SEARCH FOR AGRICULTURALLY IMPORTANT MICROORGANISMS FROM SOILS OF RIVER BASINS, FORESTS AND CROP FIELDS IN THE TERAI-DOOARS OF NORTH BENGAL AND ANALYSIS OF THEIR DIVERSITY

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Contents

Introduction

Soil is an excellent medium for the growth of microorganisms which includes bacteria, fungi, algae, actinomycetes, protozoa and various insects whose number and kinds in the soil depend mainly on the nature and depth of soil, seasonal condition, and state of cultivation, temperature, amount of organic matter, moisture content and aeration (Edwards, 1977). Soil microflora play an important role in the rhizosphere of the higher plants but are dependent to a great degree on the exudates of the roots in the rhizosphere (Tan, 1977). Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation; decomposition of organic matter; toxin removal and the cycling of carbon, nitrogen, phosphorus, and sulphur (Van, 1997). In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth, and changes in vegetation (Doran *et al.*, 1996).

Fungi are an important component of the soil microbiota. Many important plant pathogens and plant growth promoting microorganisms (ecto and endo mycorrhizae) are fungi. The saprobic fungi represent the largest proportion of the fungal species in soil and they perform a crucial role in the decomposition of plant structural polymers such as cellulose, hemicellulose, chitin and lignin thus contributing to the maintenance of the global carbon cycle. Besides, these catabolic activities enable fungi to grow on inexpensive substrates. This property, coupled with their ability to produce commercially interesting organic molecules and enzymes explains the significant interest in the biotechnological utilization of filamentous fungi (Hawksworth *et al.*, 1995).

The term rhizosphere, originally coined by Hiltner in 1904, was defined as the volume of soil adjacent to and directly influenced by plant roots. Over the years, however, the term has been redefined several times, mostly to incorporate parts of the root tissue. Rhizosphere can be divided into ecto and endo rhizosphere. The term endorhizosphere is used to describe the multi-layered microenvironment, which includes a mucoid layer on the root surface, the epidermal layer of the root tissue including the root hairs and the cortical cells (Bolton *et al.*, 1993). Rhizosphere has been regarded as 'hot spot' for microbial colonization and activity (Plate–1). Actively growing roots release organic compounds, such as sloughed off cells, secretions, lysates and exudates, into the rhizosphere (Lynch and Whipps, 1990; Bowen and Rovira, 1991).

Plate 1: Agricultural crop fields of Terai-dooars region

The activity of microbes in the rhizosphere is expected to be higher and qualitatively different in the rhizosphere as compared to microbes in bulk soil. The amount and composition of organic materials released by the plants are important factors that determine the nature of this plant-microbe interaction (Griffiths *et al.*, 1999; Jaeger *et al.*, 1999). Since such root-released products can be highly specific for a given plant species or even a particular cultivar, plants are thought to selectively enrich their rhizospheres for microorganisms that are well adapted to the utilization of specific released organic compounds (Bowen and Rovira, 1991; Lynch and Whipps, 1990).

Increased plant productivity also results from the suppression of deleterious microorganisms by antagonistic bacteria, since soil-borne pathogens can greatly reduce plant growth. As variation in microbial community structure may have effects on ecosystem processes (e.g. nutrient recycling, decomposition of organics) or plant-microbe interaction (e.g. growth of pathogens, release of plant-growth promoting rhizobacteria or genetically engineered microorganisms), understanding how community processes affect ecosystem processes is of central interest in ecology (Miethling *et al.*, 2000). Knowledge of rhizosphere microbial community also opens possibilities to promote disease suppressive microflora in the rhizosphere. The increase in shoot growth and leaf area in *Trichoderma* treated seedlings suggests a common beneficial role of *Trichoderma harzianum* in improving plant growth (Yedidia *et al.*, 2001). The mechanisms involved in increasing growth responses induced by *Trichoderma sp* might be the production of growth-stimulating compounds (Gravel *et al.*, 2006; Harman *et al.*, 2004; Yedidia *et al.*, 2001; Altomare *et al.*, 1999; Chang *et al.*, 1986).

Scientific interest has long focused on the structure of microbial communities in the rhizosphere, assessed by cultivation-based studies. These studies have shown that the microbial diversity in the rhizosphere is often extensive and that there are distinct differences in bacterial community structures between bulk (non-rhizosphere) soil and rhizosphere soil. Several studies on different plant species in different locations, using a range of cultivation-based and molecular methods have been reported.

Plate 2: River basin of Balason of Terai-Dooars region

Plate 3: Gorumara forest of Terai-Dooars region

For the past decade, PCR based techniques have been used to detect and identify pathogenic fungi in plant tissue. It is much faster than using a microscope, but it can be quite time consuming if hundreds of samples have to be analysed (Luchi *et al.*, 2004). RAPD is analyzed in a series of steps that combine PCR, restriction enzymes and gel electrophoresis (Kitts, 2001).

The term biodiversity has been defined in various ways. In microbial terms, it describes the number of different types (species) and their relative abundance in a given community in a given habitat. In molecular-ecological terms, it can be defined as the number and distribution of different sequence types present in the DNA extracted from the community in the habitat. The river basins (Plate-2) and forest areas (Plate-3) of Terai-Dooars regions have been identified as unique ecological settings with high endemism, rare flora and fauna. However diversity in microflora has not been so far explored thoroughly in this region. Studies on microbial diversity and their importance as bioprotector and biofertilizer may signify the economically agricultural based regions. Many of these microbes produce economically important compounds, such as, antibiotics, steroids, and enzymes etc. that benefit mankind in numerous ways. Many microbes have a vital role in cleaning up the environment and bioremediation and thus have direct bearing on the ecosystem. Keeping this in view, the following major objectives were undertaken to generate the possible information for utilization of microorganisms isolated from the different ecological regions of Terai-Dooars regions in North Bengal.

A) Isolation and identification of microorganisms from soil samples of different river basins, forests and major crop fields.
B) Selection of microorganisms for cellulose degradation, chitin degradation, lignin degradation and phosphate solubilization activities.
C) Screening of plant growth promoters among the isolated microorganisms.
D) Screening of antagonistic microorganisms against important plant pathogens.
E) Molecular diversity analysis of the selected agriculturally important microorganisms

Literature Review

Soil Microorganisms and their potential application in Agriculture

Agriculturally important microorganisms (AIM) are used in a variety of agro-ecosystems both under natural conditions and artificial inoculation for diverse application such as nutrient supply, biocontrol, bioremediation and rehabilitation of degraded lands. (Vessey, 2003). The successful development of AIM in stressed ecosystem poses many challenges. The last two decades of research resulted in many successful approaches to select tolerant strains for nitrogen fixation, P solubilization, plant growth promotion and biocontrol. There has been also good progress in formulation technology suitable for dryland and wetland ecosystems with the emerging application of molecular biology techniques.

Soil formation

Soil is the region on the earth's crust where geology and biology meet, the land surface that provides a home to plant animal and microbial life. Soil teems with microscopic life (bacteria, fungi, algae, protozoa and viruses) as well as macroscopic life and also with the root system of plants. The nature and conditions of all the living forms that the soil houses depends mainly on the nature and depth of soil, seasonal condition, and state of cultivation, temperature, amount of nutrient available etc. (Ogunmwonyi *et al.*, 2008).

Soils are open thermodynamic systems; soils experience a remarkable set of transformations over time, as energy, chemical elements and water are processed. Over time, primary minerals are weathered and lost. Although new secondary minerals may be formed during soil development, the soil's primary minerals are decomposed and its acid-neutralizing capacity gradually consumed. If the soil's landform is geomorphically stable, weathering of soils may proceed through a full sequence of weathering as illustrated by Jackson and Sherman (1953). Over pedogenic time, weathering consumes even large pools of primary minerals and advanced weathering-stage soils will be formed if hydrologic removals of solutes outpace renewals that can come from weatherable minerals or atmospheric deposition. Richter and Babbar (1991) have explained the possible involvement of rhizosphere in the advancement of weathering and soil formation.

Physical and chemical characteristics of rhizosphere soil

By convention (Lovejoy's 1936), the rhizosphere has been characterized as having three components (Clark 1949):

(A) Rhizoplane, the immediate surface of the root,

(B) Rhizosphere, the soil volume surrounding the rhizoplane that is immediately affected by root activity, and

(C) Bulk soil, the soil not directly affected by living roots.

Microbes in the rhizosphere are subject to an environment in which the supply of water, oxygen, and nutrients is strongly influenced by plant activity. An actively transpiring plant removes huge quantities of water from the soil. Depending in large part on the rate of water supply from the surrounding soil to the rhizosphere, the water potential in rhizosphere soil can be more than 1 MPa lower and much more variable than in the surrounding soil (Papendick and Campbell, 1975). During the daytime, rhizosphere soil is commonly measurably drier than the surrounding bulk soil. In contrast, rhizosphere soil in some terrestrial ecosystems can exhibit higher water content than that of the surrounding soil at night as a result of "hydraulic redistribution" (Caldwell and Richards, 1989). Water from deeper in the soil profile is accessed by deep roots, transported to roots in surface soil and can ultimately move out into dry surface soil at night when evapotranspiration from leaves is reduced. Both of these preceding phenomena can result in large diurnal water potential fluctuations in the soil adjacent to roots, fluctuations that likely are a critical environmental characteristic selecting rhizosphere microbial communities and influencing the rates of nitrogen-cycling occurring in this zone. The rhizosphere zone is also characterized by high rates of O_2 consumption caused by both root and microbial respiration (Sorensen, 1997). This respiration can create zones of low O_2 concentration and even anaerobic conditions depending on the diffusional resupply of O_2 into the rhizosphere from surrounding soil pores. Because diffusion of O_2 is highly dependent on soil water content, reduced water content in rhizosphere soil pores due to plant evapotranspiration can result in enhancement of O_2 diffusion. Thus, depending on the water content of soil and connections to oxygen-depleted or oxygen-replete atmosphere, the availability of O_2 in the rhizosphere soil atmosphere can be either greater or lesser than that of the surrounding soil. Plant roots are also well known to change the pH of the rhizosphere by extruding protons via H+-ATPase in epidermal cells (Hinsinger *et al.*, 2003). This can occur, for example, in response to iron deficiency (Schmidt *et al.*, 2003), since a change in pH affected by the plant can also cause the release of inorganic metals. Low molecular weight organic acids secreted by the plant can also act to lower the pH of the surrounding soil. The rhizosphere is thus a spatially and temporally patchy environment with rapid (commonly diurnal) fluctuations between potentially extreme conditions, including cycles of water stress and anaerobiosis, that microbes must respond to in order to survive and thrive

Edaphic environment and community ecology of the rhizosphere

In general, conventional agricultural practices stimulate facultative saprophytic pathogens and increase crop susceptibility to disease. The edaphic environment is modified as with high inorganic nutrient availability and low diversity carbon inputs associated with conventional agricultural systems. This profoundly influences substrate, habitat availability and microbial community dynamics (Hoitink and Boehm, 1999). These environmental modifications in conjunction with short rotations are the root of many soilborne disease problems. This is evident in intensively managed, high-value vegetable crops where reliance on fumigation, multiple tillage operations and high rates of fertilizer is often associated with compacted soils, low levels of soil microbial activity, and recurring root health problems .The use of inoculation with beneficial, biological control organisms that will colonize the rhizosphere shows some promise as a means to suppress plant disease (Cook *et al.*, 1993).

Rhizosphere microflora – direct and indirect effects on plant growth

Plants are not simply passive recipients of nutrients, but information from the environment affects their belowground allocations such as root proliferation (Hodge *et al.*, 1999), formation of symbiotic relationships e.g. mycorrhizal fungi, (Smith and Read, 1997) or N2-fixing bacteria (Ryle *et al.*, 1979), alteration in exudation rates (Bonkowski *et al.*, 2001; Wamberg *et al.*, 2003), interactions with free living bacteria (Joseph and Phillips, 2003; Mathesius *et al.*, 2003), or production of secondary defence compounds against herbivores (Cipollini *et al.*, 2003). Since root morphology is both genetically programmed and environmentally determined (Rolfe *et al.*. 1997), there must be signal transduction pathways that interpret complex environmental conditions and activate genes to enter a particular symbiosis or to form a lateral root at a particular time and place. The exchange of signals between plants and microorganisms is reciprocal (McKenzie Bird and Koltai, 2000). Phillips and Strong (2003) have introduced the concept of "rhizosphere control points" to emphasize the importance of information exchange between plants and microorganisms.

Shaik and Nusrath (1987) observed *Tricoderma viride* and *Aspergillus niger* as a part of microflora of wilt resistant cultivar while susceptible cultivar showed a predominance of *Fusarium udum* and *Fusarium* spp. during all the stages of plant growth.

Pandey *et al.* (2000) isolated four antagonistic bacterial isolates, *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas corrugata* and *P. corrugata*, from the rhizosphere of tea plants

growing in different geographical locations in India. These were tested as microbial inoculants for hardening of tissue-cultured tea plants raised in the laboratory prior to the transfer to open land. Bacterial inoculations resulted in enhanced survival up to 100, 96, and 88%, as against 50, 52, and 36% survival observed in the corresponding control plants, in rainy, winter and summer seasons, respectively. Rhizoplane and rhizosphere soil analyses showed that the major biotic factor responsible for mortality following the transfer of tissue culture raised plants to soil was fungal attack (*Fusarium oxysporum*). Bacterial inoculations also resulted in plant growth promotion of tissue culture as well as seed raised plants of tea.

Plant growth promoting rhizobacterial strains belonging to fluorescent pseudomonas were isolated from the rhizosphere of rice and sugarcane by Kumar *et al.* (2002). Among 40 strains that were confirmed as *Pseudomonas fluorescens, 18* exhibited strong antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum,* mainly through the production of antifungal metabolites. Genotyping of *P. fluorescens* strains was made by PCR-RAPD analysis, since differentiation by biochemical methods was limited.

The composition of rhizosphere microbial communities

Plant species can be important in determining the structure of rhizosphere microbial communities (Stephan *et al.*, 2000), with both positive and negative effects on different microbial groups. Within plant species, microbial communities can be affected by plant genotype (Smith *et al.*, 1999), plant nutrient status (Yang and Crowley, 2000), pathogen infection (Yang *et al.*, 2001), and mycorrhizal infection. Within root systems, microbial communities can even differ among root zones (Yang and Crowley, 2000) and at different distances from the root surface as rhizosphere soil grades into bulk soil (Marilley and Aragno, 1999). The largest numbers of bacteria in the rhizosphere have been reported to occur in the zone of root elongation (Jaeger *et al.*, 1999). Studying organisms in the rhizosphere, and more generally in soil, is not a straightforward task. A complex community of bacteria may exist at the scale of a soil aggregate, a biofilm, or a section of root surface where boundaries can be difficult to delineate (Belnap *et al.*, 2003). Physically removing microbes from soil is also non-trivial, particularly from intact rhizosphere soil. The recent development and popularity of molecular techniques to identify soil organisms has allowed us to move beyond the small subset of culturable soil organisms and begin defining populations and communities of microbes belowground. It is increasingly common to characterize complex microbial communities genotypically using the small subunit 16S ribosomal DNA gene (16S rDNA), a region that is very highly conserved, essential, subject to low homologous gene transfer and a good reflection of overall phylogenetic relatedness. A collection of 16S genes can be

analyzed partially as with the fingerprinting methods T-RFLP and DGGE, or in detail by sequencing entire populations or communities in clone libraries. Using these methods, they have begun to understand how population and community ecology concepts apply to rhizosphere microbes. Most population studies have focused on organisms that can be manipulated in agricultural settings either for biocontrol or for increased plant growth, including species of symbiotic nitrogen fixers (Carelli *et al.*, 2000), plant growth promoting rhizobacteria (Bevivino *et al.*, 1998), deleterious rhizosphere bacteria (Nehl *et al.*, 1997), pathogens (Khan and Khan, 2002) and bacteriophage (Ashelford *et al.*, 2003). Population-level studies are also common for rhizosphere bacteria useful for bioremediation.

Pathogenic communities

Ansari *et al.* (1986) have studied rhizosphere and rhizoplane mycofloa of barley infected with *Ustilago hordei* and discussed the certain biochemical changes that occur due to infection. Higher fungal population and number of fungal species were encountered in the infected plants in comparison to their healthy counterparts.

Gopinath *et al.* (1987) have reported the colonization of *Fusarium* sp. in sorghum seeds and their significance and they came with the conclusion that thirty high yielding cultivars of sorghum analyzed showed severe infection of *Fusarium moniliforme, F. oxysporum, F. semitectum* and *F. solani, F. semitectum* infected the embryonic tissue in 93% seeds, while *F. semitectum* and *F. solani* colonized the embryo in 8 and 5% seeds respectively. But *F. oxysporum* did not colonize the embryo.

Hee *et al.* (1991) have studied the selection and identification of antagonistic rhizobacteria in relation to controlling soil borne diseases of vegetables. 926 isolates of rhizobacteria from 3 different kinds of selective media and 30 isolates of plant. Pathogenic bacteria were duel cultured with 10 species of important soil borne plant pathogenic fungi, respectively and measured their antagonism by their inhibition zone the population density of rhizobacteria in the same field was different according to the crop species planted and the isolation frequency of the antagonistic bacteria from the species of plant was also markedly different according to the fields or regions where the soils were collected for the effective isolation of rhizobacteria, M523 and King's B media were more suitable than D+ medium of 926 rhizobacteria isolated from the soils with 22 plant species. Among these, 63 isolates were selected which were found to be antagonistic to *Phytophthora capsici,* while 54 isolates antagonistic to *Rhizoctonia solani* and 17 isolates antagonistic to *Fusarium oxysporum* f.sp. *lycopersici,* respectively. Of these, one isolate RB 173 was finally selected as the most

effective antagonist to the 9 species of soil borne plant pathogenic fungi and identified as *Psuedomonus flurescens.*

Yephet *et al.* (1995) have worked on *Fusarium* wilt in carnation and discussed effect of culture resistance on the propagule consists in soil they deal with six carnation cultivars with different degrees of resistance to *Fusarium oxysporum* f. sp. *dianthi* were pasted in naturally infected soil in the field and in containers with artificially infertile soil at three inoculum concentrations. Tissues and cellular location of cross reactive antigen shared by *Fusarium oxysporum*, soybean roots and *Bradyrhizobium japonicum* was carried out by Chakraborty *et al.* (1995). They have pre-inoculated the seeds of soybean cultivars with *B. japonicum* and discussed cross protection mechanism involved in reduction of disease severity.

Mathur *et al.* (2004) reported that the plant rhizosphere is an important zone where many micro-organisms both friends and foe exits. The microflora associated with a plant rhizosphere is generally influenced by the soil type, pH and temperature. Stem rot caused by *Rhizoctonia solani*, one of the diseases of chillis becomes very severe and destructive under the favourable conditions. The fungus also colonised seed, leaves and fruits of chilli. Tiwari (2004) analysed potential use of rhizosphere microorganisms of chillis for the management of stem rot disease.

Non-pathogenic communities

Parveen *et al.* (2004) discussed the mode of antagonisms of *Trichoderma viride* against *Alterneria triticina* causing leaf blight of wheat. Dual culture interaction *in vitro* revealed that mycelial strand of *T. viride* coiled around the hyphae of the test pathogen forming a rope like structure and finally inhibited the growth of *A. triticina in vitro.*

Oyeyiola (2009) isolated and identified fungi present in the rhizosphere and rhizoplane of Okra (*Hibiscus esculentus*). The fungi were *Penicillium frequetans, P. oxalicum, P. palitans, Rhizopus stolonifer, R.. oligosporus, R.. oryzae, Aspergillus niger, A. fumigatus, A. japonicus, A. clavatus, Mucor hiemalis, M. racemosus, Alternaria herbarum* and *A. triticina. R. stolonifer* were Predominant mycoflora in both the rhizosphere soil and the rhizoplane were, *A. niger and A. clavatus* while *P. oxalicum* and *A. herbarum* were predominant in the rhizosphere soil only. *Mucor hiemalis, Penicillium frequetans P. palitans, P. oxalicum, A. clavatus, and A. triticina* were present in the rhizosphere soil and/or the rhizoplane, but they were absent from the non-rhizosphere soil. The rhizosphere soil contained a greater spectrum of fungal species than either the rhizoplane or the non-rhizosphere soil. The experimental soil was sandy loam in texture. The rhizosphere effect

increased progressively with increase in plant age until the 6th week after seed sowing and then declined.

Species of *Penicillium* (*P. erythromellis*, *P. janthinellum*, *P. raistrickii*) and *Trichoderma* (*T. pseudokoningii* and *T. koningii*) were found to be dominant in the rhizosphere of established tea. While seasonal fluctuation was observed in the case of *Penicillium* spp., the population of *Trichoderma* spp. showed less variation during the year. Both species were sensitive to low temperatures. In general, fungi associated with the tea rhizosphere were found to prefer a mesophillic temperature range (15 °C to 35 °C). The dominant species of *Penicillium* and *Trichoderma* also exhibited tolerance to lower temperatures, i.e., 5 to 10 °C on agar plates. Most fungi were able to grow in a wide range of pH (4 to 12). Lowering of soil pH in the rhizosphere of tea bushes was positively correlated with the age of the bush and may have affected the development of a specific microbial community in the rhizosphere (Pandey *et al.*, 2000)

Rhizosphere of healthy pigeonpea plant was heavily colonised by *Aspergillus niger*, *Penicillum* sp., *Trichoderma viride* and *Gliocladium virens*. Resident *Trichoderma* and *Gliocladium* was highly antagositic to the pathogen (*Fusarium udum*). *T. viride* formed loops, coiling and ruptured the cell wall of the pathogen. Mechanism of parasitism between *F. udum* and *G. virens* resulted in twisting, air bubbling and disintegration of pathogen hyphae while *T. harzianum* causes shrinkage and coagulation of cytoplasm of pathogen hyphae (Pandey and Upadhyay, 2000).

Two species of *Aspergilli* and ten other fungi were isolated from rhizosphere mycoflora of onion (*Allium cepa*). *Aspergilli* in general were dominant contributing 38.59% to the total mycoflora by Kallurmath and Rajasab (2000). *A. niger* and *A. flavus* were dominant on onion bulbs with the progress of their maturity.

Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under *in situ* conditions have been discussed by Pandey *et al.* (2001). The populations of *Penicillium* and *Trichoderma* species were inversely correlated with the populations of two most dominant rhizosphere bacteria, *Bacillus subtilis* and *B. mycoides*. Both *Bacillus* species have been shown to have antagonistic activity against these two fungi under *in vitro* conditions.

Rhizosphere colonization is one of the first steps in the pathogenesis of soil borne microorganisms. It can also be crucial for the action of microbial inoculants used as biofertilizers, biopesticides, phytostimulators and bioremediators. *Pseudomonas*, one of the best root colonizers is used as a model root colonizer. Lugtenberg *et al.* (2001) focused on (a) the temporal spatial description of root colonizing bacteria as visualized by confocal laser

scanning microscopical analysis of autofluorescent microorganisms, and (b) bacterial genes and the traits used for the colonization of root and of animal tissues, indicating the general importance of a study.

Mulaw *et al.* (2010) reported the southwestern highlands forests of Ethiopia are the origin of the coffee plant *Coffea arabica*. The production of coffee in this area is affected by tracheomycosis caused by a soil-born fungus *Gibberella xylarioides*. The use of endemic antagonistic strains of mycoparasitic *Trichoderma* species would be a nature conserving means to combat this disease. We have used molecular methods to reveal that the community of *Trichoderma* in the rhizosphere of *C. arabica* in its native forests is highly diverse and includes many putatively endemic species. Among others, the putative new species were particularly efficient to inhibit growth *of G. xylarioides*. polymorphism and clone library sequencing of 16S ribosomal RNA (rRNA) gene fragments.

BENEFICIAL MICROORGANISMS

Rhizobial and mycorrhizal associations

The specificity of the legume and rhizobia association has been exploited by farmers and agricultural scientists for centuries. Application of *Rhizobia inoculum* to the seeds of leguminous species is the most widely practiced, conventional agricultural technology used to deliberately manipulate rhizosphere microorganisms. This direct biological intervention has been credited with enhancing N-fixation from 30 to 75 percent in grain legumes (Moawad *et al.*, 1998). However, indigenous strains of Rhizobia are often more effective at colonizing nodules than inoculated strains, even if the seed is inundated with Rhizobia inoculum. The interaction of focal plant with the bacterial inoculum, and the outcome in terms of colonization and development of a symbiotic organ such as nodules, are highly dependent on space and time. For instance, the community of nodule inhabitants is significantly influenced by rhizosystem architecture in inoculated soybeans (Espinosa-Victoria *et al.*, 2000). Nodules located near the central root system are developed through plant symbiotic interactions with inoculated *Rhizobium* sp., while external nodules far from the central axis are likely to be inhabited by indigenous, and often ineffective, Rhizobium. Indigenous rhizosphere populations generally resist invasion by inoculated organisms in the absence of host-microorganism specificity. This is illustrated by the widespread failure of efforts to manage arbuscular mycorrhizae in agricultural systems through inoculation-based technologies (Hamel, 1996). There are exceptions, usually involving inundation of young, uncolonized tissues in an environment with few established organisms. Examples include inoculation of seeds or mycorrhizal treatment of horticultural plantings at mine rehabilitation sites,

containerized systems or seriously degraded and fumigated soils (Jeffries *et al.*, 2003). With the notable exception of the legume–Rhizobia association, inoculation techniques have not led to consistent or persistent effects on nutrient availability in conventional agriculture. A promising area of research is to examine the potential to manage these mutualisms in low-input and organic systems that provide an energetically and biologically favorable environment for displacing or augmenting indigenous micro-flora and fauna, compared to conventional agriculture (Kumar *et al.*, 2001).

Avis *et al.*, (2008) reported that Plant growth promoting microorganisms (PGPM) and biological control agents (BCA) are shown to possess secondary beneficial effects that would increase their usefulness as bio-inoculants, regardless of the need for their primary function. Indeed, PGPM, such as *Rhizobium* and *Glomus* spp., can promote plant growth and productivity (primary effect) but have now been shown to also play a role in reducing disease (secondary effect). Conversely, BCA, such as *Trichoderma* and *Pseudomonas* spp., can control disease (primary effect) but have recently demonstrated stimulation of plant growth (secondary effect) in the absence of a pathogen. Further work shedding light onto the precise mode of action and ecophysiology of these microorganisms would assist with their timely and appropriate use and potentially unleash their full promise as beneficial rhizosphere bio-inoculants for improved growth and health of plants. The potential increased use of these microorganisms afforded by their multifaceted beneficial effects may further help in reducing problems associated with the use of synthetic chemicals in agriculture.

Arbuscular mycorrhizal fungi (AMF) are symbiotic soil fungi that are intimately associated with the roots of the majority of land plants. They colonise the interior of the roots and the hyphae extend into the soil. It is well known that bacterial colonisation of the rhizosphere can be crucial for many pathogenic as well as symbiotic plant– microbe interactions. However, although bacteria colonising the extraradical AMF hyphae (the hyphosphere) might be equally important for AMF symbiosis, little is known regarding which bacterial species would colonise AMF hyphae. Scheublin *et al.*, (2010) investigated which bacterial communities might be associated with AMF hyphae. As bacterial-hyphal attachment is extremely difficult to study *in situ*, they designed a system to grow AMF hyphae of *Glomus intraradices* and *Glomus proliferum* and studied which bacteria separated from an agricultural soil specifically attach to the hyphae.

The significance and role of plant hormones in AM symbiosis have been reviewed (Beyrle,1995) as well as the role of Mycorrhizal fungi in control of various soil borne plant diseases has been reviewed by many workers (Jalali and Jalali, 1991; Jeffries *et al.*, 2003; Aggarwal *et al.*, 2006; Sharma *et al.*, 2009).

Phosphate solubilizing microorganisms (PSM)

Phosphorus is one of major limiting factors for crop production on many tropical and subtropical soils as a result of high phosphorus fixation, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants. The concentration of soluble phosphorus (P) in tropical soil is usually very low. While most mineral nutrients in soil solution are present in millimolar amounts, phosphorus is only available in micromolar quantities or less. The majority of applied phosphorus is rapidly fixed in soil into fractions that are poorly available to plant roots. Inorganic phosphates are predominant form of inorganic phosphates in neutral or calcareous soils (Russel, 1973; Sample *et al.*, 1980; Ozanne, 1980; McLaughlin *et al.*, 1988; Dey, 1988; Sanyal and De Datta, 1991; Goldstein, 1994; Norman *et al.*, 1995; Yadav and Dadarwal, 1997; Gyaneshwar *et al.*, 2002).

Compared with the other major nutrients, phosphorus is by far the least mobile and available to plants in most soil conditions. Although phosphorus is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth. Phosphorus is added in the form of phosphatidic fertilizers, part of which is utilized by plants and the remainder converted into soluble fixed forms. To circumvent phosphorus deficiency, phosphate-solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner.

Biochemistry of Phosphate solubilizers

Pikovskaya (1948) isolated a bacterium from soil and P bearing rocks which he called P bacterium having the ability to form water soluble P from insoluble calcium phosphate. PSMs solubilize insoluble phosphates into soluble form in soil by secreting formic, acetic, propionic, lactic, glycolic, fumaric and succinic acids. These acids lower the pH and bring about solubilization. Glucose, sucrose and galactose are the best carbon source for phosphate solubilization. Decrease pH in the medium during phosphate solubilization is due to the release of organic acid by isolates. Gluconic acid is the most commonly produced acid during phosphate solubilization other mechanism like CO_2 and H_2S production and chelation of other acids are also responsible for phosphate solubilization.

Cattelan *et al.* (1999) conducted a study to identify the specific traits by which plant growth promoting rhizobacteria (PGPR) promotes plant growth. They selected 116 isolates

from bulk soil and the rhizosphere of soybean and examined them for a wide array of traits that might increase early soybean growth in non sterile soil (PGPR traits). A sub sample of 23 isolates, all but one of which tested positive for or one or more of these PPGR traits, was further screened for traits associated with biocontrol, brady- rhizobial inhibition, and rhizosphere competence. Six of eight isolates positive for 1-aminocyclopropane-1-carboxylate (ACC, a precursor of ethylene) deaminase production, four of seven isolates positive for siderophore production, three of four isolates positive for β -1,3-glucanase production and two of five isolates, positive for P solubilization increased at least one aspect of early soybean growth. One isolate which did not share any of the PGPR traits tested *in vitro* except antagonism to *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, also promote soybean growth. One of the 23 isolates changed bradyrhizobial nodule occupancy. Although the presence of a PGPR trait *in vitro* does not guarantee that a particular isolate is a PGPR, the result suggest that rhizosphere able to produce ACC deaminase and to a lesser extent, β -1,3- glucanase or siderophores or those able to solubilize P *in vitro* may increase soybean growth in non sterile soil.

As we know that Phosphorus is one of the major nutrients limiting plant growth but most of the soils throughout the world are P deficient and therefore require P to replenish the P demand by crop plants. To circumvent the P deficiency in soils, P fertilizers are applied. However, after application, a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils reported by Norrish and Roster (1983) or Ca in calcareous soils before plant roots have had a chance to absorb it. Further, the use of rock phosphate as a phosphate fertilizer and its solubilization by microbes through the production of organic acids have become a valid alternative to chemical fertilizers, investigated by Kang *et al.* (2002). Rock phosphate is widely distributed throughout the world, both geographically and geologically, confirmed by Zapata and Roy (2004), in conjugation with phosphate solubilizing microorganism. Rock phosphate provides a cheap source of P fertilizer for crop production. In this regard, several studies of Zaidi (1999), Gull *et al.* (2004) have conclusively shown that PSM solubilizes the fixed soil P and applied phosphates, resulting in higher crop yields. The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of Rhizobium and arbuscular mycorrhizal fungi by Zaidi *et al.* (2003) has shown to stimulate plant growth more then inoculation of each microorganism alone in certain situation when the soil is P Deficient.

Mikanova and Novakova (2002) reported that microbial solubilization of hardly soluble mineral phosphates in soil is an important process in natural ecosystem and in agricultural soil. Regulation of the P-solubilizing activity by the presence of soluble phosphates in medium was determined. For this reason they decided to test a number of soil bacteria showing a high P-solubilizing activity for its sensitivity to the presence of soluble dihydrogen potassium phosphate in medium. In these studies, direct determination of the solubilized phosphate in medium was masked by the presence of relatively high concentration of soluble phosphate added. Therefore, we have modified the method, determining the residual tricalcium phosphate. The effect of soluble phosphate in medium on the P-solubilizing activity of rhizosphere isolates and strains of Rhizobium were tested in liquid cultures with the addition of various concentration of soluble KH_2 PO_4. The medium was filtered after incubation and the remaining tricalcium phosphate was separated by filtrations. Filter papers with the remaining tricalcium phosphate were hydrolyzed with 2N H_2SO_4. Phosphorus was determined spectrophotometrically. The P-solubilizing activity was expressed as a difference between the tricalcium phosphate added and its reminder after the incubation. These results fully confirmed that there exist the strains, whose P-solubilizing activity is inhibited and other strains, whose P-solubilizing activity is not inhibited or is inhibited very little in the presence of soluble phosphate. The use of our adapted method was much more suitable for this type of experiments. Chemical fertilizer have played a significant role in the green revolution, but unbalanced use of them, had led to reduction in soil fertility and to environmental degradation (Gyaneshwar *et al.*, 2002). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. A survey of Indian soil revealed that 98% of soils are deficient in Phosphorous. Although P content in an average soil is 0.05% but only a fraction of this (about 0.1% of the total P present in soil) is available to the plants because of its chemical fixation and low solubility. Chemical phosphate fertilizer and their reaction products are only sparingly soluble under the condition in which they are applied to the soil. However, under such condition microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to plants. PSM include largely bacteria and fungi, which can grow on various phosphorous containing compounds such microbes not only accumulate P but a large portion of soluble phosphate is released in quantities in excess of their own requirement. Rhizosphere microorganisms also have the ability to assimilate different macro- and micro- nutrients and release them to the soils. These nutrients are utilized by the plants in a mutualistic way. Rhizosphere microorganisms have been found to solubilize the low soluble calcium

phosphates via the production of organic acids and chelating and make them available to the plants reported by Nwaga *et al.* (2000). In particular, soil micro-organisms are effective in releasing P from inorganic P through solubilization and form organic pools of total soil P by mineralization.

Twenty three isolates of *Azotobacter* were obtained from pesticide contaminated soils of cotton, sugarcane, brinjal and okra fields of HAU farm by enrichment culture technique by Suneja *et al.* (2004) All the *Azotobacter* isolates belonged to *chroococcum* species on the basis of their biochemical properties. Resistance among these isolates was studied on pesticides like endosulfan, ecalux and confidor. The result indicates that resistance to pesticides is common among *Azotobacter* soil isolates. Decreased resistance with increasing concentration of pesticide and reduced carbon was observed.

In the study carried out by Richa *et al.* (2007) *Aspergillus tubingensis* and *A. niger* were tested for their efficacy to solubilize rock phosphate (RP) and also to improve the growth of maize (*Zea mays*) in rock phosphate amended soils. Both the species was able to grow and solubilize rock phosphate and solubilize rock phosphate and soluble P levels were significantly increased in the culture medium as the concentration of RP increased. The results of nursery experiment showed that the growth of maize plants and shoot were significantly increased by these fungi compared to control soil. Soil analysis results showed that the available P, organic carbon levels were significantly increased when compared to initial soil. The soil pH was also lowered compared to initial pH of the soil. These results suggested that *A. tubingenesis* and *A. niger* serves as excellent phosphate solubilizers in alkaline soils amended with RP.

