FS/S-113), *A. melleus* (RS/P-05), *A. melleus* (RHS/R-12), *A. melleus* (FS/L-13), *A. melleus* (FS/L-17), *A. melleus* (FS/L-18), *A. clavatus* (RHS/P-38) were grown in liquid media for 4 days and mycelia were harvested, and 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 60^0C followed by centrifugation at 12,000 rpm for 15 min. The aqueous phase was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min, the aqueous phase was then extracted with equal volume of phenol : chloroform : isoamyl alcohol (23:1:1) at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and then the DNA was precipitated with chilled ehanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70 % ethanol by centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse . After incubation the sample was re-extracted with PSI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol. The yield of DNA was determined spectrophotometrically as 24 µg/g of mycelial mat. The purity of DNA genome samples of all ten PSF isolates (3 isolates of *A. niger*, 5 isolates of *A. melleus* and 1 isolate of *A. clavatus*) as indicated by A_{260}/A_{280} ratio have been presented in Table 35. The ratio was ranging from 1.42 to 2.07.

Further DNA quality was also evaluated by 0.8% agarose gel electrophoresis. 20 µl DNA was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 0.8% agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. The result revealed that RNA free DNA was yielded and the size of DNA of each isolates were ranging from 1.7-1.8 kb.

Table 35: Spectrophotometrical A_{260}/A_{280} ratio of isolated genomic DNA

Organisms	Isolate nos.	A_{260}	A_{280}	A_{260}/A_{280}
Aspergillus niger	FS/L-04	0.197	0.137	1.44
Aspergillus niger	RS/P-14	0.276	0.148	1.86
Aspergillus niger	FS/L-40	0.379	0.183	2.07
Aspergillus niger	FS/S-113	0.41	0.2	2.05
Aspergillus melleus	RS/P-05	0.38	0.188	2.02
Aspergillus melleus	RHS/R-12	0.213	0.139	1.53
Aspergillus melleus	FS/L-13	0.24	0.147	1.63
Aspergillus melleus	FS/L-17	0.523	0.259	2.02
Aspergillus melleus	FS/L-18	0.228	0.145	1.57
Aspergillus clavatus	RHS/P-38	0.319	0.182	1.75

4.11.2. RAPD patterns

The PCR conditions for RAPD analysis were optimized by investigating each factor individually. This included genomic DNA quality and concentration, primer annealing and extension temperature as well as denaturation time and temperature. Four random primers [OPD-5,OPD-2,OPB-3 andOPD6] were used for RAPD analysis (Table 36) .

Table 36 : The nucleotide sequence used for RAPD PCR

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
OPD-5	TGAGCGGACA	10	37	60%
OPD-2	TGATCCCTGG	10	34	60%
OPB-3	GATCCCCTG	10	37	70%
OPD6	GGGGTCTTGA	10	32.8	83%

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

random primer 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 s, annealing at 35°C for 60 s 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 It was found that quality of genomic DNA extracted as described here was a good template for PCR amplification. In the present investigation, four random decamer primers - OPD-5, OPD-2, OPB-3 and OPD-6 gave sufficient polymorphism among the isolates of *A. clavatus*, *A. niger* and *A. melleus* . The amplified fragments ranged from 1100 to 600 bp in size (Table 37).

Table 37 Analysis of the polymorphism obtained with RAPD markers

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPD2,	07	100	2000	0	9	100
2	OPB3	08	100	1000	0	12	100
3.	OPD5	05	100	2000	0	18	100
4.	OPD-6	14	100	1000	0	14	100

4.11.3. Phylogenetic analysis

RAPD profiles were scored by visually comparing RAPD amplification profiles and scoring the presence or absence of each band in each profile. Basically, the formation obtained from agarose gel electrophoresis was digitalized to a two - discrete - character - matrix (0 and 1 for absenwexsce and presence of RAPD - markers). UPGMA cluster analysis was carried following similarity coefficient matrix of reproducible bands using PC software NTSYSPc software (Table 38). A total of 127 polymorphic bands were obtained with an average of 31.75 bands/ primer. The second group consisting of two isolates of *A. clavatus*, two isolates of *A. melleus* and two isolates of *A. niger* showed another sub group at 50 percent similarity. The selected isolates showed three different lineages at sixty one percent similarity level (61%) (Plate 25) .

Table 38 RAPD-based genetic similarity within groups

	1	2	3	4	5	6	7	8	9	10
1	1.00									
2	1.00	1.00								
3	0.90	0.90	1.00							
4	0.81	0.81	0.90	1.00						
5	0.58	0.58	0.66	0.75	1.00					
6	0.50	0.50	0.58	0.66	0.90	1.00				
7	0.58	0.58	0.66	0.75	1.00	0.90	1.00			
8	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00		
9	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00	1.00	
10	0.	.63	0.63	0.72	0.66	0.90	0.80	0.90	0.90	0.90 1.00

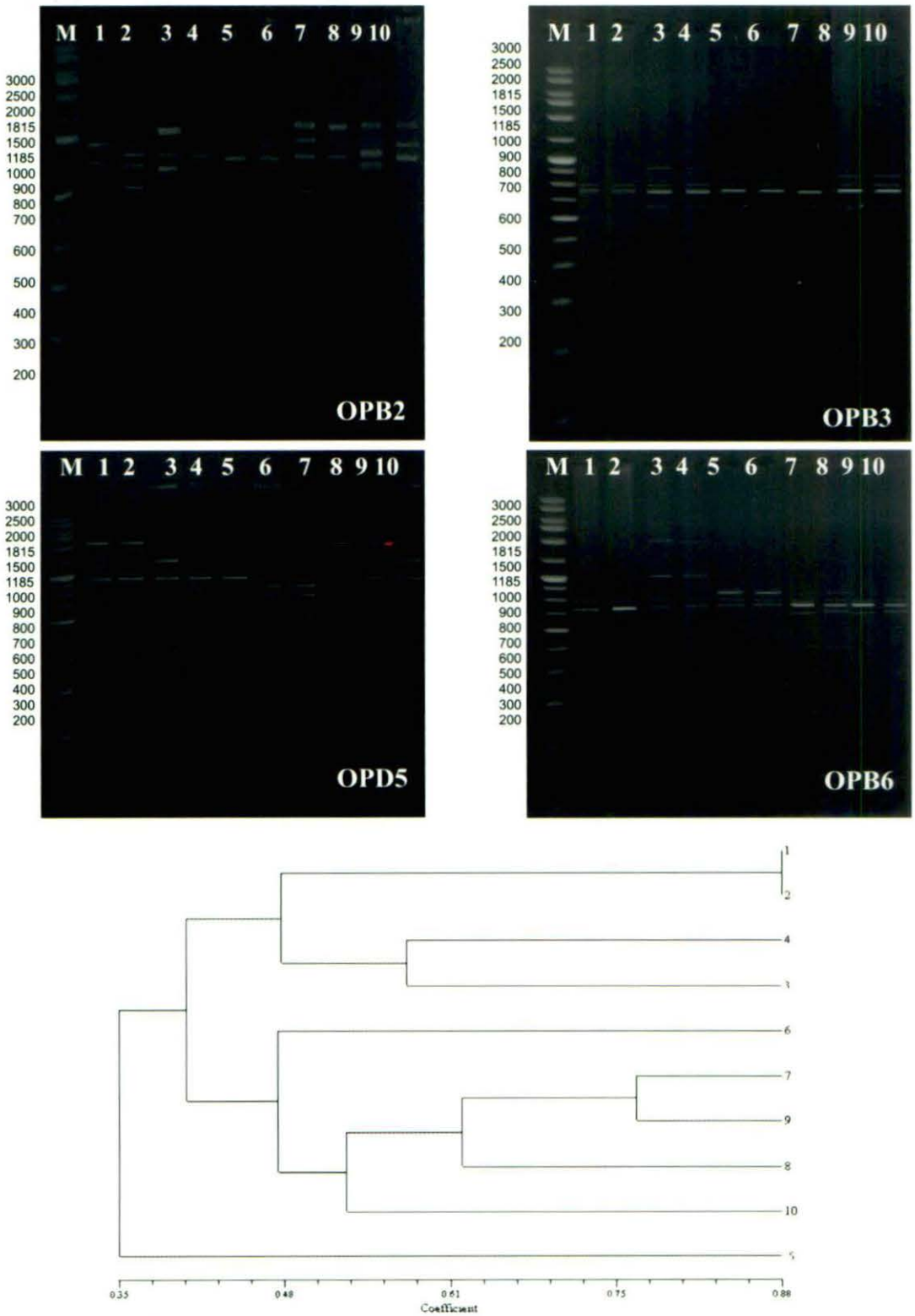


Plate 25 Plate : RAPD amplified products of phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* using four random primers Lane M: Low range DNA marker, Lane 1: *A. niger* (FS/L-04), Lane 2: *A. niger* (RS/P-14), Lane 3: *A. niger* (FS/L-40), Lane 4: *A. niger* (FS/S-113) , Lane 5: *A. melleus* (RS/P-05), Lane 6: *A. melleus* (RHS/R-12), Lane 7: *A. melleus* (FS/L-13), Lane 8: *A. melleus* (FS/L-17), Lane 9: *A. melleus* (FS/L-18), Lane 10: *A. clavatus* (RHS/P-38) and Dendrogram showing the genetic relationships among 10 Phosphate solubilizing fungal isolates based on RAPD analysis [1: *A. niger* (FS/L-04), 2: *A. niger* (RS/P-14), 3: *A. niger* (FS/L-40), 4: *A. niger* (FS/S-113) , 5: *A. melleus* (RS/P-05), 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)]

4.12. Diversity analysis of bacterial isolates

4.12.1 Isolation and purification of genomic DNA from bacterial isolates

The broth cultures of phosphate solublizing bacterial isolates (*Bacillus cereus* (B/FS/B21), *Bacillus cereus* (B/FS/B5), *Bacillus cereus* (B/FS/B6), *Bacillus cereus*(B/FS/S19), *Bacillus pumilus* (B/RHS/P22), *Bacillus sp* (B/FS/J29), *Bacillus sp.* (B/FS/B14), *Bacillus sp.* (B/FS/C35), *Bacillus sp.* (B/FS/C36), *Bacillus sp.* (B/FS/C37), *Bacillus sp.* (B/FS/C38), *Bacillus sp.* (B/FS/G43), *Bacillus sp.* (B/FS/M24), *Bacillus sp.* (B/FS/T10), *Bacillus sp.* (B/FS/T4), *Bacillus sp.* (B/FS/T5), *Bacillus sp.* (B/RS/M 57), *Bacillus sp.*(B/FS/J28), *Pseudomonas sp* (B/RHS/P91), *Pseudomonas sp.* (B/FS/C33), *Pseudomonas sp.* (B/FS/C34), *Pseudomonas sp.* (B/FS/G44), *seudomonas sp.* (B/FS/J33), *Pseudomonas sp.* (B/RHS/P88), *Pseudomonas sp.* (B/RS/M 51), *Pseudomonas sp.* (B/RS/M 54), *Pseudomonas sp.* (B/RS/M 55), *Pseudomonas sp.* (B/RS/M 56), *Pseudomonas sp.*(B/FS/S12), *Pseudomonas sp.*(B/RHS/P89)) were centrifuged at 10,000 rpm at 28C for 5 mins and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5ml of CTAB buffer) was added and incubated at 37C for 3 hrs. Then 10 µl proteinase K solution (20mg/ml) was added and it was allowed to incubate at 65C for 3min. The lysate was extracted with equal volume of tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 mins at 4^{O}C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4C.

Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse . After incubation the sample was re-extracted with PSI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol. The yield of DNA was determined spectrophotometrically as 24 µg/g of mycelial mat. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio (Table 39) and DNA quantity was evaluated by 0.8% agarose gel electrophoresis.The quantity and quality of the genomic DNA, isolated from thirty different isolates was checked on 0.8% agarose gel electrophoresis. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

Table 39: Spectrophotometrical A_{260}/A_{280} ratio of isolated genomic DNA

Organisms	A_{260}	A_{280}	A_{260}/A_{280}	Organisms	A_{260}	A_{280}	A_{260}/A_{280}
Pseudomonas sp. (B/FS/S12)	0.197	0.137	1.44	*Bacillus sp.* (B/FS/C36)	0.379	0.183	2.07
Bacillus cereus (B/FS/S19)	0.276	0.148	1.86	*Bacillus sp.* (B/FS/C37)	0.276	0.148	1.86
Bacillus sp. (B/FS/T4)	0.379	0.183	2.07	*Bacillus sp.* (B/FS/C38)	0.197	0.137	1.44
Bacillus sp. (B/FS/T5)	0.41	0.2	2.05	*Bacillus sp.* (B/FS/G43)	0.276	0.148	1.86
Bacillus sp. (B/FS/T10)	0.197	0.137	1.44	*Pseudomonas sp.* (B/FS/G44)	0.197	0.137	1.44
Bacillus sp. (B/FS/B14)	0.276	0.148	1.86	*Pseudomonas sp.* (B/RS/M 51)	0.276	0.148	1.86
Bacillus cereus (B/FS/B5)	0.379	0.183	2.07	*Pseudomonas sp.* (B/RS/M 54)	0.379	0.183	2.07
Bacillus cereus (B/FS/B6)	0.276	0.148	1.86	*Pseudomonas sp.* (B/RS/M 55)	0.276	0.148	1.86
Bacillus cereus (B/FS/B21)	0.197	0.137	1.44	*Pseudomonas sp.* (B/RS/M 56)	0.379	0.183	2.07
Bacillus sp. (B/FS/M24)	0.276	0.148	1.86	*Bacillus sp.* (B/RS/M 57)	0.276	0.148	1.86
Bacillus sp. (B/FS/J28)	0.379	0.183	2.07	*Bacillus pumilus* (B/RHS/P22)	0.197	0.137	1.44
Bacillus sp. (B/FS/J29)	0.379	0.183	2.07	*Pseudomonas sp.* (B/RHS/P88)	0.276	0.148	1.86
Pseudomonas sp. (B/FS/J33)	0.197	0.137	1.44	*Pseudomonas sp.* (B/RHS/P89)	0.379	0.183	2.07
Pseudomonas sp. (B/FS/C33)	0.276	0.148	1.86	*Pseudomonas sp.* (B/RHS/P91)	0.276	0.148	1.86
Pseudomonas sp. (B/FS/C34)	0.379	0.183	2.07	*Bacillus sp.* (B/FS/C35)	0.276	0.148	1.86

4.12.2 RAPD patterns

All isolates were taken up for RAPD-PCR amplification. Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100µl, containing 78µl deionized water, 10µl 10X taq polymerase buffer, 1µl of 1U Taq polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template DNA. Three random decamers (OPA1, OPA4, A11) were used to prepare the RAPD profiles of the isolates (Table 40). PCR was programmed with an initial denaturing

at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, 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. After RAPD-PCR amplifications, all amplified DNA products were resolved by electrophoresis on agarose gel(2%) in TAE(1X) buffer, stained with ethidium bromide and photographed. After that all reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed in to similarity matrix using NTSYS computer program to prepare a dendrogram

Table 40: The nucleotide sequence used for RAPD PCR

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
OPA1	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%
A-11	AGGGGTCTTG	10	31.8	76%

Table 41 Analysis of the polymorphism obtained with RAPD markers

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPA1	04	100	2000	0	4	100
2	OPA-4	05	100	1000	0	5	100
3.	A-11	07	100	2000	0	7	100

4.12.3. Phylogenetic analysis

The genetic relatedness among isolated thirty phosphate solubilizer bacterial isolates were analysed by three random primers (OPA1, OPA4, A11) to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of isolates. RAPD profiles showed that primer A11 scored highest bands 7 (Table 41). Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix. The dendogram was generated by unweighted pair- group methods with arithmetric mean (UPGMA) using NTSYSpc software (Plate 26). Similarity co-efficient ranged from 0.65-.00. Based on the results obtained all nine isolates can be grouped into 8main clusters.