Nenwani *et al.* (2010) reported that a fungal species namely F1 was isolated from the rhizosphere on the basis of its ability to form halos (zone of solubilization) on Pikovskaya's agar. F1 was assessed for phosphate solubilization, titratable acidity (TA), gluconate concentration and change in pH over incubation period of 21 days and other plant growth promoting traits. F1 solubilized maximum inorganic phosphorus (662 g P ml-1) from tricalcium phosphate present in the Pikovskaya's broth on 18th day. The TA followed a similar trend as that of P solubilized, except on day 21 when the value for TA was highest. A similar pattern was also observed with production of gluconic acid, for which a constant value of 8.96 × 10-4 g% was observed till 18th day of incubation. However, gluconic acid was not the only organic acid produced in the culture broth, because the amount of gluconic acid produced did not relate to the high values observed for P solubilized and TA. Efficiency of the F1 to solubilize phosphate from organic reserves was determined by performing assays of

phosphatases and phytases. The culture F1 produced 1.86 and 1.90 EU of enzymes alkaline and acid phosphatase, respectively and phytase activity was 28 mU. The concentration of catechol and hydroxymate type siderophores produced by F1 was 4.50 and 4.55 μgml^{-1} respectively and it also produced 11.45 μgml^{-1} of IAA which is significantly high. Some fungi may possess traits associated with biocontrol of plant pathogens such as production of enzyme chitinase which was 0.037 EU for F1. On the basis of cultural and microscopic features, the isolate F1 could be *Absidia* spp. and has potential of being a competent bioinoculant.

Antifungal activities

Antifungal metabolites produced by *Bacillus pumilus* in potato dextrose broth were isolated from culture supernatant fluid by precipitation with ammonium sulphate by Munimbazi *et al.* (1998), which inhibited mycelial growth of many species of *Aspergillus, Penicillum* and *Fusarium*. They also inhibited production of aflatoxins, cyclopiazonic acid, ochratoxin A and patulin. Their activity was stable over wide range of temperature and pH (2-10). The metabolites were also resistant to hydrolysis by various proteases, peptidases and other enzymes, so it has the potential to use as fungicide but more investigations is needed with regard to their inexpensive large scale production, evaluation for toxicity and degradation in the environment.

Tarafdar *et al.* (2003) isolated seven efficient phosphatase producing fungi (PPF) and identified as *Aspergillus rugulosus. A. fumigatus, A. terreus, A. niger, A. parasiticus, Pseudeurotium zonatum and Trichoderma harzianum.* Their efficiency to hydrolyze different compounds of organic phosphorus (mono-and hexa) was examined. The fungi reduced the pH of the medium, which was maximum with *A. niger*. A significant negative correlation of pH with development of fungal mats was observed ($r=0.39$, $n=28$, $p<0.05$). The maximum secretion of acid phosphatase by PPF was at 21 d and alkaline phosphatase at 14d. Acid phosphatase produced by PPF was three times higher than alkaline phosphatase. The intracellular phosphatase activity was significantly higher than extra cellular activity. The efficiency to hydrolyze mono- phosphate by phosphatases released from the PPF was 4-times higher than hexa phosphate. *T. harzianum* was found to be most efficient organic P mobilize as compared to the other fungi, tested. The efficiency per unit of enzyme produced by different fungi was different and that indicated the isoenzymes being of different types.

Trivedi *et al.* (2005) studied the microbial diversity of Indian Himalaya and based on a detailed study conducted to isolate microbes from soil samples collected from various tea gardens located in region, two bacteria namely *Bacillus subtilis* and *Pseudomonas corrugate* have been selected as promising inoculants for field application in tea gardens. Bioassays based on the inoculation of seed raised and tissue culture raised tea plants had earlier indicated the biocontrol and growth promotion properties of selected bacteria. With a view to introduce these bacterial isolates eventually in the gardens, suspension cultures were raised and applied in the rhizosphere region of both seedling and cutting raised young tea plants under net-house conditions. Monthly enumeration of bacterial, fungal and actinomycetes populations up to a period of one year, indicated excellent rhizosphere colonization by the inoculated bacteria. The presence of introduced bacteria in the rhizosphere was confirmed by the use of antibiotic markers.

Molecular identification and diversity analysis

Variation within 16S rRNA gene sequences of different bacterial species has significantly increased our understanding of the diversity and ecology of soil bacterial communities. In contrast to bacteria, taxonomic identification of fungi based on sequences of the eukaryotic ribosomal small subunit, the 18S rRNA, is more problematic, with identification commonly limited to genus or family level. This is primarily due to the relative lack of variation within 18S rRNA genes between closely related fungal species as a result of the relatively short period of evolution of the kingdom fungi compared with bacteria (Hugenholtz and Pace, 1996).

Chen (2006) advocated that the ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants. In this study, isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria (PSB) from Central Taiwan were carried out. Mineral phosphate solubilizing (MPS) activities of all isolates were tested on tricalcium phosphate medium by analyzing the soluble-P content after 72 h of incubation at 30 °C. Identification and phylogenetic analysis of 36 isolates were carried out by 16S rDNA sequencing. Ten isolates belonged to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium*. In addition, four strains namely, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. are

being reported for the first time as phosphate solubilizing bacteria (PSB) after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by secreting organic acids. P-solubilizing activity of these strains was associated with the release of organic acids and a drop in the pH of the medium. HPLC analysis detected eight different kinds of organic acids, namely: citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three unknown organic acids from the cultures of these isolates. An inverse relationship between pH and P solubilized was apparent from this study. Identification and characterization of soil PSB for the effective plant growth-promotion broadens the spectrum of phosphate solubilizers available for field application.

Ecology and diversity of phosphate solubilizing microorganisms in 20 soil samples comprising organic and non-organic farming, virgin and barren soils of Gujarat, India were studied by Haque and Dave (2005). No considerable seasonal variation in population densities of various phosphate solubilizers was observed. Out of 40 phosphate solubilizing microorganisms, *Pseudomonas* spp., *Bacillus* spp., *Saccharomyces* spp. and *Aspergillus niger* were found to be most prevalent as they were present in more then 50% of the soil samples. The phosphate solubilization index for different cultures varied between 104 to 240. When the isolates, which gave good phosphate solubilization on solid medium, were further explored for phosphate solubilization in liquid medium, the phosphate solubilization ability decreased to as low as 8% and increased to as high as 99% by these cultures. Moreover, phosphate solubilization in the range of 18.1 to 16.63% was recorded when microbial consortia in the form of soil suspension were inoculated, inspite of semi arid nature of the ecosystem, considerable phosphate solubilizing microbial activity observed indicate the fertility status of the soil in terms of phosphate mobilization.

Biocontrol agents (BCAs)

The adverse effect to the environment due to indiscriminate use of chemical pesticides is of great concern and hence development of alternate control strategies such as biological control as substitute for chemicals or as a key component in integrated disease management system is gaining momentum (Harman *et al.* 2004, Mathivannan *et al.*, 2006; Mukerji *et al.*, 1999; Mukhopadhyay, 2009). Biological control has been developed as an academic discipline during 1970s and is now a mature science supported by both the public and private sectors (Baker, 1988; Mukhopadhyay, 2009) which involves the use of beneficial organisms, their genes and or gene products, such as metabolites, that reduce the negative effects of plant pathogens and promote positive responses by the plant (Vinale *et al.*, 2008)).

Biological control is the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens. *Trichoderma* is the most widely exploited fungal genus as biocontrol agent (BCA) in the field of agriculture for the management of crop diseases caused by a wide range of fungal pathogens.

Trichoderma species : Most potent antagonistic fungi

Species of the fungal genus *Trichoderma* are typically soil dwellers, existing as anamorphs belonging to the sub-division Deuteromycotina (fungi imperfecti) (Hawksworth, *et al.*, 1983). As a rule, *Trichoderma* species are fast growing fungi which are commonly found in a variety of soil types, such as, agricultural, prairie, forest, salt marsh and desert soils in all climatic zones (Brewer *et al.*, 1971; Danielson and Davey, 1973; Domsch *et al.*, 1980). Danielson and Davey (1973) found that *Trichoderma* species constituted up to 3% of the total fungal propagules in a wide range of forest soils and Brewer *et al.* (1971) found that they made up 1.5% of the fungi in pasture soils. Many *Trichoderma* isolates are of great economic importance, producing hydrolytic enzymes (chitinases, cellulases and xylanases) (Schirmbock *et al.*, 1994; Worasatit *et al.*, 1994) biochemicals and antibiotics (Ghisalberti and Sivasithamparam, 1991; Okuda *et al.*, 1982) - products which have been applied to fields such as food processing and pulp bleaching (Buchert *et al.*, 1992; Nigam, 1994). In addition, some species produce heterologous proteins (Cheng *et al.*, 1990; Nevalainen *et al.*, 1991) and others have been successfully used as biological control agents against a range of phytopathogens (Chet, 1987; Chet and Inbar, 1994; Papavizas, 1985; Taylor, 1986; Wells, 1988). However, in addition to their usefulness in industry, some species of *Trichoderma* also pose a threat to the horticultural industry. For example, reduction in mushroom yields has been attributed to *Trichoderma* infection (Seaby, 1997).

Species of *Trichoderma* were found to dominate the rhizosphere of established tea bushes in a detailed study conducted from various tea growing locations in India and the population of *Trichoderma* spp. showed less variation (Pandey and Upadhyay, 2000). In general, fungi associated with the tea rhizosphere were found to prefer a mesophillic temperature range (15°C to 35°C). The dominant species of *Trichoderma* exhibited tolerance to lower temperatures, i.e., 5 to 10 °C on agar plates. Most fungi were able to grow in a wide range of pH (4 to 12). Lowering of soil pH in the rhizosphere of tea bushes was positively correlated with the age of the bush and may have affected the development of a specific microbial community in the rhizosphere. *Trichoderma* species were also isolated by

Chakraborty *et al.* (2010) from different rhizosphere soils collected from various locations of North Bengal region.

Taxonomy of the Genus Trichoderma

The genus *Trichoderma* was first proposed by Persoon (Persoon, 1794) and consisted of four macroscopically similar fungi described as appearing like mealy powder enclosed by a hairy covering. The four species proposed by Persoon were named *T. viride*, *T. nigrescens*, *T. aureum* and *T. roseum*. These were distinguished from each other by their different coloured conidiation. However, these four species are now considered to be unrelated to each other and are presently known as *Trichoderma viride*, *Xylohypha nigrescens* (Pen. ex. Fr.) Mason, *Sporotrichum aureum* Pers ex Fr and *Trichothecium roseum* (Pers.) Link ex S.F. Gray respectively. The name *Trichoderma* is now applied to the most frequently encountered green forms typified by the original *T. viride* species as described by Persoon (1794).

Rifai (1969) provided the first real generic description of *Trichoderrma*, based on colony growth rate and microscopic characters and the genus was split into nine species, distinguished from each other primarily by conidiophore branching patterns and conidium morphology. However, Rifai recognized that the groupings that he defined were made up of more than one genetic entity. These could not be distinguished with his proposed morphological characters and consequently he recommended that they be referred to as species-aggregates. The nine species-aggregates proposed by Rifai were (i) *T. pituliferum*, (ii) *T. polysporum* (iii) *T. hamatum*, (iv) *T. koningii*, (v) *T. aureoviride* (vi) *T. harzianum*, (vii) *T. longibrachiatum* (viii) *T. pseudokoningii* and (ix) *T. viride*. There were, however, problems associated with Rifai's key since significant variation remained to be defined within each of the nine aggregate taxa- for example, *T. harzianum* isolates were found to differ in their ability to attack *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium aphanidermatum* (Elad, 1982). Furthermore, Meyer and Plaskowitz (1989) were able to further divide the *T. viride* aggregate into two groups which had completely different conidial ornamentation types. These groups were referred to as *T. viride* groups I and II. Another problem with Rifai's key was that a number of newly described isolates did not fit into any of the species-aggregates. These isolates were subsequently named as new species (Domsch *et al.*, 1980; Hammill, 1970; Veerkamp *et al.*, 1983). Yet another complication is that five of Rifai's species-aggregates (*T. harzianum*, *T. longibrachiatum*, *T. piluliferum*, *T. polysporum* and *T. pseudoconigii*) appear to be narrowly defined groups, each consisting of isolates with a small range of variation. In contrast, each of the remaining aggregates (*T. hamatum*, *T. koningii* and

T. viride) appears to accommodate isolates with a much broader range of morphological characters. Bissett (Bissett 1984, Bissett 1991a, Bissett 1991b, Bissett 1991c, Bissett 1992) revised the genus in an attempt to address the problems experienced with use of Rifai's key. In 1984, Bissett made a partial revision of the genus *Trichoderma* and established *Longibrachiatum* as a section of the genus in which he included *T. pseudokoningii*, *T. longibrachiatum* and added two new species *T. citrinoviride* and *T. atroviride*. Later, the species *T. parceramosum* and the anamorph of *Hypocrea schweinitzii* (Fr.:Fr.) Sacc were also added to the section *Longibrachiatum*.

Following this, Bissett (1991a) proposed a sectional classification recognising the following five sections within the genus: *Pachybasium*, *Trichoderma*, *Saturnisporum*, section *Longibrachiatum* and *Hypocreanum*. As with the section *Longibrachiatum*, Bissett also revised the section *Pachybasium* (Bissett, 199lb). Section *Pachybasium* consists of the five species *T. hamatum*, *T. polysporum*, *T. piluliferum*, *T. harzianum* and *T. virens* that were already recognised in the genus *Trichoderma*. Also included were the species *T. flavofuscum*, which had been transferred from the genus *Gliocladium*, the anamorphs of *Hypocrea gelatinosa*, *H. semiorbrs* and two unnamed *Hypocrea* species. Ten new species which had been separated from the *T. hamatum* species-aggregate proposed by Rifai (1969), were also included in this section. Since Bissett's key used different and more specific characters in the initial separation of species into sections, some of Rifai's aggregates were not only split up but the members were distributed amongst different sections. For example, where Rifai (1969) placed all isolates with warted conidia in the *T. viride* aggregate, Bissett (1991a, b) placed these in a number of species within a number of sections because the nature of their conidiophore branching pattern takes priority over the presence of warts on their conidia. Although most taxa hold up well under Bissett's classification system, a number of discrepancies do exist. For example, observations made by Samuels *et al.* (1994) did not support the synonym of *T. longibrachiatum* and *T. reesei* proposed by Buchert (1992). Samuels *et al.* (1994) found that the two species could be distinguished from one another by the size of their conidia, their different growth rates and isoenzyme data. Meyer *et al.*, (1991, 1992) and Morarvetz *et al.*, (1992) also demonstrated differences between *T. reesei* and *T. longibrachiatum* using DNA restriction fragment length polymorphisms (RFLPs). In addition to the existence of discrepancies, some of the species (*T. harzianum*) proposed by Bissett were still recognised as species-aggregates rather than species (Bissett 1991b). Since the introduction of the revised key of Bissett, its use has increased in popularity, so that it is now used in preference to the key of Rifai (1969). The genus *Trichoderma* was described by Bissett (1984) as rapidly growing colonies bearing tufted or pustulate, repeatedly branched

conidiophores with lageniform phialides and hyaline or green conidia borne on slimy heads. Teleomorphs were proposed to belong to the genus *Hypocrea* and other closely related genera (Bissett 1991a, Rifai, 1969). Under Bissett's key there are now 30 species of *Trichoderma* described and recognised. However, it is suspected that as many as 150 species may exist. This point of view is supported by the fact that more than 70 species of *Hypocrea* have been described, the vast majority of which have *Trichoderma* anamorphs (Bissett, 1984; Rifai, 1969).

Eighty-nine species of *Trichoderma* have been named, and several species of *Hypocrea* have been linked to unnamed *Trichoderma* anamorphs. Eighty-three taxa of *Trichoderma* and their teleomorphs, *Hypocrea* spp., have been included in phylogenetic analyses, including 11 species of *Hypocrea* with unnamed *Trichoderma* anamorphs. Phylogenetic analyses show that *Trichoderma* and *Hypocrea* are congeneric. *Trichoderma* species not linked to *Hypocrea* teleomorphs are derived from among species that are linked to teleomorphs, indicating sexual and asexual lineages are not independent of each other. Many more species remain to be discovered and described. Molecular phylogenetic analyses have revealed the existence of more species than have been recognized on the basis of morphology alone. A suggestion is made to modify the International Code of Botanical Nomenclature to enable adoption of a single generic name for *Trichoderma/Hypocrea*, with *Trichoderma* being the older and more utilitarian name. As increasing numbers of species are studied, the few morphological characters of anamorph and teleomorph have reached their limit for defining species. DNA-based characters have assumed an indispensable role. Exploration of new niches, such as within tree trunks and new geographic locations, have resulted in a substantial increase in the number of species of *Trichoderma*. *Trichoderma* is usually considered a genus of free-living soil fungi but evidence suggests that *Trichoderma* species may be opportunistic, avirulent plant symbionts as well as parasites of other fungi. Members of the genus *Trichoderma* are universally present in soils, although individual species may be either cosmopolitan (*T. harzianum*) or limited (*T. viride*) in their geographic distribution. To facilitate identification of species, a list of correctly identified strains of *Trichoderma* and their GenBank numbers for sequences of translation-elongation factor EF-1α and internal transcribed spacer rDNA has been provided (Samuels *et al.*, 1998).

Due to the commercial importance of some *Trichoderma* strains, it is important to be able to distinguish these isolates from other *Trichoderma* isolates for the purpose of patent verification. Moreover, if the growing body of knowledge on various aspects of these *Trichoderma* isolates is to be of any use on a global scale, the identification of species and strains must be consistent between different laboratories. The differentiation of *Trichoderma*

species has traditionally been based on morphological features such as colony growth rate, conidiophore arrangement and orientation, colour, size and surface texture of conidia and other such characters observed through light microscopy and scanning electron microscopy studies.

Molecular Systematics

In more recent years, a number of molecular studies have also been employed to characterise *Trichoderma* species. Molecular techniques differentiate between isolates by differences in their DNA and RNA. Techniques focusing on DNA have an advantage over biochemical techniques in that an isolate's DNA content is not affected by external factors such as age and growth medium. Furthermore, extraction of DNA and the identification of differences in the DNA content of different isolates tend to be faster and provide more information than the alternative biochemical techniques. Molecular techniques investigated for their usefulness in differentiating the genus *Trichoderma* include electrophoretic karyotyping of chromosomes and the assignment of specific genes to chromosomes, restriction fragment analysis, restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction based rapid amplification of polymorphic DNA (Williams *et al.*, 1990) analysis and comparison of DNA sequence.

Electrophoretic karyotyping

Development of the pulse field gel electrophoresis technique has permitted the resolution of chromosome sized DNA fragments up to 10 Mb in size and consequently allowed the karyotyping of organisms such as filamentous fungi (Skinner *et al.*, 1991; Smith *et al.*, 1987). Typically, individuals are differentiated by the differences in the number and sizes of their chromosomes on these gels. Further resolution can be achieved by performing southern (Southern, 1975) analysis on the separated chromosomes using gene specific probes. The first karyotype reported for a member of the genus *Trichoderma* was in 1991 when Gilly and Sands (1991) produced a karyotype for a single strain of *T. reesei.* Soon after, Carter *et al.* (1992) successfully used electrophoretic karyotyping, combined with Southern analysis, to differentiate between individual strains of *T. reesei* and several of its mutated derivatives. This result indicated that this technique was capable of distinguishing between very closely related individuals. Similarly, Hayes *et al.* (1993) were able to differentiate between two isolates of the same *T. harzianum* species. Henera-Estrella *et al.* (1993) found that electrophoretic karotyping allowed the differentiation of a phyopathogenic strain of *T. reesei* from two individuals (*T. harzianum and T. viride* strain) with good biological control activity.

Furthermore, the karyotypes of the two biocontrol species were found to be very similar to one another. Southern analysis on the electrophoretically separated chromosomes, using a series of five genes cloned from *T. harzianum* and *T. viride* as hybridisation probes, revealed further differences between the isolates. For example, the prbl gene, encoding a proteinase expressed during the mycoparasitic interaction of *T. harzianum* against phytopathogenic fungi (Geremia *et al.*, 1993), hybridized strongly to chromosomes of *T. harzianum* and *T. viride*. In contrast, *T. reesei* showed a weak hybridization to this probe suggesting that *T. reesei* was evolutionarily more distant from *T. harzianum* and *T. viride* than they are from each other. Poor hybridization of the prbl gene probe to the genome of *T. reesei* is consistent with *T. reesei* not possessing mycoparasitic activity (Herrera-Estrella *et al.*, 1993). Results such as these suggest that electrophoretic karyotype profiles, combined with Southern analysis, may provide a powerful tool for differentiating between closely related strains of *Trichoderma*, as well as, differentiating between isolates with different biological activities.

Restriction fragment analysis

DNA is extracted from an individual and digested to completion with a restriction endonuclease that cleaves the DNA. The resulting fragments are separated by electrophoresis in agarose or polyacrylamide gels and stained with a dye such as ethidium bromide. Differences in DNA fragment patterns following electrophoresis are used to differentiate between individuals. Variation in restriction fragments results from gain or loss of particular sites. This can result from base pair changes at restriction sites or DNA rearrangements between sites. A difference between two organisms in the size of restriction fragments at a definable genetic locus is termed a restriction fragment length polymorphism. Restriction fragment analysis is limited to the investigation of small genomes where the number of fragments generated is not numerous (mitochondrial DNA or plasmid DNA). For example, restriction analysis of mitochondrial DNA (mtDNA) has been widely used for evolutionary studies in fungi and individual isolates have been differentiated to the subspecies level (Martin, 1990; Smith *et al.*, 1994; Taylor, 1986). Plasmids are of more limited usefulness in taxonomic studies. Although they are extremely common in fungi (Samac and Leong, 1989), they tend to be neither universally present nor conserved in sequence, and may be horizontally transmitted (Collins and Saville, 1990; May and Taylor, 1989). To date, there has been only one report where mtDNA and plasmids have been investigated in relation to the classification of *Trichoderma* species. Meyer *et al.*, (1991) purified the mtDNA from isolates of *T. viride* and found that eight isolates had plasmids in their mtDNA preparations. No phenotype could be associated with the presence of a plasmid and isolates with similar

plasmids did not have similar mtDNA restriction patterns. In contrast to plasmid DNA analysis, restriction endonuclease digestion of isolate mtDNA produced patterns in which the presence or absence of certain fragments correlated with the classification of the strains into *T. viride* groups I or II. Based on conidial ornamentation, these groups had previously been described by Meyer and Plaskowitz (1989). Moreover, all but two strains produced unique fragment patterns indicating that this technique was useful in subdividing *Trichoderma* species.

RFLP analysis

In contrast to mitochondrial or chloroplast DNA restriction digests, digestion of total genomic DNA often generates digestion patterns that are extremely complex. The digest often resembles a smear and discrete fragment bands are usually not evident. In such cases, variation in restriction fragment length may only be detected by using labeled hybridization probes in Southern analysis to sample a subset of the genome. This process of restriction digestion followed by Southern hybridization is referred to as RFLP analysis and, like restriction fragment analysis, yields a band pattern for a specific genotype (Epplen, 1988; Jeffreys *et al.*, 1985). Meyer *et al.*, (1991) were the first to apply RFLP analysis to *Trichoderma* and successfully differentiated the genus from isolates of the two genera *Penicillium* and *Aspergillus*. Following this, Meyer *et al.* (1991, 1992) employed RFLP analysis to investigate genomic differences between nine species of *Trichoderma* and three strains of *T. reesei* (wildtype and two mutants). Each of the species could be distinguished from one another while one of the mutant *T. reesei* strains was found to be distinct from the wildtype and the other mutant. They proposed the reclassification of the *Trichoderma* genus into the five groups: (I) *T. reesei*, *T. todica* (II) *T. polysporum*, *T. longibrachiatum*, *T. koningii*, *T. psuedokoningii* (III) *T. virgatum* (IV) *T. saturnispornm* and (V) *T. harzianum*. This new classification system was consistent with results from a study where these isolates were characterised by the relative position of two cellulase genes (chl and cbhll) on restriction fragments (Morarvetz *et al.*, 1992). High cellulase producing *Trichoderma* species were found in groups I and II only. Although cellulase genes were present in the species *T. harzianum*, *T. saturnisporum* and *T. virgatum*, these species did not produce associated proteins and always produced a different restriction fragment banding pattern from the cellulose producing species. In addition, both Meyer *et al.*, (1991, 1992) and Morawetz *et al.*, (1992) found that *T. reesei* strains were clearly distinguished from *T. longibrachiatum*, to which they were for a long time considered to be identical (Bissett 1984, Rifai 1969). This was also confirmed by isoenzyme studies conducted by Samuels *et al.*, (1994). Both studies

also found that *T. todica* and *T. reesei* produced identical band patterns, suggesting they may belong to the same species-aggregate. However, conclusions drawn from Meyer *et al.*, (1991, 1992) and Morawetz *et al.*, (1992) should be considered with caution since each species-aggregate was represented by only one isolate. It, therefore, appears that RFLP analysis also provides a powerful tool for the differentiation of *Trichoderma* species.

RAPD PCR analysis

RAPD PCR is a technique where a single oligonucleotide primer (typically 10 bases in length) is used in conjunction with a DNA polymerase enzyme, such as *Taq* polymerase, to amplify regions of genomic DNA. A DNA amplification product is generated for each genomic region that is flanked by a pair of priming sites (in the appropriate orientation) and the resulting amplification products are analysed by elecrophoresis in an agarose or polyacrylamide gel. A particular DNA fragment which is generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker.

The RAPD technique has been successfully used to distinguish subgroups within 23 strains of *T. harzianum* and 19 strains of *T. viride* (Zimand *et al.*, 1994). However, in the same study, no genetic variation was found between five strains of *T. hamatum*. Zimand *et al.* (1994) also found that RAPD based subgroups of *T. harzianum* correlated with isolates geographical origins with strains from the same area producing identical band patterns. The RAPD technique has also been successfully employed to eliminate duplicate strains when screening for metabolite production in *Trichoderma* strains (Fujimori and Okuda, 1994). In addition, Arisan-Atac *et al.* (1995) recently discovered that isolate RAPD band patterns correlated well with an isolate's ability to inhibit the growth of the chestnut blight fungus *Cryphonectria parasitica* (Munill) Barr in dual culture.

The RAPD technique does have several advantages over other molecular techniques since only very small amounts of DNA are required, radioactive probes are not required and it is faster and easier to carry out with deca primers (Table 1). Furthermore, there are a large variety of commercially available primers which increases the possibility of detecting DNA polymorphisms for very closely related strains.

Nucleotide sequence comparison

The region of sequence most commonly used to differentiate between organisms is that of the ribosomal RNA gene complex (rDNA). This is because rDNA exists in numerous copies within the genome and contains areas of sequence that differ in their level of variation between organisms. This variability allows the differentiation of individuals at the genus,

species or sub-species level, depending on which area of rDNA sequence is compared (Qu *et al.*, 1983; White *et al.*, 1990). Sequence data is usually obtained by either sequencing of cloned rDNA or direct sequencing of amplified DNA fragments generated from rDNA templates. The data are often employed to determine gene phylogenies which are subsequently used to infer species phylogenies. Phylogenetic studies provide information about the evolutionary relationships of a group of isolates, information which can be useful in their characterization. Nucleotide sequence comparison has been used to differentiate between morphologically identical strains of *Trichoderma* (Muthumeenakshi *et al.*, 1994).

Table : 1 Some RAPD primer sequences (5' – 3') used for *Trichoderma* spp.

Code	Sequences	Code	Sequences
A-01	CAG GCC CTT C	A-5	AGG GGT CTT G
A-20	GTT GCG ATC C	AA-3	TTA GCG CCC C
AA-04	AGG ACT GCT C	AA-06	GTG GGT GCC A
AA-09	AGATGG GCA G	AA-15	ACG GAA GCC C
AA-17	GAG CCC GAC T	AC-03	CAC TGG CCC A
F-3	CCT GAT CAC C	OPA10	GTGATCGCAG
OPA17	GACCGCTTGT	211	GAAGCGCGAT
220	GTCGATGTCG	232	CGGTGACATC
238	CTGTCCAGCA	OPH-19	GACCAGCC
OPE-16	GGTGACTGTG	D-06	ACCTG AACGG
A-4	AAT CGG GCT G	D-09	CTCTGGAGAC
AA-11	AGA CGG CTC C	OPA1	CAGGCCCTTC
AA-7	CTA CGC TCA C	203	CACGGCGAGT
AA-14	AAC GGG CCA A	230	CGTCGCCCAT
AA-18	TGG TCC AGC C	241	GCCCGACGCG
F-12	ACG GTA CCA G	OPH-20	GGAGACATC
D-03	TCTGGTGAGG		

Rehner and Samuels (1994) found that phylogenetic analysis of sequence data from the 28S ribosomal gene supported the transfer of *Gliocladium virens* to *Trichoderma virens* as was proposed by Von Arx (1987) and Bissett (1991a). In addition, the *T. virens* isolates formed a clade with isolates from the two *Hypocrea* species *H. gelatinosa* and *H. lutea*, supporting the proposed teleomorphic/anamorphic relationship between the two genera. The main advantage of using sequence data over other molecular techniques is that a large number of characters are compared between individuals which can substantially increase isolate resolving power. Furthermore, results from different laboratories can be directly compared, and the publication of sequences and their deposition in electronic databases (GENBANK, EMBL) facilitates the confirmation of results and their application to other taxa without the need to obtain strains or clones, or to repeat experiments.

Studies employing a combination of molecular techniques

There have been a limited number of studies where more than one biochemical and molecular technique were employed to differentiate between isolates of *Trichoderma*. In all instances there was good correlation between the results obtained with each technique. In the first study, four *T. harzianum* isolates (including one wildtype and gamma-ray induced mutants) and one *T. reesei* isolate were subjected to RFLP analysis, RAPD PCR analysis and to analysis by comparison of sequence from the ITSI and ITS2 regions of rDNA (Schlick *et al.*, 1994). Both RFLP analysis and RAPD PCR allowed identification and differentiation of the individual *Trichoderma* strains and mutants. In contrast, sequence comparison of the ITSI and ITS2 regions of the rDNA gene complex provided differentiation at the species level only. In the second study, Muthumeenakshi *et al.* (1994) analysed T. *harzianum* strains using a number of techniques and found good correlation between isolate morphology, pathogenicity on commercially grown mushrooms, RFLP analysis, RAPD analysis and ITS1 sequence data. When these were analysed by RFLP analysis, their rDNA and mtDNA separated them into three major groups 1, 2 and 3 (Muthumeenakshi *et al.*, 1994). RAPD analysis on 30 randomly chosen isolates from the original, were generally consistent with the three groups identified by RFLP analysis. Similarly, nucleotide sequence determination of the ITS I region of rDNA for 18 of the isolates revealed three distinct ITS types which were consistent with the three RFLP groupings. Furthermore, the three molecular based groupings (Muthumeenakshi *et al.*, 1994) correlated well with the three biological forms described by Seaby (1997) and Doyle (1991). These biological forms were differentiated by their growth rates and time and pattern of sporulation when grown under specified cultural conditions.

The three molecular techniques clearly distinguished group 2 isolates, the aggressive colonisers of mushroom compost, from the isolates belonging to the other two groups. There has also been a study where metabolite production was combined with RAPD analysis (Fujimori and Okuda, 1994). In that study, RAPD band patterns of 74 strains of *Trichoderma* were found to correlate well with strain morphological and cultural properties, metabolite production profiles (isonitriles) and ecological data. For example, *T. harzianum* could be split into two groups, *T. harzianum* 1and 2, based on morphology, metabolite production and RAPD band patterns.

Phylogenetic studies

Phylogenetics is the study of relationships (or classification) based on closeness of evolutionary descent and usually involves the construction of a branch-like diagram known as a phylogenetic tree. Phylogenetic analysis can sometimes provide additional information

about a group of isolates not immediately obvious from the raw data. For example, RAPD data generated from *T. viride* strains, two strains of the teleomorph *Hypocrea rufa* and 13 other strains of *Trichoderma* with three primers revealed that most of the isolates could be differentiated from one another, and those that produced the same band pattern were identified as the same morphological species. However, when these data were subjected to parsimony analysis, it was further revealed that all isolates with biocontrol activity formed distinct clusters from the biocontrol-negative strains, thus, indicating a relationship between those isolates with biological control activity. Furthermore, teleomorphic strains of *H. rufa* clustered with the biocontrol-negative *T. viride* strains, which was consistent with their inability to antagonise the mycelium of the chestnut blight causing fungus *Cryphonectria parasitica* (Arisan-Atac *et al.*. 1995). In addition, phylogenetic analysis of isoenzyme data also identified core groups of strains within each of the morphological species (Stasz *et al.*, 1989).

A polymerase chain reaction-amplified DNA containing the internal transcribed spacer (ITS)-1, 5.8S, and ITS-2 regions of the nuclear ribosomal DNA transcriptional unit was sequenced for 81 isolates of *Trichoderma* spp. associated with mushroom culture or used for biological control of plant pathogens. Phylogenetic analyses revealed that the biocontrol isolates were more closely related to an isolate of *T. harzianum* biotype 1 (Th1) than to the aggressive biotypes 2 and 4. Th1 has been isolated from mushroom compost but is not the cause of widespread green mold epidemics that have occurred during the last 12 years in Europe and North America. Three isolates of *T. harzianum* obtained from shiitake (*Lentinula edodes*; Shi1B and S3-96) and maitake (*Grifola frondosa*; Mai1) substrates were placed within the biocontrol group. Authors also found evidence suggesting that some isolates of *T. harzianum* originally identified as Th4 from Pennsylvania were more closely related to Th2 from Europe. Finally, considering the wide range in sequence distribution of their samples, it was proposed that the consensus sequence found in their investigation be used as the reference sequence for further studies involving the identification and taxonomy of *T. harzianum*.

The genus *Trichoderma* has been reported to be strains of *Trichoderma virens*, *T. harzianum*, and *T. viride*. Since *Trichoderma* BCAs use different mechanisms of biocontrol, it is very important to explore the synergistic effects expressed by different genotypes for their practical use in agriculture. A certain degree of polymorphism was detected in hybridizations using a probe of mitochondrial DNA. Sequencing of internal transcribed spacers 1 and 2 (ITS1 and ITS2) revealed three different ITS lengths and four different sequence types. Phylogenetic analysis based on ITS1 sequences, including type strains of

different species, clustered the 17 biocontrol strains into four groups: *T. harzianum-T. hamatum* complex, *T. longibrachiatum*, *T. asperellum*, and *T. atroviride-T. koningii* complex. ITS2 sequences were also useful for locating the biocontrol strains in *T. atroviride* within the complex *T. atroviride, T. koningii*. None of the biocontrol strains studied corresponded to biotypes Th2 or Th4 of *T. harzianum*, which cause mushroom green mold. Correlation between different genotypes and potential biocontrol activity was studied under dual culturing of 17 BCAs in the presence of the phytopathogenic fungi *Phoma betae*, *Rosellinia necatrix*, *Botrytis cinerea*, and *Fusarium oxysporum* f. sp. *dianthi* in three different media (Hermosa *et al.*, 2000).

Analysis of internal transcribed spacer -1 region of the rDNA can be used to detect species level of *Trichoderma harzianum*. Internal transcribed spacer- 1 region (ITS 1) of the ribosomal DNA was amplified by polymerase chain reaction (PCR). The PCR purification products were proved possible to amplify the ITS 1 region of all *Trichoderma* strains. The amplified DNA was sequenced and aligned against using ex-type strains sequencings from *TrichoBLAST* /GenBank and established *Trichoderma* taxonomy.

Thirty-six isolates were positively identified as *Trichoderma harzianum* (32 strains) *Trichoderma virens* (3 strains) and *Trichoderma longibrachiatum* (1 strain) formed clearly defining phylogenetic analysis. *T. virens* and *T. longibrachiatum* which were used as an outgroup in these analyses. To this end, it was proposed that the ITS-1 region sequences be used as the reference sequence for future study involving the identification and taxonomy of *Trichoderma harzianum* with dendogram presentation. Amplification of ITS 1 region of the rDNA has showed potential as a rapid technique for identifying *Trichoderma harzianum* successfully fungi in all cases (Shafiquzzaman *et. al.*, 2007)

***In vitro* inhibition of Phytopathogens and Biological control of plant diseases**

There have been numerous reports on the ability of *Trichoderma* species to antagonise a wide range of commercially important plant pathogens combined with their ability to reduce the incidence of disease caused by these pathogens in a wide range of crops. The use of biological control agents provides an alternative to the use of chemicals for pest and disease control. Biological control has certain advantages over the use of chemicals. These include greater public acceptance of produce and a reduction in the risk of chemical residue contamination of the environment. The fact that biological control agents are a renewable resource, and their production is relatively inexpensive is also advantageous. Furthermore, biological control agents are usually target specific and by using these agents in conjunction with fungicides, the level of fungicide applied can be reduced. A reduction in the use of

fungicide is desirable because it reduces the risk of the build up of resistant pathogenic strains, as well as, the risk of accelerated microbial degradation of fungicides (Papavizas 1985; Wells, 1988; Chet, 1987; Chet and Inbar, 1994). Pandey and Upadhyay (2000) reported that rhizosphere of healthy pigeonpea plant was heavily colonised by resident *Trichoderma* and *Gliocladium* which were highly antagositic to the pathogen. *T. viride* formed loops, coiling and ruptured the cell wall of the pathogen. Mechanism of parasitism between Fusarium *udum* and *G. virens* resulted in twisting, air bubbling and disintegration of pathogen hyphae while *T. harzianum* causes severe vaculation, shrinkage and coagulation of cytoplasm of pathogen hyphae.

In vitro evaluation of antagonists revealed that 38.42% and 32.78% inhibition of *Rhizoctonia solani* was achieved by the application Trichodrma *longibrachiatum* and *T. harzianum* respectively (Sharma and Gupta, 2003). Parveen *et al.*, (2004) reported the mode of antagonisms of *Trichoderma viride* against *Alterneria triticina* causing leaf blight of wheat which was studied *in vitro* by employing dual culture technique. *T. viride* inhibited the growth of the pathogen; its mycelial strands coiled around the hyphae of the test pathogen forming a rope like structure and finally desintegrating the test pathogen, *A. triticina*.

Singh *et al.* (2004) evaluated *Trichoderma viride*, *Trichoderma harzianum*, *Gliocladium virens* and *Aspergillus nidulans* as seed, soil and combined seed and soil treatment for the control of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* in green house. *Trichoderma viride*, *Trichoderma harzianum* and *Gliocladium virens* as seed treatment @10g/kg seed were effective in controlling seedling mortality upto 85% and were as per with carbendazin. Sharma and Champawat (2004) reported that *Aspergillus niger*, *Trichoderma harzianum*, *Trichoderma viride*, and *Penicillum aurantiogrisem* and the bactrerium (B1) and *Bacillus subtilis* were isolated from the rhizosphere while *A. nidulens* ver *acristatus*, *Drechslera specifera*, *Gliocladium virens*, *Fusarium solani*, *Fusarium moniliforme*, *Fusarium oxysporum* and the bacteria (B2) were isolated from rhizoplane. Amongst the various rhizospheric microorganisms, *Trichoderma viride* and from rhizoplane microorganisms *Gliocladium virens* and bacterium (B2) proved effective against *Fusarium oxysporum* Schlecht under experimental condition. The spore of *F. oxysporum* Schlecht germinated minimum in association with rhizospheric *Trichoderma viride*. The rhizoplane microorganisms *G. virens* and bacterium (B2) exhibited minimum spore germination of *Fusarium oxysporum*. In a study by Mulaw *et al.* (2010) using molecular methods it was revealed that the community of *Trichoderma* in the rhizosphere of *Coffea arabica* in its native forests is highly diverse and includes many putatively endemic species. Among others, the putative new species were particularly efficient to inhibit growth *of Gibberella xylarioides*.

Bioformulations of Trichoderma

Species of *Trichoderma* exhibiting good biological control activity have also proved to be particularly amenable to studies because, as a rule, they are ubiquitous, easy to isolate and culture and grow rapidly on many substrates. These species compete well for food and site, grow well on root surfaces, produce a wide range of antibiotics and act as mycoparasites utilizing an enzyme system capable of attacking a wide range of plant pathogenic fungi. Those *Trichoderma* isolates that have exhibited good biological control activity most frequently belong to one of four species-aggregates: *T. hamatum*, *T. harzianum*, *T. koningii* and *T viride*. Biological control activity of these isolates has been demonstrated *in vitro* and glasshouse studies where the environment is controlled and in numerous field trials (Table 2).

Table 2. Examples of the successful field control of phytopathogens by *Trichoderma* species.

Trichoderma	Crop	Pathogen	References
Trichoderma	Citrus trees, kiwi fruit vines, pine trees	*Armillaria* species	Bliss (1951), Cutler and Hill (1994), Munnecke (1972), Ohr *et al.* (1973)
Trichoderma	Apple, strawberry, kiwifruit	*Botryrts cinerea*	Sutton and Peng (1993), Tronsmo and Raa (1977)
Trichoderma	stone-fruit and other crops	*Chondrostereum purpureum*	Dubos and Ricard (1974), Meyer and Plaskowitz (1989)
Trichoderma	Tomato, bean, iris, sugarbeet, cotton	*Sclerotium rolfsii*	Elad *et al.* (1980), Latunde-Dada (1993), Upadhyay and Mukhopadhyay (1986)
Trichoderma	Tomato	*Fusarium oxysporum*	Marois *et al.* (1981), Sivan (1987)
Trichoderma	Apples	*Nectria galligena*	Corke and Hunter (1979)
Trichoderma	Sugarbeet	*Phoma* betae	Grondona *et al.* (1992)
Trichoderma	strawberry, cucumber, potato, tomato, cotton	*Rhizoctonia solani*	Beagle-Ristaino and Papavizas (1985), Chet (1987), Lewis and Papvizas (1980)
Trichoderma	Chrysanthemum	*Sclerotiana sclerotiorum*	Delgado De Kallman and Arbelaez Torres (1990)
Trichoderma	Onion	*Sclerotiun cepivorum*	Abd-El-Moity and Shatla (1981)
Trichoderma	Maize, melon	*Macrophomina phaseolina*	Elad and Chet (1986)
T. harzianum T-39, T. atroviride P1	Bean, tomato, pepper, Tobacco, lettuce,	*Botrytis cinerea*	De Meyer *et al.* (1998)
Trichoderma GT3-2	Cucumber	Green-mottle mosaic virus	Lo *et al.* (1998)
T. harzianum T-22	Tomato	*Alternaria solani*	Seaman (2003).
T. asperellum T-203	Cucumber	*Colletotrichum orbiculare*	Koike (2001)
T. harzianum	Apple	*Phytophthora capsici*	Ahmed *et al.* (2000).
Trichoderma virens	Gladiolus	*Fusarium oxysporum*	Mishra *et al.* (2005)
Trichoderma harzianum	Chickpea	*Fusarium oxysporum*	Mukhopadhyay (1992)

However, despite extensive research over the last 70 years on the biological control capabilities of *Trichoderma* species, few isolates have been commercialized. Three examples of where preparations of *Trichoderma* have been commercialised include the marketing of *Trichoderma* biocontrol agents by Binab Corporation (Sigtuna, Sweden) and the use of *Trichoderma*-based biofungicide products (Agrimm Technologies Limited, New Zealand) in the New Zealand horticultural industry for the control of a range of plant pathogens and the marketing of a preparation called Trichodex (Abbot Laboratories, Australia) for the control of Botrytis bunch rot of grapes.

The fact that there are relatively few examples of commercialisation can be attributed to the lack of consistency observed in the control of phytopathogens by *Trichoderma* species. In an attempt to address this problem, research in the field of biological control is now focused on understanding how disease control is achieved and how the factors that affect its efficiency can be optimised. For instance, research is being directed towards understanding the mode of action of *Trichoderma* biological control agents, with a view to enhancing biological control activity via either mutation or genetic manipulation of genes associated with biological control activity (Papavizas *et al.*, 1982, Faull and Graeme-Cook, 1992; Harman *et al.*, 1980; Hayes *et al.*, 1993). Research conducted at HortResearch, using 14 0f the 50 strains of *Trichoderma*, has found that those that produced high quantities of the antimicrobial secondary metabolite 6-penryl-a-pyrone (PAP) and other active compounds exhibited the greatest biological control activity (Robert Hill pen comm.). Also, direct injection of kiwi fruit vines with either PAP (extracted from one of the *Trichoderma* isolates) or synthetic 6-amyl-cr-pyrone, increased the survival rate of vines when under natural *Armillaria* induced disease conditions. However, injection with formulations containing propagules of *Trichoderma* more effective. Moreover, when pastes containing propagules of *Trichoderma* were applied directly to areas of infection, vines were completely healed, even in situations where as much as four fifths of the vascular cambium had been destroyed. The spread of *Armillaria* within kiwi fruit orchards was also inhibited when formulations, consisting of mixed populations of *Trichoderma* strains were used to coat old tree stumps within the orchard, or added to barrier trenches (physical barriers separating kiwi fruit vines from infectious *Armillaria* sites) or the soil (Cutler and Hill, 1994). The same *Trichoderma* strains were also tested in pine tree field trials. The survival and vigour of pine trees were determined after 15 months. Those trees which had had their seedling root systems immersed in a slurry containing mixed populations of *Trichoderma* strains prior to planting had both a higher survival rate and were significantly more vigorous when compared to trees that were not treated (Cutler and Hill, 1994).

In addition to controlling *Armillaria* induced disease, these same *Trichoderma* strains have also provided good control of *Chondrostereum purpureum* (silver leaf) induced disease of pip fruit, stone fruit and *Leucadendron* as well as *Corticium rolfsii* (sclerotium) disease in capsicum.

Some of the currently available commercial bioformulations of *Trichoderma* world-wide are - Biofungus (Belgium), Bineb-T (Sweden,U.K.) , Planterbox (U.S.A.), Rootpro, Trichodex, Trichoderma 2000 (Israel), Supresivit (Denmark) Trichopel, Trichodowels (New Zealand), Talc based formulations - Biocure F, Biogourd, Funginil, Echoderma, Trieco, Trishul, Trichodermin- 6(Indian market).

Mode of action

The mechanisms by which disease control is achieved by *Trichoderma* species are not clear, they undoubtedly involve one or more of the following; mycoparasitism and hyphal lysis, antibiosis, competition for nutrients and space, and SAR (Baker, 1988; Chet, 1987; Henis, 1984; MacKenzie *et al.*, 1995; Papavizas, 1985).

Mycoparasitism and hyphal lysis

Trichoderma species have been shown to be capable of parasitising and killing a wide range of plant pathogenic fungi from genera such as *Alternaria, Colletotrichum, Diaporthe, Endothia, Fusarium, Fusicladium, Helminthosporium, Phytophthora, Pythium, Rhizoctonia, Rhizopus, Sclerotinia, Sclerotium, Venturia* and *Verticillium* (Chet, 1987; Chet and Inbar, 1994; Papavizas, 1985; Wells, 1988). Evidence of mycoparasitism by *Trichoderma* species has largely been provided by fluorescence microscopy studies, SEM studies and by the production of enzymes (chitinase, p-1, 3-glucanase) capable of degrading fungal cell walls at the site of invasion. Fluorescence microscopy and SEM studies have shown *Trichoderma* hyphae coiled around the hyphae of the phytopathogen, penetration and growth into the phyopathogen's hyphae and, in some cases, the lysis of the hyphae (Elad *et al.*, 1983). Furthermore, removal of the coiled hyphae has revealed partial degradation of the phytopathogen's hyphal wall, outlining the area of former contact. Elad *et al.* (1983) observed that enzymatic activity was reduced in the presence of cycloheximide, indicating that β-1, 3-glucanase was excreted by *Trichoderma* species at the sites of contact. It appears that mycoparasitism may not be a random phenomenon as Chet *et al.* (1987) found that the hyphae of *T. hamatum* grew directly toward hyphae of *Rhizoctonia solani. Trichoderma* species have also been shown to be capable of attacking rhizomorphs, sclerotia and fruiting structures of numerous fungal species (Dumas and Boyonoski, 1992; Stewart and Hanison,

1988; Wells, 1988). For example, light microscopy and SEM studies conducted by Stewart and Harrison (1988) revealed that the sclerotia of the onion pathogen *Sclerotium cepivorum* were penetrated, colonised and killed by an isolate of *T. virens* (Samuels and Rehner, 1993).

Antibiosis

The production of antifungal metabolites by species of *Trichoderma* is proposed to aid in biological control activity by either killing the plant pathogenic fungi or inhibiting their growth, thus, giving the *Trichoderma* a competitive advantage over these fungi. *Trichoderma* species have been shown to produce a wide variety of antibiotics, several of which have demonstrated antifungal properties *in vitro* (Claydon *et al.*, 1987; Dennis and Webster, 1971; Dunlop *et al.*, 1989; Ghisalberti and Sivasithamparam, 1991; Okuda *et al.*, 1982; Scarselletti and Faull, 1994; Simon *et al.*, 1988). However, at this stage it can only be assumed that these compounds are produced, to some extent, under natural conditions and contribute to the colonizing potential and biological control activity of *Trichoderma* species. To date, the most convincing evidence for the importance of antibiosis in biological control has been provided by the production and study of antibiotic deficient mutants (Faull and Graeme-Cook, 1992; Howell and Stipanovic, 1983). Howell and Stipanovic (1983) found that UV light induced mutants of *T. virens* (*Gliocladium virens*), deficient in the production of the antibiotic gliovirin, had concurrently lost their ability to provide biological control of *Pythium* induced damping-off disease of cotton seedlings. Furthermore, another mutant with enhanced gliovirin production was more inhibitory to *P. ultimum* in culture than the wildtype. Similarly, Faull and Graeme-Cook (1992) found that a UV light induced mutant of *T. harzianum*, deficient in the production of the antifungal compound 6-pentyl-a-pyrone, also had reduced biological control activity.

Competition

The ability of *Trichoderma* species to compete well for space and nutritional resources that could potentially be utilized by phytopathogens is proposed to result in a reduction in inoculum levels of the less competitive phytopathogenic fungi and, therefore, reduce the level of plant disease. The fact that *Trichoderma* species occur frequently in agricultural and natural soils throughout the world suggests that they are excellent competitors for space and nutritional resources. Furthermore, *Trichoderma* species display good competitive advantage in their ability to recolonise rapidly fumigated and partially sterilized soils (Bliss, 1951; Evans, 1955; Hubbard *et al.*, 1983). This competitive ability of *Trichoderma* species does appear to be dependent on soil properties such as temperature

(Elad *et al.*, 1982; Harman *et al.*, 1980), pH (Chet *et al.*, 1981; Marshall, 1982) iron availability (Hadar *et al.*, 1984; Hubbard *et al.*, 1983) and soil moisture levels (Liu and Baker, 1980). It is also affected by the level of antagonism directed towards the *Trichoderma* by other soil borne micro-organisms. There is evidence to suggest that these interactions play a major role in the regulation of potential inoculum levels of *Trichoderma* in the soil (Papavizas, 1985).

Plant growth Promotion

It has been hypothesised that *Trichoderma* species may also reduce disease by promoting the growth and development of the plant, thus, giving the plant a competitive advantage over potential pathogens. For example, application of *Trichoderma* species to the soil was associated with a reduction in the germination period of pepper seed, thus, shortening the length of time that these plans were susceptible to damping-off diseases (Chang *et al.*, 1986). Furthermore, soil applications of *Trichoderma* were also associated with an increase in the number of blooms per plant on ornamentals (Chang *et al.*, 1986; Ousley *et al.*, 1994) and with an increase in weight and height of ornamental plants, vegetable crops (Chang *et al.*, 1986; Inbar *et al.*, 1994; MacKenzie *et al.*, 1995; Ousley *et al.*, 1994) and pine trees (Kleifeld and Chet, 1992).

The increase in shoot growth and leaf area in *Trichoderma* treated seedlings suggests a common beneficial role of *Trichoderma harzianum* in improving plant growth (Yedidia *et al.*, 2001). The mechanisms involved in increasing growth responses induced by *Trichoderma* sp. might be the production of growth-stimulating compounds (Chang *et al.*, 1986; Gravel *et al.*, 2006; Harman *et al.*, 2004; Yedidia *et al.*, 2001).

SAR

Induced-resistance systems in plants are complex, but have been partially elucidated in several model plant systems. The figure shows a model of induced resistance in tomato. There are three generally recognized pathways of induced resistance in plants. Two of these pathways involve the direct production of pathogenesis-related (PR) proteins; in one pathway, the production of PR proteins is generally the result of attack by pathogenic microorganisms, and in the other pathway, PR proteins are generally produced as a result of wounding, or necrosis-inducing plant pathogens - for example, herbivory by insects although both pathways can be induced by other mechanisms. Typically, the pathogen-induced pathway relies on salicylic acid produced by the plant as a signalling molecule, whereas the herbivory-

induced pathway relies on jasmonic acid as the signalling molecule. These compounds, and their analogues, induce similar responses when they are applied exogenously, and there is considerable crosstalk between the pathways (Bostock, 2001). The terminology that is associated with these two pathways is confusing, and depends on the tradition of individual researchers (Hammerschmidt *et al.*, 2000). The jasmonate-induced pathway is designated as induced systemic resistance, and this term is also used to refer to the quite different process that is initiated by rhizobacteria (Bolar, 2000).

The jasmonate- and salicylate-induced pathways are characterized by the production of a cascade of PR proteins. These include antifungal chitinases, glucanases and thaumatins, and oxidative enzymes, such as peroxidases, polyphenol oxidases and lipoxygenases. Low-molecular-weight compounds with antimicrobial properties (phytoalexins) can also accumulate. The triggering molecules in the *Trichoderma* responses are not clear, but possibly may result in the direct accumulation of PR proteins or phytoalexins as systemic acquired resistance (SAR). The third type of induced resistance has been best-described as being induced by non-pathogenic, root-associated bacteria, and rhizobacteria-induced systemic resistance (RISR). It is phenotypically similar to the jasmonate- and salicylate-induced systems, as it results in systemic resistance to plant diseases. However, it is functionally very different, as the PR proteins and phytoalexins are not induced by root colonization by the rhizobacteria in the absence of attack by plant-pathogenic microorganisms. However, once pathogen attack occurs, the magnitude of the plant response to attack is increased and disease is reduced. Thus, RISR results in a potentiation of plant defence responses in the absence of the cascade of proteins that is typical of the jasmonate- or salicylate-induced systems (Gary *et al.*, 2004).

3.1. Griding of study area

North Bengal has a total area of 21763.0 sq km stretching from $24^0$40'28'' N to $27^0$13' N Latitudes and $87^0$45'50'' to $89^0$54'35'' E Longitudes. The entire area comprises of six districts and three important ecological zones (Plate 4).

3.1.1. Terai-Dooars region

The Terai (moist land) is a belt of marshy grasslands, savannas and forests at the base of the Himalaya range stretching southwards to about 38 km. Above the Terai belt lies the Bhabhar, a forested belt of rock, gravel and soil eroded from the Himalayas. The Terai zone is composed of alternate layers of clay and sand with a high water table that creates many springs and wetlands. The terai zone is inundated yearly by the monsoon-swollen rivers of the Himalaya. The Terai-Dooars savanna and grasslands is an ecoregion that stretches across the middle of the Terai belt. The Terai-Dooars savanna and wetlands are a mosaic of tall grasslands, savannas, 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, Raidak, Jaldhaka, Sankosh, Balason, Atrai and several other small rivulets. The Teesta has divided the area into two parts- the western part is known as the Terai whereas the eastern part is known as the Dooars. The Dooars region can be further subdivided into the Siliguri or Western Dooars, the middle or Jalpaiguri Dooars and the eastern or Alipur Dooars. North Bengal plain start from the south of Terai region and continues up to the left bank of the Ganges. The southern parts of the district Jalpaiguri, North Dinajpur baring some extreme northern regions, South Dinajpur, Malda and southern part of Cooch Behar districts constitute this geographical region. The narrow land mass in the North Dinajpur district is known as Mahananda Corridor. This corridor runs north to south joining Malda with the plains of Jalpaiguri and Cooch Behar. The entire part of North and South Dinajpur is silt laden plain.

Mahananda river divides the district of Malda into two parts. The eastern part consists of undulating plains and some tilas and is made up of old alluvium and is a part of the Ganges delta. It is also known as Barind or Barendrabhumi. In contrast to the eastern part, the western part is made up of new alluvium and in this part river Kalindi joins the Mahananda river. The part of Malda lying to the north of river Kalindi is known as Tal.

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..

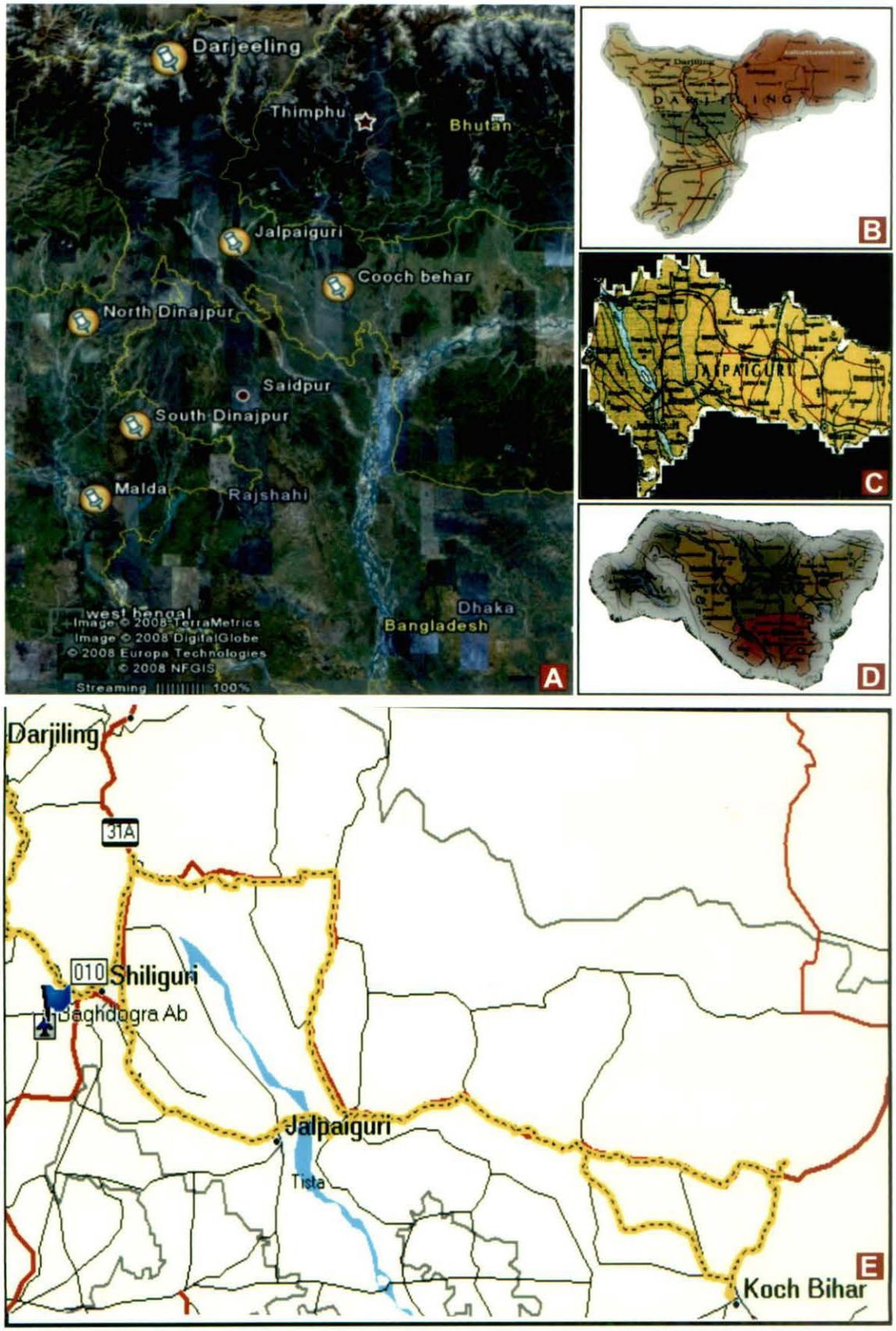


Plate 5: Satellite image of North Bengal (A), Map of Darjeeling (B) Jalpaiguri (C) Cooch behar (D) and map of study area of Terai-Dooars region by GPS tool (E)

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

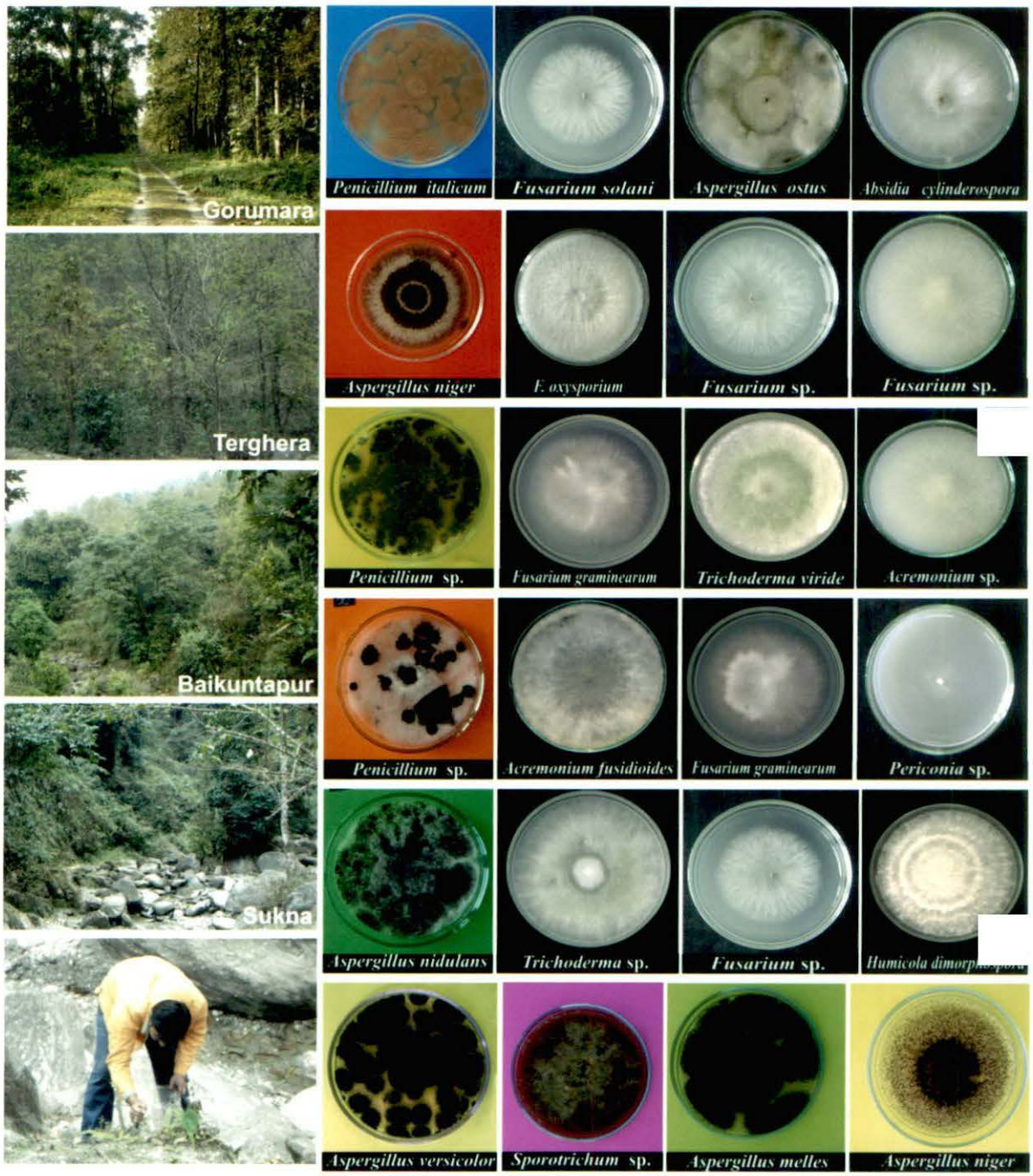


Plate 7 : Radial growth patterns of different fungi isolated from forest soil in PDA

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.

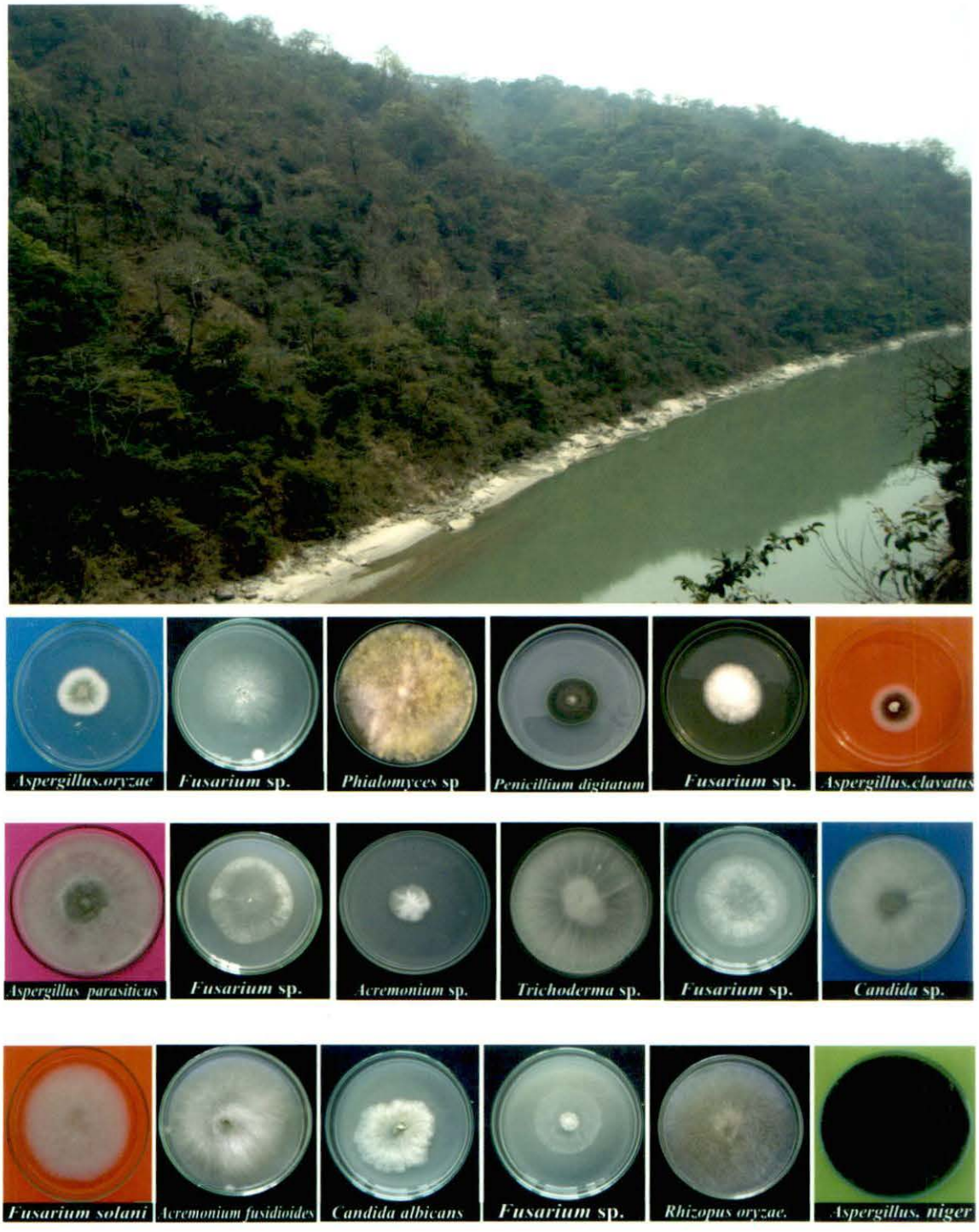


Plate 8 : Radial growth patterns of different fungi isolated from riverine soil in PDA

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..

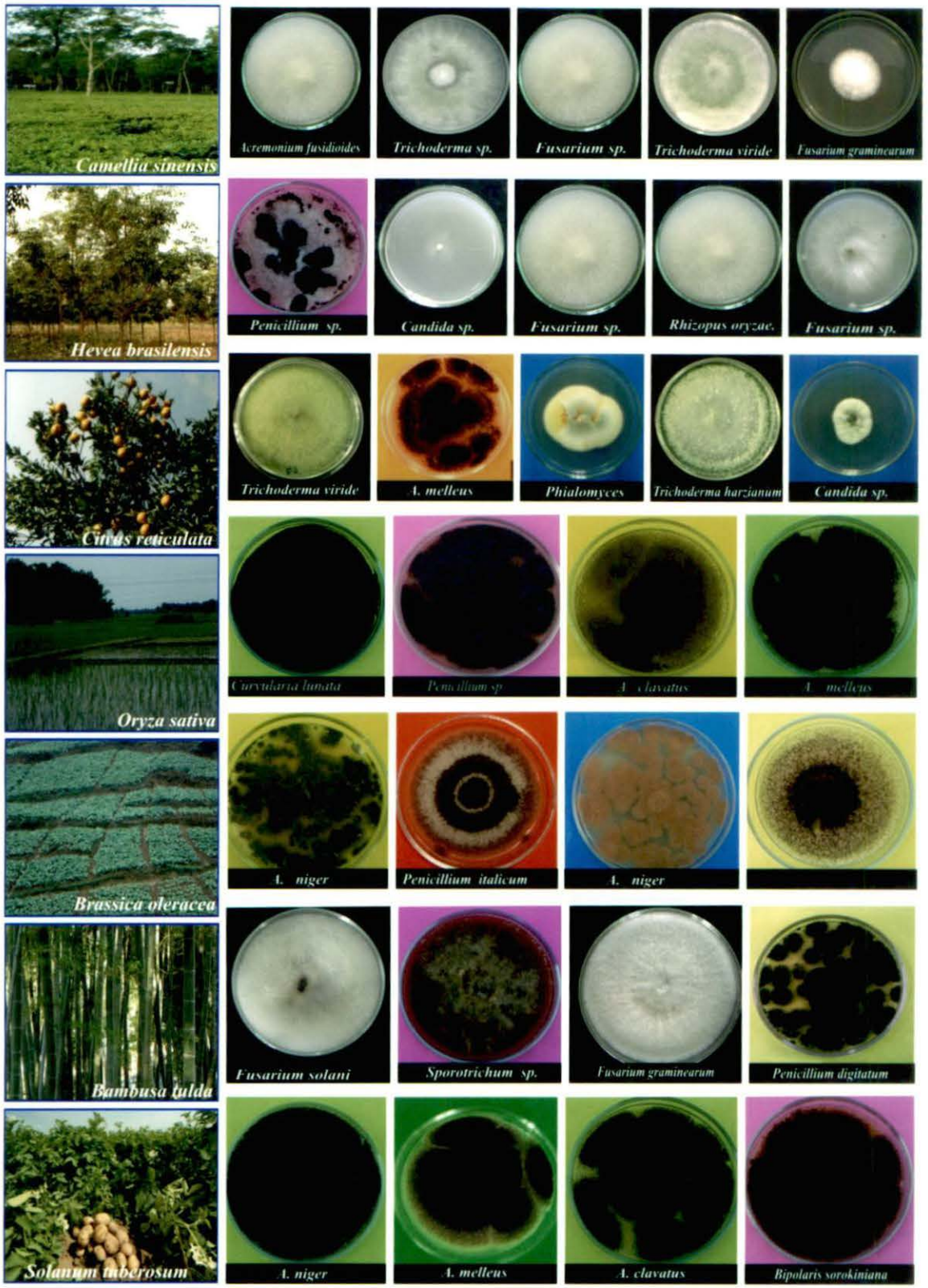


Plate 9 : Radial growth patterns of different fungi isolated from rhizosphere soil of different crop fields in PDA

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.