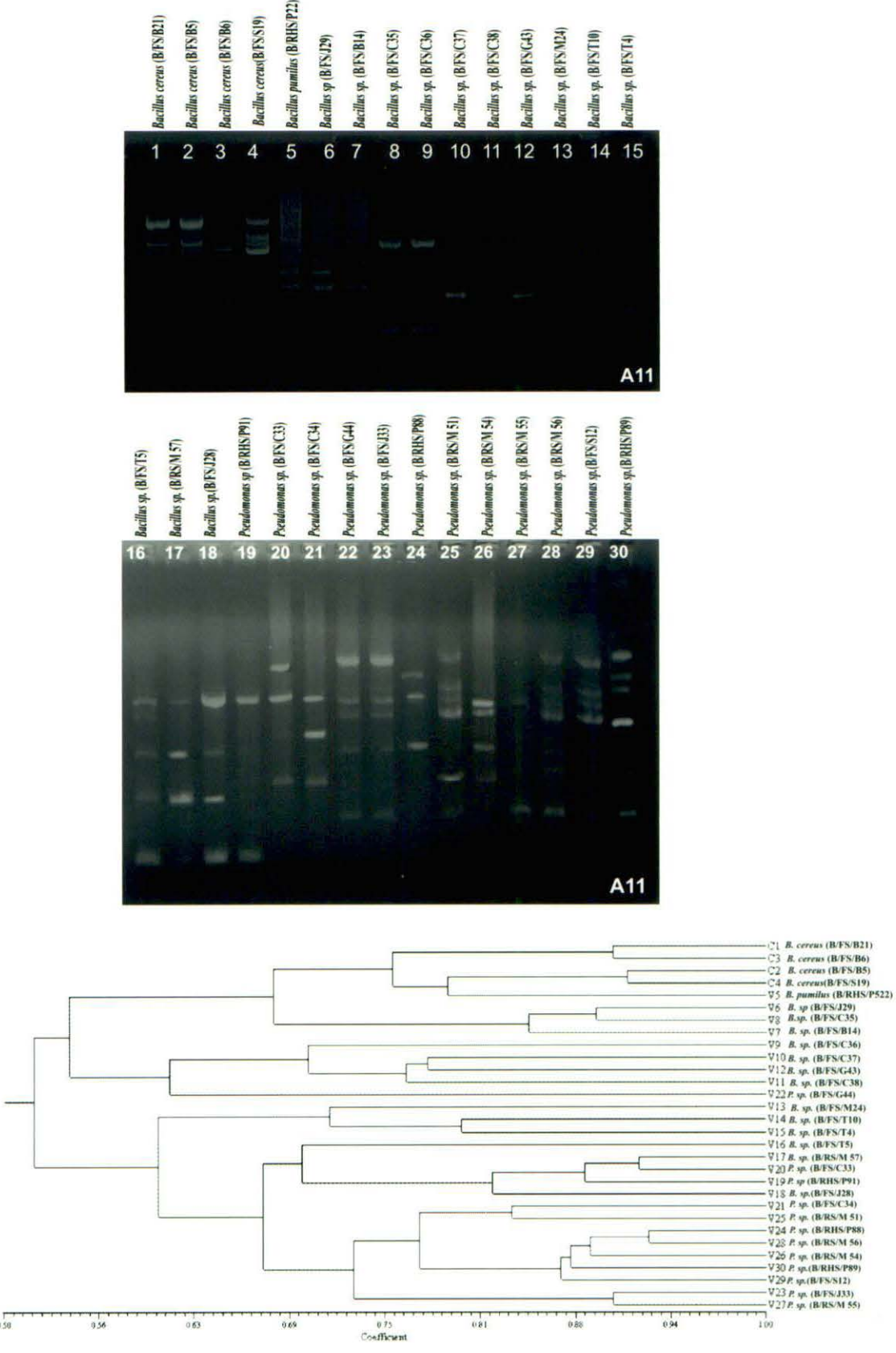


Plate 26: RAPD analysis and dendogram of phosphate solubilizing bacterial isolates obtained from forest soil, riverine soil and rhizosphere of agricultural fields.

4.13 Diversity analysis of actinomycetes isolates

4.13.1 Isolation and purification of genomic DNA from actinomycetes isolates

The broth cultures of actinomycetes isolates showing phosphate solubiling activities *in vitro* [*Streptomyces viridis,* isolates A/FS/S1, A/FS/J2, *Streptomyces griseus* isolates A /RHS/R79, A /RHS/PO26, A/FS/B14, A/FS/B21, *Streptomyces.* sp.A/FS/J28, A /RHS/P92, A /RHS/P93,A /RHS/P94] were centrifuged at 10,000 rpm at 28°C for 5 mins and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5ml of lysis solution (100mM Tris Hcl, pH 7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme). To it 5 µl of RNase (50mg/ml) was added and incubated at 37°C for 3 hrs. Then 10 µl proteinase K solution (20mg/ml) was added and it was allowed to incubate at 65C for 3min. The lysate was extracted with equal volume of tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 mins at 4°C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4°C.

Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse. After incubation the sample was re-extracted with PSI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol. The yield of DNA was determined spectrophotometrically as 24 µg/g of mycelial mat. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio (Table 42) and DNA quantity was evaluated by 0.8% agarose gel electrophoresis. The quantity and quality of the genomic DNA, isolated from nine different isolates was checked on 0.8% agarose gel electrophoresis. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

Table 42: Spectrophotometrical A_{260}/A_{280} ratio of isolated genomic DNA

Organisms	A_{260}	A_{280}	A_{260}/A_{280}
S. viridis. (A/FS/S12)	0.276	0.148	1.86
S. viridis (A/FS/J29)	0.379	0.183	2.07
S. griseus (A/FS/B14)	0.379	0.183	2.07
S. griseus (A /RHS/PO26)	0.197	0.137	1.44
S. griseus(A /RHS/R79)	0.276	0.148	1.86
S. griseus (A/FS/B21)	0.276	0.148	1.86
Streptomyces. sp. (A /RHS/P92)	0.276	0.148	1.86
Streptomyces. sp.(A /RHS/P94)	0.276	0.148	1.86
Streptomyces. sp.(A/FS/J28)	0.197	0.137	1.44
*Streptomyces.*sp.(A /RHS/P93)	0.379	0.183	2.07

4.13.2 RAPD patterns

All isolates were taken up for RAPD-PCR amplification. Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100µl, containing 78µl deionized water, 10µl 10X taq polymerase buffer, 1µl of 1U Taq polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template DNA. Three random decamers (OPA1, OPA4) (Table 43) were used to prepare the RAPD profiles of the isolates. PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, 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. After RAPD-PCR amplifications, all amplified DNA products were resolved by electrophoresis on agarose gel(2%) in TAE(1X) buffer, stained with ethidium bromide and photographed. After that all reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed in to similarity matrix using NTSYS computer program to prepare a dendrogram.

Table 43 : The nucleotide sequence used for RAPD PCR

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
OPA1	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%

Table 44 Analysis of the polymorphism obtained with RAPD markers

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPA1	8	100	2000	0	8	100
2	OPA4	12	100	1000	0	12	100

4.13.3. Phylogenetic analysis

The genetic relatedness among isolated isolates were analysed by three random primers (OPA1, OPA4) to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of isolates. RAPD profiles showed that primer OPA4 scored highest bands (12) (Table 44). Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix. The dendogram was generated by unweighted pair- group methods with arithmetric mean (UPGMA) using NTSYSpc software (Plate 27). Similarity co-efficient ranged from 0.65-1.00. (table 45)Based on the results obtained all isolates can be grouped into 4 main clusters.

Table 45 RAPD-based genetic similarity within groups

1	2	3	4	5	6	7	8	9	10
1.0000000									
0.2857143	1.0000000								
0.1428571	0.8000000	1.0000000							
0.4000000	0.3333333	0.1666667	1.0000000						
0.6000000	0.2857143	0.3333333	0.4000000	1.0000000					
0.1666667	0.6000000	0.7500000	0.2000000	0.4000000	1.0000000				
0.2857143	1.0000000	0.8000000	0.3333333	0.2857143	0.6000000	1.0000000			
0.5714286	0.7142857	0.5714286	0.4285714	0.5714286	0.4285714	0.7142857	1.0000000		
0.4000000	0.3333333	0.4000000	0.2000000	0.4000000	0.2000000	0.3333333	0.4285714	1.0000000	
0.6000000	0.2857143	0.3333333	0.1666667	0.6000000	0.4000000	0.2857143	0.5714286	0.4000000	

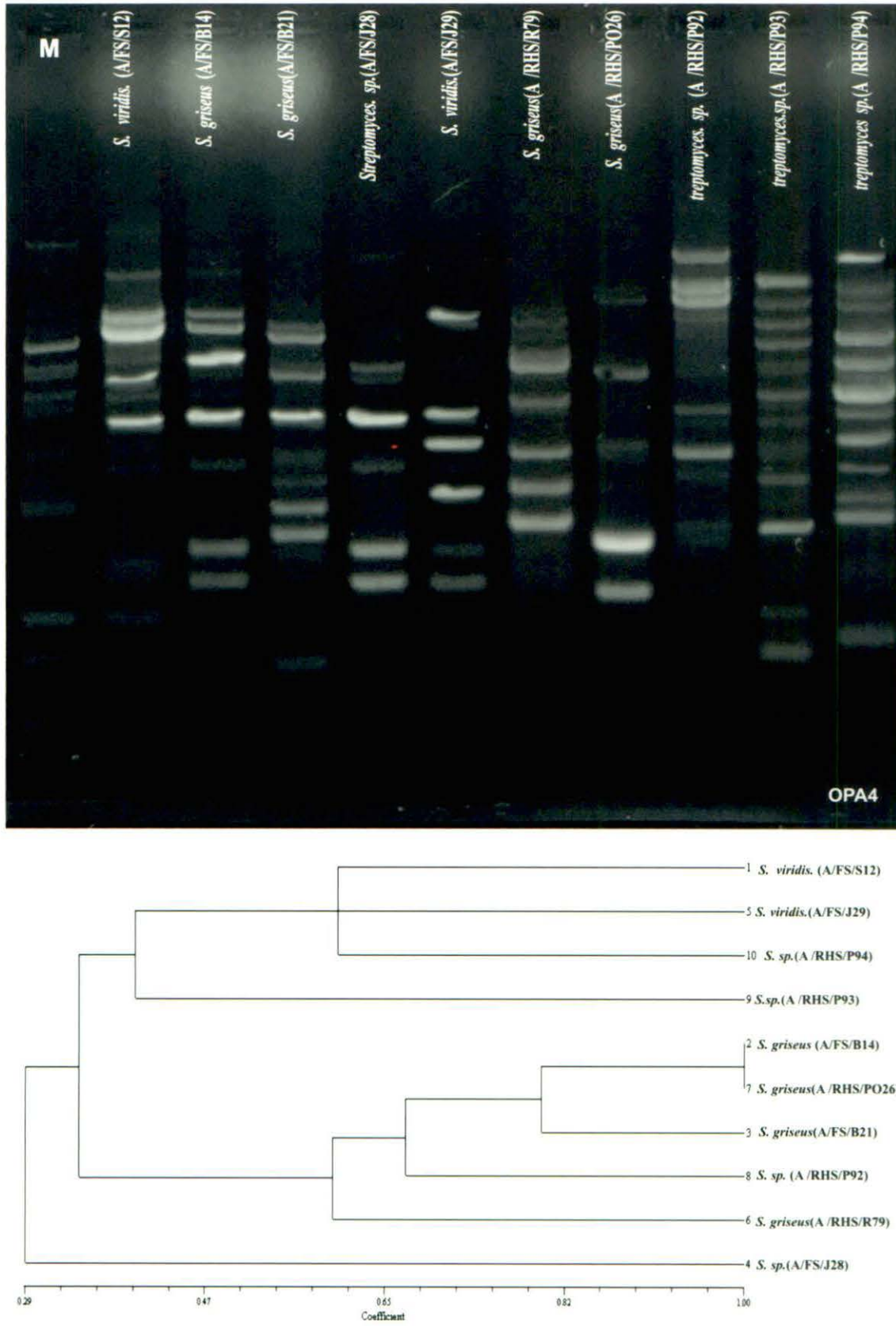


Plate 27: RAPD analysis and dendogram of phosphate solubilizing actinomycetes isolates obtained from forest soil, riverine soil and rhizosphere of agricultural fields.

4.14 Genomic DNA preparation and quantification of isolates of *Trichoderma harzianum* and *Trichoderma viride*

Genomic DNA was prepared from of isolates of *T. viride* (FS/L-20, FS/S-473, FS/S-474, FS/S-475, FS/S-478, RHS/T- 460, RHS/T- 463, RHS/T- 472) and *T. hatzianum* (FS/C-90, FS/S-455, FS/S-458, RHS/T- 477, RHS/AC480, RHS/AC481, RHS/AC482, RHS/AC483, Ag/S476, Ag/S471, Ag/S479). The mycelia 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 60^{0}C followed by centrifugation at 12,000 rpm for 15 min. 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 centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ehanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

Quantitative estimation of DNA

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 (Table 46). The DNA from all isolates produced clear sharp bands, indicating good quality of DNA. The result revealed that RNA free DNA was yielded and the size of DNA of each isolates ranging from 1.5-1.8 kb.

Table 46: Spectrophotometrical A_{260}/A_{280} ratio of isolated genomic DNA

Organisms	Ioslate Code	NAIMCC acc.no.*	A_{260}	A_{280}	A_{260}/A_{280}
T. viride	FS/L-20	NAIMCC-F-01949	0.356	0.191	1.86
	FS/S-473	NAIMCC-F-0157	0.456	0.243	1.88
	FS/S-474	NAIMCC-F-01958	0.647	0.259	2.50
	FS/S-475	NAIMCC-F-01959	0.247	0.165	1.50
	FS/S-478	NAIMCC-F-01960	0.348	0.173	2.01
	RHS/T- 460	NAIMCC-F-01953	0.244	0.16	1.53
	RHS/T- 463	NAIMCC-F-01954	0.319	0.182	1.75
	RHS/T- 472	NAIMCC-F-01956	0.272	0.173	1.57
T. harzianum	FS/C-90	NAIMCC-F-01950	0.197	0.137	1.44
	FS/S-455	NAIMCC-F-01955	0.276	0.148	1.86
	FS/S-458	NAIMCC-F-01952	0.379	0.183	2.07
	RHS/T- 477	NAIMCC-F-01962	0.41	0.2	2.05
	Ag/S476	NAIMCC-F-01966	0.38	0.188	2.02
	Ag/S471	NAIMCC-F-01967	0.213	0.139	1.53
	Ag/S479	NAIMCC-F-01968	0.24	0.147	1.63
	RHS/AC480	NAIMCC-F-01961	0.523	0.259	2.02
	RHS/AC481	NAIMCC-F-01963	0.228	0.145	1.57
	RHS/AC482	NAIMCC-F-01964	0.275	0.16	1.72
	RHS/AC483	NAIMCC-F-01965	0.424	0.24	1.77

*National Agriculturally Important Microbial Culture Collection (NAIMCC)

4.15. PCR-RAPD analysis of isolates of *Trichoderma harzianum* and *T. viride*

Six random primers i.e. OPA-1; OPD-6; OPA-4; A-5; AA-04 and AA-11 were selected for the diversity analysis of *Trichoderma harzianum* and *T. viride* (Table-47). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles 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. 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 by horizontal electrophoresis.

Scoring and data analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostastiscs, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. 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. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc . The genetic relatedness among isolates of eight *Trichoderma viride* and isolates of eleven *Trichodema hurzianum* were analyzed by six random primers OPA-1, OPD-6, OPA-4, A-5, AA-04 and AA-11 to generate reproducible polymorphisms. All amplified products with the primers had shown

polymorphic and distinguishable banding patterns which indicate the genctic diversity of *Trichoderma* isolates. A total of 73 reproducible and scorable polymorphic bands ranging from approximately 100bp to 2000bp were generated with six primers among the nineteen *Trichoderma* isolates (Table B). RAPD profiles showed that primer A-5 scored highest bands which ranged between 100bp to 2000bp. Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix (Table 48). The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using BIO Profil 1D image software for each primer (Plate 28-31) and NTSYSpc software (Plate 32& 33.) (table 49). Based on the results obtained all the nineteen isolates can be grouped into two main clusters. One cluster represents *T.viride* and other *T harzianum*. Again the *T.viride* cluster is also subgrouped into two. First subgroup with four isolates and second one is with seven isolates of two subclusters. The cluster of *T harzianum* divided into two different cluster contains four different isolates (Fig 8).

Table 47 : The nucleotide sequence used for ITS and RAPD PCR

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%

Table 48 Analysis of the polymorphism obtained with RAPD markers in 19 *Trichoderma* isolates.