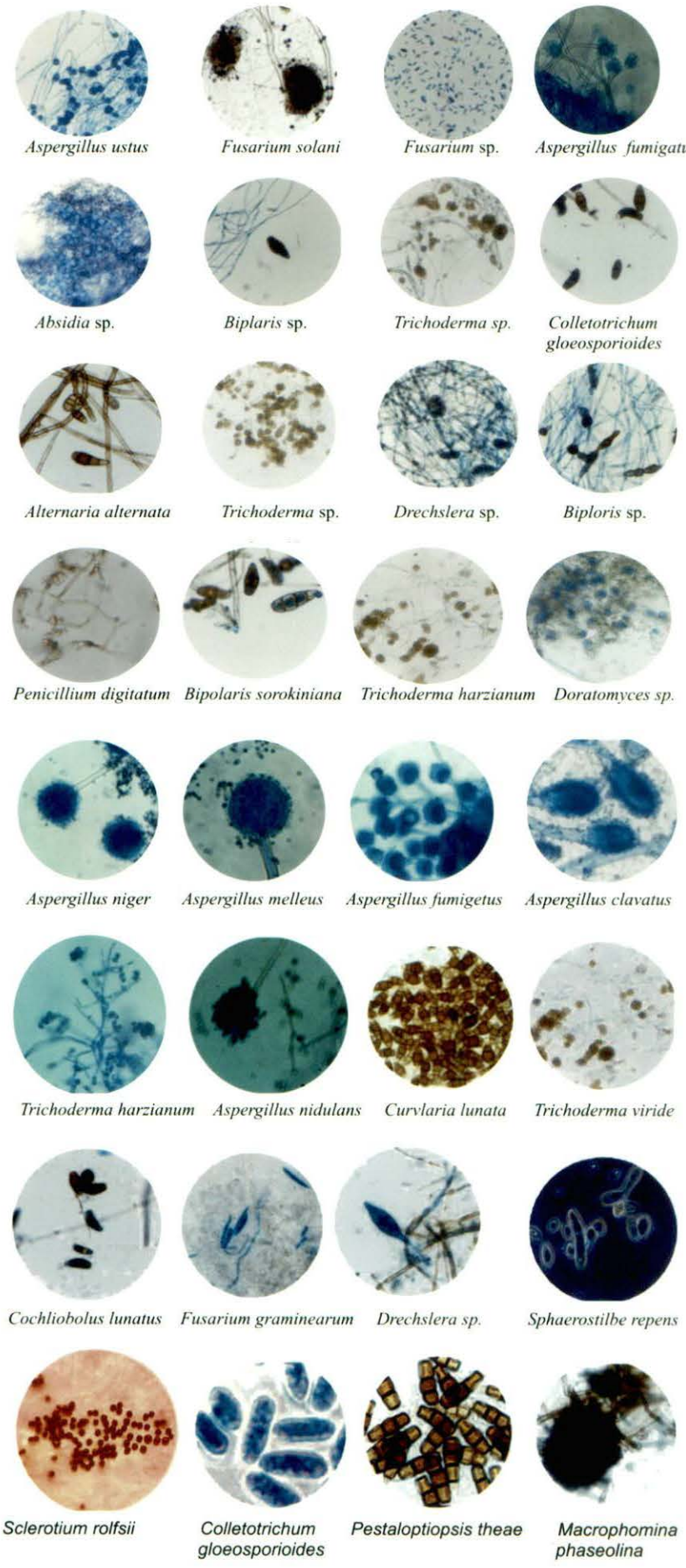


Plate 10 : Light microscopic views of different fungi isolated from forest, riverine and rhizosphere soil

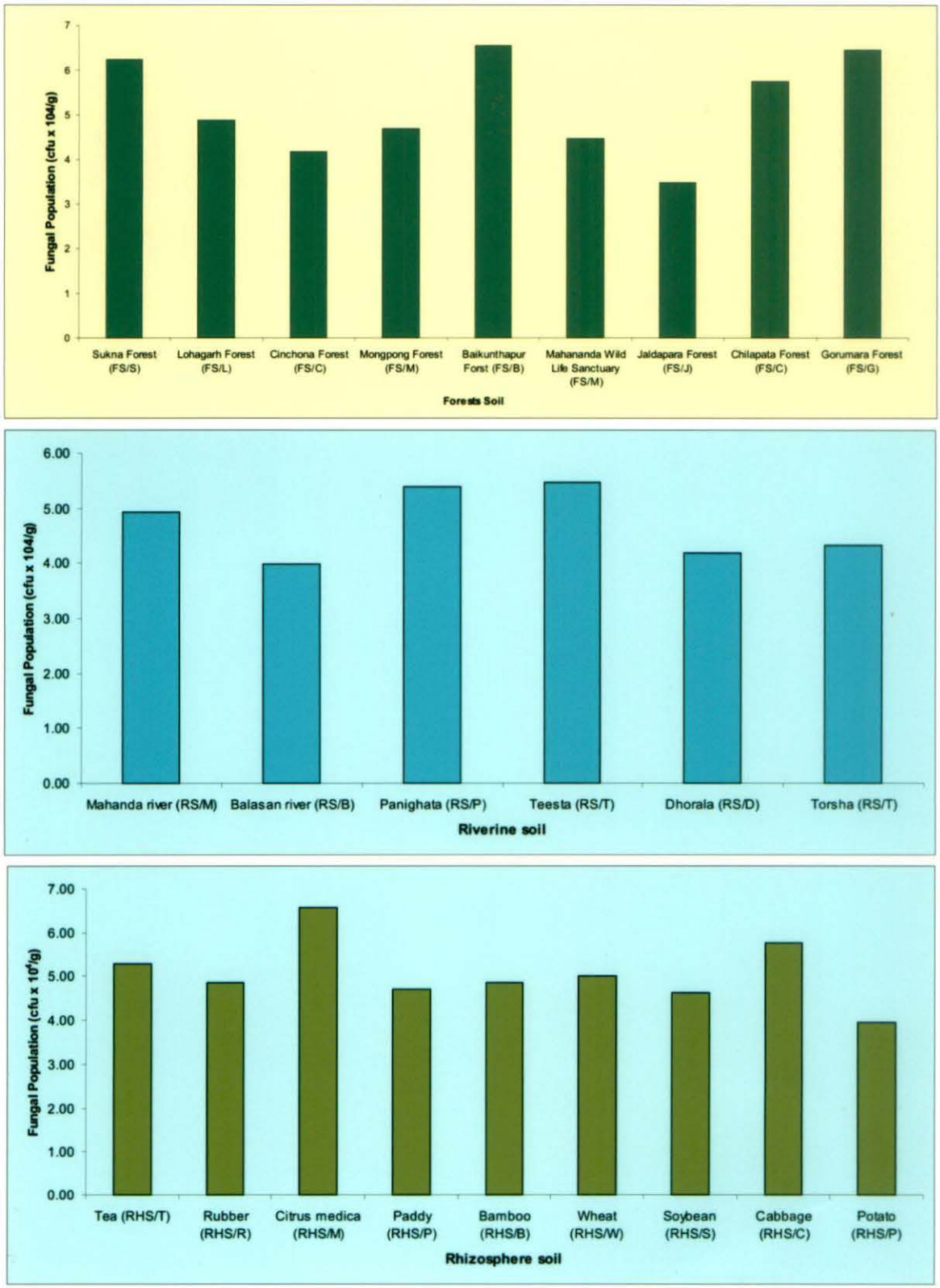


Figure 1: Fungal population of forest soil, riverine soil and rhizosphere soil

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'

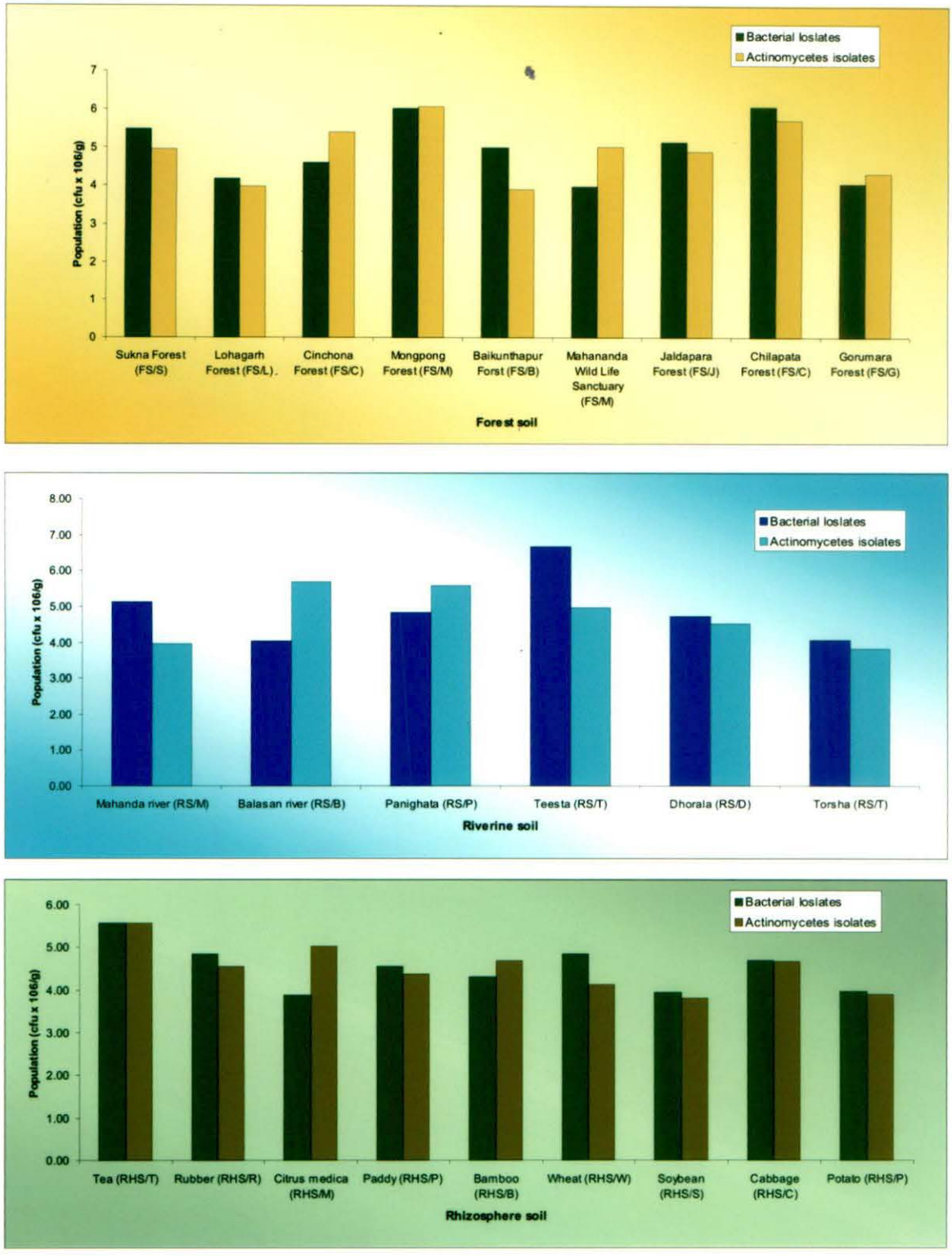


Figure 2: Microbial population of forest soil, riverine soil and rhizosphere soil

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.*

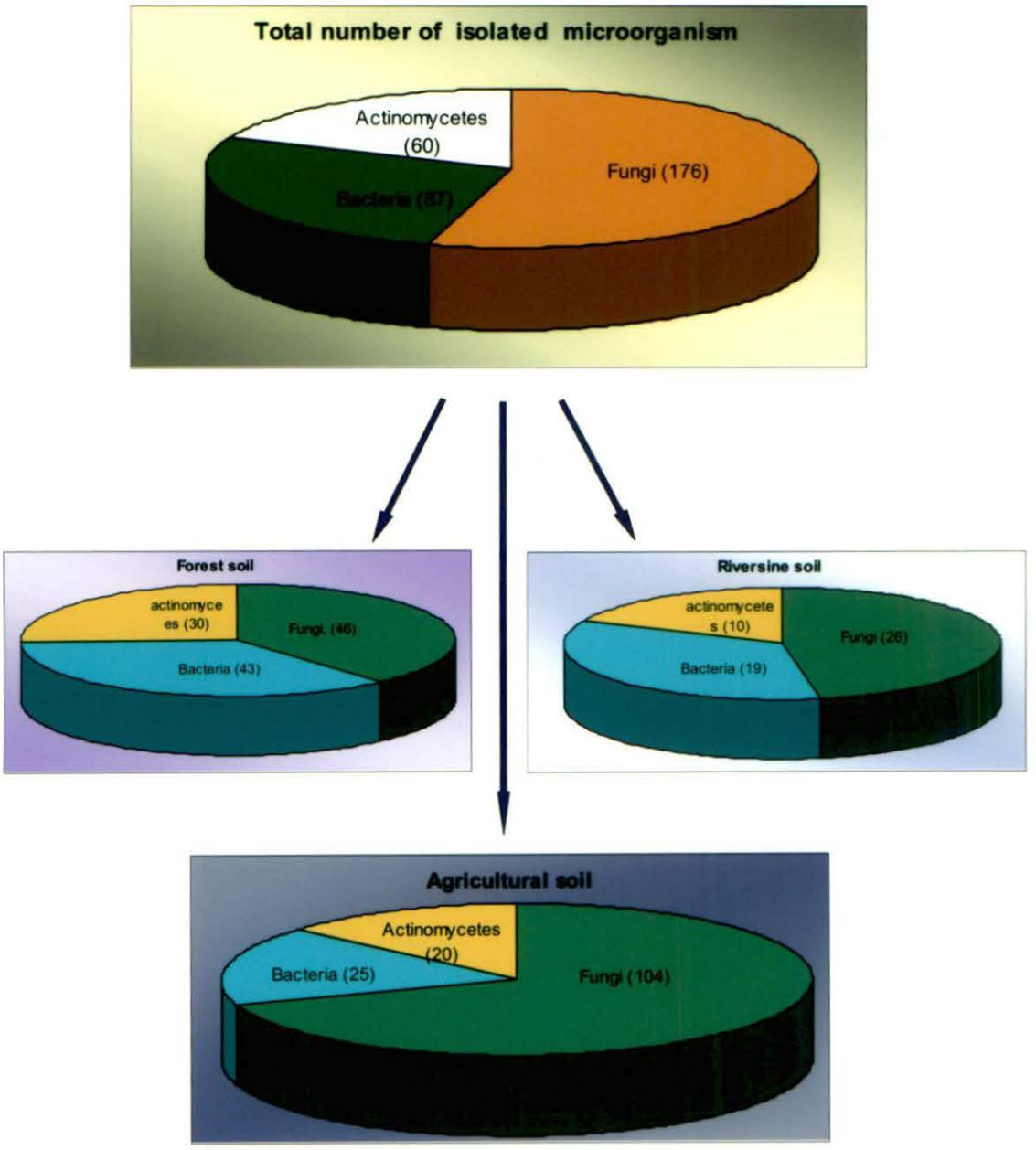


Figure 3: Number of microorganisms isolated from Terai-Dooars regions

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).

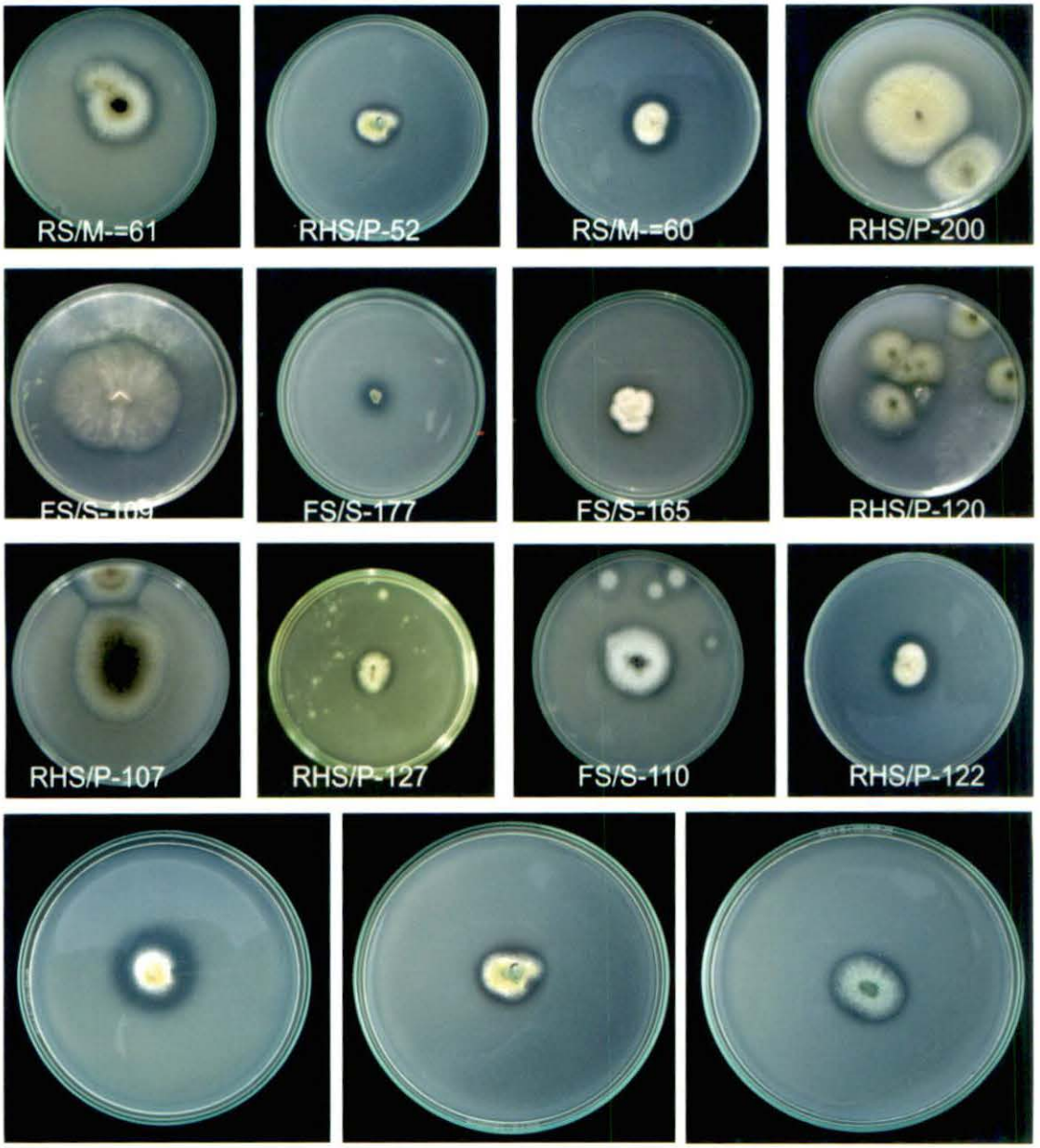
4.2.2. Evaluation in liquid medium

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.

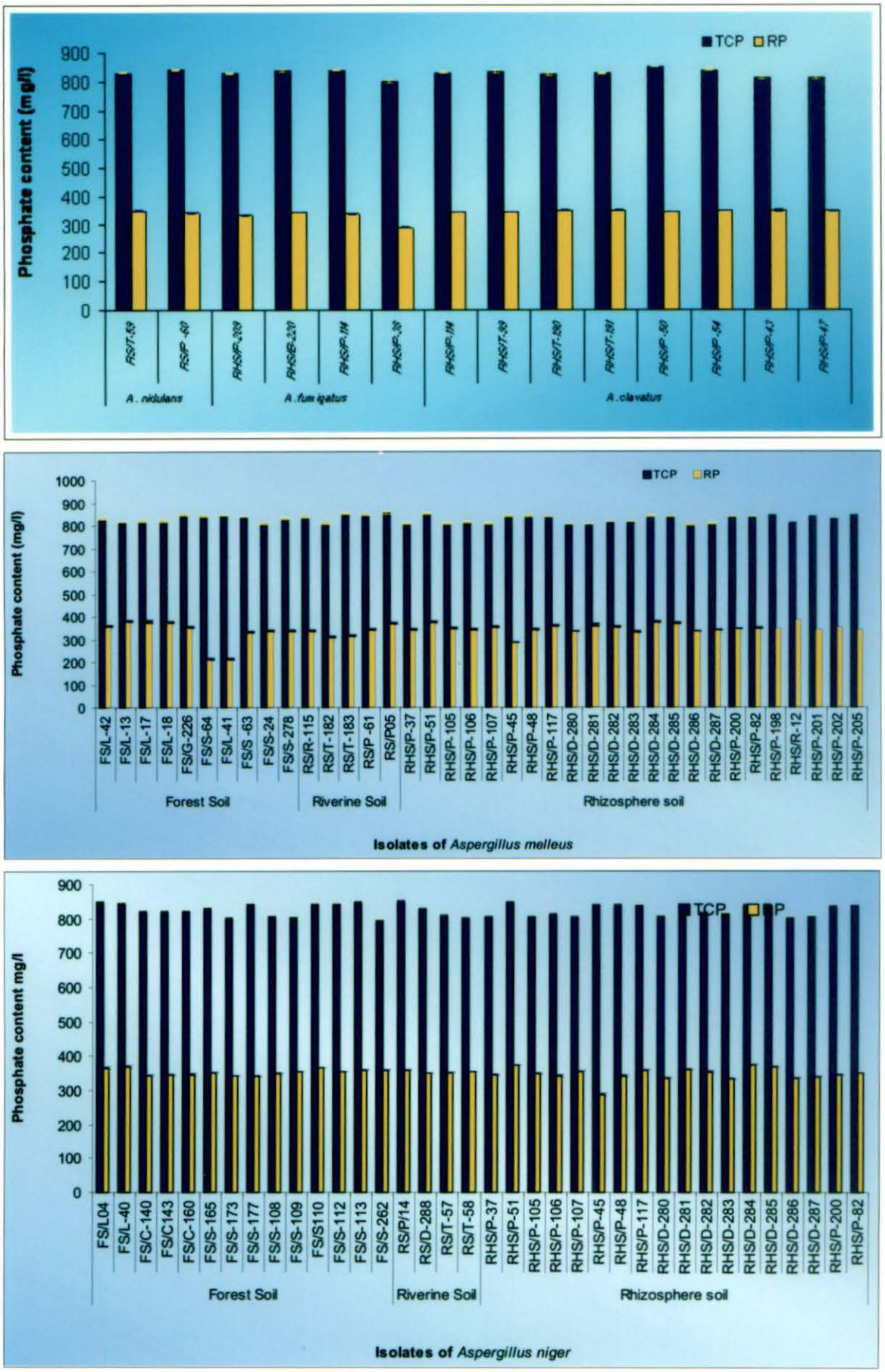


Figure 4: Estimation of phosphate solubilized by fungal isolates *in vitro*

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

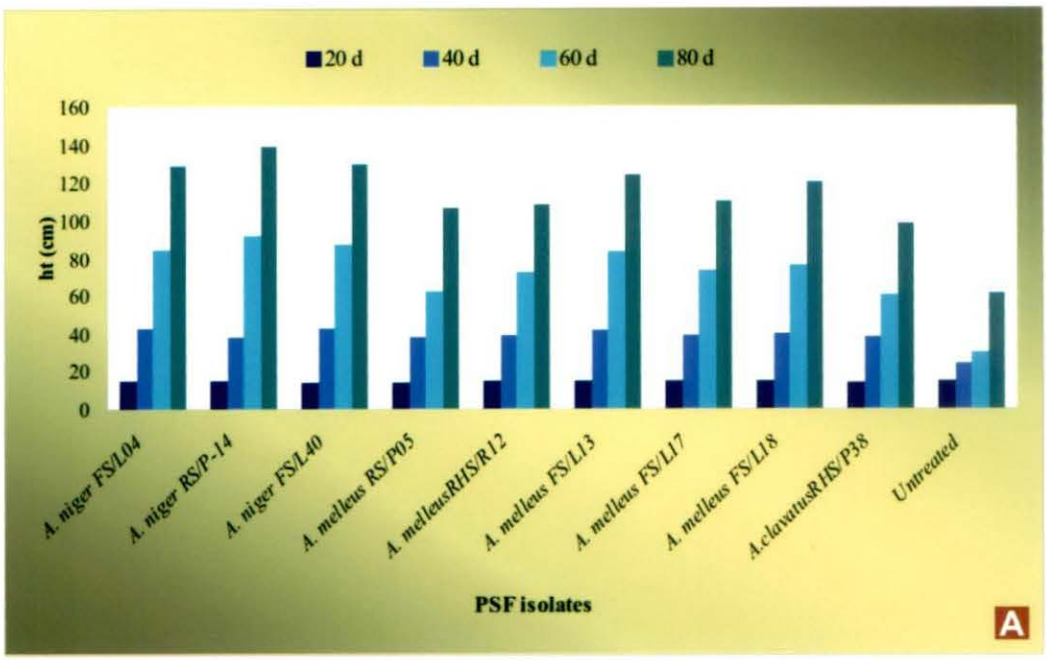
Among seventy phosphate solubilizer identified, nine PSF of which three isolates of *A.niger*, five *A. melleus* and one isolates of *A. clavatus* were selected on the basis of their greater phosphate solubilizing activity *in vitro* for their further evaluation on plant growth. PSF isolates were grown 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



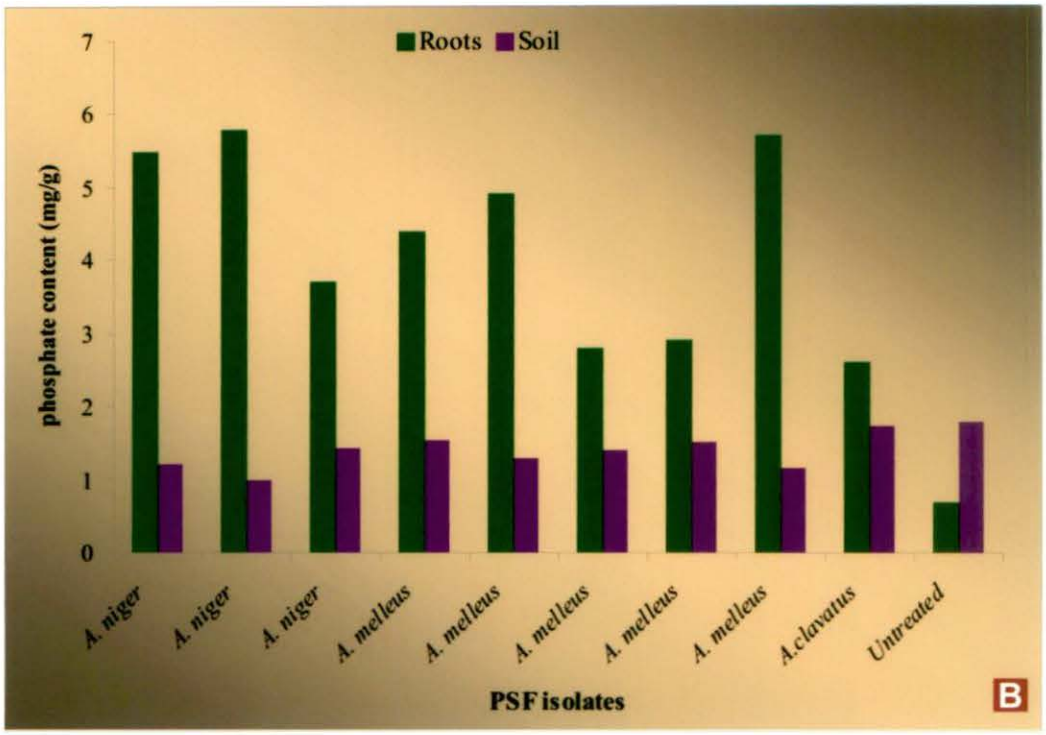


Figure 5:) Height of soybean plants after amendment of PSF isolates in the soil (A) Phosphate content of root and soil (B)

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

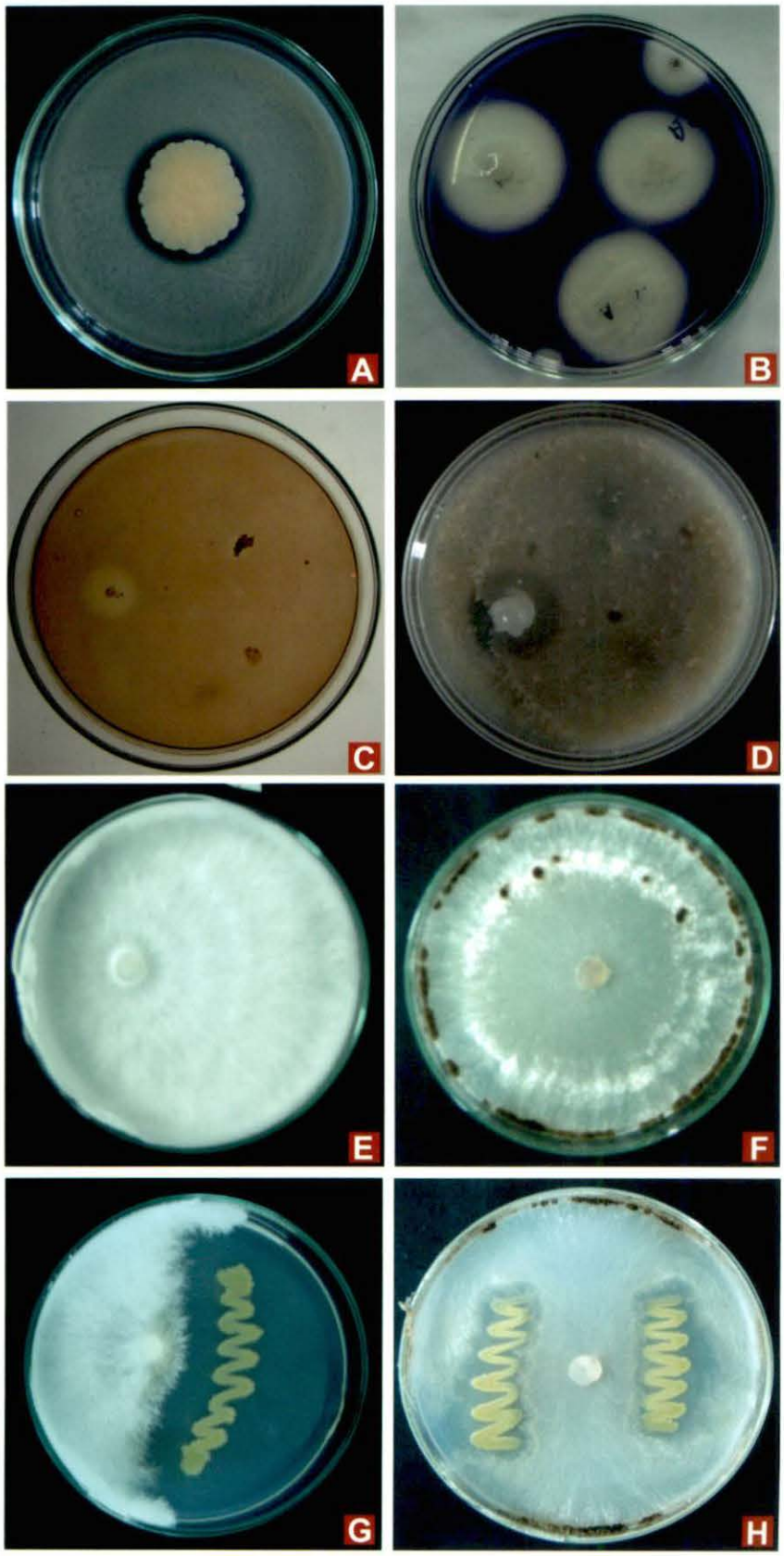


Plate13 : Screening for phosphate solubilizing activity (A)starch hydrolysis (B), chiniase activity (C), protease activity (D) and antifungal activity of *Bacillus pumilus* B/RHS/P 22 (MTCC 10653) against *Sclerotium rolfsii* (G) and *Rhizoctonia solani* (H). Control plates (E & F) respectively for (G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

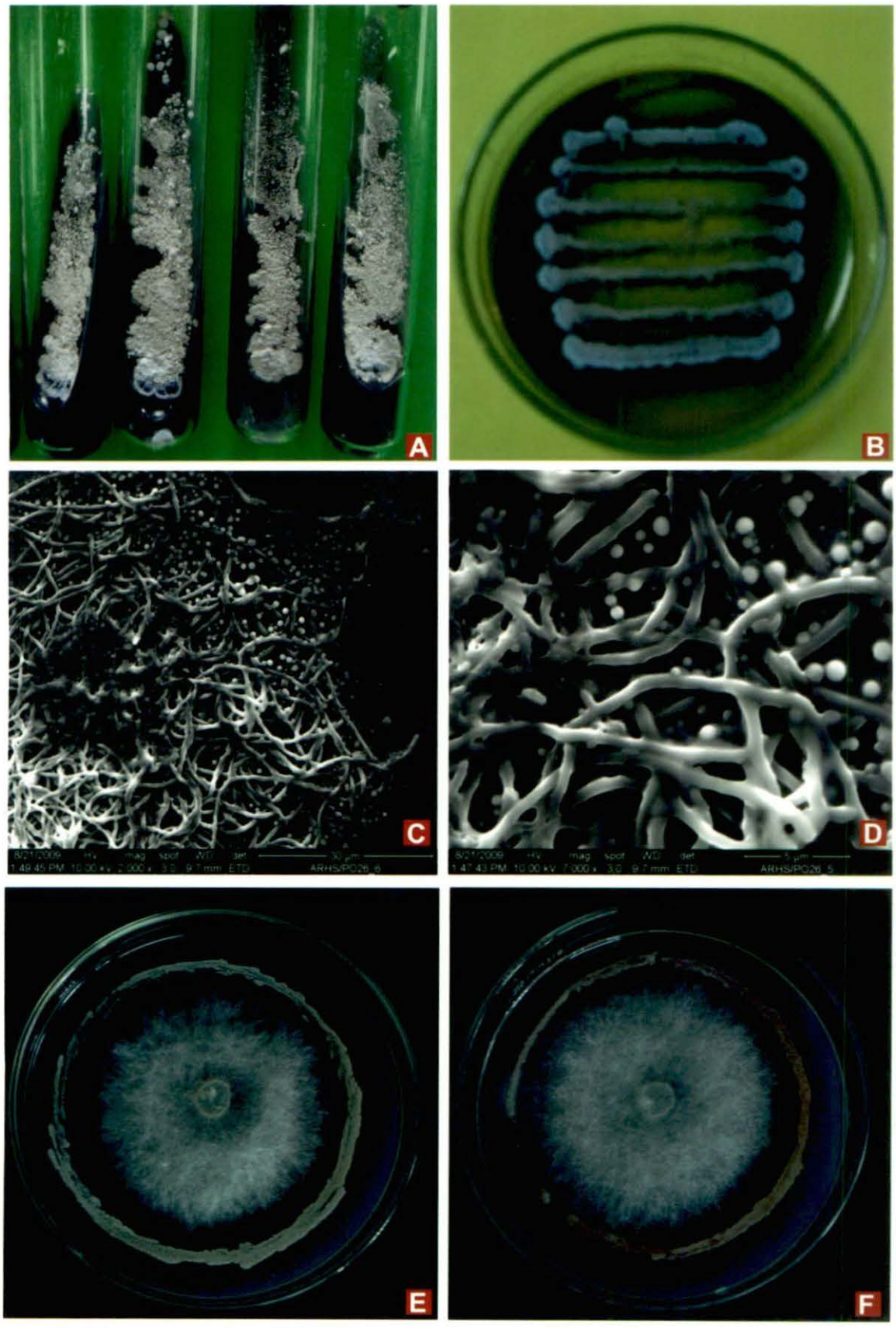


Plate 15 : *Streptomyces griseus* ARHS/PO 26 (NCFT 2578.08) in SCN medium (A&B) SEM view (C&D) and its antifungal activity against *Sclerotium rolfsii* (E) and *Rhizoctonia solani* (F)

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

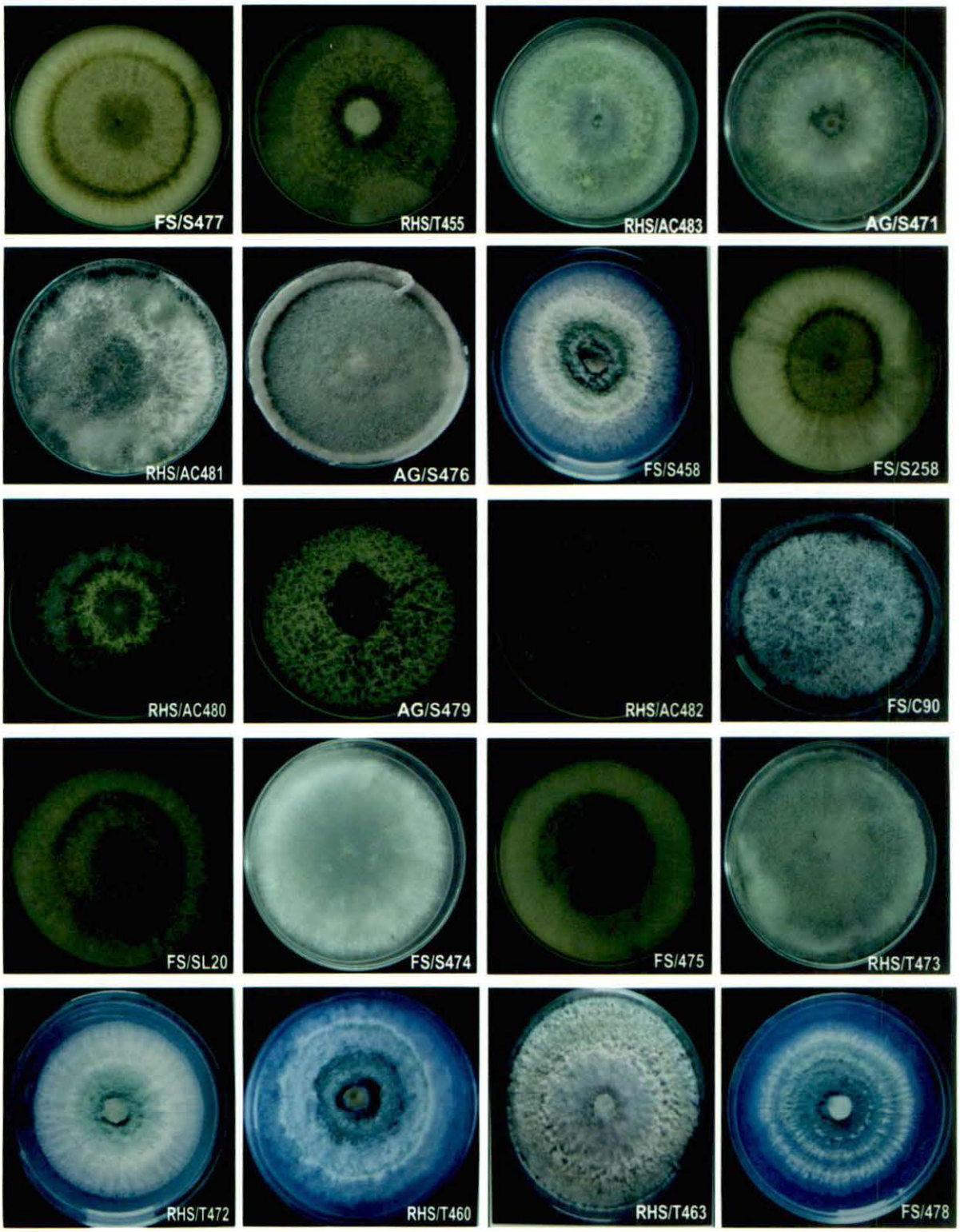


Plate 17: Radial growth of different isolates of *Trichoderma* obtained from forest, riverine and agricultural soil.