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	A-11	09	100	2000	0	9	100
2	OPA-4	12	100	1000	0	12	100
3.	A-5	18	100	2000	0	18	100
4.	OPD-6	14	100	1000	0	14	100
5.	AA-04	11	100	1000	0	11	100
6.	OPA1	09	100	1000	0	09	100
	Total	73			0	73	100

Table 49: RAPD-based genetic similarity within groups

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.00																		
2	1.00	1.00																	
3	0.90	0.90	1.00																
4	0.81	0.81	0.90	1.00															
5	0.58	0.58	0.66	0.75	1.00														
6	0.50	0.50	0.58	0.66	0.90	1.00													
7	0.58	0.58	0.66	0.75	1.00	0.90	1.00												
8	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00											
9	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00	1.00										
10	0.63	0.63	0.72	0.66	0.90	0.80	0.90	0.90	0.90	1.00									
11	0.54	0.54	0.63	0.58	0.80	0.88	0.80	0.80	0.80	0.88	1.00								
12	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.285	0.30	1.00							
13	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00						
14	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00	1.00					
15	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00	1.00	1.00				
16	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00			
17	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00		
18	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00	1.00	
19	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00	1.00	1.00

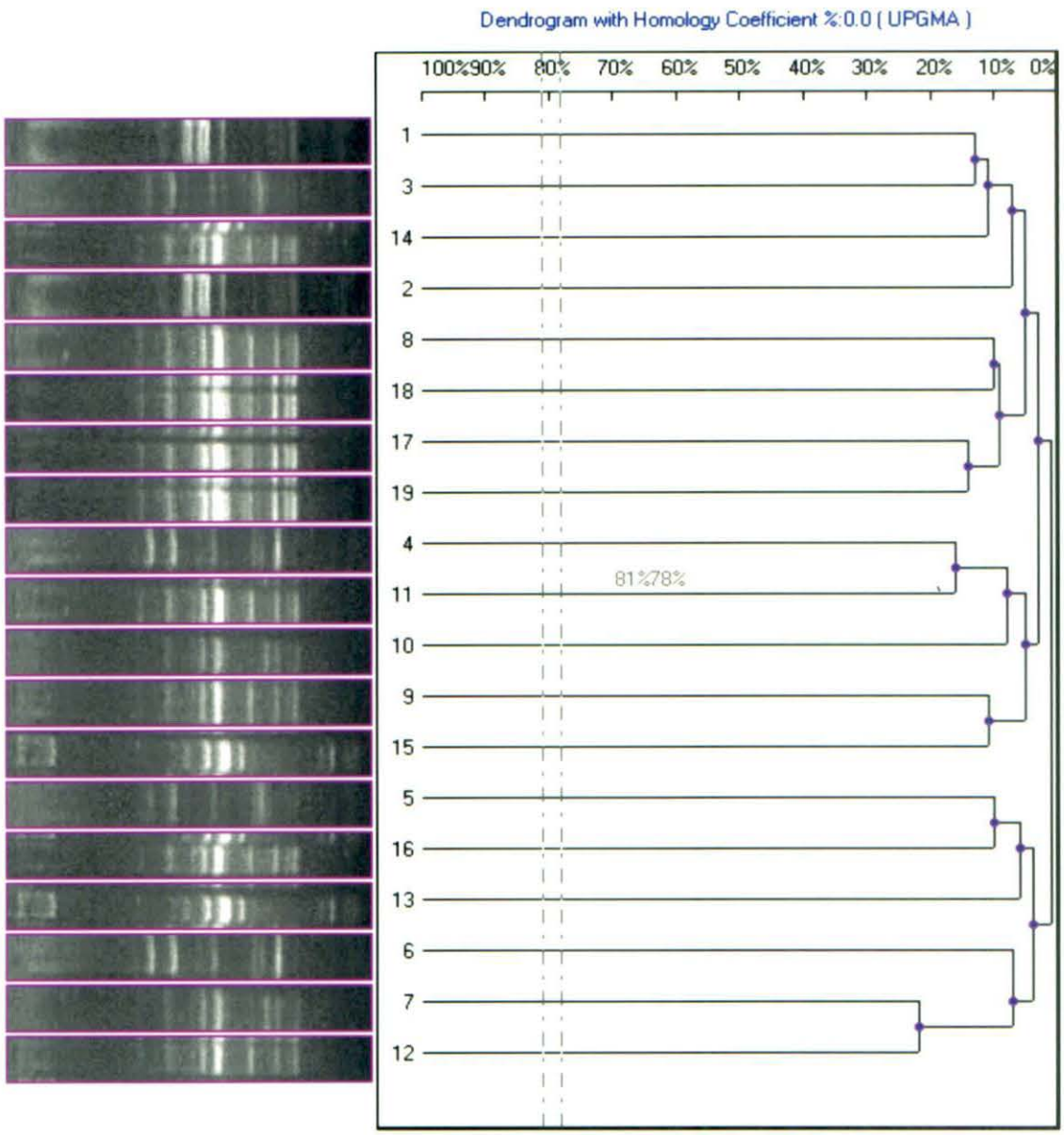


Plate 28: RAPD analysis of *Trichoderma* isolates with A-04 primer in BioProfile software.(1. FS/L-20, 2. FS/S-473,3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T- 460, 7. RHS/T- 463,8. RHS/T- 472, 9. FS/C-90,10.FS/S-455,11.FS/S-458,12.RHS/T-477,13.Ag/S476, 14.Ag/S471, 15.Ag/S479, 16.RHS/AC480, 17.RHS/AC481, 18.RHS/AC482, 19. RHS/AC483)

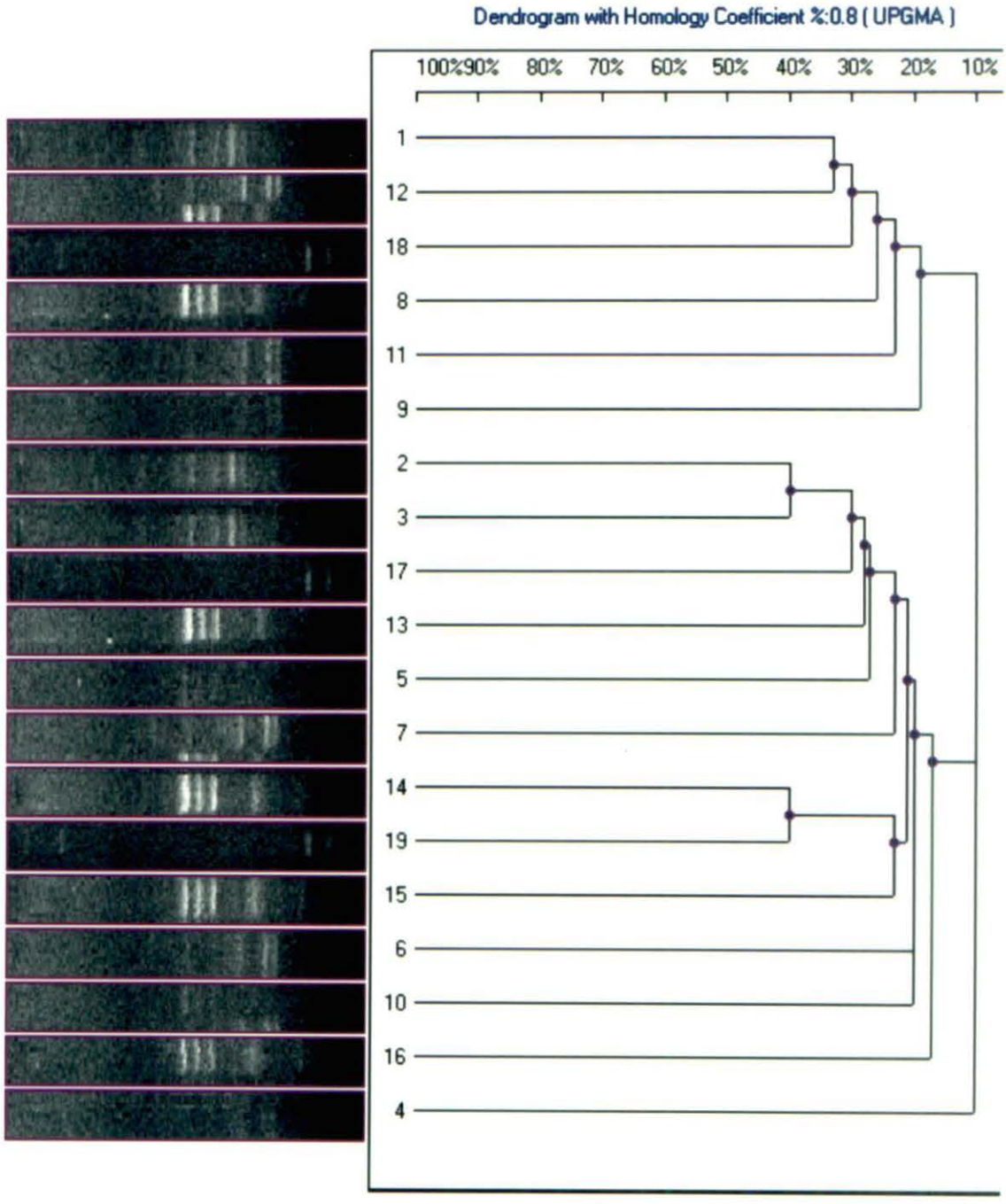


Plate 29: RAPD analysis of *Trichoderma* isolates with OPD6 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T-460, 7. RHS/T- 463, 8. RHS/T- 472, 9. FS/C-90,10. FS/S-455, 11. FS/S-458, 12. RHS/T-477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480, 17. RHS/AC481, 18. RHS/AC482, 19. RHS/AC483)

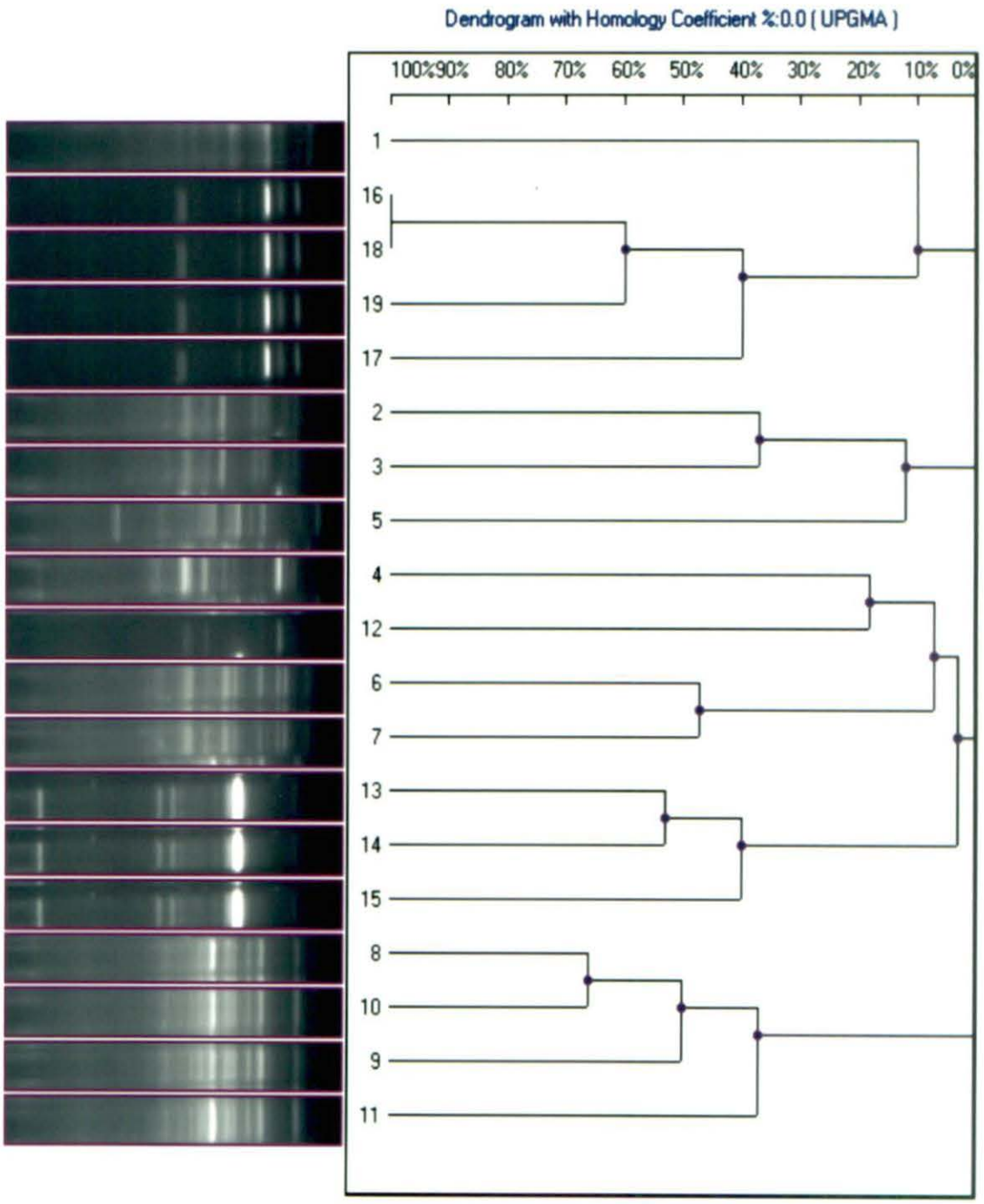


Plate 30: RAPD analysis of *Trichoderma* isolateswith A5 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T- 460, 7. RHS/T- 463,8. RHS/T- 472, 9. FS/C-90, 10. FS/S-455, 11. FS/S-458, 12. RHS/T- 477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480, 17. RHS/AC481, 18. RHS/AC482, 19. RHS/AC483)

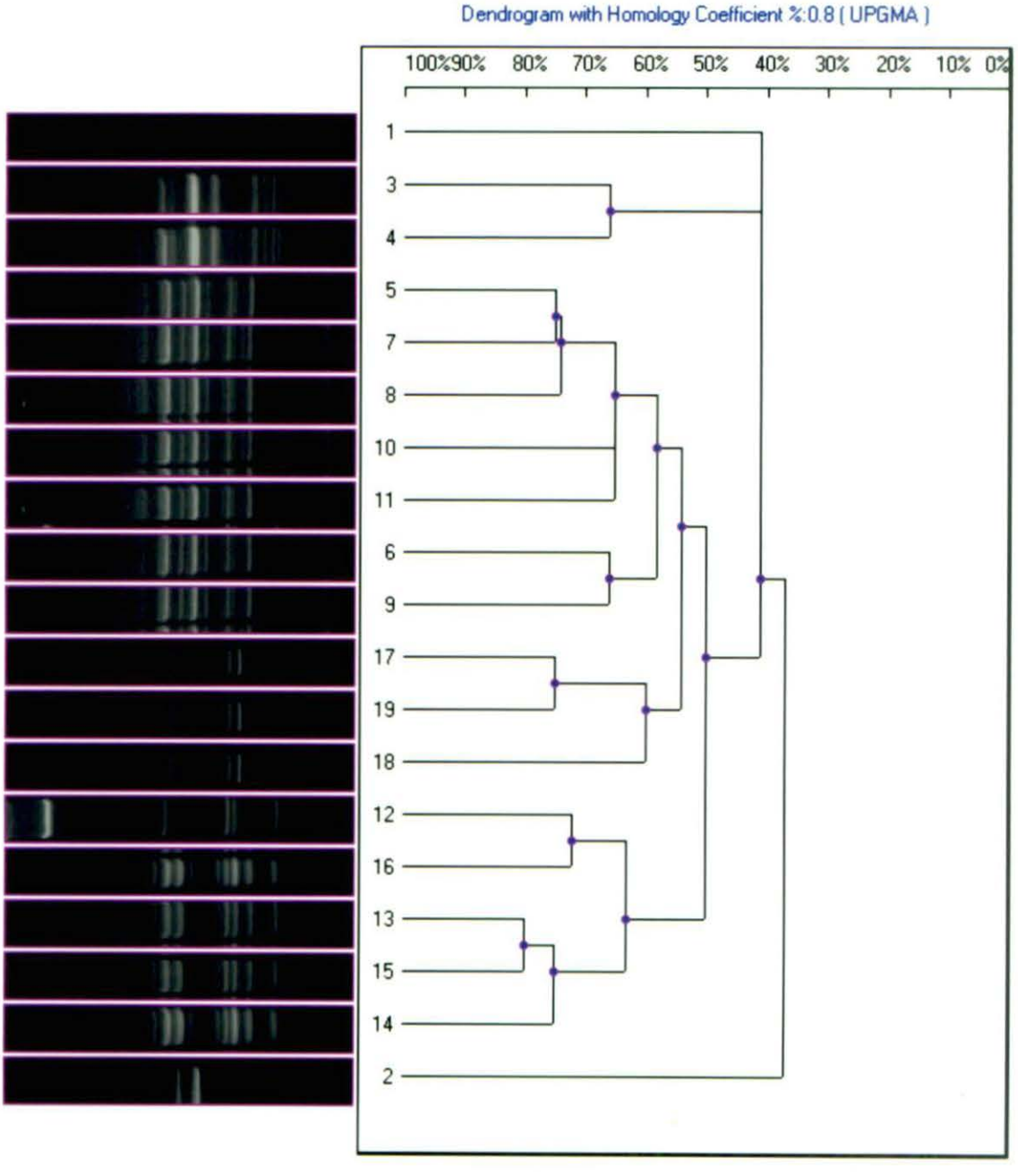


Plate 31: RAPD analysis of *Trichoderma* isolates with AA-11 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T-460, 7. RHS/T- 463, 8. RHS/T- 472, 9. FS/C-90, 10. FS/S-455, 11. FS/S-458, 12. RHS/T-477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480, 17. RHS/AC481,18. RHS/AC482, 19. RHS/AC483)