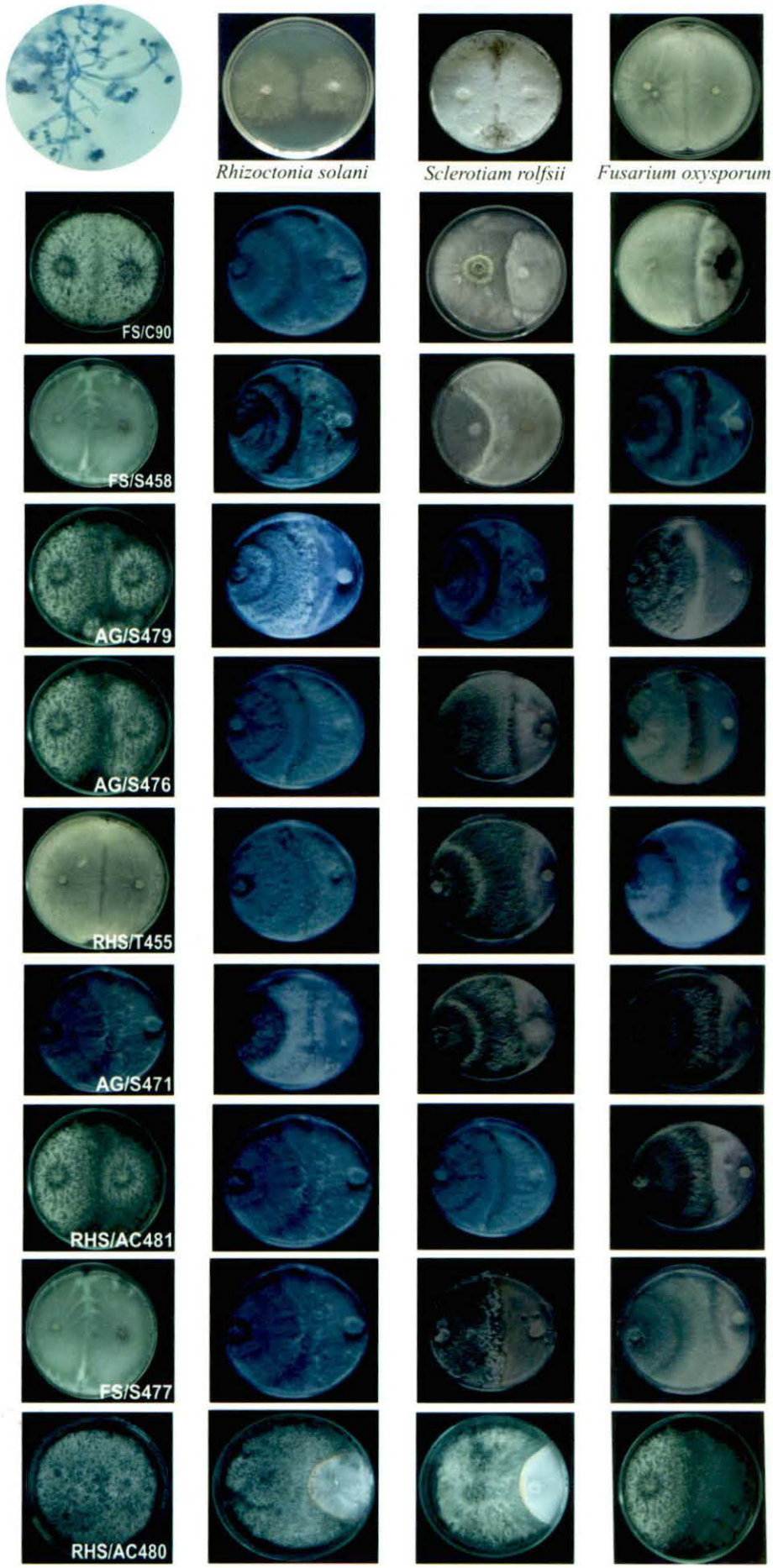


Plate 18 : Antagonistic activity of isolates of *Trichoderma harzianum* against *Rhizoctonia solani*, *Scleoratium rolfsii* and *Fusarium oxysporum*

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

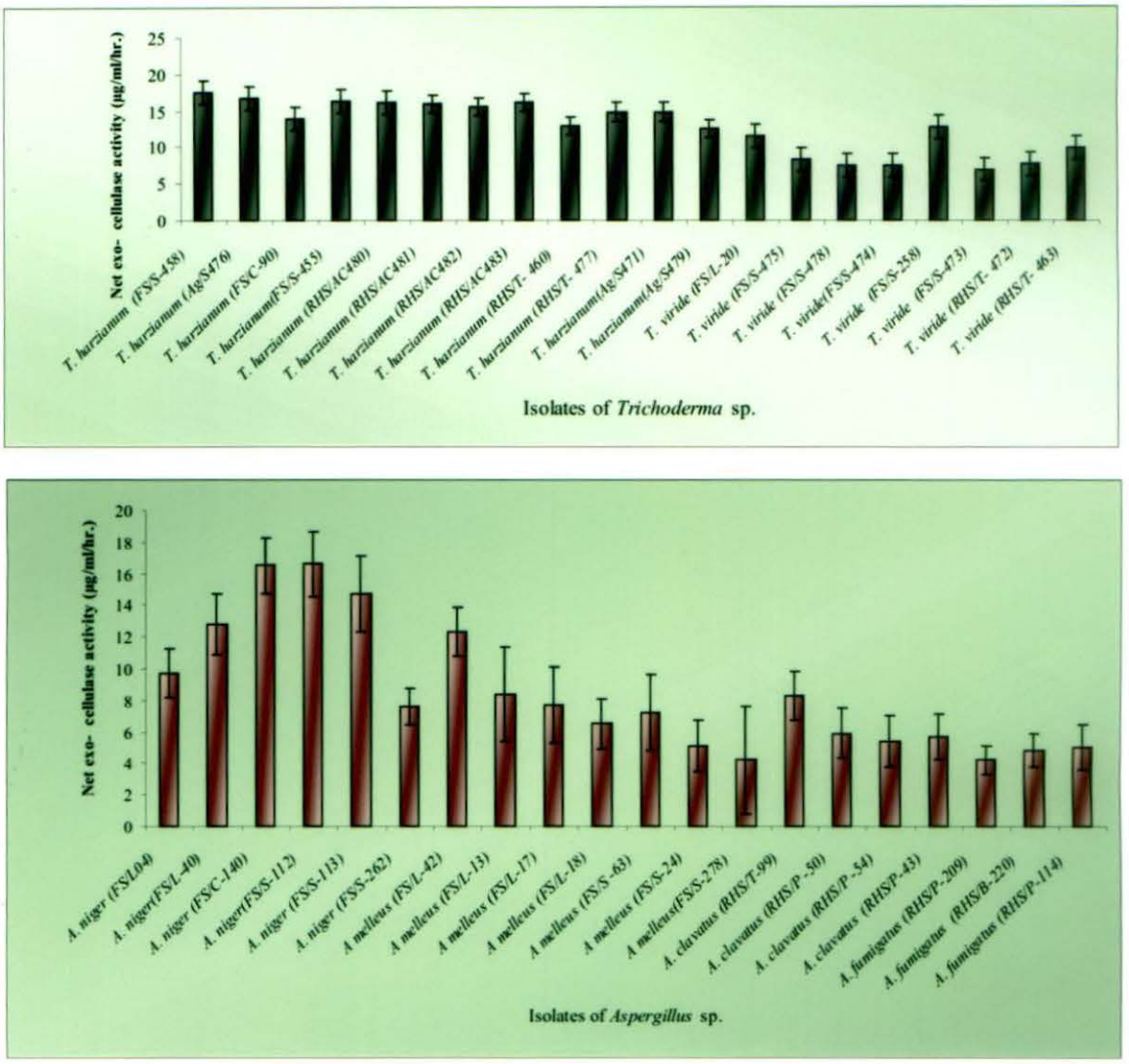


Figure 6: Cellulase activity of fungal isolates

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.

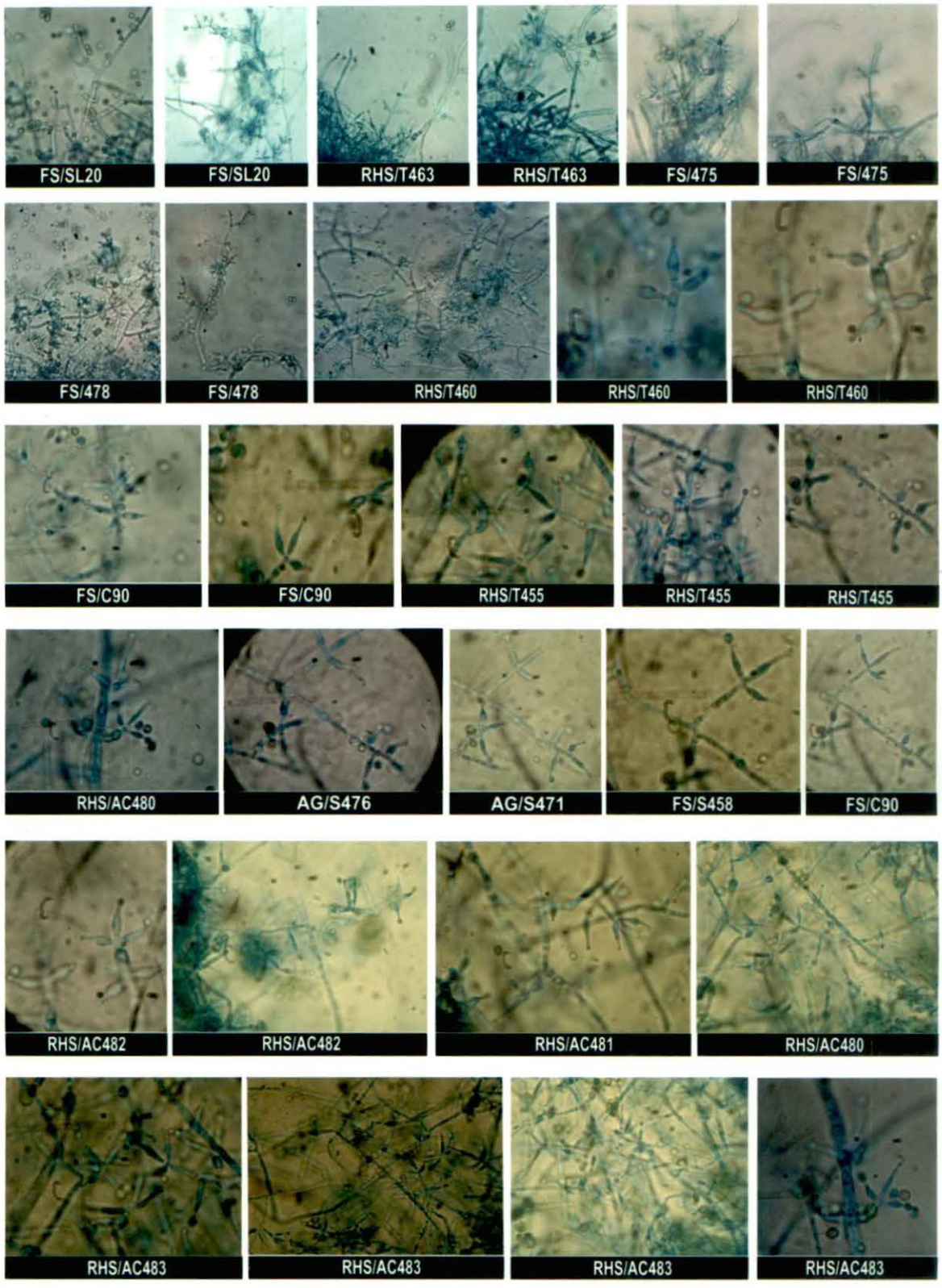


Plate 19: Microscpical views of the isolates of *Trichoderma harzianum and T. viride*

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)

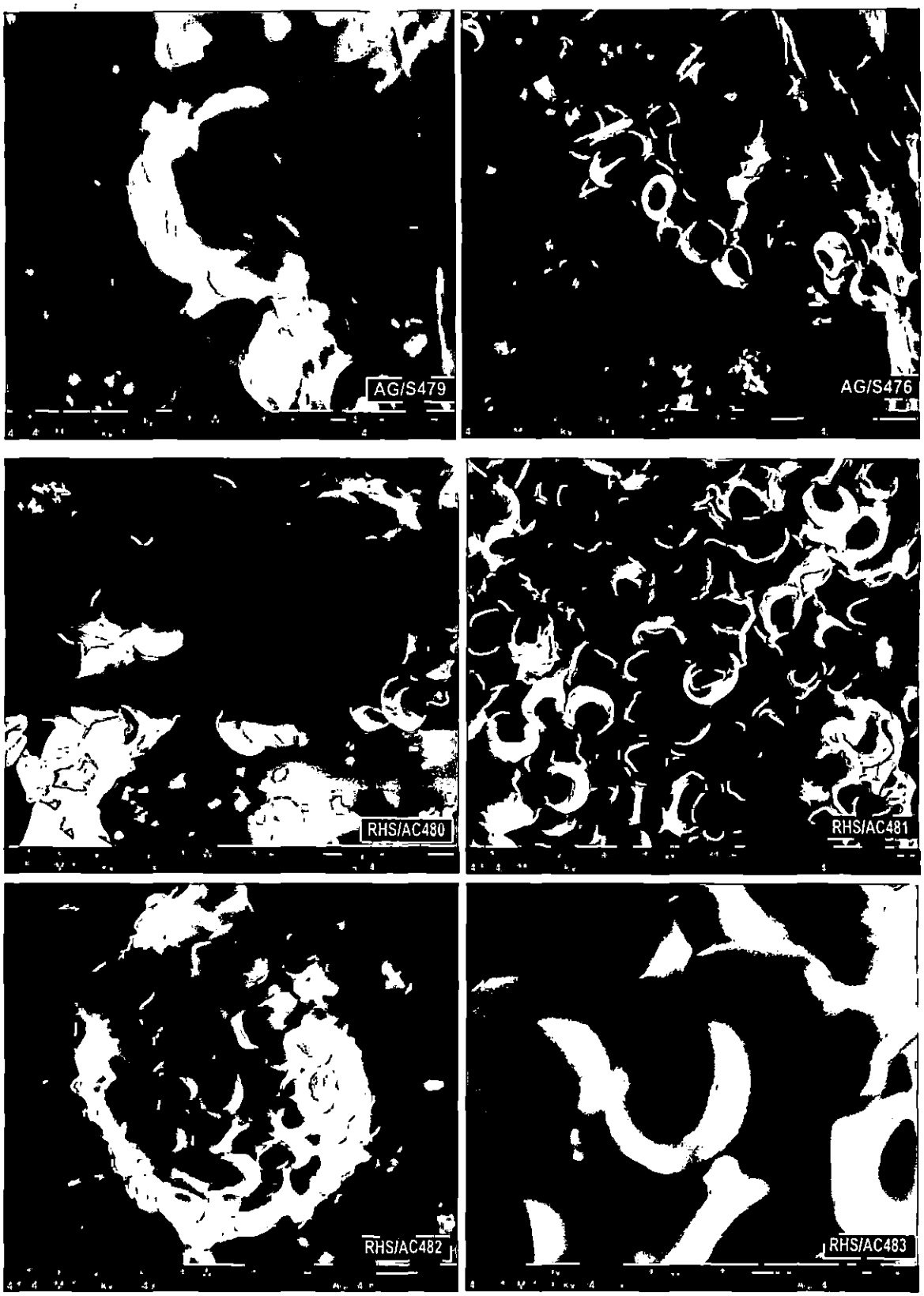


Plate 20: SEM of spores of *Trichoderma harzianum* isolates

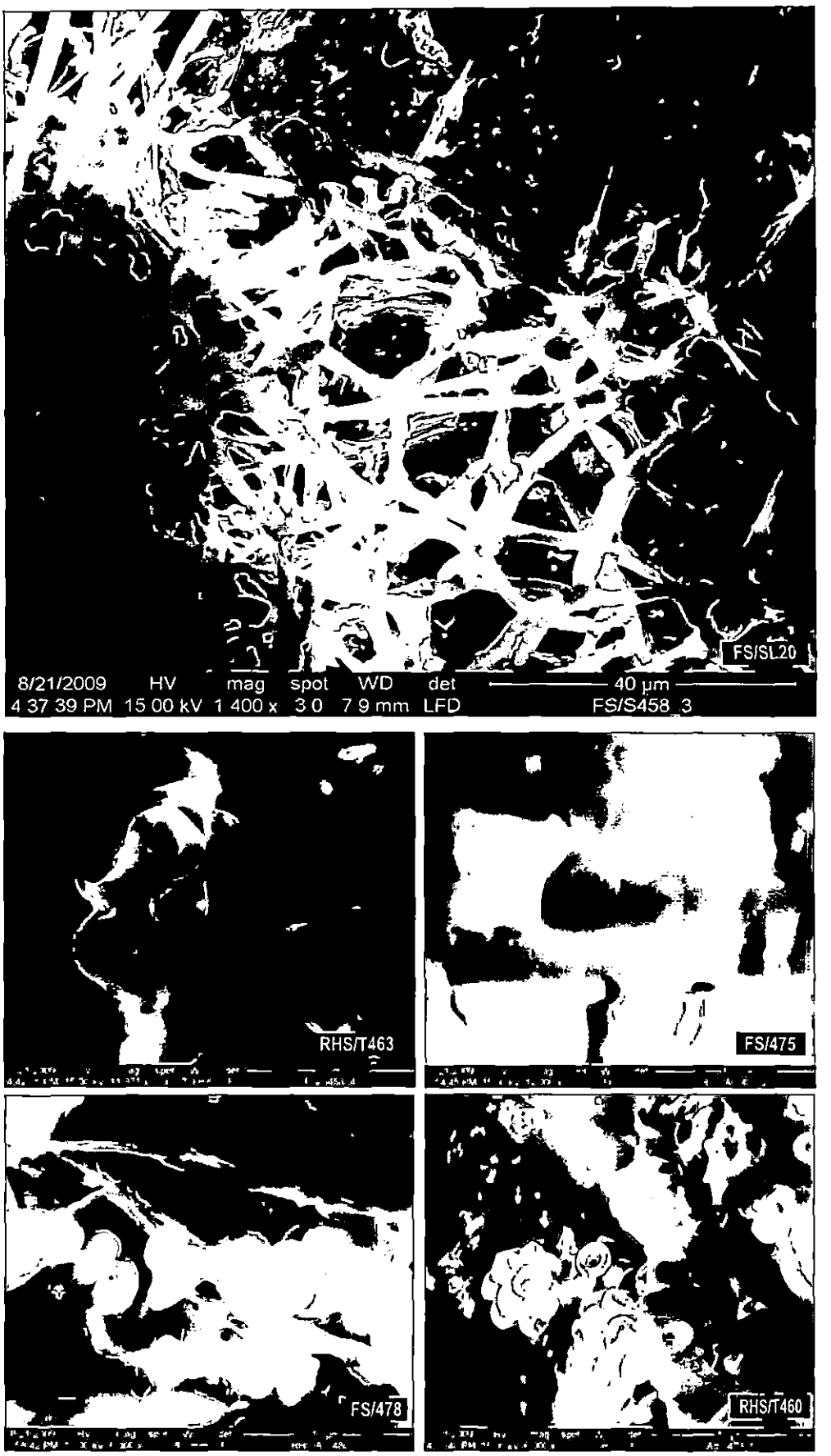


Plate 21: SEM hyaphe and spores of *Trichoderma viride* isolates

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)

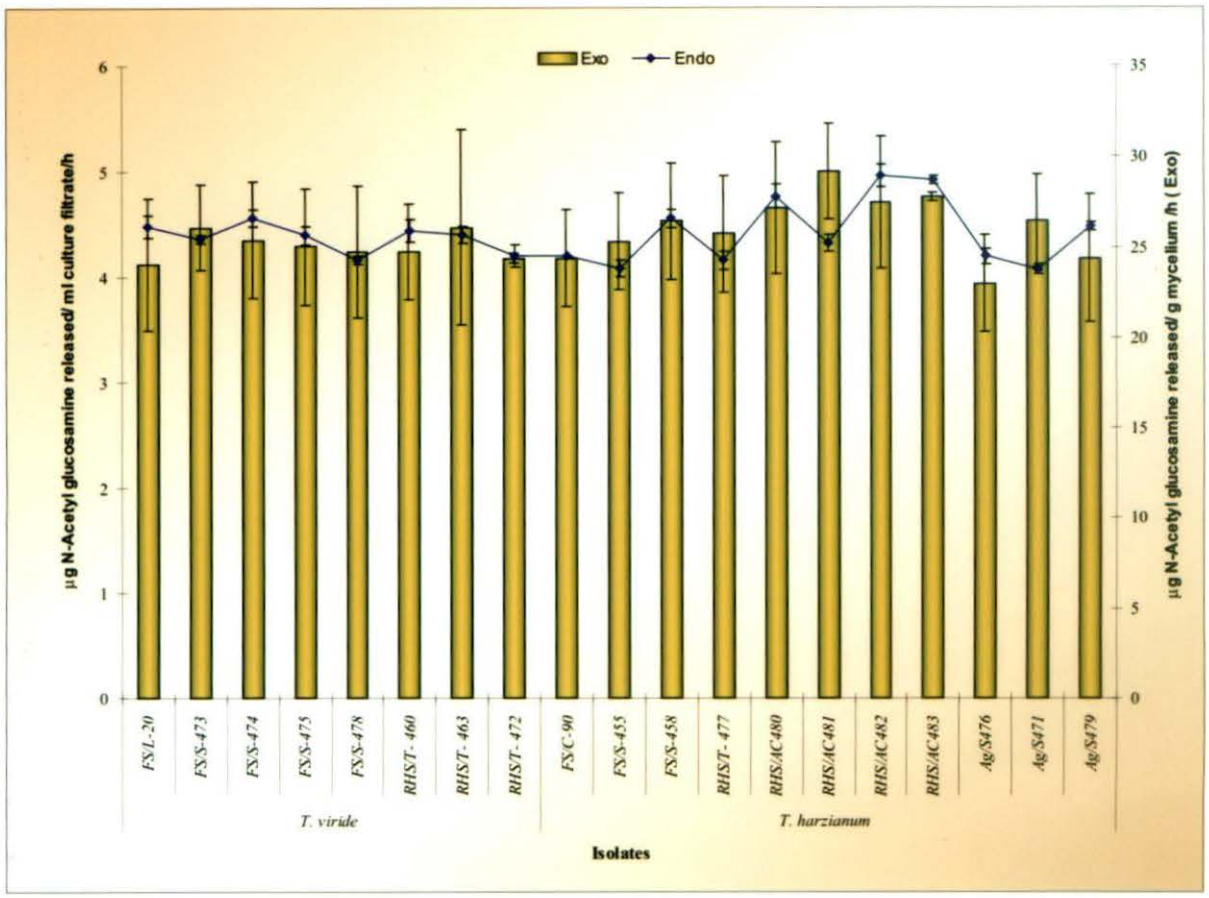


Figure 7: Chitinase assay of *Trichoderma* isolates

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

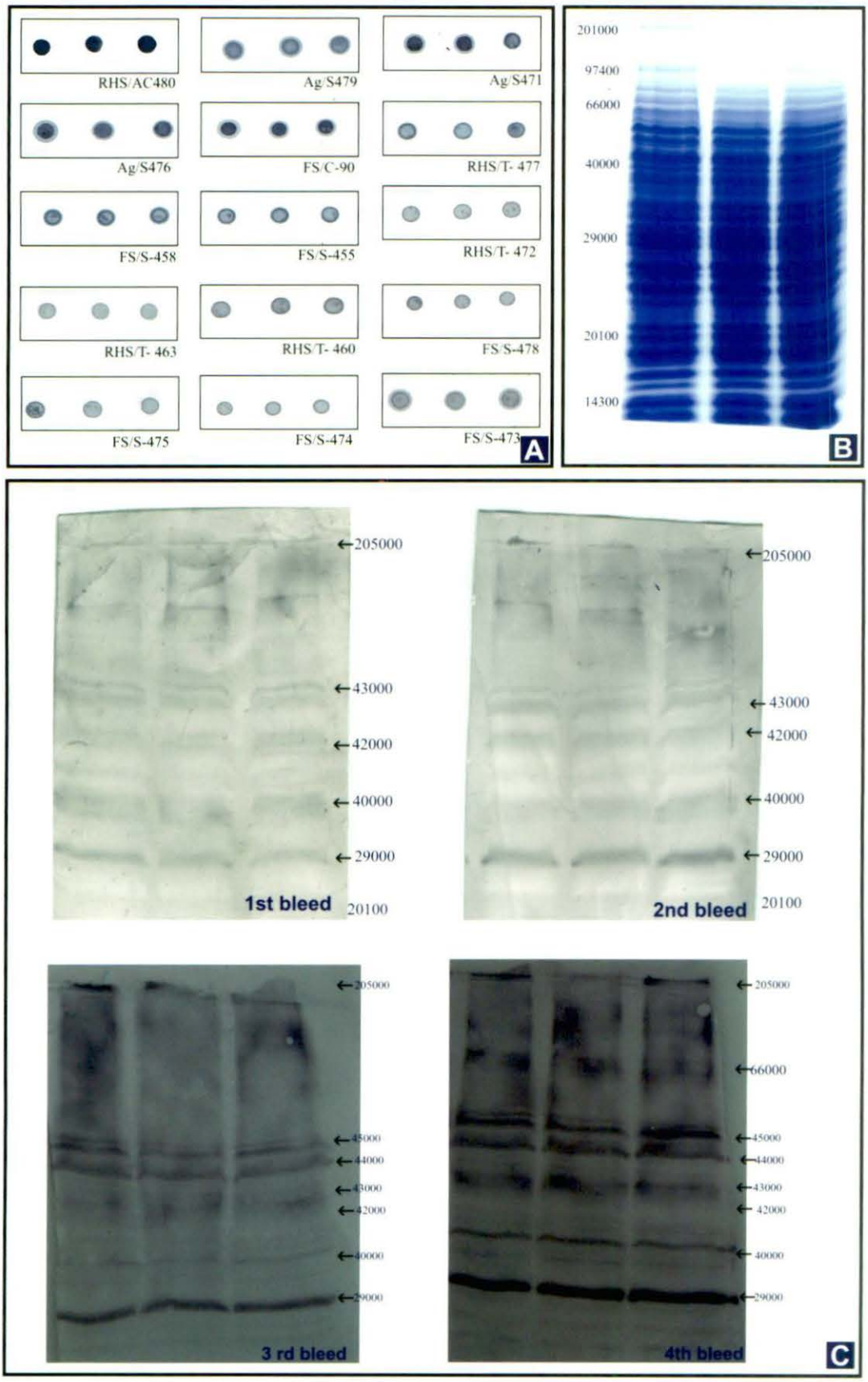


Plate 22: Dot immunobinding assay (A) on nitrocellulose paper with PAb of *Trichoderma harzianum* (RHS/AC480) against mycelial antigen of *T.harzianum* and *T. viride*. SDS-PAGE (B) and Western blot analysis (C)of *T. harzianum* (RHS/AC480)

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) .