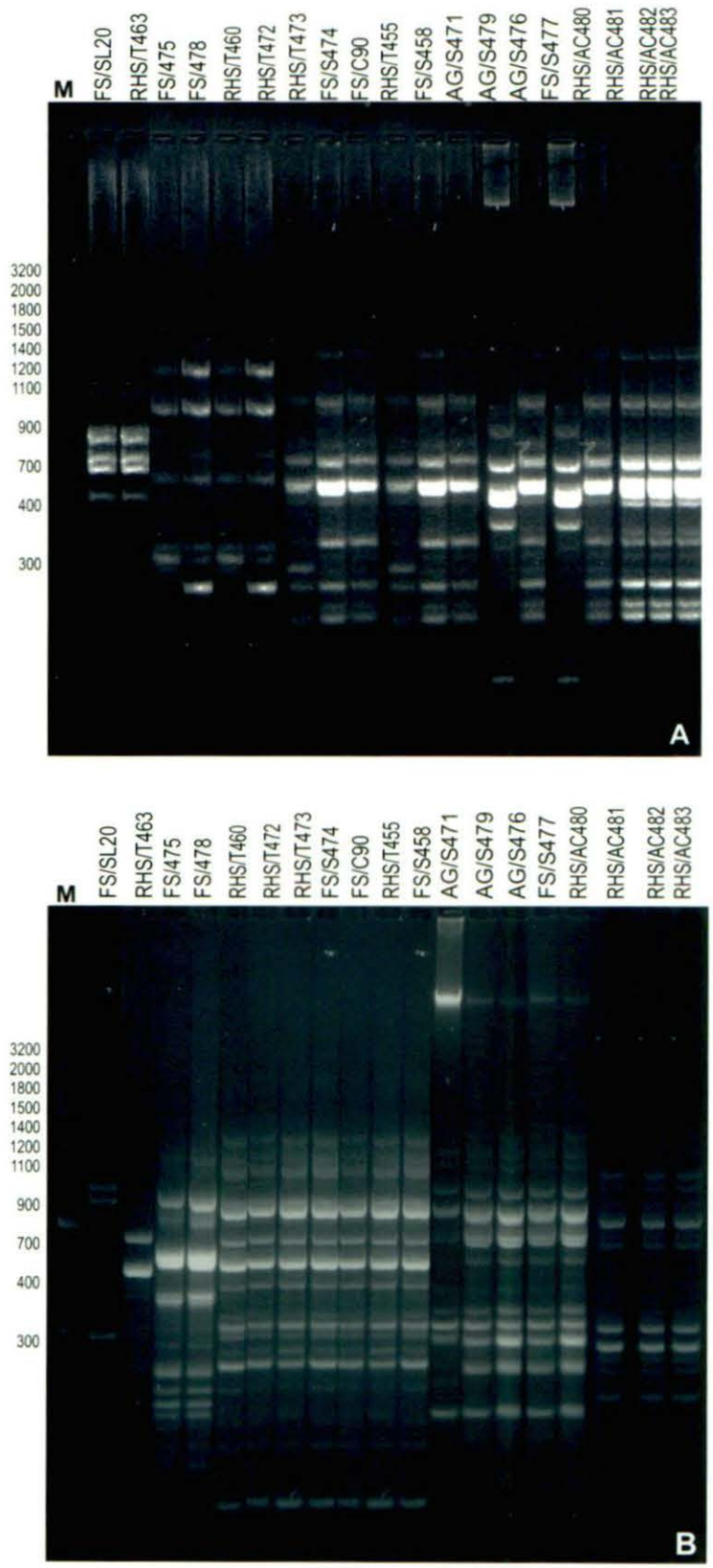


Plate 32: RAPD analysis of 19 *Trichoderma* isolates using A-5 primer (A) and DPD-6 primer (B)

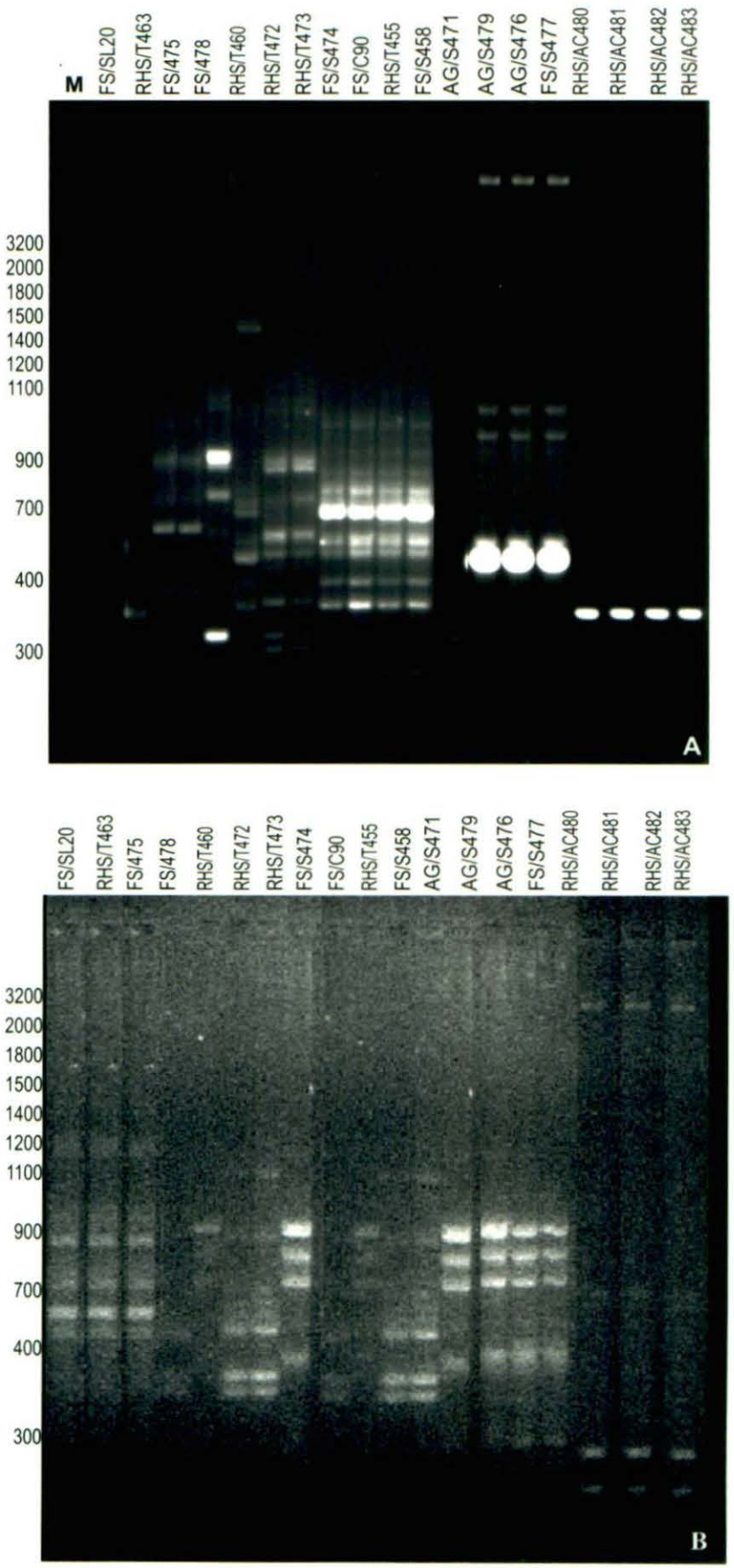


Plate 33: RAPD analysis of 19 *Trichoderma* isolates using A-11 primer (A) and OPA Primer (B)

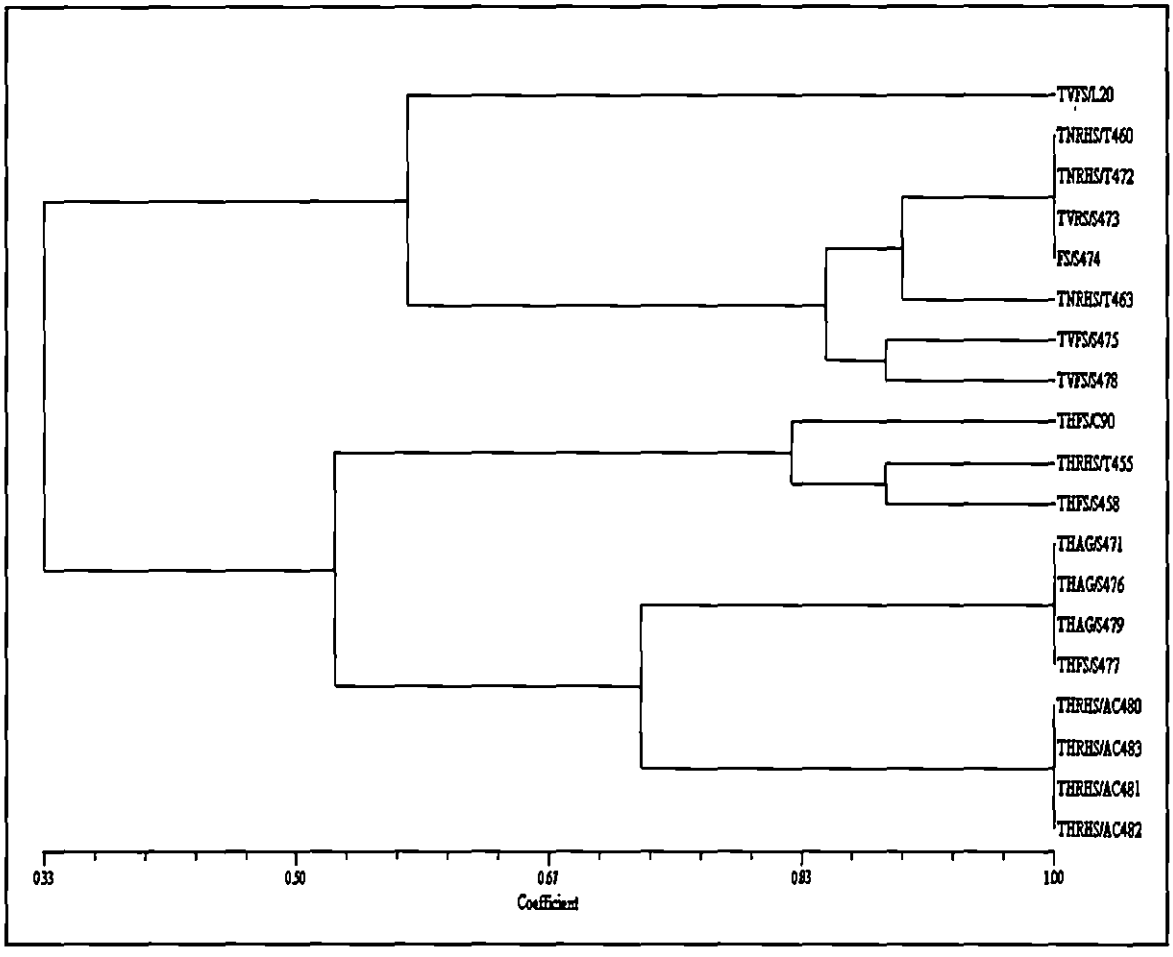


Figure 8: RAPD analysis by Dendogram of *Trichoderma* isolates

4.16. Internal Transcribed Spacer (ITS) amplification of *Trichoderma* isolates and their analyses

Nineteen isolates of *Trichoderma sp.* [*T. viride* (FS/L-20, FS/S-473, FS/S-474, FS/S-475, FS/S-478, RHS/T- 460, RHS/T- 463, RHS/T- 472) and *T. hatzianum* (FS/C-90, FS/S-455, FS/S-458, RHS/T- 477, RHS/AC480, RHS/AC481, RHS/AC482, RHS/AC483, Ag/S476, Ag/S471, Ag/S479] of which were taken up for ITS-PCR amplification. 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. For amplification of the ITS1–5.8S–ITS2 region of *Trichoderma* isolates, the primer pair T/ITS1 TCTGTAGGTGAACCTGCGG and T/ITS4 TCCTCCGCTTATTGATATGC was used. 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 examining by with horizontal electrophoresis. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 600bp was produced by the primers. (Plate 34). A single distinct DNA bands was observered on the gel for each isolates. Midium range of DNA rular (Genei, Bangalore) was used in the marker lane. The purified PCR products of seven isolates of *Trichoderma harzianum* (NAIMCC-F-01950, NAIMCC-F-01955, NAIMCC-F-01952, NAIMCC-F-01962, NAIMCC-F-01966, NAIMCC-F-01967, NAIMCC-F-01968) were sequenced bidirectionally in Applied Biosystems by Bangalore Genei. Partial sequence of ITS region of rDNA of above mentioned seven isolates of *T. harzianum* have been presented in Figures 9 to 15.

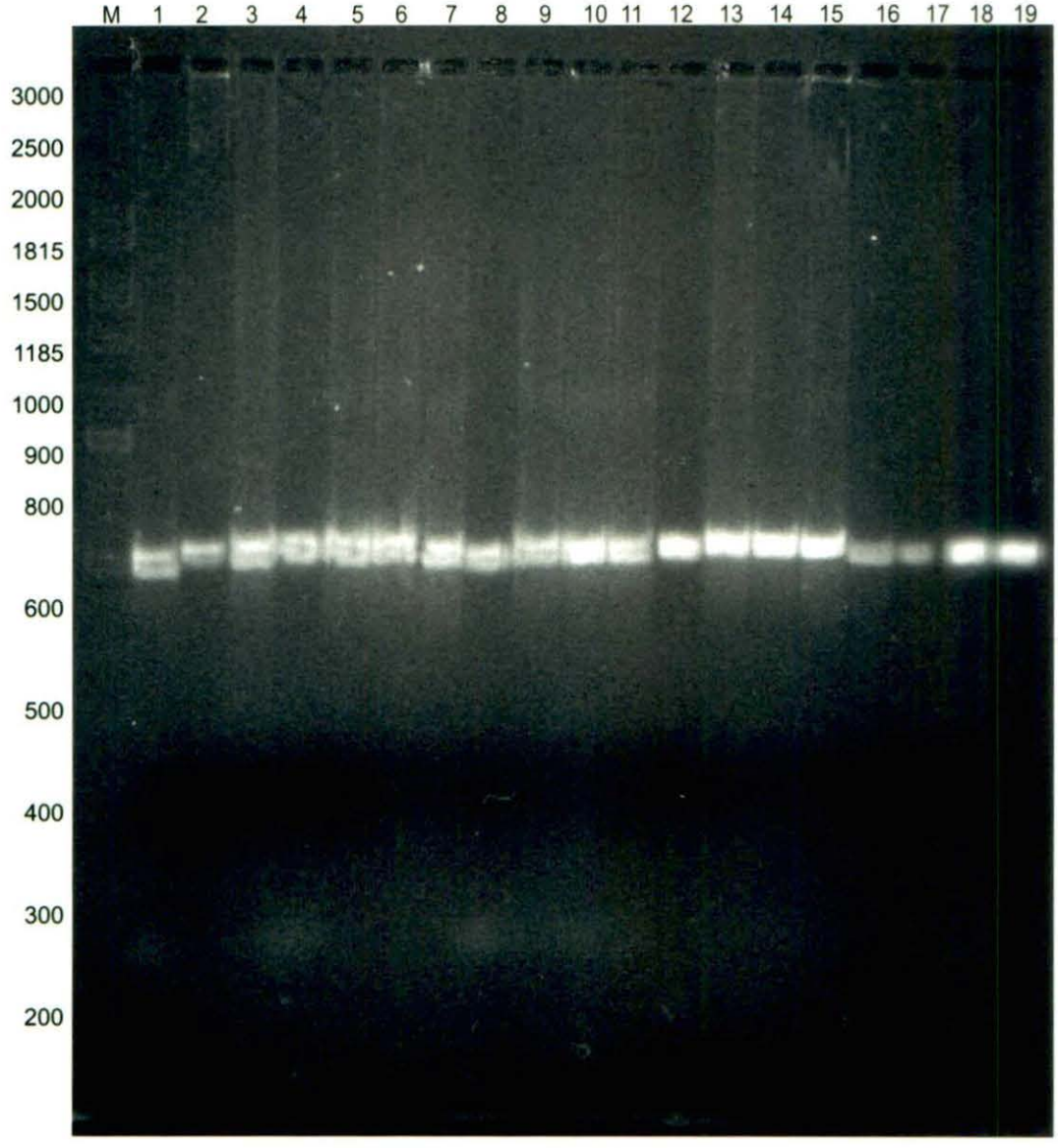
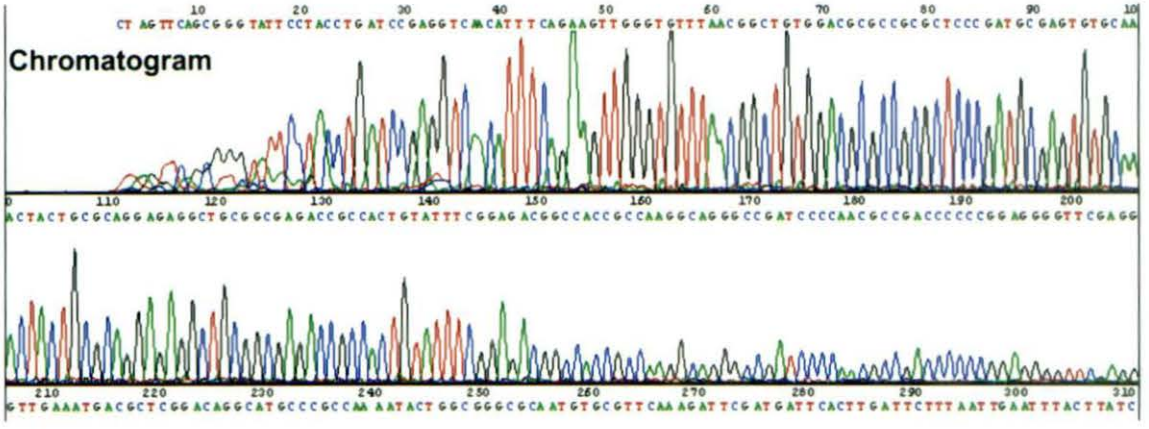


Plate 34: ITS PCR *Trichoderma* isolates (Marker (M), (1) FS/L-20, (2) FS/S-473, (3) FS/S-474, (4) FS/S-475,(5) FS/S-478, (6) RHS/T- 460, (7) RHS/T-463, (8) RHS/T- 472, (9) FS/C-90, (10) FS/S-455, (11) FS/S-458, (12) RHS/T-477, (13) Ag/S476, (14) Ag/S471, (15) Ag/S479, (16) RHS/AC480, (17) RHS/AC481, (18) RHS/AC482, (19) RHS/AC483)



Partial sequence of ITS 4 region of rDNA

CTAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACATTTCAGAAGTTGG
GTGTTTAACGGCTGTGGACGCGCCGCGCTCCCGATGCGAGTGTGCAAACTA
CTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGACGGCC
ACTGCCAAGGCAGGGCCGATCCCCAACGCCGCCCCCCCGGAGGGGTTCGA
GGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAAAATACTGGCGGGCGC
AATGTGCGTTCAAAGATACGATGATTCACTTGATTCTTTAATTGAATTTACTT
ATCTTATTTCGCTCATTTTCGTCCGCCATGATTCAGGGCGGGAGATGCTTGTG
CTAACTTTTTTTCCACCCTCCAAACCCCTCGGGGGGAGGCGCTTAGAATATT

Sequence Deposited: NCBI

ACCESSION: GU564469

VERSION: GU564469.1

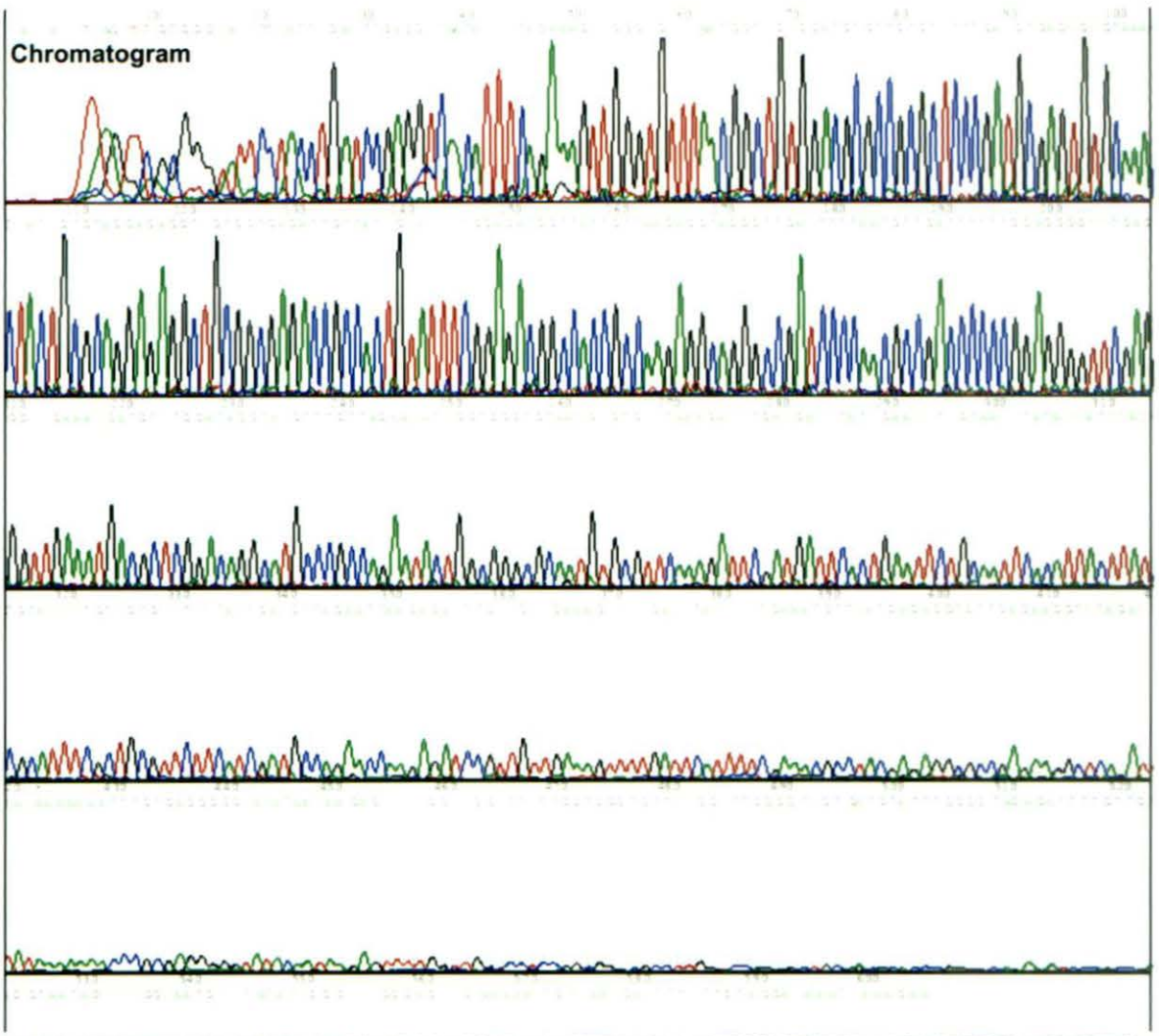
GI:291194458

DNA linear : 521 bp

Title : *Hypocrea lixii* strain AG/S471 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

```
ORIGIN
        1 tttaccggcg ggggactccc ccccccgggg ggtggaagcc ccagaaccag gggggggggg
       61 gagggagcca acccaactat ttatttttagg ccccccaccc atataggtga atattctaag
      121 cgcctccccc cgaggggttt ggagggtgga aaaaaagtta gcacaagcat ctcccgccct
      181 gaatcatggc ggacgaaaat gagcgaaata agataagtaa attcaattaa agaatcaagt
      241 gaatcatcgt atctttgaac gcacattgcg cccgccagta ttttggcggg catgcctgtt
      301 cgagcgtcat ttcaacccctc gaaccccctcc gggggggcgg cgttggggat cggccctgcc
      361 ttggcagtgg ccgtctccga aatacagtgg cggtctcgcc gcagcctctc ctgcgcagta
      421 gtttgcacac tcgcatcggg agcgcggcgc gtccacagcc gttaaacacc caacttctga
      481 aatgttgacc tcggatcagg taggaatacc cgctgaacta g
```

Figure 9 : Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01967)



AACGAGTTCGCGGGTATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTAACGGCTGTGGACGCGCCG
CGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGACGGCCTC
CGCCAAGAGGCAGGGCCGATCCCCAACCCCGACCCCCCGGAGGGGTTCGAGGGTTGAAATGACGATCGGACAGGCAT
GCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATC
GCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTCGAAACGCCTAC

Sequence Deposited: NCBI

ACCESSION: GU187914

VERSION: GU187914.1

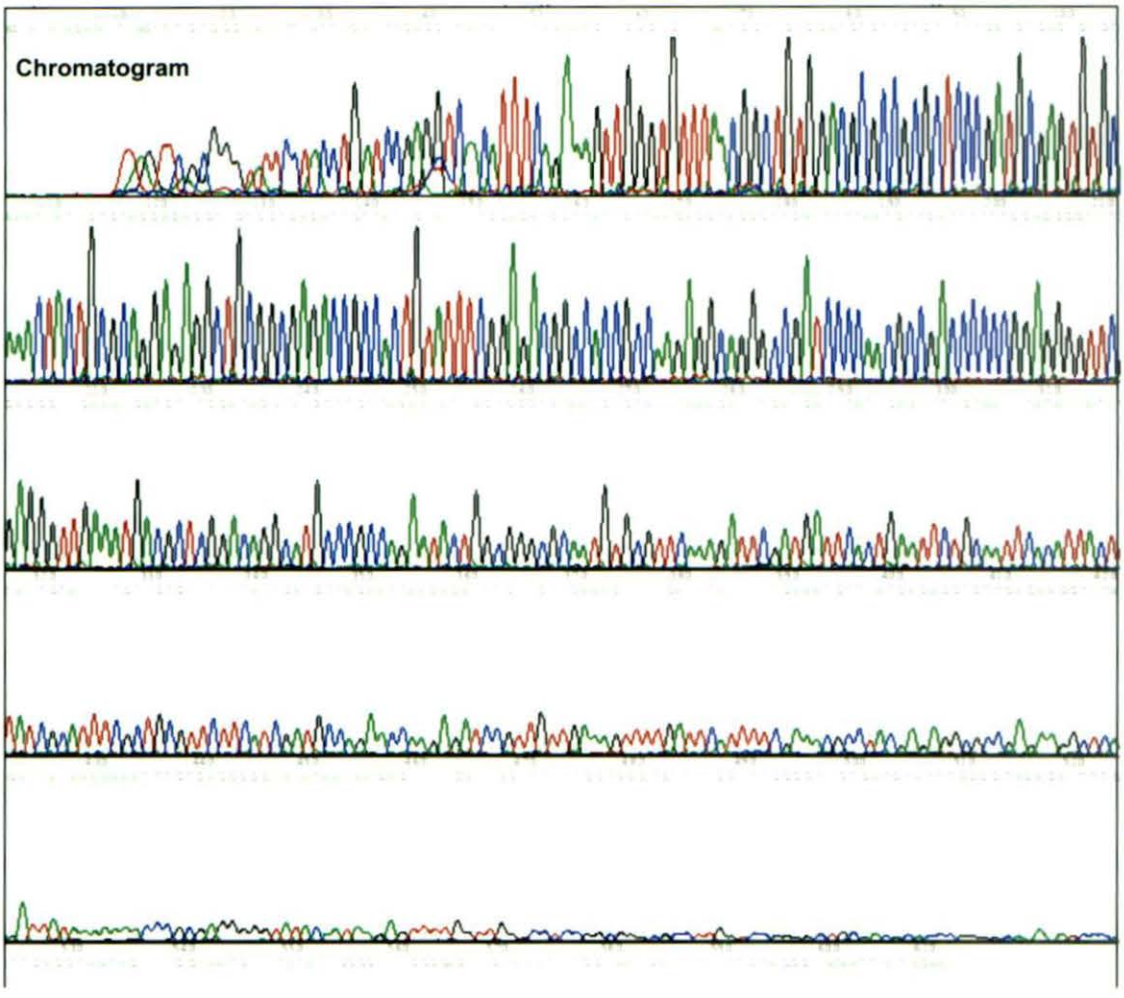
GI:270271228

DNA linear : 604 bp

Title : *Hypocrea lixii* strain FS/C-90 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed

```
  1 ttctttagtt tatcctgcgg agagatcatt agcgattttt acaactccca aacccaatgt
 61 gaacgttacc aaaactgttg cctcgtcggg acctctgccc cgggtgcgtc gcagccccgg
121 accaaggcgc ccgcctgagg accaaccaaa actcttattg tatacccct cgcgggttat
181 tttataatct gagccttctc ggcgcctctc gtaggcgttt cgaaaatgaa tcaaaacttt
241 caacaacgga tctcttggtt ctggcatcga tgaagaacgc aacgaaatgc gataagtaat
301 gtgaattgca gaattcagtg aatcatcgaa tctgtgaacg cacattgcgc ccgccagtat
361 tctggcgggc atgcctgtcc gatcgtcatt tcaaccctcg aacccctccg gggggtcggg
421 gttggggatc ggccctgcct cttggcggcg gccgtctccg aaatacagtg gcggtctcgc
481 cgcagcctct cctgcgcagt agtttgcaca ctcgcatcgg gagcgcggcg cgtccacagc
541 cgttaaacac ccaactttct gaaatgttga cctcggatca ggtaggaata cccgcgaact
601 cgtt
```

Figure 10: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01950)



AGAGAGAACTAGTTCGCGGGTATTCCTACCTGATCCGAGGTCACATTTCAGAAAGTTGGGTGTTTAACGGCTGTG
GACGCGCCGCGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTT
CGGAGATGGCCACCGCCAAGAGGCAGGGCCGATCCCCAACGCCGACCCCCCGGAGGGGTTCGAGGGTTGAAAA
GACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGATCAAAGATTCGATGATTCACTGAAT
TCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGT

Sequence Deposited: NCBI

Title : *Hypocrea lixii* isolate FS/S455 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA

ACCESSION: HM107420

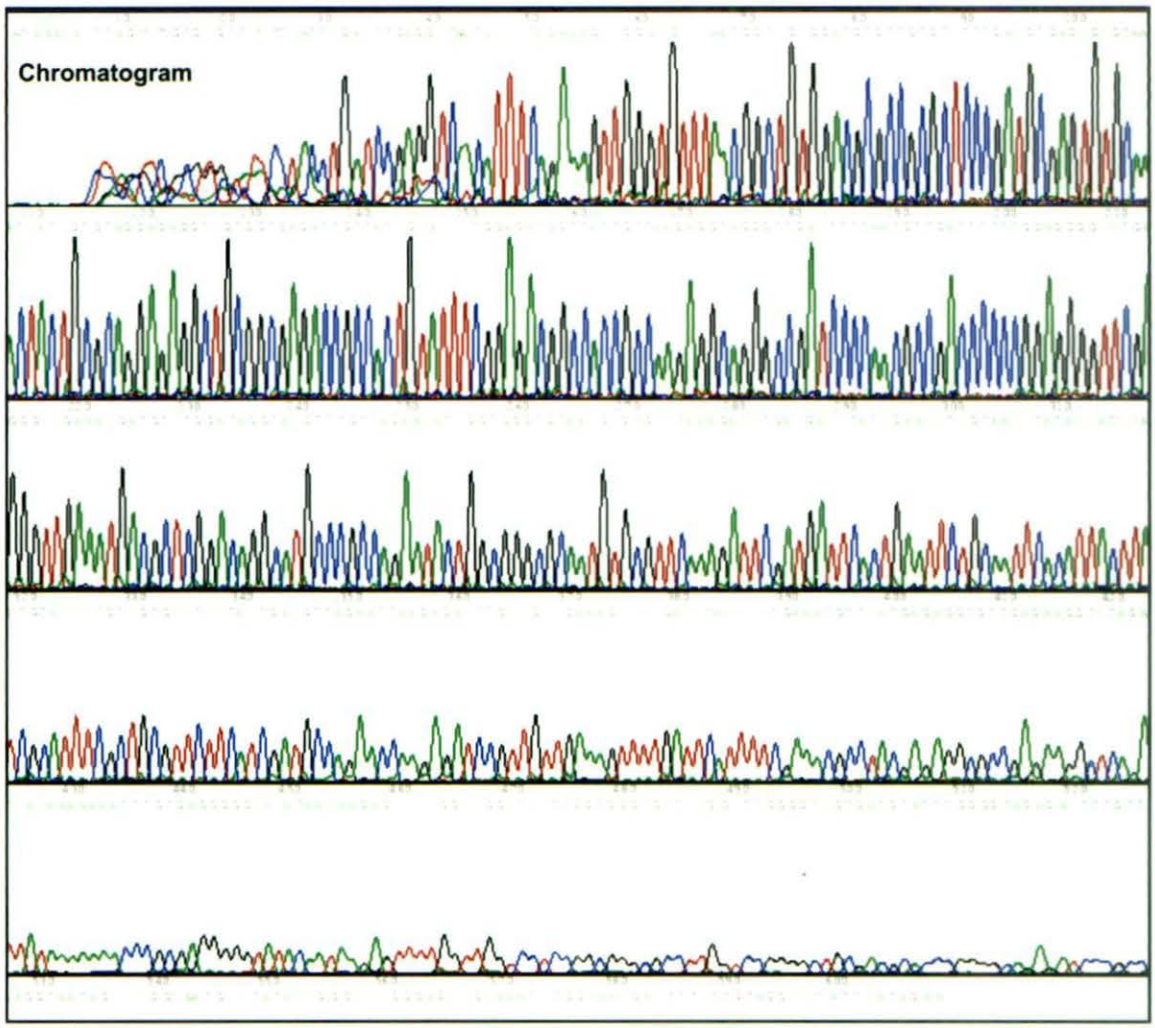
VERSION: HM107420.1

GI:298364291

DNA linear : 611 bp

```
ORIGIN
        1 ttctgtaggt tgtaccctgc ggagggatca ttaccgagtt tacaactccc caaaccccaa
       61 tgtgaacgtt accaaactgt tgcctcggcg ggttctctcc cccgggtgcg tcgcagcccc
      121 ggaccaaggc gcccgccgga ggaccaacca aaactcttat tgtatacccc ctcgcgggtt
      181 tttttataat ctgagccttc tcggcgcctc tcgtaggcgt ttcgaaaatg aatcaaaact
      241 ttcaacaacg gatctcttgg ttctggcatc gatgaagaac gcagcgaaat gcgataagta
      301 atgtgaattg cagaattcag tgaatcatcg aatctttgat cgcacattgc gcccgccagt
      361 attctggcgg gcatgcctgt ccgagcgtct tttcaaccct cgaacccctc cgggggggtcg
      421 gcgttgggga tcggccctgc ctcttggcgg tggccatctc cgaaatacag tggcggtctc
      481 gccgcagcct ctcctgcgca gtagtttgca cactcgcatc gggagcgcgg cgcgtccaca
      541 gccgttaaac acccaacttt ctgaaatgtg acctcggatc aggtaggaat acccgcgaac
      601 tagttctctc t
```