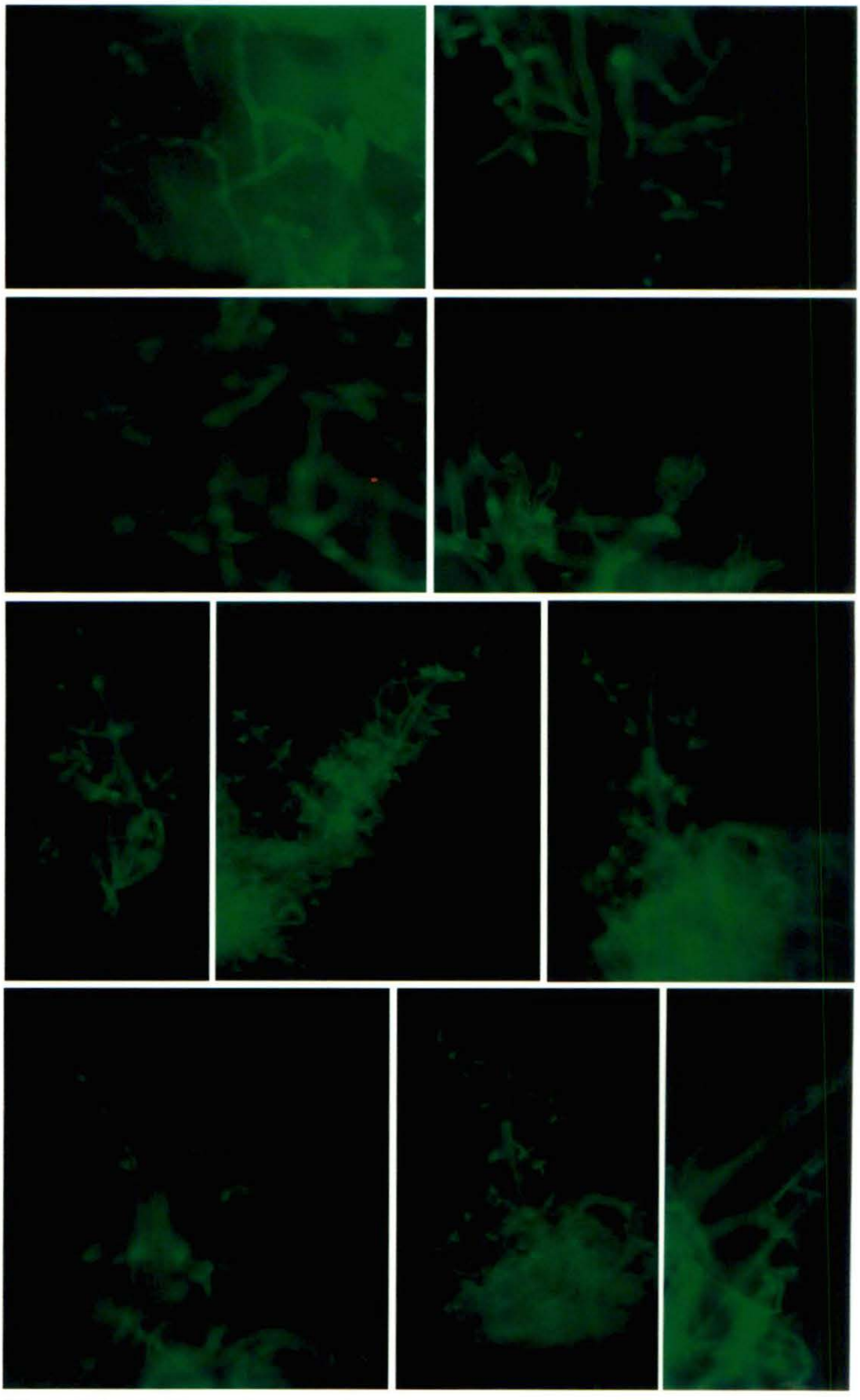


Plate 23: Indirect immunofluorescence of hyphae and phialides of *Trichoderma harzianum* (RHS/AC480) (treated with PAb and reacted with FITC labelled antibodies of goat specific for rabbit globulin)

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

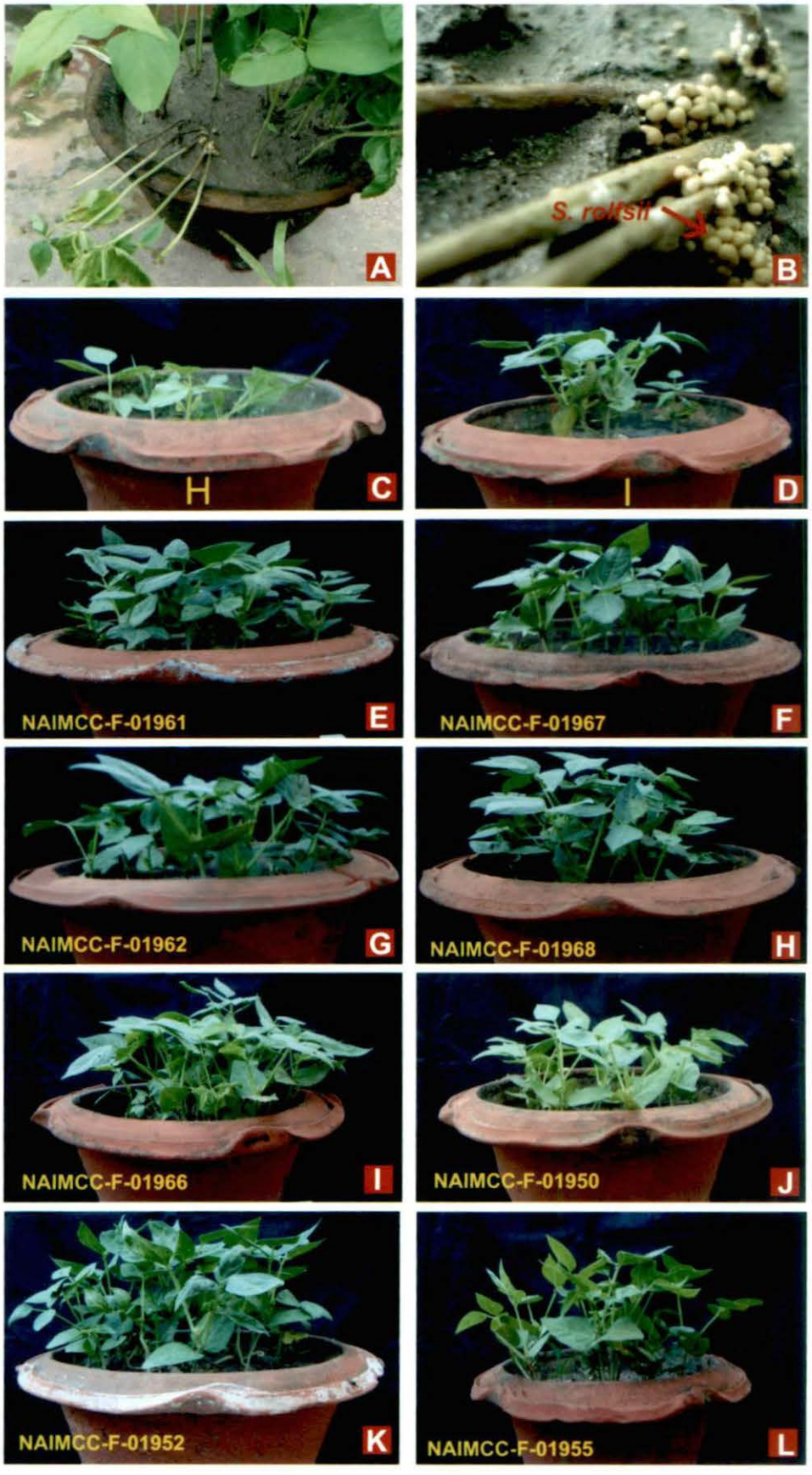


Plate 24: Evaluation of eight isolates of *Trichoderma harzianum* on the development of sclerotial blight incidence of *Phaseolus vulgaris*.
(A&B) Sclerotial blight, (C) Untreated control, (D) Inocualted with *S. rolfsii,* (E-L) treated with *T. harzianum* and inoculated with *S. rolfsii*

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

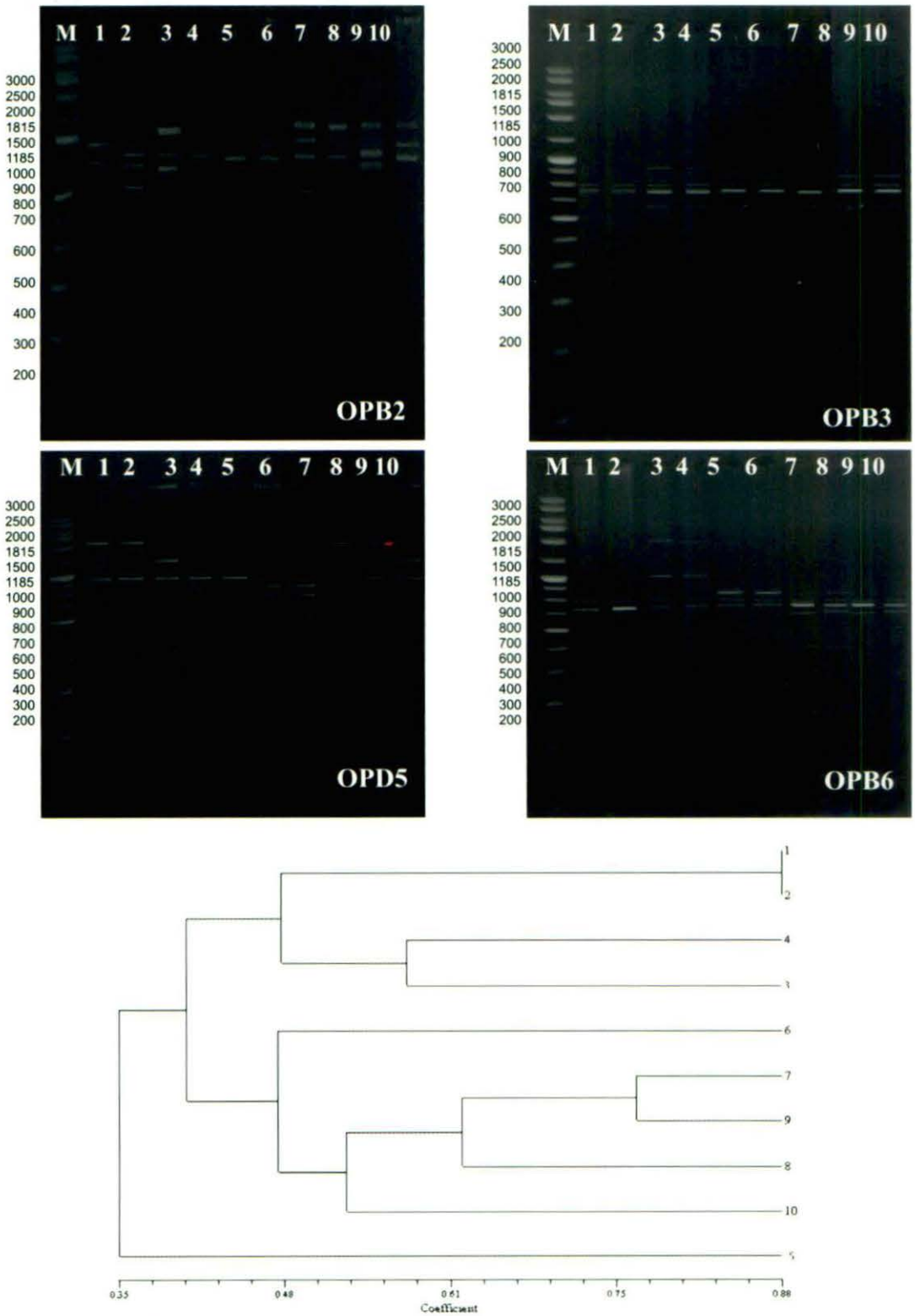


Plate 25 Plate : RAPD amplified products of phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* using four random primers Lane M: Low range DNA marker, Lane 1: *A. niger* (FS/L-04), Lane 2: *A. niger* (RS/P-14), Lane 3: *A. niger* (FS/L-40), Lane 4: *A. niger* (FS/S-113) , Lane 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.

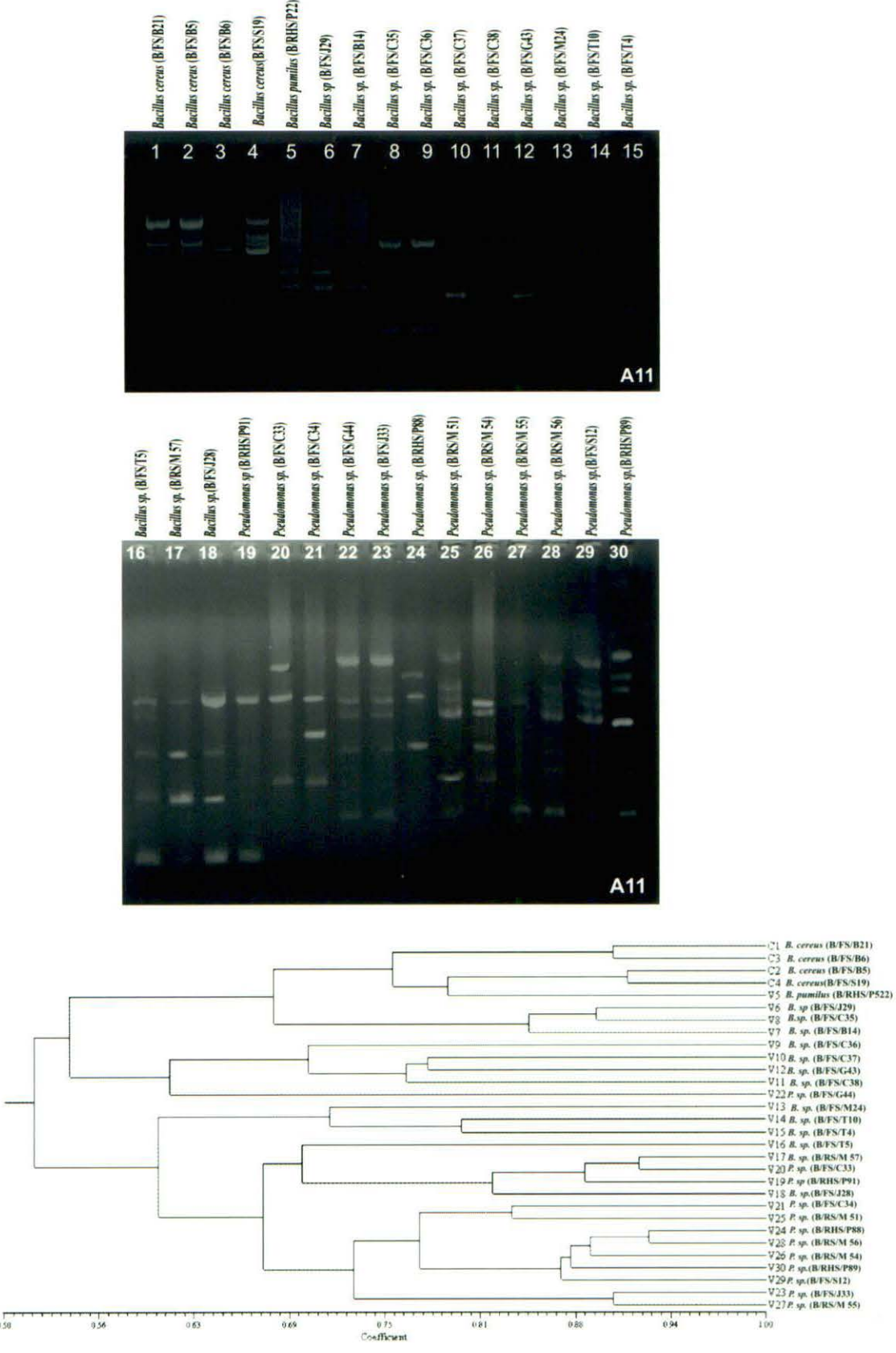


Plate 26: RAPD analysis and dendogram of phosphate solubilizing bacterial isolates obtained from forest soil, riverine soil and rhizosphere of agricultural fields.

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	

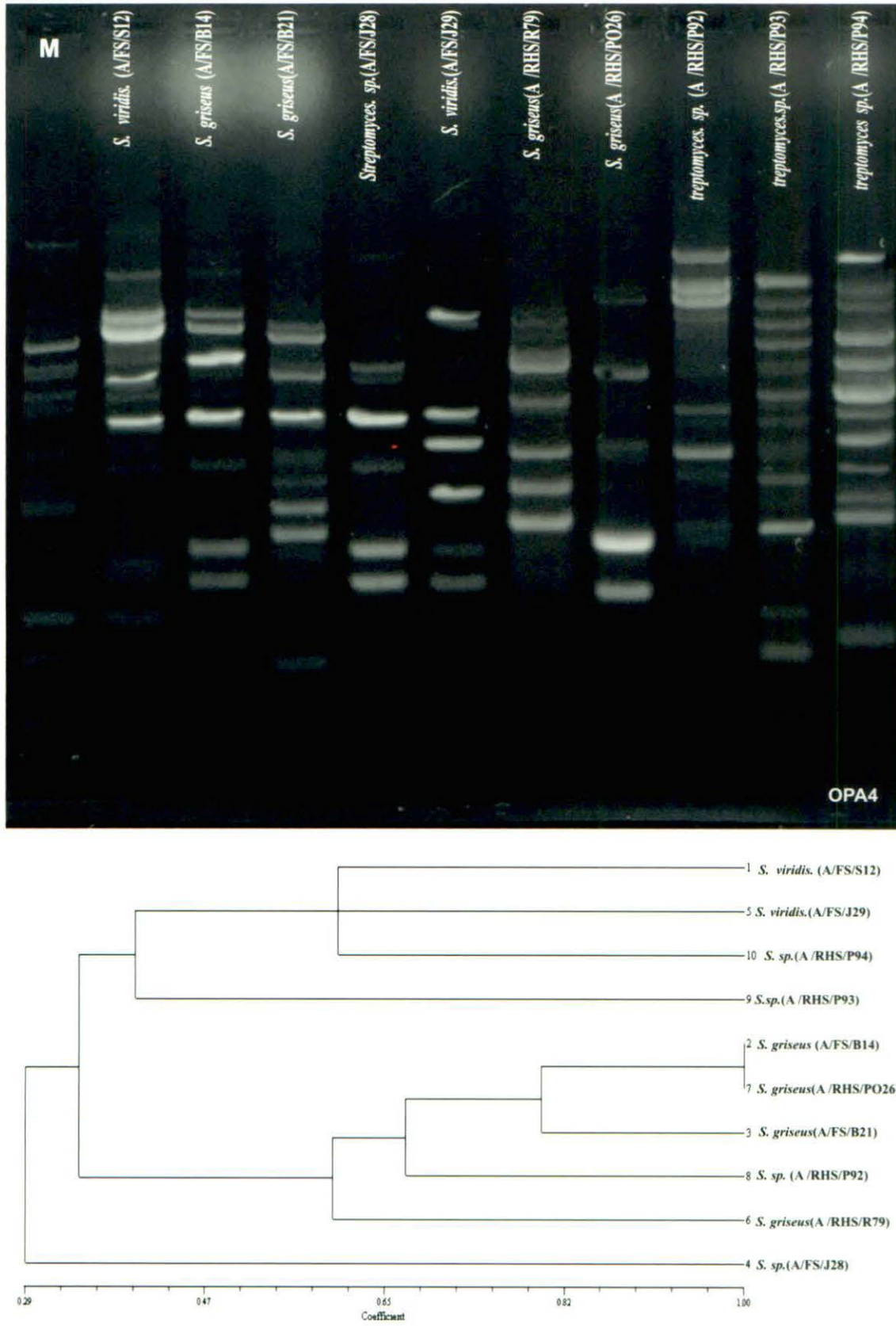


Plate 27: RAPD analysis and dendogram of phosphate solubilizing actinomycetes isolates obtained from forest soil, riverine soil and rhizosphere of agricultural fields.

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

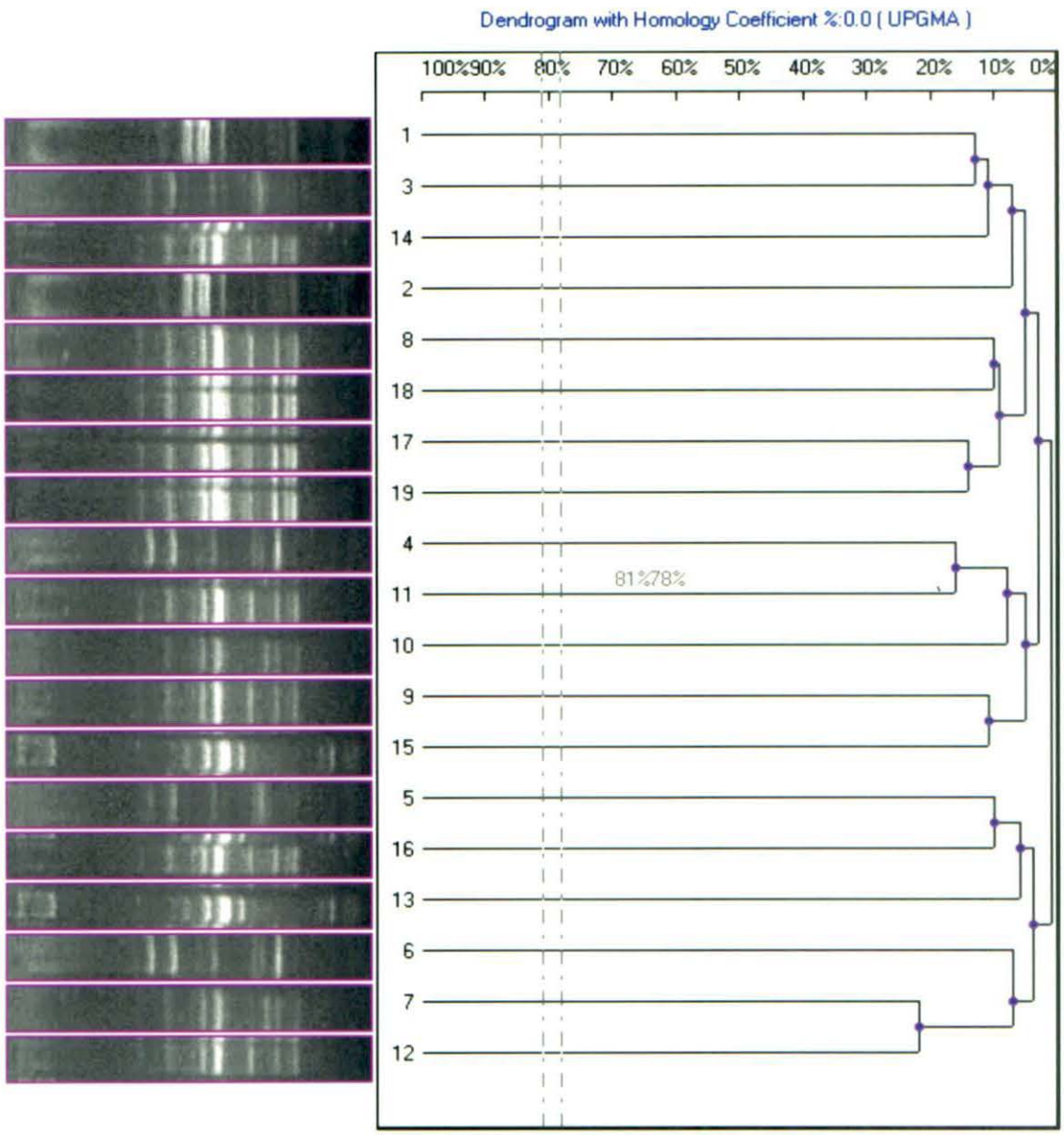


Plate 28: RAPD analysis of *Trichoderma* isolates with A-04 primer in BioProfile software.(1. FS/L-20, 2. FS/S-473,3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T- 460, 7. RHS/T- 463,8. RHS/T- 472, 9. FS/C-90,10.FS/S-455,11.FS/S-458,12.RHS/T-477,13.Ag/S476, 14.Ag/S471, 15.Ag/S479, 16.RHS/AC480, 17.RHS/AC481,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

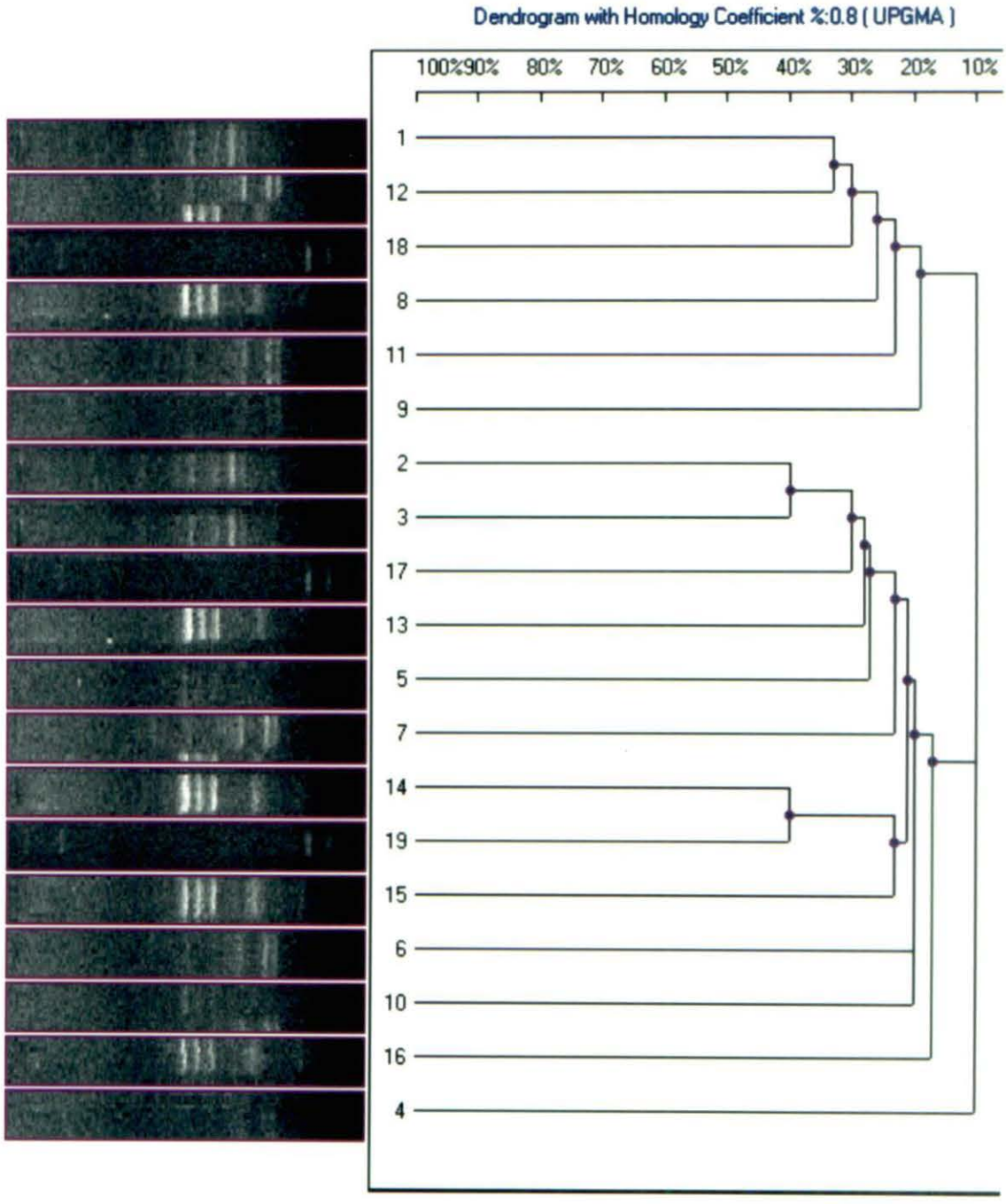


Plate 29: RAPD analysis of *Trichoderma* isolates with OPD6 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T-460, 7. RHS/T- 463, 8. RHS/T- 472, 9. FS/C-90,10. FS/S-455, 11. FS/S-458, 12. RHS/T-477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480,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

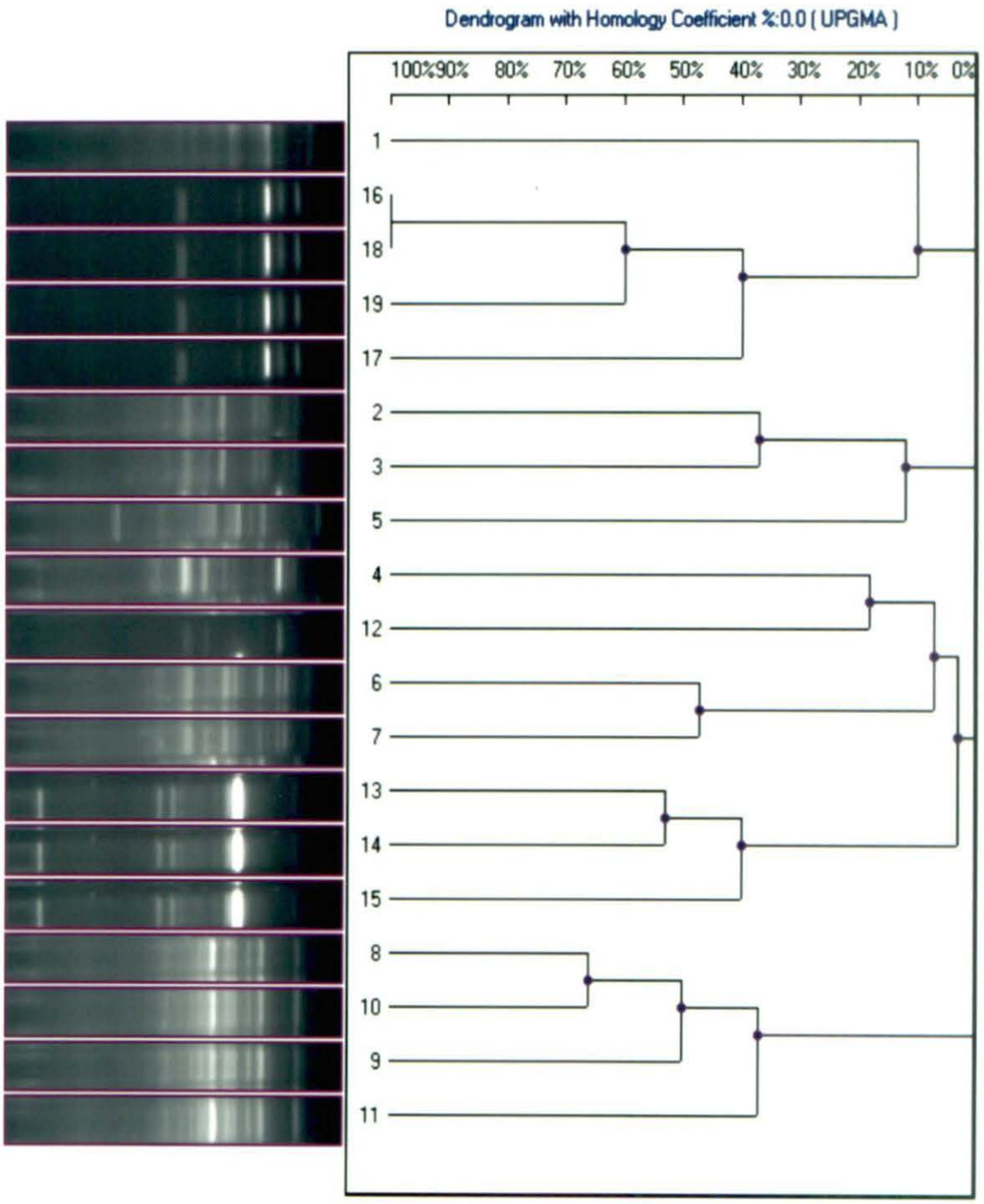


Plate 30: RAPD analysis of *Trichoderma* isolateswith A5 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T- 460, 7. RHS/T- 463,8. RHS/T- 472, 9. FS/C-90, 10. FS/S-455, 11. FS/S-458, 12. RHS/T- 477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480,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

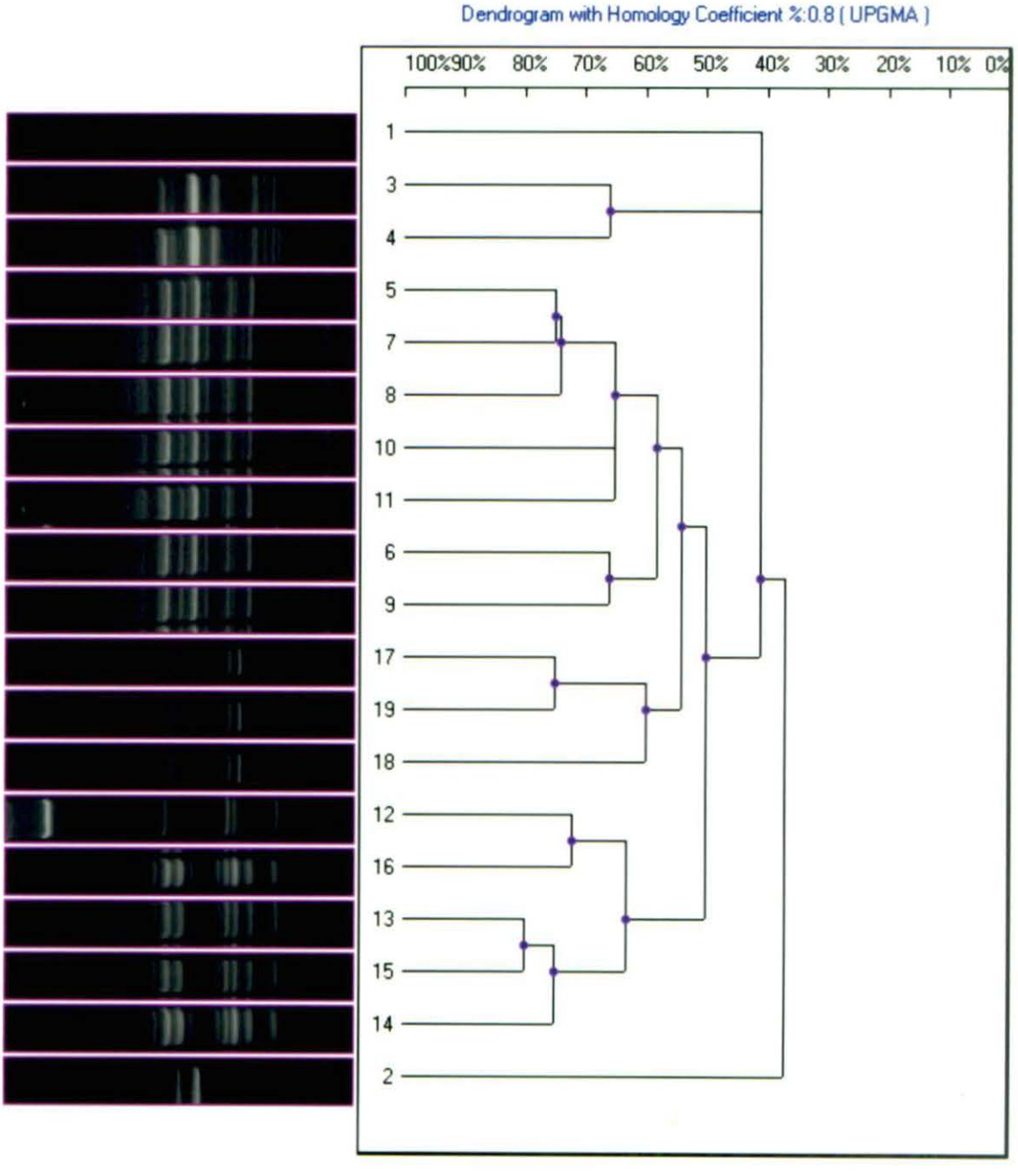


Plate 31: RAPD analysis of *Trichoderma* isolates with AA-11 primer in BioProfile software. (1. FS/L-20, 2. FS/S-473, 3.FS/S-474, 4.FS/S-475, 5. FS/S-478, 6. RHS/T-460, 7. RHS/T- 463, 8. RHS/T- 472, 9. FS/C-90, 10. FS/S-455, 11. FS/S-458, 12. RHS/T-477, 13.Ag/S476, 14. Ag/S471, 15. Ag/S479, 16. RHS/AC480,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

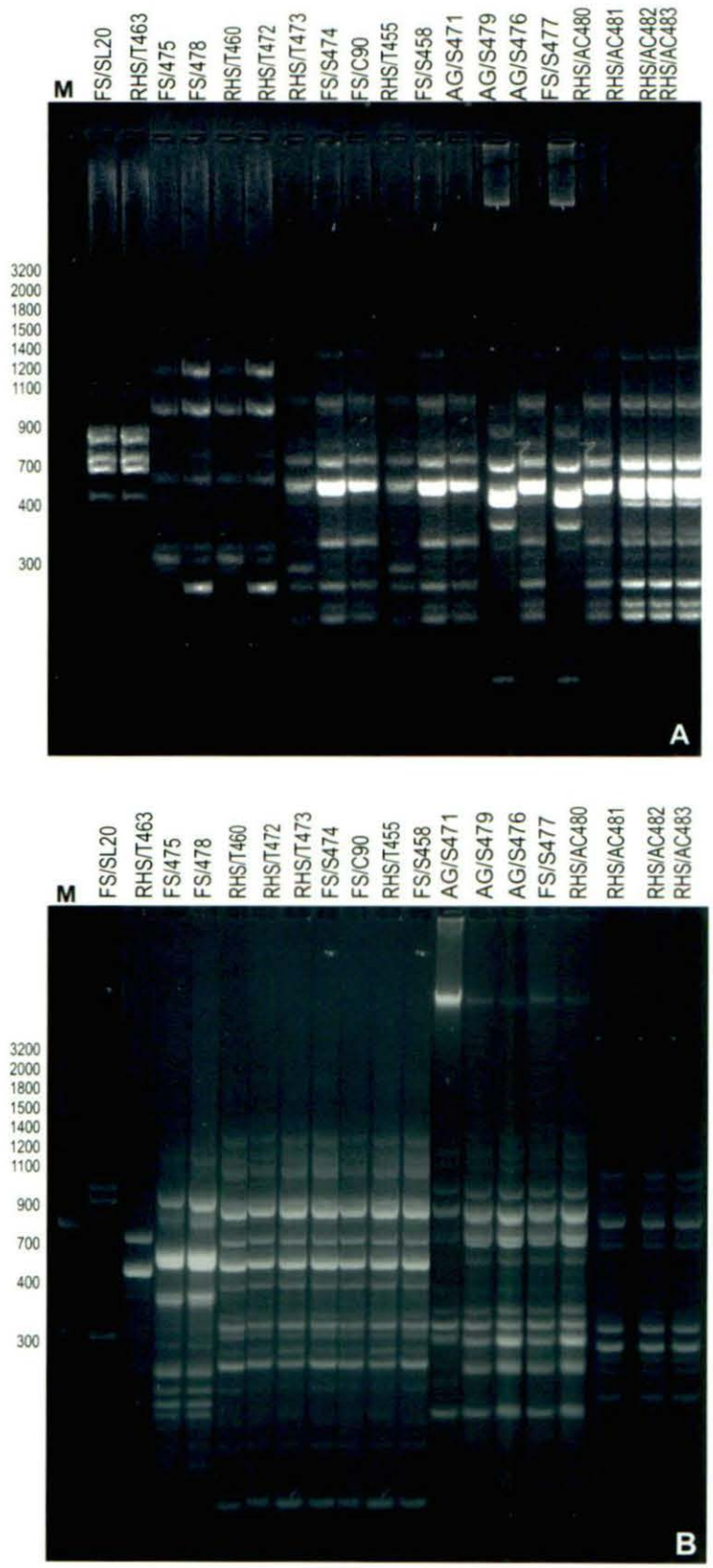


Plate 32: RAPD analysis of 19 *Trichoderma* isolates using A-5 primer (A) and DPD-6 primer (B)