Figure 11: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain



Partial sequence of ITS 4 region of rDNA

ACAGAACCAGTTCTGCGTGTCTTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTAACGGCTGTGGAC
GCGCCGCGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGA
TGGCCACCGCCAAGAGGCAGGGCCGATCCCCAACGCCGACCCCCCCGGAGGGGTTCGAGGGTTGAAAAGACGCTCGG
ACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGATCAAAGATTCGATGATTCACTGAATTCTGCAATTCACA
TTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTCGA
AACGCCTACGAGAGGCGCCGAGAAGGCTCAGATTATAAAAAAACCCGCGAGGGGGTATACAATAAGAGTTTTGGTTGG
TCCTCCGGCGGGCGCCTTGGTCCGGGGCTGCGACGCACCCGGGGGAGAGAACCCGCCGAGGCAACAGTTTGGTAACG
TTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAGGTTTCACCTACAGAA

Sequence Deposited: NCBI

ACCESSION: HM107421

VERSION: HM107421.1

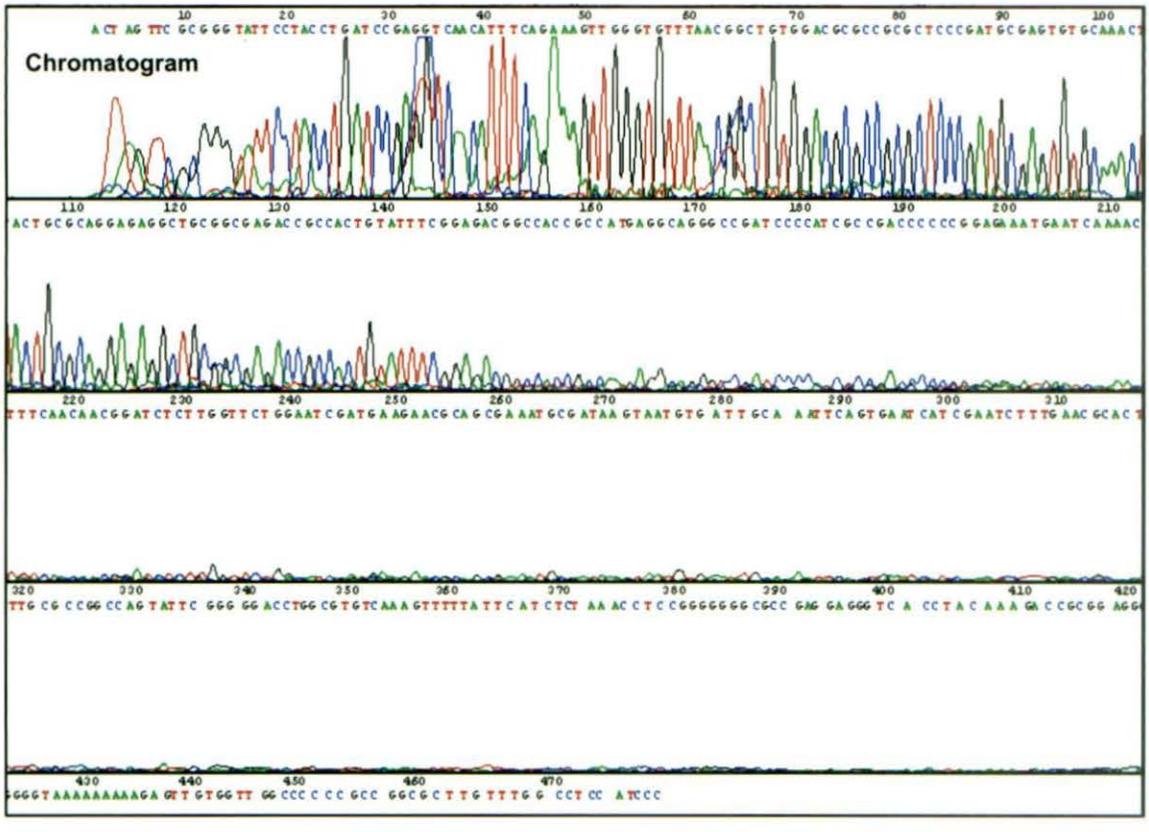
GI:298364292

DNA linear :608 bp

Title : *Hypocrea lixii* isolate FS/S458 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,

```
ORIGIN
        1 ttctgtaggt gaaacctgcg gagggatcat taccgagttt acaactccca aacccaatgt
       61 gaacgttacc aaactgttgc ctcggcgggt tctctccccc gggtgcgtcg cagccccgga
      121 ccaaggcgcc cgccggagga ccaaccaaaa ctcttattgt atacccctc gcgggttttt
      181 ttataatctg agccttctcg gcgcctctcg taggcgtttc gaaaatgaat caaaactttc
      241 aacaacggat ctcttggttc tggcatcgat gaagaacgca gcgaaatgcg ataagtaatg
      301 tgaattgcag aattcagtga atcatcgaat ctttgatcgc acattgcgcc cgccagtatt
      361 ctggcgggca tgcctgtccg agcgtctttt caaccctcga acccctccgg gggtcggcg
      421 ttggggatcg gcctgcctc ttggcggtgg ccatctccga aatacagtgg cggtctcgcc
      481 gcagcctctc ctgcgcagta gtttgcacac tcgcatcggg agcgcggcgc gtccacagcc
      541 gttaaacacc caactttctg aaatgttgac ctcggatcag gtaggaagac acgcagaact
      601 ggttctgt
```

Figure 12: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01952)



Partial sequence of ITS 4 region of rDNA

ACTGGTTCGCGGGTATTCCTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTAACGGCTGTGGACGCGCCG
CGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGACGGCCA
TCGCCATGAGGCAGGGCCGATCCCCATCGCCGACCCCCCCGGAGAAAGGAATCAAAACTTTCAACAAAGGATCTCTTG
GTTCTGGAATCGATGAAGAACGCAGCGAAATGCGATAAGAAATGTGATTGCAAATTCAGTGAATCATCGAATCTTTGA
ACGCACTTTGCGCCGGCCAGTATTCGGGGGACCTGGCGTGTCAAAGTTTTTATTCATCTCTAAACCTCCGGGGGGGCG
CCGAGGAGGGTCACCTACAAAGACCGCGGAGGGGGGTTAAAAAAAAAGAGTTGTGGTTGGCCCCCCGCCGGCTCTTG
TTTGGCCTCCATCCC

Sequence Deposited: NCBI

ACCESSION: HM117840

VERSION: HM117840.1

GI:298104179

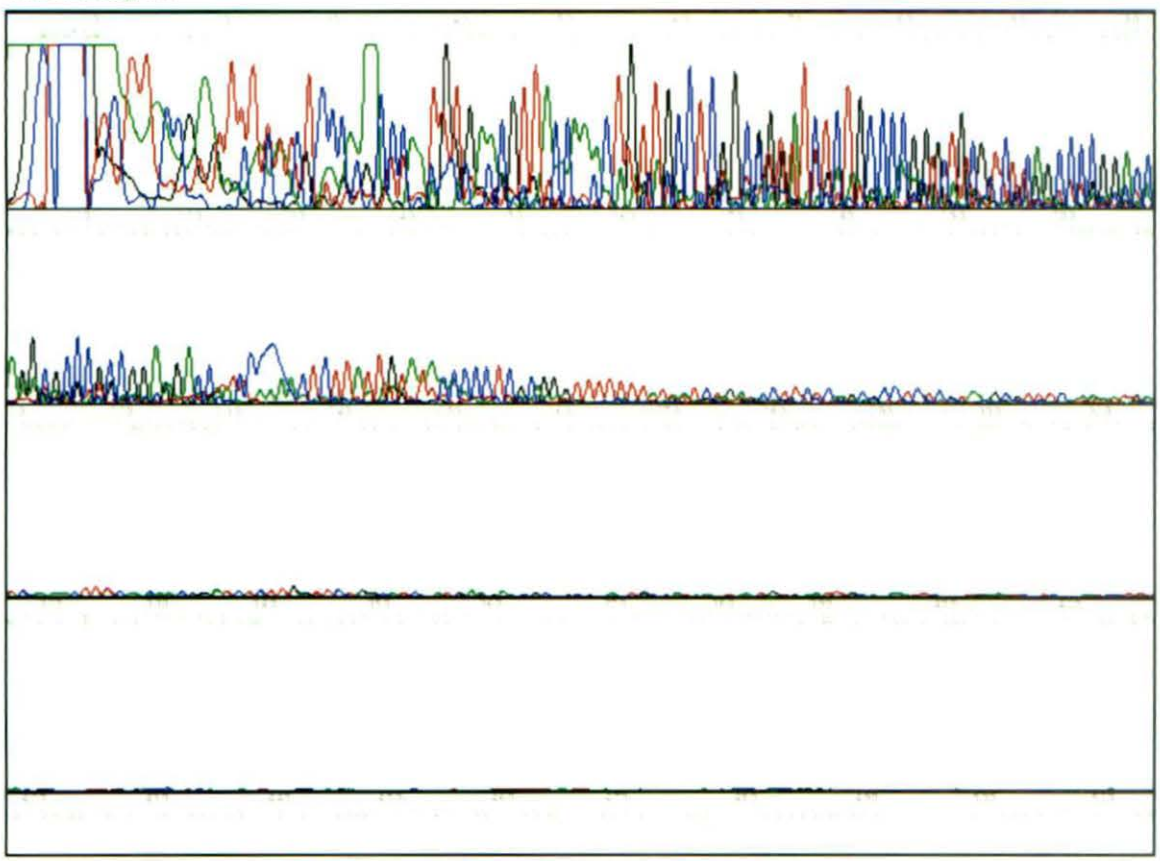
DNA linear :479 bp

Hypocrea lixii isolate FS/S477 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 gggatggagg ccaaacaaga gccggcgggg ggccaaccac aactcttttt tttaaccccc
       61 ctccgcggtc tttgtaggtg accctcctcg gcgccccccc ggaggtttag agatgaataa
      121 aaactttgac acgccaggtc ccccgaatac tggccggcgc aaagtgcgtt caaagattcg
      181 atgattcact gaatttgcaa tcacatttct tatcgcattt cgctgcgttc ttcatcgatt
      241 ccagaaccaa gagatccttt gttgaaagtt ttgattcctt tctccggggg gtcggcgatg
      301 gggatcggcc ctgcctcatg gcgatggccg tctccgaaat acagtggcgg tctcgccgca
      361 gcctctcctg cgcagtagtt tgcacactcg catcgggagc gcggcgcgtc cacagccgtt
      421 aaacacccaa ctttctgaaa tgttgacctc ggatcaggta ggaatacccg cgaaccagt
```

Figure 13: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01962)

Chromatogram



Partial sequence of ITS 4 region of rDNA

TAAGACTGAACTTACCGATTTTTTTCTCCCCACCCACGTGAACGTTACCAAACTGTTGCCTCGGCGGTATCTCTGCCCCG
GGTGCGTCTCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCCAAACTCTTATTGTATACCCCCTCGCGGGTTTTT
TTATTTTCTGAGCCTTCTCGGCGCCCCTCGTAGGCGTTTCGAGAATGAATCAAAACTTTCAGAAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAAAATTCTATGAATCATCGTATCCTTGAACGC
ACGTTGCGCCCTTCAGTAAATTGACAGGCCAGCCGGTCTTATTTAATTCATCCTCCAACCCCCCCCCGCGGCGCCGAGT
GGGCACCTTTCCTAATGCCGCGAGGCGTATAATAAAAGTTGTGTTGGAGCCCCCGCCCGGCTAGAGTTTGGAGCTCGAA
TCCAGGGGGAGAATCCCCCCCATTAAAACCTTATTTGAGAAAAGATAGCTTTGAAAGGAAAAAAAACACCTGACCTACC
AAATCTTAACGAGGAGAAACCAAGGGA

Sequence Deposited: NCBI

ACCESSION: GQ454925

VERSION: GQ454925.1

GI:257815518

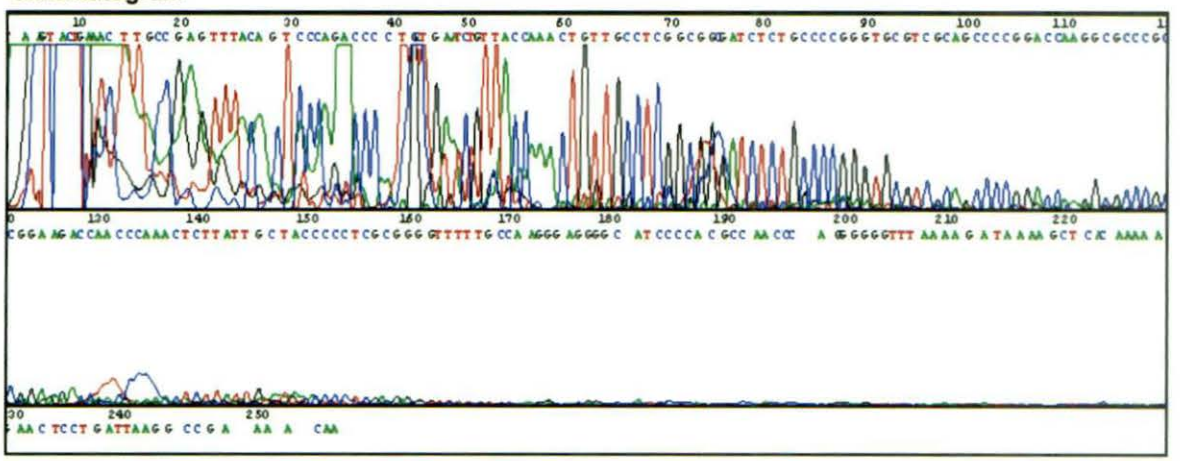
DNA linear : 605 bp

Title : *Hypocrea lixii* isolate AG/S476 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

```
ORIGIN
        1 ctctctcccg ctttcatggg gttaggtccg ggcggggttt ttccacctgc ccagagccca
       61 acctttttcag aaaccaaacg gttttccccc gcggggactt ccccccccggt cggtggattc
      121 cccaaacaat ggggcgggga gggggggca accaaaacta attttttata gcccctcgcc
      181 ggctatgagt aagtgtggtc caactcggcg ccccccggag gggttgagga aaagaattaa
      241 cactcgcaca ggcagtcctg ccaaatttct agagggcaca gaatgccgtc aaggattcgg
      301 aatttcagaa atttgtgcaa atcataatct tttatcgcac atcgcggccg ccagtattgt
      361 ggcgggcatg caggtccgag cgtcatttca ttcctcgaac ccctccgggg ggtcggcgtt
      421 ggggatcggc cctgcctctt ggcggtggcc gtctccgaaa tacagtggcg gtctcgccgc
      481 agcctctcct gcgcagtagt ttgcacactc gcatcgggag cgcggcgcgt ccacagccgt
      541 taaacaccca actttctgaa atgtgacctc ggatcaggta ggaatacccg cgaactagtt
      601 tttta
```

Figure 14: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01966)

Chromatogram



Partial sequence of ITS 4 region of rDNA

TAAGTACTGAAACTTGCCGAGTTTACAGTCCCAGACCCCTGCTGAATCTGTTA
CCAAACTGTTGCCTCGGCGGCGATCTCTGCCCCGGGTGCGTCGCAGCCCCGG
ACCAAGGCGCCCGCCGGAAGACCAACCCAAACTCTTATTGCTACCCCCTCTC
GGGGTTTTTGCCAAGGGAGGGGCATCCCCACGCCAACCCAGGGGGGTTTAA
AAGATAAAAGCTCACAAAAAGAACTCCTGATTAAGGCCGAAAACAA

Sequence Deposited: NCBI

ACCESSION: GU564470

VERSION: GU564470.1

GI:291194459

DNA linear : 254 bp

Title : *Hypocrea lixii* strain AG/S479 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.

```
ORIGIN
        1 taagtactga aacttgccga gtttacagtc ccagacccct gctgaatctg ttaccaaact
       61 gttgcctcgg cggcgatctc tgccccgggt gcgtcgcagc cccggaccaa ggcgcccgcc
      121 ggaagaccaa cccaaactct tattgctacc ccctctcggg gtttttgcca agggagggggc
      181 atccccacgc caacccaggg gggtttaaaa gataaaagct cacaaaaaga actcctgatt
      241 aaggccgaaa acaa
```

Figure 15: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01968)

Data analysis:

After direct sequencing of the PCR products, a total of 8 isolates of *Trichoderma* PCR products produced sequences that could be aligned and showed satisfactory homology with ex-type strain (THVA) of *T. harzianum* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 4 region. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-4 region that were closely related and similar sequence indicated. These 7 *Trichoderma* isolates were used in the pair wise and multiple sequence alignment. From the sequence alignment, variations were observed between *T.harzianum* isolates and *Trichoderma viride* isolates. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma harzianum* isolates. The evolutionary history was inferred using the UPGMA and Neibourhood-Joining (N J)method (Fig 16).

The optimal tree with the sum of branch length = 1.84709756 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 the Kimura 2-parameter 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 189 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

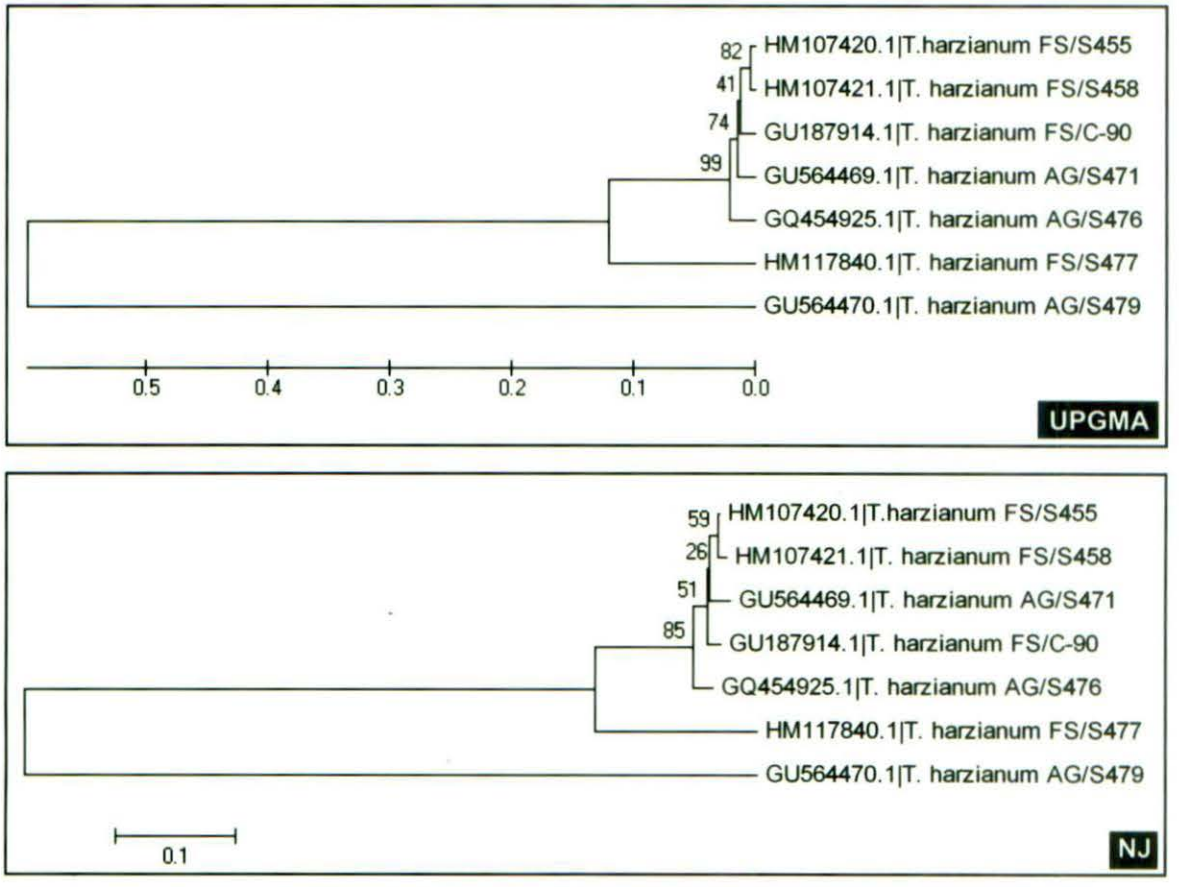


Figure 16: Phenogram of seven isolates of *Trichoderma harzianum* by UPGMA and NJ method

4.17 Denaturing gradient gel electrophoretic (DGGE) analyses of *Trichoderma harzianum*

Trichoderma harziamum isolates, FS/C-90 [NAIMCC-F-01950]; FS/S-455 [NAIMCC-F-01955], FS/S-458 [NAIMCC-F-01952] , [NAIMCC-F-01962] RHS/T- 477, Ag/S476 [NAIMCC-F-01966], Ag/S471 [NAIMCC-F-01967], Ag/S479 [NAIMCC-F-01968] were used in the present study. For this, 18S rDNA (320 bp with GC clamp) of each isolates of *T. harzianum* were amplified with the forward primer containing GC clamp at NS1 (5'-GTAGTCATATGCTTGTCTC-3') and GCfung (5'-CGCCCGCCGCGCCCCGCGCCC GGCCCG CCGCCCCCGCCCCA TTCCCCGTTAC CCGTTG-3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM $MgCl_2$ (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 4 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 10 sec, and 72°C for 2 min then followed by a last extention at 72°C for 8 min.

DGGE was performed with "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). The gels contained 10% (wt/vol) of acrylamide (acrolamide/bisacrolamide 37.5:1) and a range of denaturant concentration from 0% to 100% (formamide and urea). The gels were run at 110 V for 06 hours in 1X TAE buffer (pH 8.0) at 60°C. DGGE gels were stained with ethidium bromide in 1x TAE for 20 min DNA bands on the DGGE gels were excised under UV trans-illumination. The gel photographs were taken and analysied. In this uniform gradient gel of 0% to 100% and shorter run time could not separate the individual bands so as to optimize in suitable concentration for the runing time was changed to 12h at 110V which resulting the a minimum separation of bands in the 40% region of denaturant gel. So, finally 20 to 60% denaturant was found optimal for the best result in 110V for 8h (Plate 35). The profile obtained after 8 hours of run time from 20-60 % gradient showed all the bands have co migrated however the profile obtained 12 hours of run time showed a close variation in presence or absence of dominant bands. The DGGE analysis demonstrated that all the corresponding three bands on DGGE gels belonged to the isolates of *Trichoderma harzianum*. A similar type of distinct band was formed for all selected isolates but two separate bands were formed in the gel due to their G+C variation in their ITS region of rDNA.

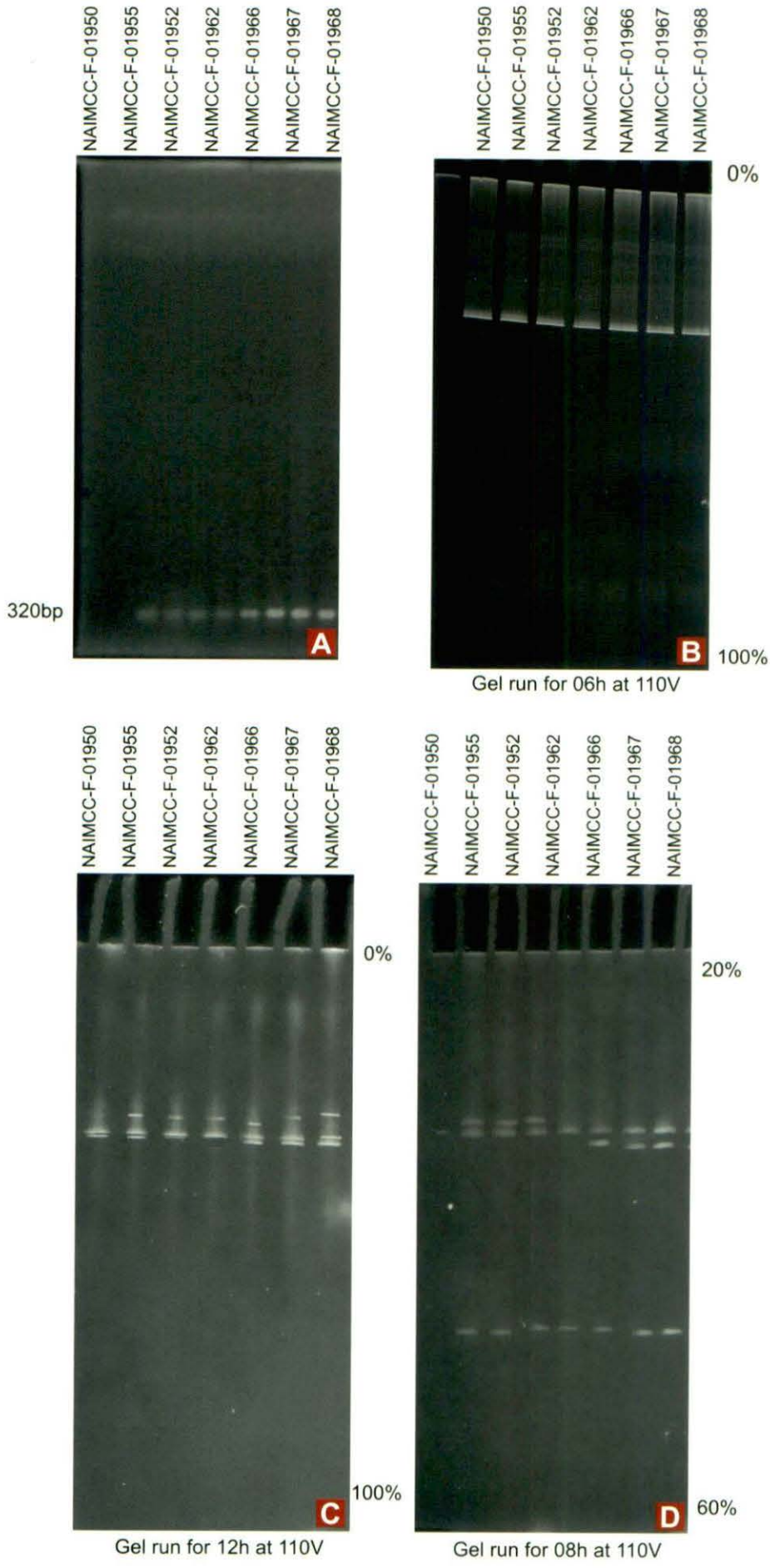


Plate 35: DGGE analysis of seven isolates of *Trichoderma harzianum*

4.18 Sequencing of rDNA region of *T. harzianum* and their submission in NCBI database

A total of 7 isolates of *Trichoderma harzianum* PCR products produced sequences that could be aligned and showed satisfactory homology with ex-type strain of *T. harzianum* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 1 region. ITS1 showed the highest number of nucleotide substitutions, and it was used for the phylogenetic study. Although studies involving biocontrol isolates of *T. harzianum* revealed that the 5.8S rRNA gene is as variable as ITS1 regions.). The sequence information was then analysed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *Trichoderma harzianum* with 100% similarity . The sequence alignment of our biocontrol isolates show variation into this gene . These sequences were deposited to NCBI genebank to get accession numbers (Table 50).

Table 50. Accesssion no. of submitted *Trichoderma* isolates

Organism	Strains	NAIMCC acc. No.	NCBI acc. No.	Sequences (bp)
T. harzianum	FS/C-90	NAIMCC-F-01950	GU187914.1	604
	FS/S-455	NAIMCC-F-01955	HM107420	611
	FS/S-458	NAIMCC-F-01952	HM107421	608
	RHS/T- 477	NAIMCC-F-01962	HM117840	479
	Ag/S476	NAIMCC-F-01966	GQ454925.1	605
	Ag/S471	NAIMCC-F-01967	GU564469	521
	Ag/S479	NAIMCC-F-01968	GU564470	254

4. 19 Analyses of rDNA gene sequences of *Trichoderma harzianum*

Further analysis of the ITS sequences of seven isolates of *Trichoderma harzianum* obtained from tarai-dooars regions were conducted using online Bioinformatic tools. On the first approach all the conserved regions of 18S r DNA sequences of these isolates were analyzed using the bioinformatics tool BioEdit. A multiple sequence alignment was carried out that included the ITS region, including gaps and the complete sequences align. 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 whether the isolates were closely related (Fig 17.) Further, hundred ex -type sequences of potential biocontrol agent *Thrichoderma harzianum* obtained from NCBI Gene bank database (Table 51) were aligned with these seven sequences for their phylogenetic placemet and a phenogram was developed using UPGMA method by Mega4 software (Fig 18). The sequence alignment revealed the presence within both ITS regions, which supports the distribution of all *Trichoderma harzianum* strains. In general terms, ITS1 showed the highest number of nucleotide substitutions, and it was used for the phylogenetic study. The sequence alignment of our biocontrol isolates show variation into this gene. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-4 region that were closely related and similar sequence indicated. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 32.82032529 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 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 from the dataset (Complete deletion option). There were a total of 45 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [4].

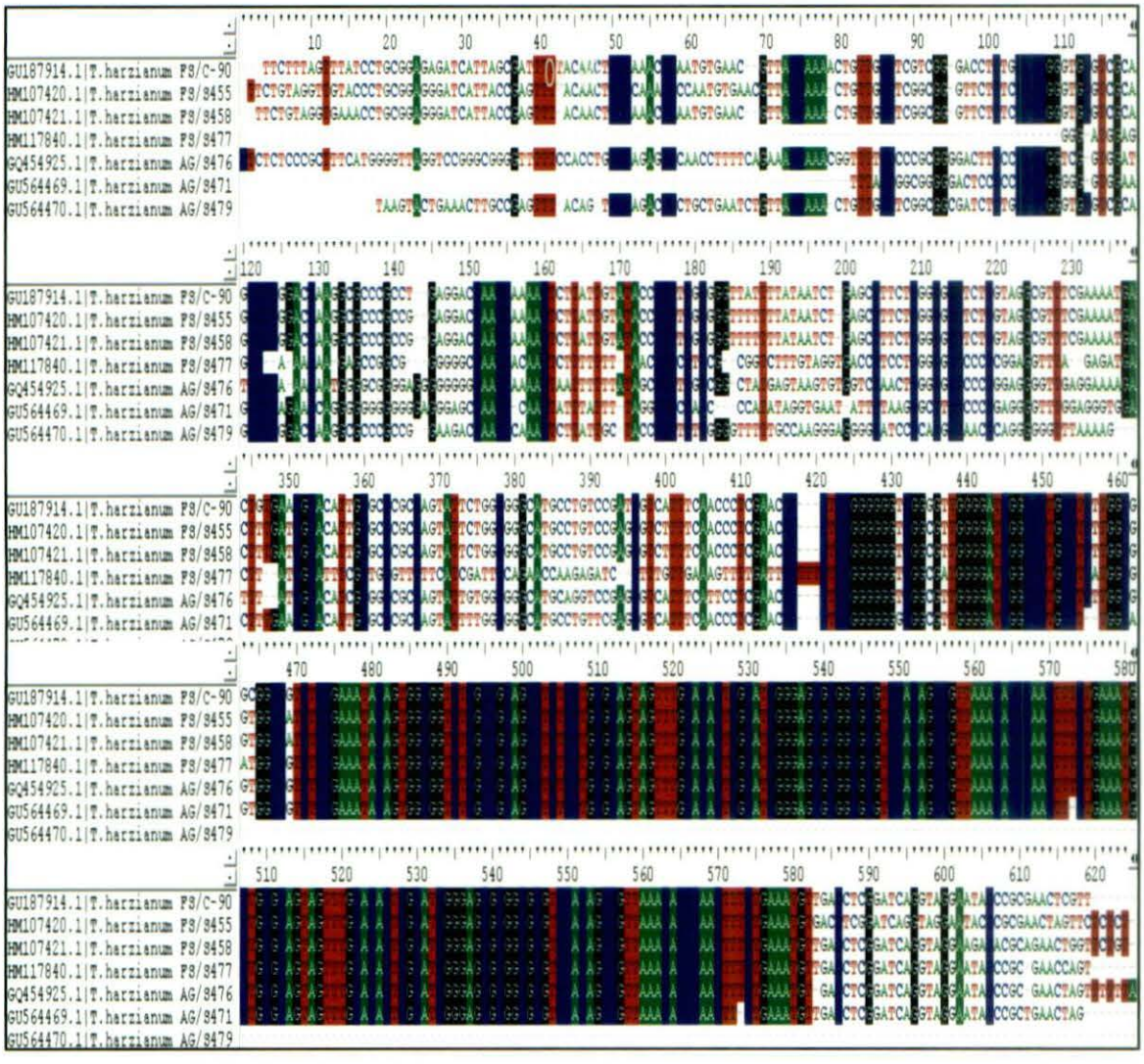


Figure 17: 18S rDNA sequence alignments of *T.harzianum*
Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in different colour

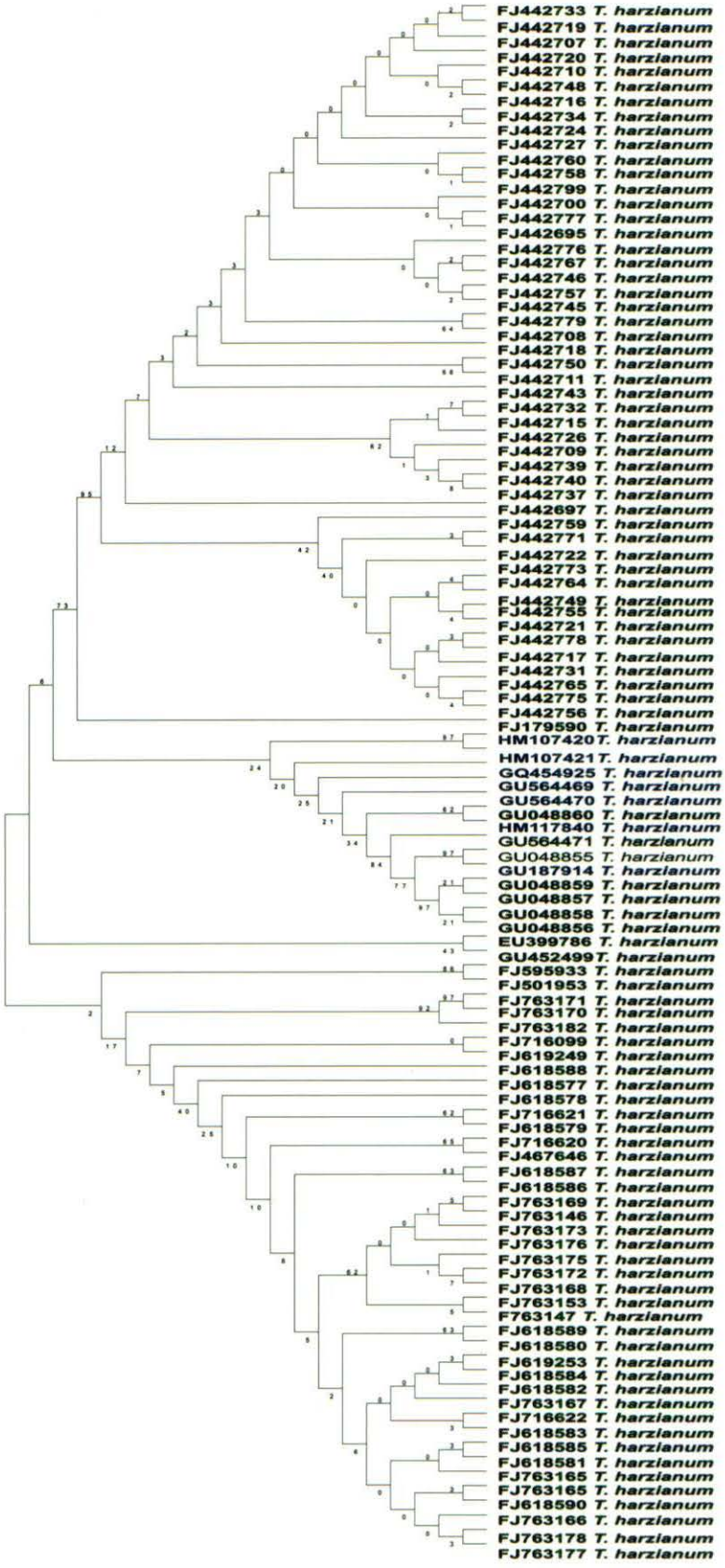


Figure 18: Phylogenetic placement of seven isolates of *Trichoderma harzianum* with extype strains from NCBI genebank

Table 51. Identified *Trichoderma harzianum* and comparison with referred NCBI GenBank

Strain No	GeneBank accession no	Identified as	Country of Origin	Identity (%)
ETS-323	GU452499.2	*T. harzianum*	-	100
GJS 92-120	FJ467646.1	*T. harzianum*	USA	100
C.P.K. 1934	FJ179590.1	*T. harzianum*	-	100
-	FJ501953.1	*T. harzianum*	-	100
GJS 04-71	FJ442779.1	*T. harzianum*	-	100
DIS 314F	FJ442778.1	*T. harzianum*	-	100
GJS 98-183	FJ442777.1	*T. harzianum*	-	100
GJS 94-53	FJ442776.1	*T. harzianum*	USA	100
DIS 221E	FJ442775.1	*T. harzianum*	Ecuador	100
DIS 389A	FJ442773.1	*T. harzianum*	Cameroon	100
GJS 97-106	FJ442771.1	*T. harzianum*	Thailand	100
GJS 04-212	FJ442767.1	*T. harzianum*	Italy	100
DIS 220K	FJ442765.1	*T. harzianum*	Ecuador	100
DIS 167E	FJ442764.1	*T. harzianum*	Brazil	100
DIS 246E	FJ442760.1	*T. harzianum*	Ecuador	100
DIS 264V	FJ442759.1	*T. harzianum*	Ecuador	100
DIS 246K	FJ442758.1	*T. harzianum*	Ecuador	100
GJS 91-138	FJ442757.1	*T. harzianum*	USA	100
DIS 253B	FJ442756.1	*T. harzianum*	Ecuador	100
GJS 00-18	FJ442750.1	*T. harzianum*		100
DIS 221F	FJ442755.1	*T. harzianum*	Ecuador	100
DIS 94D	FJ442749.1	*T. harzianum*	Peru	100
DIS 314B	FJ442748.1	*T. harzianum*	-	100
DIS 375G	FJ442746.1	*T. harzianum*	-	100
GJS 05-101	FJ442745.1	*T. harzianum*	-	100
GJS 00-08	FJ442743.1	*T. harzianum*	-	100
GJS 04-197	FJ442740.1	*T. harzianum*	Peru	100
DIS 55F	FJ442739.1	*T. harzianum*	Ghana	100
DIS 55I	FJ442737.1	*T. harzianum*	Ghana	100
DIS 354A	FJ442734.1	*T. harzianum*	Ecuador	100
DIS 218H	FJ442733.1	*T. harzianum*	Ecuador	100
DIS 55J	FJ442732.1	*T. harzianum*	Ghana	100
DIS 217P	FJ442731.1	*T. harzianum*	Ecuador	100
GJS 06-113	FJ442727.1	*T. harzianum*	Cameroon	100
GJS 00-24	FJ442726.1	*T. harzianum*	Mexico	100
GJS 04-67	FJ442724.1	*T. harzianum*	Italy	100
DIS 218F	FJ442722.1	*T. harzianum*	Ecuador	100
DIS 217H	FJ442721.1	*T. harzianum*	Ecuador	100
DIS 337F	FJ442720.1	*T. harzianum*	Panama	100
DIS 314D	FJ442719.1	*T. harzianum*	Cameroon	100
GJS 85-119	FJ442718.1	*T. harzianum*	Indonesia	100
DIS 233G	FJ442717.1	*T. harzianum*	Ecuador	100
GJS 06-124	FJ442716.1	*T. harzianum*	Cameroon	100
GJS 06-111	FJ442715.1	*T. harzianum*	Cameroon	100
GJS 04-70	FJ442711.1	*T. harzianum*	Italy	100
GJS 92-100	FJ442710.1	*T. harzianum*	USA	100
GJS 04-193	FJ442709.1	*T. harzianum*	Peru	100
GJS 05-107	FJ442708.1	*T. harzianum*	Italy	100
DIS 169C	FJ442707.1	*T. harzianum*	Brazil	100
GJS 07-19	FJ442700.1	*T. harzianum*	Ghana	100
DIS 386AI	FJ442699.1	*T. harzianum*	Cameroon	100
GJS 92-61	FJ442697.1	*T. harzianum*	Australia	100
DIS 246J	FJ442695.1	*T. harzianum*	Ecuador	100

Strain No	GeneBank accession no	Identified as	Country of Origin	Identity (%)
PPRC-ET47	FJ763182.1	*T. harzianum*	-	100
PPRC-ET41	FJ763178.1	*T. harzianum*	-	100
PPRC-ET40	FJ763177.1	*T. harzianum*	-	100
PPRC-ET39	FJ763176.1	*T. harzianum*	-	100
PPRC-ET38	FJ763175.1	*T. harzianum*	-	100
PPRC-ET34	FJ763173.1	*T. harzianum*	-	100
PPRC-ET33	FJ763172.1	*T. harzianum*	-	100
PPRC-ET32	FJ763171.1	*T. harzianum*	-	100
PPRC-ET31	FJ763170.1	*T. harzianum*	-	100
PPRC-ET29	FJ763169.1	*T. harzianum*	-	100
PPRC-ET28	FJ763168.1	*T. harzianum*	-	100
PPRC-ET27	FJ763167.1	*T. harzianum*	-	100
PPRC-ET26	FJ763166.1	*T. harzianum*	-	100
PPRC-ET25	FJ763165.1	*T. harzianum*	-	100
PPRC-ET24	FJ763164.1	*T. harzianum*	-	100
PPRC-ET13	FJ763153.1	*T. harzianum*	-	100
PPRC-ET2	FJ763147.1	*T. harzianum*	-	100
PPRC-ET1	FJ763146.1	*T. harzianum*	Ethiopia	100
CBS 110080	FJ716622.1	*T. harzianum*	-	100
PPRC RW20	FJ716621.1	*T. harzianum*	-	100
DAOM 229978	FJ716620.1	*T. harzianum*	-	100
-	EU399786.1	*T. harzianum*	-	100
-	GU048860.1	*T. harzianum*	-	100
-	GU048859.1	*T. harzianum*	-	100
-	GU048858.1	*T. harzianum*	-	100
-	GU048857.1	*T. harzianum*	-	100
-	GU048856.1	*T. harzianum*	-	100
	GU048855.1	*T. harzianum*	-	100
T88	FJ716099.1	*T. harzianum*	-	100
OY1107	FJ619253.1	*T. harzianum*	-	100
OY3207	FJ619249.1	*T. harzianum*	-	100
-	FJ618590.1	*T. harzianum*	-	100
-	FJ618589.1	*T. harzianum*	-	100
-	FJ618588.1	*T. harzianum*	-	100
-	FJ618587.1	*T. harzianum*	-	100
-	FJ618586.1	*T. harzianum*	-	100
-	FJ618585.1	*T. harzianum*	-	100
-	FJ618584.1	*T. harzianum*	-	100
-	FJ618583.1	*T. harzianum*	-	100
-	FJ618582.1	*T. harzianum*	-	100
-	FJ618581.1	*T. harzianum*	-	100
-	FJ618580.1	*T. harzianum*	-	100
-	FJ618579.1	*T. harzianum*	-	100
--	FJ618578.1	*T. harzianum*	-	100
	FJ618577.1	*T. harzianum*	-	100
	FJ595933.2	*T. harzianum*	-	100

Next 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 (Table 52).

Table 52: rDNA gene status and nucleotide percentage of rDNA sequence of *T.harzianum* isolates

	FS/C90	FS/S455	FS/S458	FS/S477	AG/S476	AG/S471	AG/S479
Nucleotide	Percentage:	Percentage:	Percentage:	Percentage:	Percentage:	Percentage:	Percentage:
G	24.88	25.2	25.82	25.89	27.93	28.6	24.02
A	22.06	20.95	21.55	21.29	20.33	23.22	25.98
T	23.71	23.9	23.52	23.59	22.48	19.77	18.5
C	29.35	29.95	29.11	29.23	29.26	28.41	31.5
Gg	6.81	7.21	7.41	7.95	11.26	11.35	8.3
Ga	5.48	5.08	5.44	6.28	4.14	5.38	5.14
Gt	5.15	5.41	5.44	4.39	5.13	4.23	3.56
Gc	7.48	7.54	7.58	7.32	7.45	7.5	7.11
Ag	3.65	3.93	4.12	4.81	4.97	5.77	5.53
Aa	7.64	7.05	7.41	6.49	6.62	7.88	11.46
At	5.65	5.08	4.94	5.23	4.47	5.38	2.37
Ac	5.15	4.92	5.11	4.81	4.14	4.23	6.32
Tg	5.65	5.57	5.93	5.02	4.14	4.62	4.74
Ta	3.32	3.28	2.8	2.3	2.98	4.04	3.56
Tt	6.48	6.56	6.59	8.58	7.95	5.58	5.53
Tc	8.14	8.36	8.07	7.53	7.45	5.58	4.74
Cg	8.8	8.52	8.4	7.95	7.62	6.92	5.53
Ca	5.65	5.57	5.93	6.28	6.62	5.96	5.93
Ct	6.31	6.72	6.43	5.44	4.97	4.42	6.72
Cc	8.64	9.18	8.4	9.62	10.1	11.15	13.44
g,c	54.23	55.16	54.93	55.11	57.19	57.01	55.51
a,t	45.77	44.84	45.07	44.89	42.81	42.99	44.49

Open reading frame (ORF) number were searched using the website http://www.ualberta.ca/~stothard/javascript/orf_find.html for each seven sequences with their translation are as follows

1. NCBI ACC. No GU187914.1: *T. harzianum* FS/C-90 [NAIMCC-F-01950]
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

ORF Finder results
Results for 604 residue sequence "Untitled" starting "TTCTTTAGTT"