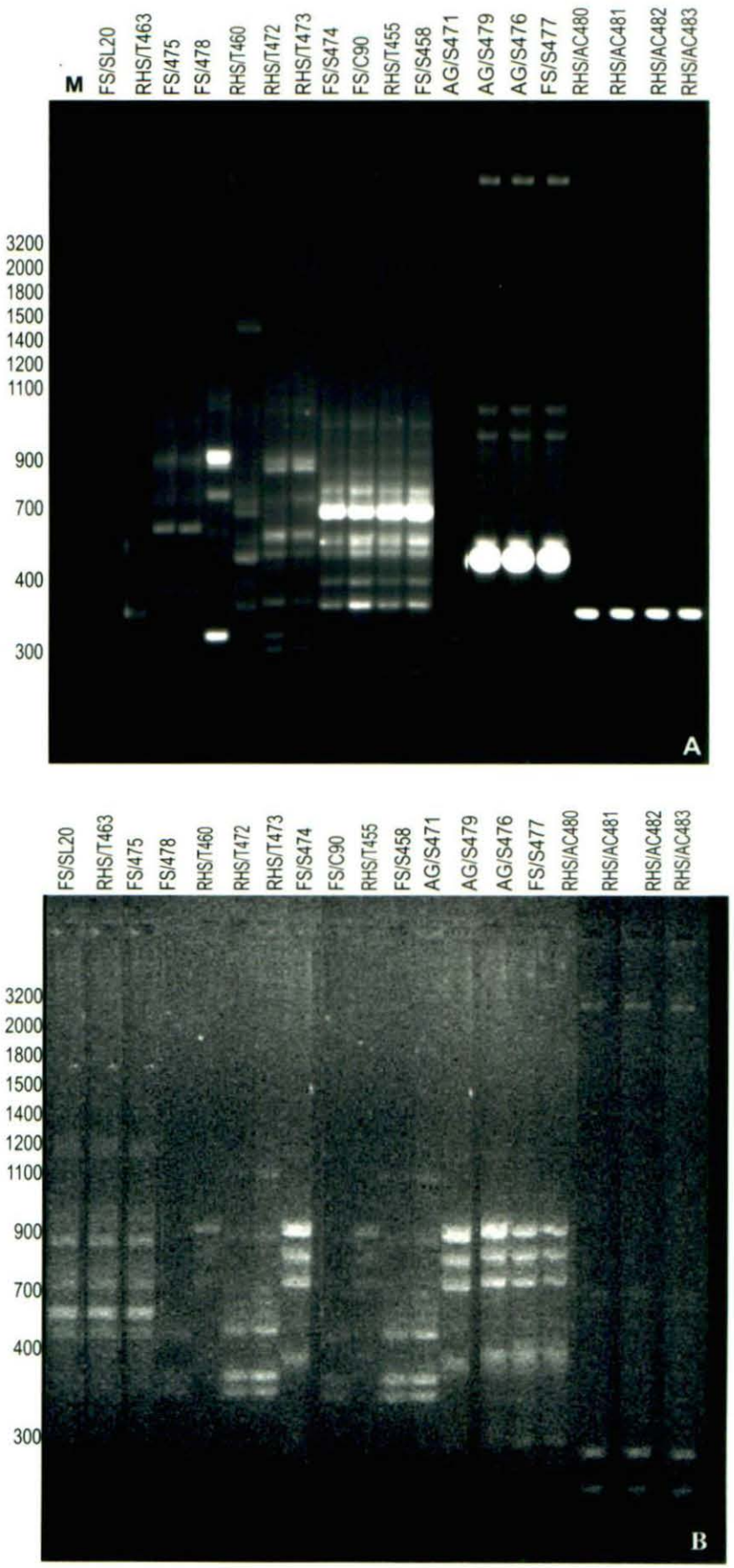


Plate 33: RAPD analysis of 19 *Trichoderma* isolates using A-11 primer (A) and OPA Primer (B)

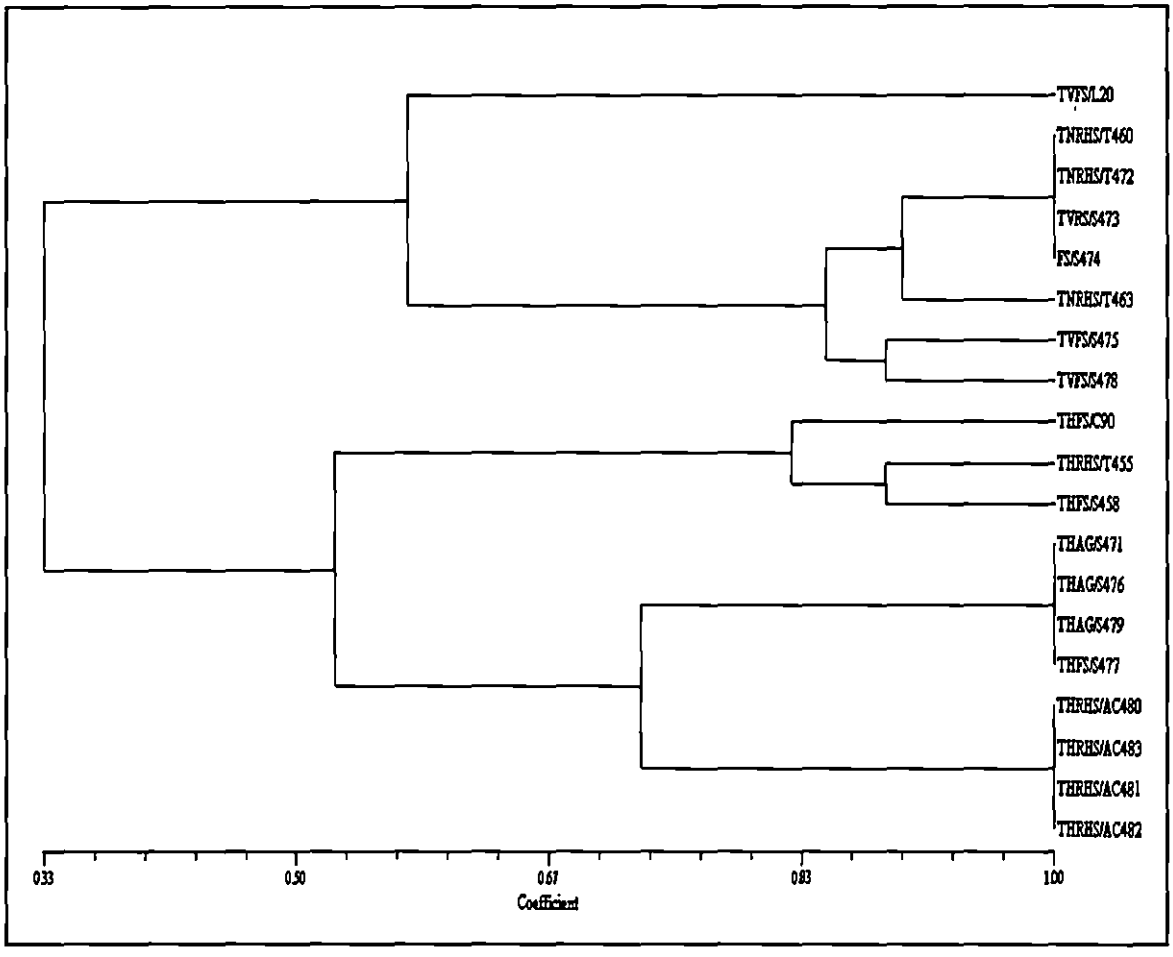


Figure 8: RAPD analysis by Dendogram of *Trichoderma* isolates

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.

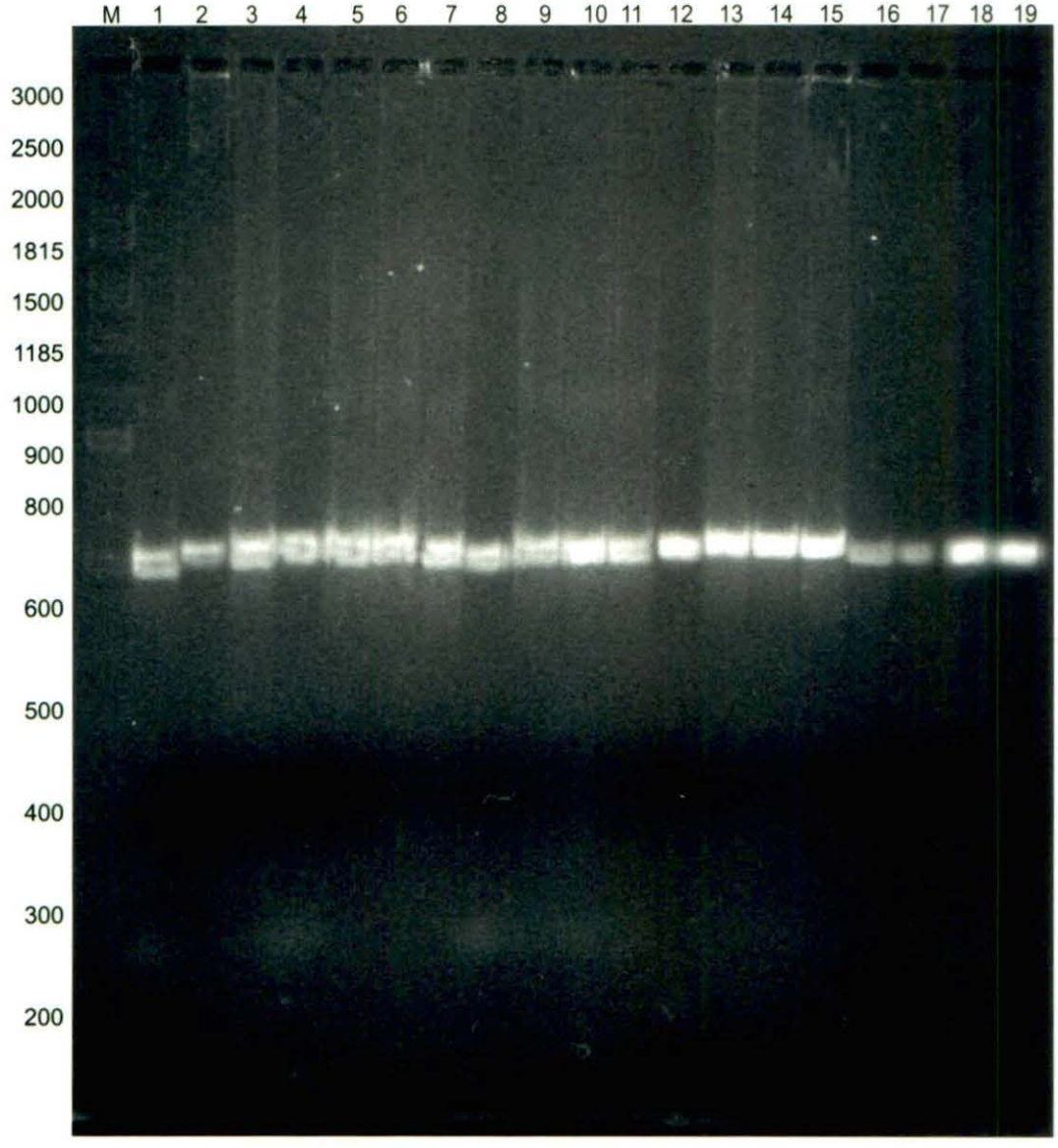
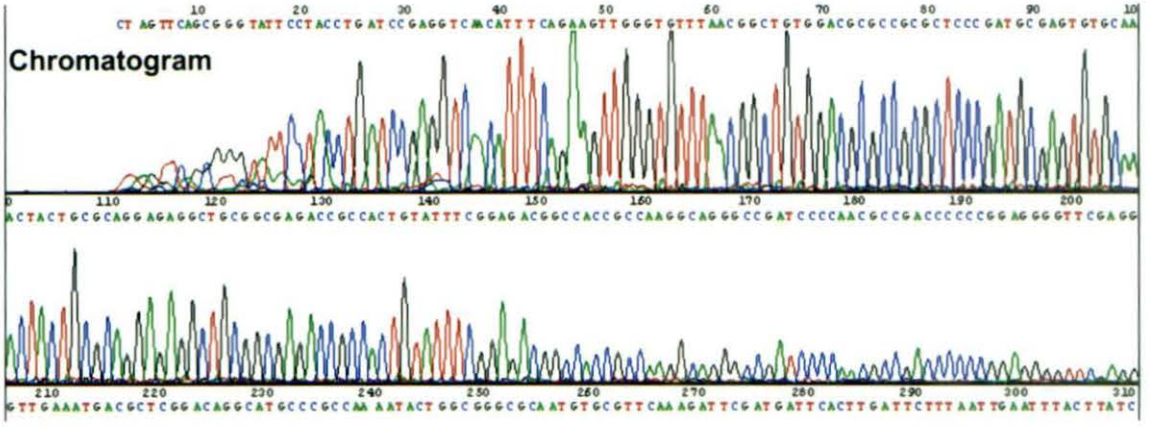


Plate 34: ITS PCR *Trichoderma* isolates (Marker (M), (1) FS/L-20, (2) FS/S-473, (3) FS/S-474, (4) FS/S-475,(5) FS/S-478, (6) RHS/T- 460, (7) RHS/T-463, (8) RHS/T- 472, (9) FS/C-90, (10) FS/S-455, (11) FS/S-458, (12) RHS/T-477, (13) Ag/S476, (14) Ag/S471, (15) Ag/S479, (16) RHS/AC480, (17) RHS/AC481,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