```
>ORF number 1 in reading frame 1 on the direct strand extends from base
1 to base 138.
TTCTTTAGTTTATCCTGCGGAGAGATCATTAGCGATTTTTACAACTCCCAAACCCAATGT
GAACGTTACCAAAACTGTTGCCTCGTCGGGACCTCTGCCCCGGGTGCGTCGCAGCCCCGG
ACCAAGGCGCCCGCCTGA

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

FFSLSCGEIISDFYNSQTQCERYQNCCLVGTSAPGASQPRTKAPA*

>ORF number 2 in reading frame 1 on the direct strand extends from base 322 to base 546.
ATCATCGAATCTGTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCG
ATCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGGGTTGGGGATCGGCCCTGCCTC
TTGGCGGCGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTA
GTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAA

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

2. NCBI ACC. No HM107420: *T. harzianum* FS/S455 [NAIMCC-F-01955]
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

ORF Finder results
Results for 611 residue sequence "Untitled" starting "TTCTGTAGGT"
>ORF number 1 in reading frame 1 on the direct strand extends from base 1 to base 189.
TTCTGTAGGTTGTACCCTGCGGAGGGATCATTACCGAGTTTACAACTCCCCAAACCCCAA
TGTGAACGTTACCAAACTGTTGCCTCGGCGGGTTCTCTCCCCCGGGTGCGTCGCAGCCCC
GGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTT
TTTTTATAA

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

>ORF number 2 in reading frame 1 on the direct strand extends from base 340 to base 504.
TCGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCTTTTCAACCC
TCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCGGTGGCCATCT
CCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAG

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

3. NCBI ACC. No HM107421 : *T. harzianum* FS/S458 [NAIMCC-F-01952]
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

ORF Finder results
Results for 608 residue sequence "Untitled" starting "TTCTGTAGGT"
>ORF number 1 in reading frame 1 on the direct strand extends from base 13 to base 186.
AACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCAATGTGAACGTTACCAA
ACTGTTGCCTCGGCGGGTTCTCTCCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCG
CCGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAA

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

>ORF number 2 in reading frame 1 on the direct strand extends from base 337 to base 501.

TCGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCTTTTCAACCC
TCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCGGTGGCCATCT
CCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAG

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

4. NCBI ACC. No HM117840 **:** ***T. harzianum*** **FS/S477 [NAIMCC-F-01962]**
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

ORF Finder results
Results for 479 residue sequence "Untitled" starting "GGGATGGAGG"
>ORF number 1 in reading frame 1 on the direct strand extends from base 193 to base 378.
ATTTGCAATCACATTTCTTATCGCATTTCGCTGCGTTCTTCATCGATTCCAGAACCAAGA
GATCCTTTGTTGAAAGTTTTGATTCCTTTCTCCGGGGGGTCGGCGATGGGGATCGGCCCT
GCCTCATGGCGATGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCG
CAGTAG

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

5. NCBI ACC. No GQ454925.10 : ***T. harzianum*** **Ag/S476 [NAIMCC-F-01966]**
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

ORF Finder results
Results for 605 residue sequence "Untitled" starting "CTCTCTCCCG"
>ORF number 1 in reading frame 1 on the direct strand extends from base 1 to base 171.
CTCTCTCCCGCTTTCATGGGGTTAGGTCCGGGCGGGGTTTTTCCACCTGCCCAGAGCCCA
ACCTTTTCAGAAACCAAACGGTTTTCCCCCGCGGGGACTTCCCCCCCGGTCGGTGGATTC
CCCAAACAATGGGGCGGGGAGGGGGGGGCAACCAAAACTAATTTTTTATAG

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

>ORF number 2 in reading frame 1 on the direct strand extends from base 328 to base 543.
TCTTTTATCGCACATCGCGGCCGCCAGTATTGTGGCGGGCATGCAGGTCCGAGCGTCATT
TCATTCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCGGTG
GCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACA
CTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAA

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

6. NCBI ACC. No GU564469: *T. harzianum* Ag/S471 [NAIMCC-F-01967]
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

ORF Finder results
Results for 521 residue sequence "Untitled" starting "TTTACCGGCG"

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>ORF number 1 in reading frame 1 on the direct strand extends from base
1 to base 216.
TTTACCGGCGGGGGACTCCCCCCCCCGGGGGGTGGAAGCCCCAGAACCAGGGGGGGGGGG
GAGGGAGCCAACCCAACTATTTATTTTAGGCCCCCACCCCATATAGGTGAATATTCTAAG
CGCCTCCCCCCGAGGGGTTTGGAGGGTGGAAAAAAAGTTAGCACAAGCATCTCCCGCCCT
GAATCATGGCGGACGAAAATGAGCGAAATAAGATAA

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

>ORF number 2 in reading frame 1 on the direct strand extends from base
259 to base 465.
ACGCACATTGCGCCCGCCAGTATTTTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCC
TCGAACCCCTCCGGGGGGGGCGGCGTTGGGGATCGGCCCTGCCTTGGCAGTGGCCGTCTCC
GAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCG
GGAGCGCGGCGCGTCCACAGCCGTTAA

>Translation of ORF number 2 in reading frame 1 on the direct strand.
THIAPASILAGMPVRASFQPSNPSGGAALGIGPALAVAVSEIQWRSRRSLSCAVVCTLAS
GARRVHSR*
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7. NCBI ACC. No GU564470: *T. harzianum* Ag/S479 [NAIMCC-F-01968]
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

ORF Finder results
Results for 254 residue sequence "Untitled" starting "TAAGTACTGA"

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>ORF number 1 in reading frame 1 on the direct strand extends from base
46 to base 252.
ATCTGTTACCAAACTGTTGCCTCGGCGGCGATCTCTGCCCCGGGTGCGTCGCAGCCCCGG
ACCAAGGCGCCCGCCGGAAGACCAACCCAAACTCTTATTGCTACCCCCTCTCGGGGTTTT
TGCCAAGGGAGGGGCATCCCCACGCCAACCCAGGGGGGTTTAAAAGATAAAAGCTCACAA
AAAGAACTCCTGATTAAGGCCGAAAAC

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