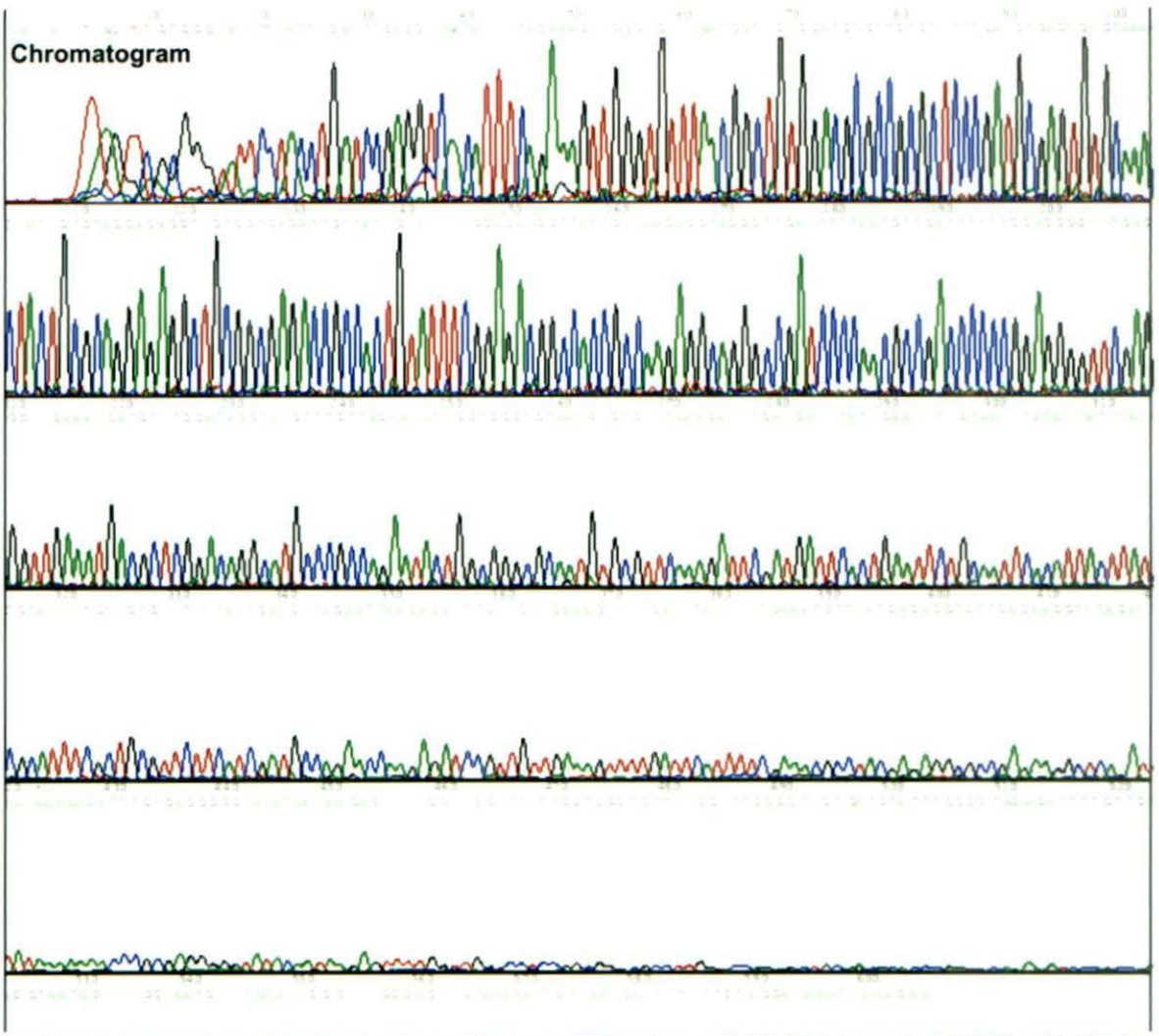
GI:291194458

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

```
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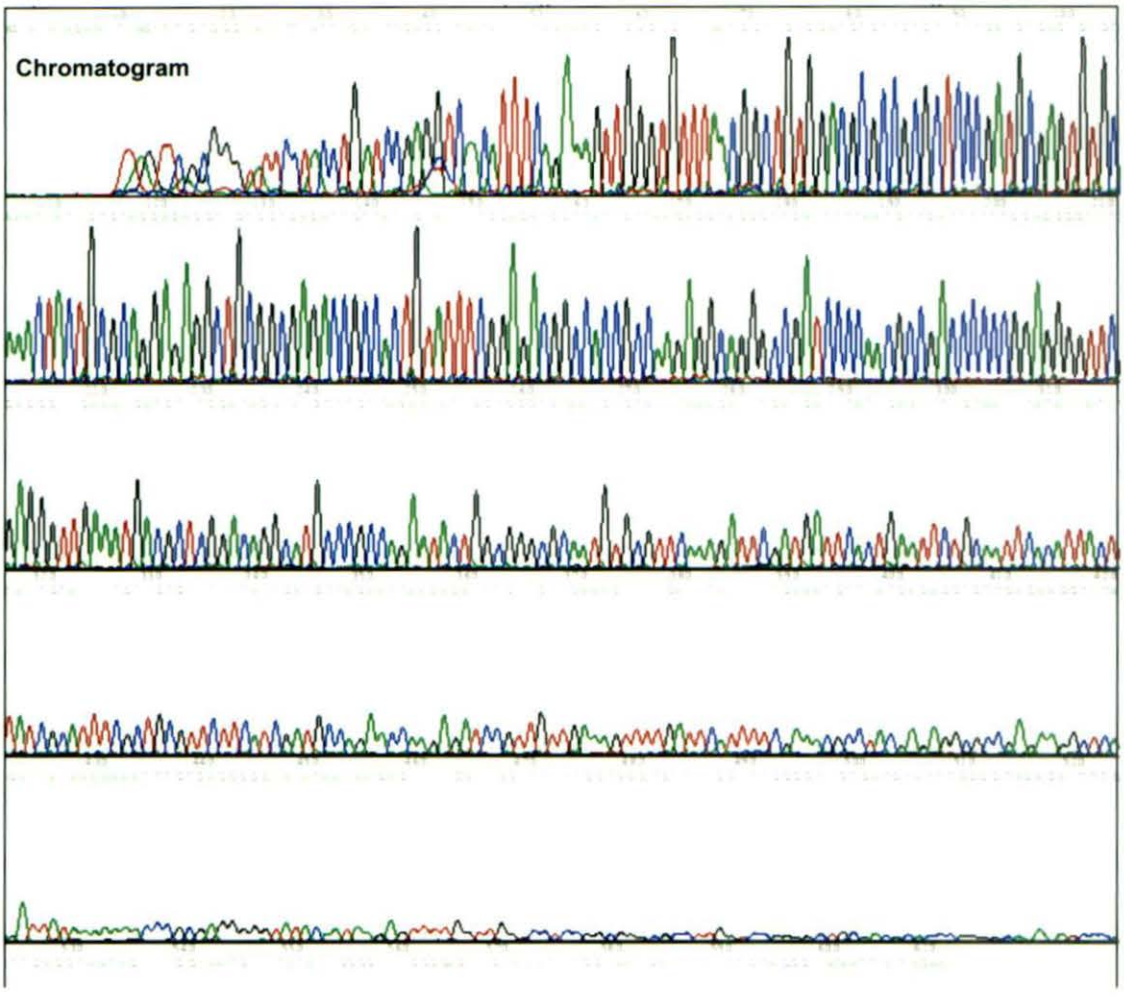
 61 gaacgttacc aaaactgttg cctcgtcggg acctctgccc cgggtgcgtc gcagccccgg
121 accaaggcgc ccgcctgagg accaaccaaa actcttattg tatacccct cgcgggttat
181 tttataatct gagccttctc ggcgcctctc gtaggcgttt cgaaaatgaa tcaaaacttt
241 caacaacgga tctcttggtt ctggcatcga tgaagaacgc aacgaaatgc gataagtaat
301 gtgaattgca gaattcagtg 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)



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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

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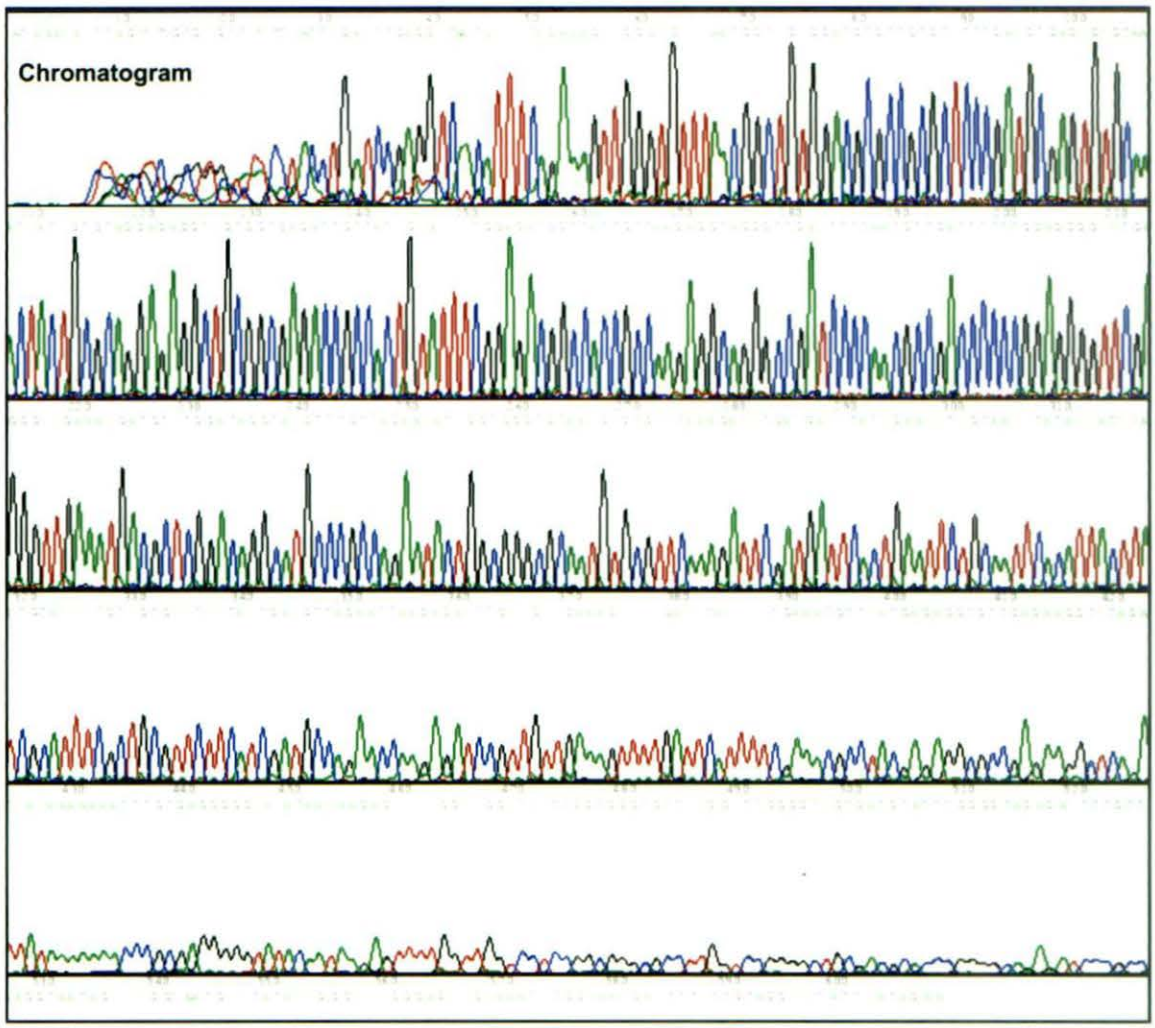
Figure 11: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain



Partial sequence of ITS 4 region of rDNA

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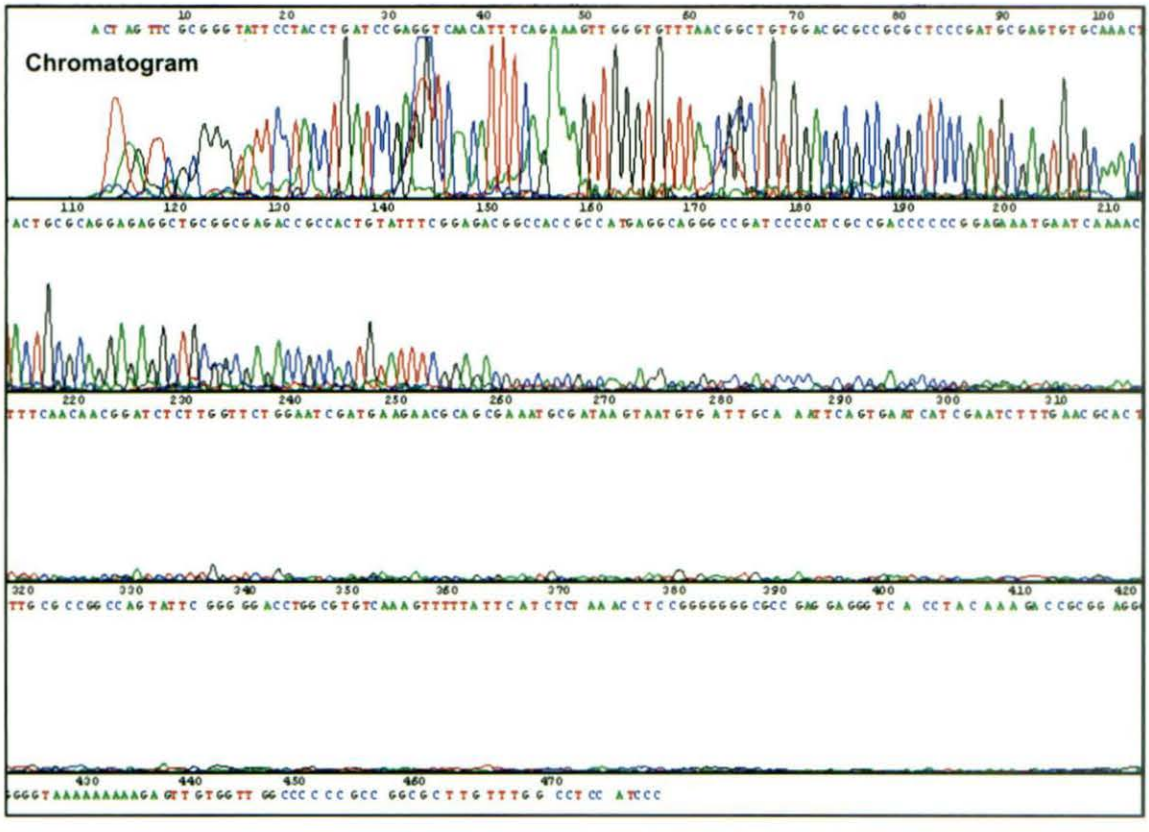
Figure 12: Chromatogram and sequence deposition of ITS region of *T. harzianum* strain (NAIMCC-F-01952)



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

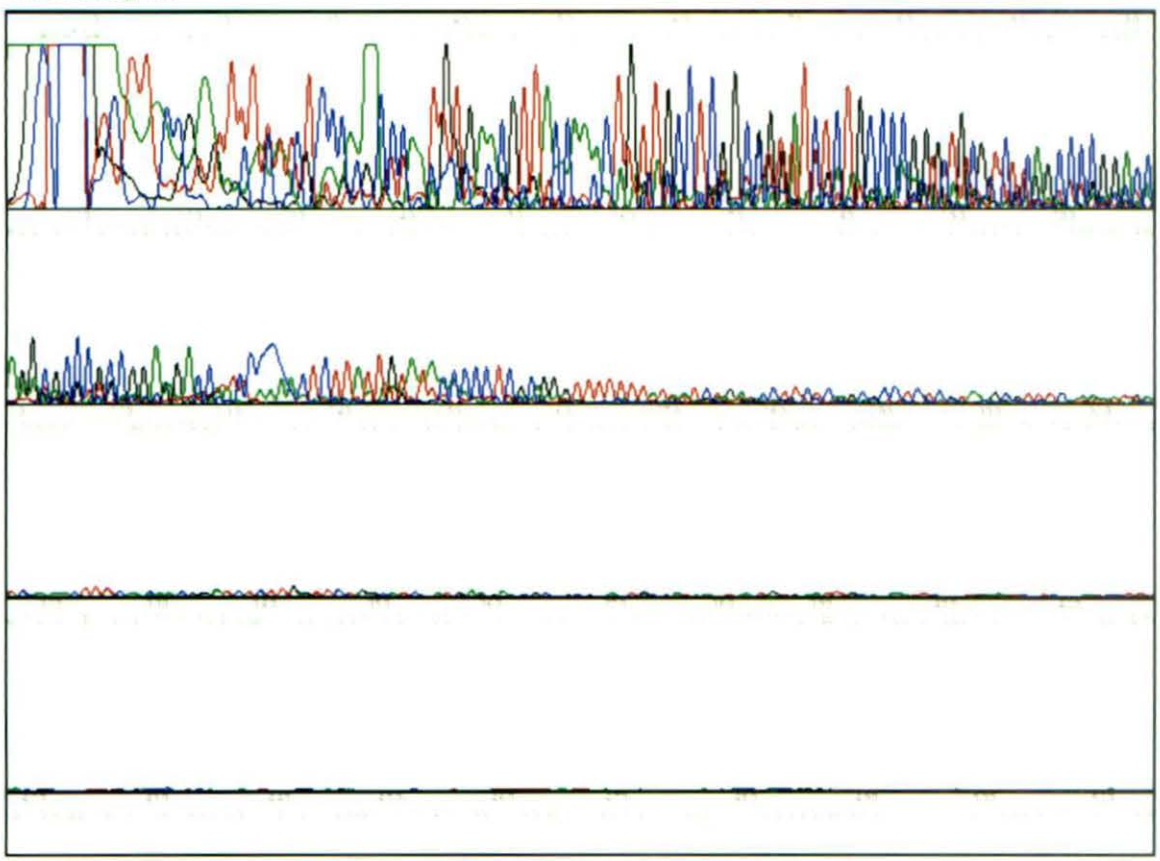
DNA linear :479 bp

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

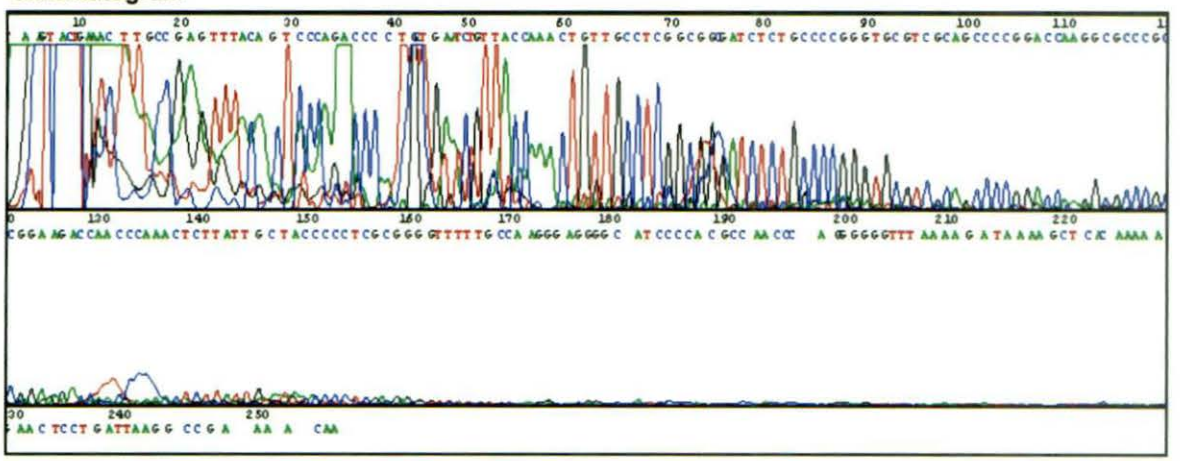
DNA linear : 605 bp

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.

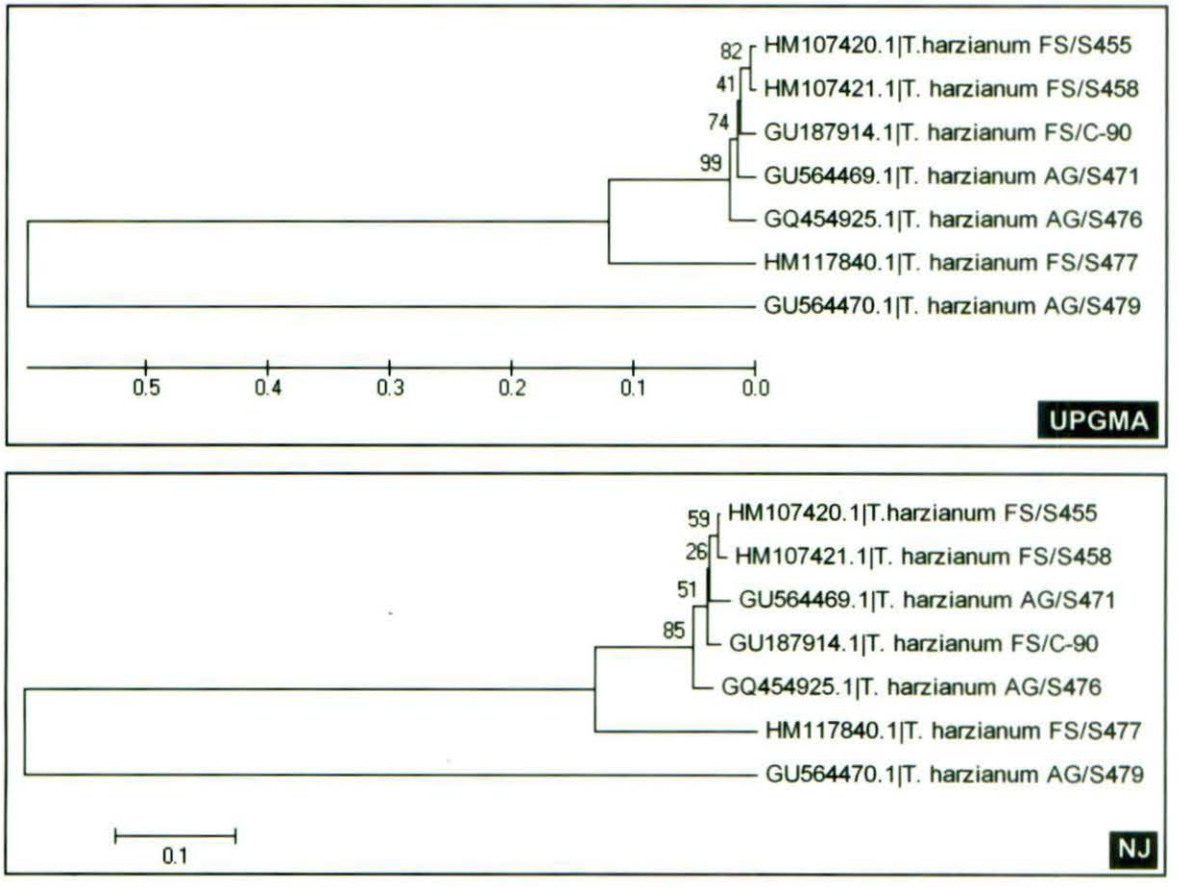


Figure 16: Phenogram of seven isolates of *Trichoderma harzianum* by UPGMA and NJ method

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.

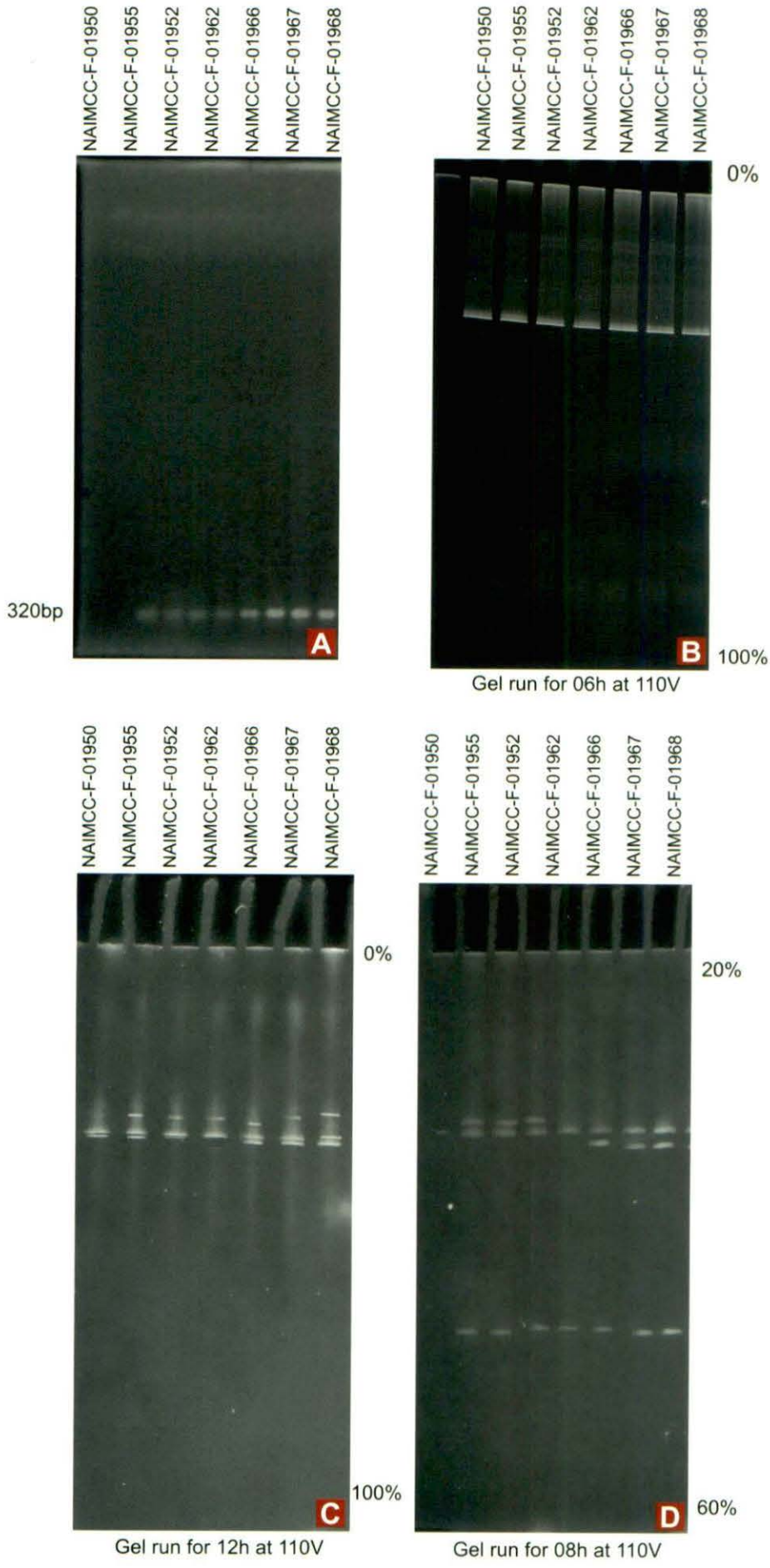


Plate 35: DGGE analysis of seven isolates of *Trichoderma harzianum*

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].

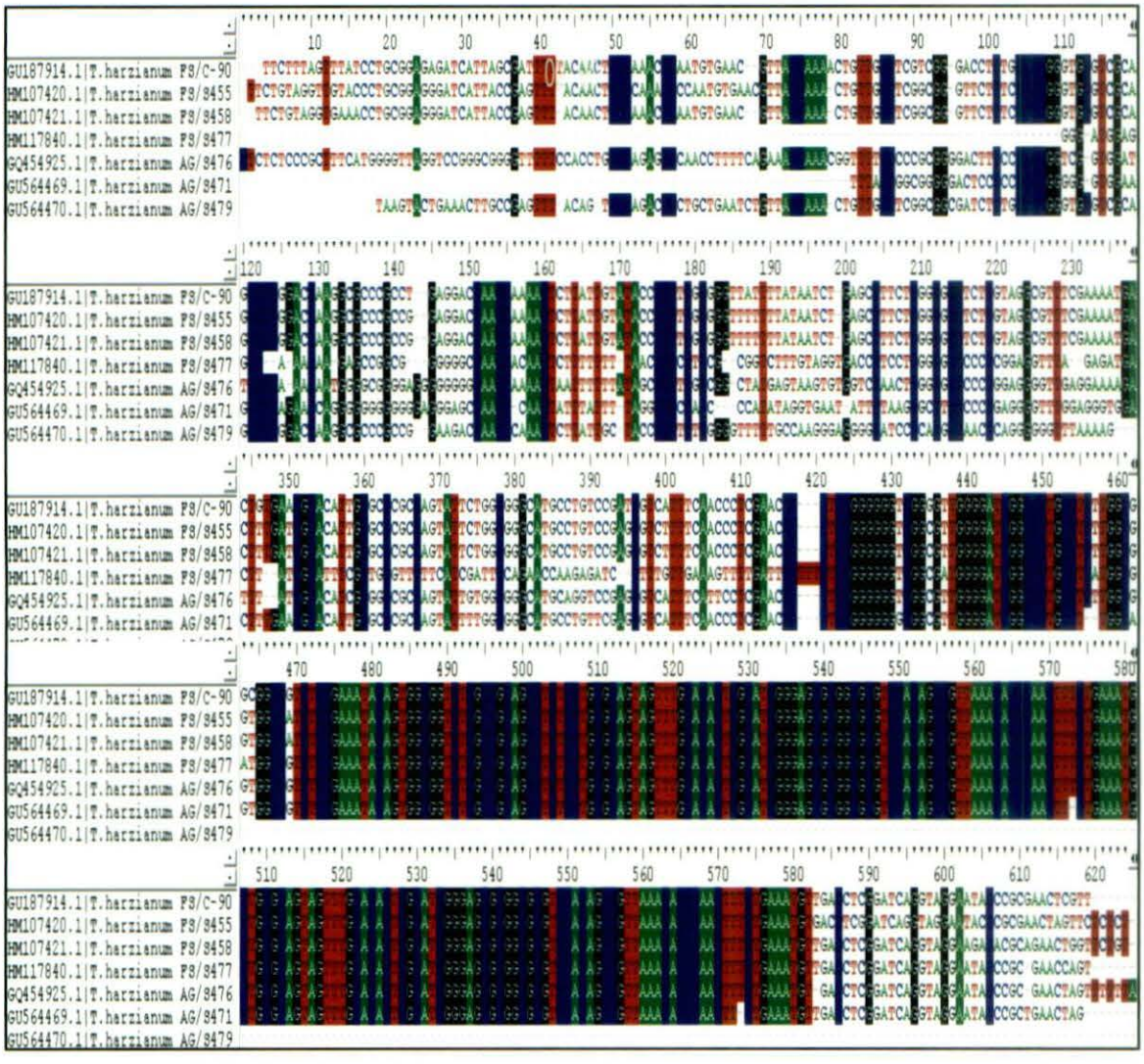


Figure 17: 18S rDNA sequence alignments of *T.harzianum*
Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in different colour

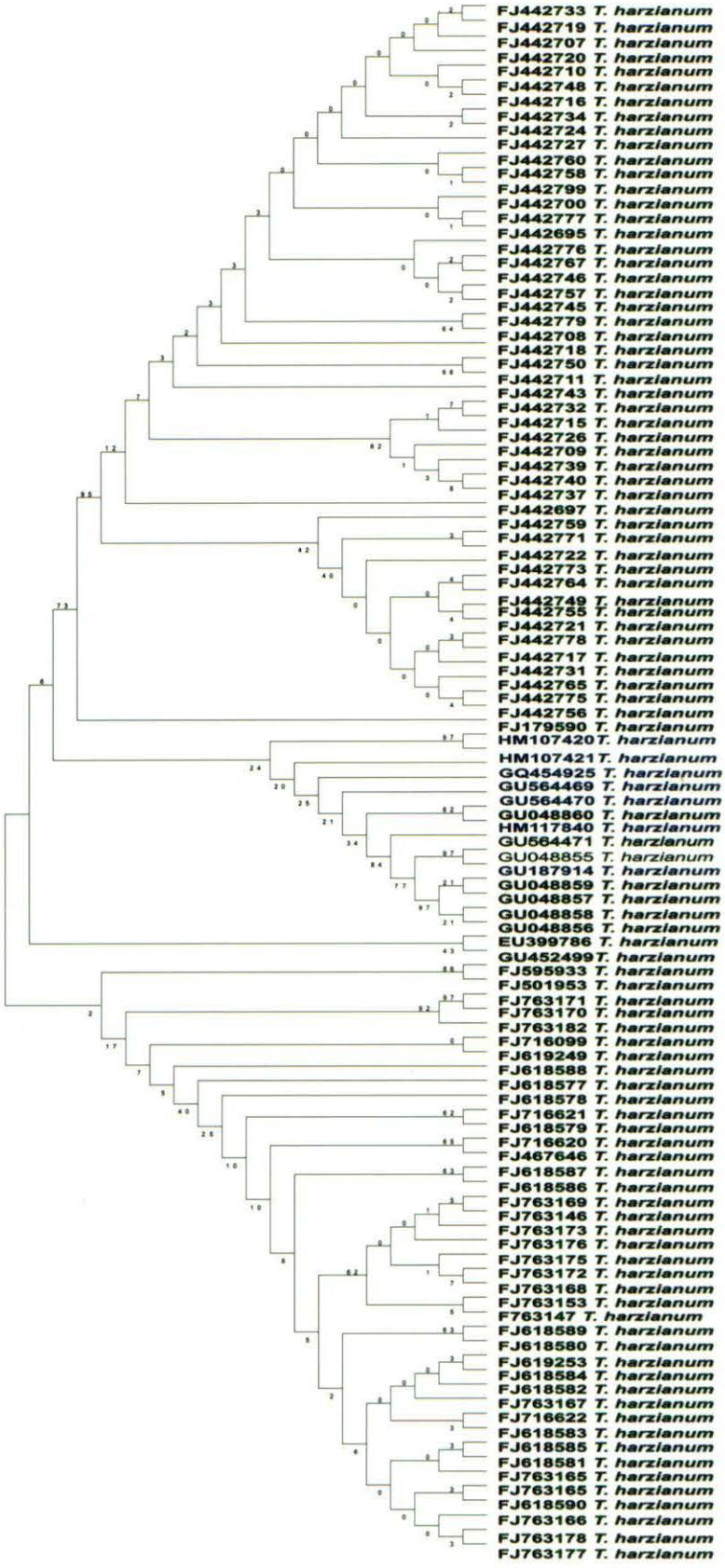


Figure 18: Phylogenetic placement of seven isolates of *Trichoderma harzianum* with extype strains from NCBI genebank

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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Discussion

Rhizosphere fungi, bacteria, and actimomycetes can have a profound effect on plant health. Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms, but also is crucial in the application of microorganisms for beneficial puposes; most significant among these applications are biofertilization, phytostimulations, biocontrol and phytoremediation (Lugtenberg et al., 2001). The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field (Basnayake and Brich, 1995). Microorganisms contribute to the availability and mobilization of nutrients, production of growth regulators, phytotoxic substances or by suppression of pathogens and pollutants added to soil. Nearly 5-23% of all photosynthetically fixed carbon is being transferred to the rhizosphere through root exudates (Marschner, 1995);an increase in root exudation of organic solutes could affect the rate of phyto-siderophore release. In turn, rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Yang *et al.*, 2001).

Although non chemical control of pests and diseases seems an attractive option in sustainable agricultural systems, its practical application is severely constrained by lack of reproducible results in comparison to the use of agrochemicals. The biotic and abiotic factors in the environment, which influence the efficiency of pathogen suppression, are not well understood. Nevertheless it is known that some soil become suppressive to fungal, bacterial and nematode pests when susceptible host plants are grown continuously for several years and there is evidence that this is due to the development of soil biological communities which inhibit pest survival or infection of the hosts.

In the present study a large number of microorganisms were isolated from the forest soil, riverine soil and rhizosphere soil of different regions and heir molecular abnd functional diversity studied.

The present study on qualitative and quantitative evaluation of various soil factors and fungal, bacterial and actinomycetes biomass with special reference to phosphate solubilizer, biocontrol agents indicated several interesting facts. As generally observed in our study, a high fungal population was known to be associated with high organic carbon content of the soil, acidic pH like agricultural soil, forest soil. Such contradictory results are probably due to the fact that the microbial population of the soil is influenced by various factors of the soil and just not only organic and inorganic nutrients in the soil. All

the soil samples found to be acidic to alkaline (pH 3.8 to 5.6) which indicated their suitability for plantation and agriculture. The biological population depends on the physical and chemical properties of the soil whereas these properties in turn, are continuously modified by the activities of biological population (Kennedy and Papendick, 1995). The physical, chemical and biological properties of any soil are the indicators of the quality of that soil (Drinkwater *et al.*, 2002). Fungi are aerobic microorganisms found more in the upper layers of the soils. Quality and quantity of organic matter present in the soil controls the number of fungi in soil since the fungi are heterotrophic in nature. Fungi are dominant in acidic soils but are also present in alkaline soils. Composition of the fungal communities in soil is also affected by other factors like soil aeration, soil density, soil structure, salinity and also the water holding capacity of the soil.

A total of 176 fungal isolates, 87 bacterial isolates and 60 isolates of actinomycetes were obtained from soil samples collected from Darjeeling, Jalpaiguri and Cooch Behar districts of North Bengal. Source of soil samples from forest, riverine and agricultural land (rhizosphere of plantation and agricultural crops) yielded 46, 26 and 104 fungal isolates respectively. Cultural characteristics of the isolated fungi were studied and microscopic observations were made for identification of these isolates. On the basis of their morphological characters 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.*

The abundance, diversity, and distribution of native population and inoculant strains in agricultural fields have been characterized using a variety of methods. Screening of rhizosphere micro flora for antagonism against pathogenic fungi in order to select suitable biocontrol agents has also been previously reported by a large number of workers. Kobayashi *et al.* (2000) isolated three bacteria showing antagonism to *Rhizoctonia solani* from the rhizosphere soil of different crops which they identified as *B. pumilus*. In the present study, Bacillus *pumilus* isolates were evaluated as phosphate solubilizer of which one strain (B/RHS/P 22) was found to be most effective against *Fusarium oxysporum and Sclerotium rolfsii.*

Soil microbial communities are often difficult to fully characterize, mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity. With

respect to the latter, bacterial populations in soil top layers can go up to more than 109 cells per g soil (Torsvik, 2002), and most of these cells are generally unculturable. The fraction of the cells making up the soil microbial biomass that have been cultured and studied in any detail are negligible, often less than 5% (Borneman and Triplett, 1997; Torsvik *et al.*, 1990). As direct DNA-based methods offer the possibility to assess the total microbial diversity present, thus by passing the limitations of cultivation-based studies, there has been a rapid development of cultivation-independent methods for analyzing the microbial communities in soil (Akkermans *et al.*, 1995; Ovreas *et al.*, 1998). Traditionally, methods to analyze soil microorganisms have been based on cultivation and isolation (Van Elsas, 1998); a wide variety of culture media has therefore been designed to maximize the recovery of diverse microbial groups. A Biolog-based method for directly analyzing the potential activity of soil microbial communities, denoted community-level physiological profiling (CLPP), was introduced (Garland, 1996). Unfortunately, as a result of biases favoring copiotrophic organisms, the resulting metabolic fingerprints are unlikely to represent accurately the *in situ* functional diversity in a natural microbial community (Smalla *et al.*, 1998). Cultivation-based methods are limited in that only a small fraction of the microbial cells in soil are accessible to study, although a recent study claimed that this percentage can be raised substantially by using special cultivation techniques (Janssen *et al.*, 2002). Scientific interest has long focused on the structure of microbial communities in the rhizosphere, assessed by cultivation-based studies. These studies have shown that the microbial diversity in the rhizosphere is often extensive and that there are distinct differences in bacterial community structures between bulk (nonrhizosphere) soil and rhizosphere soil. Recently, several studies on different plant species in different locations, using a range of cultivation-based and molecular methods, indicated that plant type is indeed a major factor influencing the structure of microbial communities. In specific cases, bacterial communities were shown also to be influenced by plant genotype, root zone, or plant age.

Since phosphate solubilization is an important trait of soil microorganism for improving crop productivity, the present study isolates were further taken up for evaluation of phosphate solubilization potential in liquid medium, *A. niger* (RS/P 14) showed maximum solubilization when medium was amended with tricalcium phosphate, whereas isolate *A. melleus* (RHS/R 12, FS/L 13 and FS/L 17) showed maximum amount of phosphate solubilization when the medium was amended with rock phosphate, with an

average drop in the pH from 7 to 3.5 (Chakraborty *et al.*, 2008, 2010 a). Acid production and drop in the pH of the medium have been reported in the earlier studies (Abd Alla, 1994; Whitelaw, 2000), however no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium. In a similar study a total of 70 fungal isolates obtained from different sources showed phosphate solubilizing activities as detected in Pikovskaya's agar medium showing halo zones after 4-5 days of incubation in a study carried out by Pradhan and Sukla (2005). The phosphate solubilization potential of *Aspergillus* isolates was determined in three different liquid medium where PVK medium showed maximum phosphate solubilization. Some researchers have suggested that of the medium increasing P concentration in the phosphate solubilizing fungus containing medium was related to metabolities, which should correlate with the pH (Illmer and Schinner, 1992). Many studies have showed the ineffectiveness of rock phosphate use due to low solubility of its P content. Phosphate solubilizing fungus (PSF) has demonstrated the utilization of these poorly soluble phosphate source and PSFs were used as bioactivators of poorly soluble rock phosphate (Didiek *et al.*, 2000).

There are previous reports that leaf area index of plants are affected by the availability of the nutrients in the soil. Increased content of soluble phosphate in the soil due to the activity of PSF increases the leaf area of these plants. Nodulation of leguminous plants is directly affected by the availability of the nutrients in the soil (Zaidi and Khan, 2006). Increased content of soluble phosphate content in the soil due to the activity of PSF accelerates the rate of nodulation formation on these plants. Results of the present study also indicated that the number of nodules was found to be more in case of the plants treated with the three isolates of A. *niger*. Average number of nodules in the roots of these plants is also an indicator of the role of PSF in improving the plant health status of the soybean plants.

In a study carried out to determine the role of dual PSF on the growth of soybean, it was found that when inoculated with FYM were in soil mixed with rock phosphate significant increase in the growth in terms of plant height, number of leaves and dry weight of shoot and root were noted (Bora *et al.*, 2003). In another study it was reported that *A. niger* was one of the most efficient phosphatase producing fungi among many PSFs screened for efficiency for phosphatase production and it was observed that the reduction of the pH of

the medium was maximum with the *A. niger* isolate which efficiently hydrolyzed different compounds (mono- and hexa-) of organic phosphorous (Tarafdar *et al.*, 2003).

It is apparent from the results of the present study as well as studies by a large number of previous workers that PGPR and PSFs have the ability to promote growth in plants, which in many cases is associated with pathogen suppression in soil (Chanway *et al.*, 2000; Castillo *et al.*, 2002). PGPR and PSFs secrete one or more metabolites in the soil which then elicits the observed response in the host. Thus, these microorganisms or their products have the ability to elicit responses at molecular level which would include activation of a number of metabolic pathways in the host, the end product of which is finally expressed as increased growth of plants or reduced disease. Induced Systemic Resistance (ISR) is effective against different types of pathogens but differs from Systemic Acquired Resistance (SAR) in that the inducing PGPR does not cause visible symptoms on the host plant (Loo *et al.*, 1998). Pieterse *et al.* (2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. The phenomenon of SAR suggests that there is a signal that originates at the site of elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns the compatible plant–pathogen interaction into an incompatible (Uknes *et al.*, 1992) one. This resistance was correlated with the accumulation of pathogenesis related (PR) proteins, generally assumed to be markers of defense response added by (Ward *et al.*, 1991).

The major components analyzed in leaves in present study were defense enzymes, In all tested varieties defense related enzymes viz. chitinase (CHT), β-1,3- glucanase (GLU), peroxidase (PO) and amylase increased significantly, especially in presence of the pathogen. In a similar study, one isolates of *B. pumilus* (B/RHS/P-22) and *Streptomyces gresious* (A/RHS/PO 26) were reported to be best plant growth promoters and biocontrol agents downy mildew disease in pearl millet (Niranjana *et al.*, 2003). They also reported increased activities of PAL, PO and β-1, 3- glucanase (GLU), but not of (CHI) activity. Induction of defense related enzymes by *P. flurescens* in black pepper and *Phytopthora capsici* pathosystem was reported by (Paul and Sharma, 2003). They obtained increased level of PO, PAL, PPO and Catalase in leaves apart from root of treated plants indicating the systemic protection offered to black pepper by PGPR strains. Peroxidase and polyphenol oxidase are important components of the defense mechanism of plants against pathogens. Chakraborty *et al.* (2010d) reported that *Serratia marcescens* (TRS-1), as

aqueous suspensions, promoted growth in tea seedlings as evidenced by increase in height, emergence of new leaves and branches, as well as increase in leaf biomass. Survival of *S. marcescens* in soil after application was determined by ELISA and Dot-Blot using PAb raised against the bacterium. *S. marcescens* solubilized phosphate *in vitro* and *in vivo*. Following application of the bacterium, soil P content decreased, root and leaf phosphate increased, and soil phosphatase activities were also enhanced. In another study, survival of *Pseudomonas sp.* SF4c and *Pseudomonas sp.* SF10b (two plant-growth-promoting bacteria isolated from wheat rhizosphere) was investigated in microcosms. Spontaneous rifampicin resistant mutants derived from these strains (showing both growth rate and viability comparable to the wild-strains) were used to monitor the strains in bulk soil and wheat rhizosphere. Studies were carried out for 60 days in pots containing non-sterile fertilized or non-fertilized soil. The number of viable cells of both mutant strains declined during the first days but then became established in the wheat rhizosphere at an appropriate cell density in both kinds of soil. Survival of the strains was better in the rhizosphere than in the bulk soil. Finally, the antagonism of *Pseudomonas spp.* against phytopatogenic fungi was evaluated *in vitro*. Both strains inhibited the mycelial growth (or the resistance structures) of some of the phytopathogenic fungi tested, though variation in this antagonism was observed in different media. This inhibition could be due to the production of extracellular enzymes, hydrogen cyanide or siderophores, signifying that these microorganisms might be applied in agriculture to minimize the utilization of chemical pesticides and fertilizers (Fischer *et al.*, 2000). Treatment with *Ochrobactrum anthropi* decreased brown root rot of tea, caused by *Phellinus noxius* (Chakraborty *et al.*, 2009). Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in tea plants was observed on application of *O. anthropi* to soil followed by inoculation with *Phellinus noxius*. A concomitant increase in accumulation of phenolics was also obtained. Further, Chakraborty *et al.* (2010d) also reported that *Serratia marcescens* (TRS 1) showed antagonism to a number of fungal pathogens *in vitro*. It also reduced brown root rot of tea caused by *Fomes lamaoensis*. Significant increase in phenolics, as well as peroxidise, chitinase, β-1,3-glucanase and phenylalanine ammonia-lyase, were observed in tea plants on application of *S. marcescens* alone or followed by *F. lamaoensis*. *Ochrobactrum anthropi* TRS-2, isolated from tea rhizosphere could solubilize phosphate, produce siderophore and IAA *in vitro* and also exhibited antifungal activity against six test pathogens. Application of an aqueous suspension of *O. anthropi* to the rhizosphere of nursery grown tea seedlings of

five varieties of tea (TV-18, T-17, HV-39, S-449, UP-3 and) led to enhanced growth of the treated plants, as evidenced by increase in height, in the number of shoots and number of leaves per shoot (Chakraborty *et al.*, 2009).

Among the various isolates obtained from soil in this study, most efficient phosphorus solubilizers are species of *Aspergillus*. The strains with maximum phosphate solubilizing activities also showed good plant growth promoting activity. Such microorganisms could be used in the field as efficient biofertilizers.

Accumulation of defense enzymes such as PAL, PPO, PO, in *Phaseolus vulgaris* plants following inoculation with *S. rolfsii* were determined. PPO usually accumulated following inoculation of plants. Among all the stress related enzymes, the role of peroxidese has been most thoroughly worked out. PO is a metallo- enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. The induction of PO activity by pathogens and methyl jasmonate and existence of multiple molecular forms of peroxidase in tea has also been reported (Sharma and Chakraborty, 2004). Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosnthesis such as PAL are involved in defense reaction in plants. Bhattacharya and Ward (1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma.* Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolics compounds have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicators of the activation of defense enzymes.

Nineteen isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil, forest soil and agricultural field which were identified as *Trichoderma viride* and *Trichoderma harzianum.* Assay of both endo and exo chitinase activities of the different isolates of *T. harzianum* and *T. viride* revealed that RHS/AC480 had maximum activity while FS/C-90 had minimum. Results of *in vitro* pairing tests of *Trichoderma* isolates with *S. rolfsii* for antagonistic activity were recorded at different intervals from 4th day onwards. Both *T. harzianum* and *T. viride* inhibited growth of *S. rolfsii*. Among the isolates, RHS/AC480 was the most antagonistic. It seems therefore, that the isolate producing highest chitinase activity also exhibited maximum antagonistic activity. These

observations, together with the fact that chitin, β1,3-glucan and protein are the main structural components of most fungal cell walls, confirms the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens (Chet and Baker, 1981). *Trichoderma* spp. (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their biocontrol activity and host range (Sivan and Chet, 1992).

The overall results of the present study have shown that phosphate solubilizing microorganisms isolated from forest soil, riverine soil and rhizosphere soil could induce plant growth promotion and disease reduction in tea as well as in other crops. Different species of *Bacillus, Trichoderma* and *Aspergilius* are now widely used in other crops as plant growth promoters as well as biocontrol agents (BCA). Development of better formulations to ensure survival and activity in the field and compatibility with chemical and biological seed treatments is another area of focus; approaches include optimization of growth conditions prior to formulation and development of improved carries and application technology. Mulaw *et al.* (2010) reported the southwestern highlands forests of Ethiopia are the origin of the coffee plant *Coffea arabica*. The production of coffee in this area is affected by tracheomycosis caused by a soil-born fungus *Gibberella xylarioides*. The use of endemic antagonistic strains of mycoparasitic *Trichoderma* species would be a nature conserving means to combat this disease. Authors used molecular methods to reveal that the community of *Trichoderma* in the rhizosphere of *C. arabica* in its native forests is highly diverse and includes many putatively endemic species. Among others, the putative new species were particularly efficient to inhibit growth *of G. xylarioides*. polymorphism and clone library sequencing of 16S ribosomal RNA (rRNA) gene fragments.

In order to determine whether the applied microorganisms are sustainable in the soil, immunological techniques such as Dot- blot, Western blot etc. are used in detection. In the present study, antibodies raised against antigens from mycelia of *T. harzianum* were tested by the various immunological testes and show positive result.

The growing importance of many *Trichoderma* strains has made their identification and distinction from other *Trichoderma* isolates crucial. This is particularly true in relation to the commercialization of strains where the distinction of these strains from all other *Trichoderma* strains is essential for the purpose of patenting. Of equal importance, is the need for consistency in the naming of *Trichodema* strains between laboratories. This is

especially significant when compiling the information available on a given species to produce a species specific profile, e.g. Ghisalberti & Sivasirhamparam (1991) compiled metabolite production profiles for a number of species using results from several laboratories. Studies such as these are important in determining the level of diversity present within the genus and, thus, resolving the best way in which to characterize individual isolates. Dennis & Webster (l97l) assessed the reliability of morphological characters and three alternative techniques and their ability to resolve individuals of the genus *Trichoderma.* Prior to the initiation of their work, three other techniques had already been investigated in relation to the characterisation of this genus. These techniques included the construction of strain specific metabolite production profiles (Dennis & Webster l97l a,b; Taylor 1986; Ghisalberti & Sivasithamparam, l99l), the use of specific antisera assays (Barak *et al.*, 1985a, b). All three techniques successfully differentiated between strains of the same species-aggregates as defined by the taxonomic key of Rifai (1969). However, these techniques shared the disadvantage that results were often affected by differences in colony growth conditions and the age of the colony at the time of assessment. The of the genus *Trichoderma* is concerned, it appears that the new revised key of Bissett (1984, l99l a, b, c, 1992) is now the most widely accepted key for characterizing individuals of the genus *Trichoderma* based on morphological and cultural characteristics. However, the results of this study suggest that the use of these biological characteristics alone was insufficient for the delineation of the genus *Trichoderma* to the most informative level. Results from both this study and several related studies, have demonstrated that a number of biochemical and molecular techniques can provide easily obtained and repeatable characters which will differentiate between strains of *Trichoderma* at a number of different levels. A number of these characters have also been successfully used to estimate phylogenetic relationships within the genus *Trichoderma,* at both the species and strain level of variation. However, to date, only a relatively small sample of isolates from the genus *Trichoderma* have been tested using these techniques and there has been little coordination in relation to the species and strains investigated between such studies. The versatility of such a key could be further enhanced by storing the information in a data base. New information could be added to the data base as it came to hand, thus, building up a complete profile for each strain (i.e. metabolite profiles and commercially exploitable activities). In addition, any new strains, with say unique RAPD profiles or ITS 1 sequence, could also be added to the data base and their profiles developed. This would provide an up to date profile of the members of the genus

Trichoderma which could be accessed by researcher's world wide for comparison with other strains. Furthermore, as the profiles of individual type species and strains developed patterns may emerge leading to a better understanding of the level of diversity within the genus and the intergeneric relationships of its members.

The selected isolates of BCA showed three different lineages at sixty one percent similarity level (61%). During the past few years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic diversity among species and within fungi populations and plant populations. In studies conducted with the toxin producing *Aspergillus* strains, it was found that *A. flavus* and *A. parasiticus* did not show any relationship between RAPD-based band profile and toxin production. Results of the present study suggest that these random RAPD markers can be used for identification of phosphate solubilizers. The variation obtained in terms of their genetic make-up gives an idea of improving the marker based selection of this beneficial group of organisms.

The genetic relatedness among isolates of eight *Trichoderma viride* and eleven isolates of *Trichoderma harzianum* 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 genetic 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. 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. The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using BIO Profil 1D image software for each primer and NTSYSpc software. Based on the results obtained all the nineteen isolates can be grouped into two main clusters. *T. viride* cluster is also sub grouped into five, first subgroup with four isolates and second one with seven isolates of two sub clusters. The cluster of *T. harzianum* could be divided into four different clusters containing different isolates (Chakraborty *et al.*, 2010 b). These results are in agreement with those of Muthumeenakshi (1994) Latha *et al.* (2002), and Venkateswarlu (2008) who studied genetic variability among the isolates of *Trichoderma* by RAPD using random primers. Use of more RAPD primers belonging to different operon series would further generate

more polymorphisms among the isolates. However, the present study may be useful in linking a specific amplified RAPD fragment to its antagonistic activity, and would help in developing Sequence Characterized Amplified Region (SCAR) marker linked to a potential antagonist *Trichoderma* isolate.

Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al.*, 1995). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.*, 1991; Yao *et al.*, 1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Therefore we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* spp. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 600bp were produced by the primers. The results are in accordance with Mukherjee (2002) who studied the identification and genetic variability of the *Trichoderma* isolates. These results are in accordance with several workers viz. Muthumeenakshi (1994), Ospiana (1999), Lieck-Feedt (1999) and Venkateswarlu (2008) who observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in *Trichoderma.* The ITS-PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates.

After direct sequencing of the PCR products, a total of 13 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 ITS 4 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 *Trichoderma* isolates were used in the pair wise and multiple sequence alignment. From the sequence alignment, variations were observed between

T.harzianum isolates. In conclusion, above results strongly indicated a "*Trichoderma* aggregate species" with similar "DNA-based sequence", which could be accommodated with similar forms as referred in the species identifying concept. Seven *Trichoderma* isolates comprise the largest group and similar gene sequence with respect to phylogenetic analyses and have been assigned to *T. harzianum.* For proper identification of the isolates all the PCR products were sequenced following direct sequencing of the PCR products. of sequences were obtained that could be aligned and showed satisfactory homology with ex-type strains of *Trichoderma* sequences from the NCBI Genbank data base as analysed by BLAST.

In the present investigation PCR products of *Trichoderma harzianum* were sequenced bidirectionally through the BigDye terminator technology (applied biosystem, at Bangalore Genei, Bangalore India). The sequence information was then analysed through BLSATn program which indicated that the sequences (604, 611, 608, 479, 605, 521, 254 bp) contains the genetic information of internal transcribe spacer region of rDNA gene of Thrichoderma harzianum (Accession no. GU187914, HM107420, HM107421, HM117840, GQ454925.1, GU564469, GU564470) (Chakraborty *et al.*, 2010 c).
When the three alternative techniques were assessed in combination with morphological data, all three could differentiate isolates within these morphologically defined groups. That is, morphologically identical isolates possessed differences in their nucleotide sequence, produced different RAPD band patterns. As with the results of this study, other similar studies also found ITS I sequence data, and RAPD data metabolite production data capable of distinguishing between morphologically identical isolates of *Trichoderma* (Ghisalberti and Sivasithamparam, 1991; Fujimori and Okuda, 1994; Muthumeenakshi et al., 1994). However, these studies did not investigate the possibility of producing a hierarchical classification system based on characters generated from a number of different techniques. The results of the study indicate four main levels of variation may be achievable using the characters investigated herein. The RAPD PCR technique provided the greatest resolution of isolates, permitting the distinction of 19 isolates of *Trichoderma.* We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database.

This analysis could have other applications in DNA barcoding besides in cluster analysis. The determination of frequencies of DNA strings would enable easy identification of taxon-specific strings that can be used as taxonspecific probes in DNA chip for species identification In conclusion, above results indicated that the *Trichoderma harzianum* under study could be identified very easily on the basis of available similar "r DNA-based sequence" from the available database .

A second level of variation was achieved with both morphological characters and sequence data from the ITS 1 region of the ribosomal gene complex. In general, sequence data from the ITS 1 region of the seven isolates tested were able to distinguish by DGGE analysis. Because DGGE is a suitable method that can be applied in future studies to estimate fungal diversity, but it has several drawbacks. The primer pair NS1 and GCFung, as described by May *et al.* (2001) amplifies less than 400 nucleotides and it appears to be specific to ascomycetes. In addition, the region 134 amplified and sequenced (partial 18S rDNA gene) is rather conserved and therefore not appropriate to properly identify taxa at the genus or species level. Further studies should consider primers that are more universal (for fungi) and that give better phylogenetic resolution at generic or species level. Although DGGE is a significant advancement for ecological research, attention must also be given to the sources of bias with this technique. These include differential amplification, the variation in different copy numbers, and difficulty in detection of minor species (Muyzer, 1999). In addition, the concentration of the DGGE gel and running time for electrophoresis should always be adjusted based on the GC content of the target DNA, these procedures will eventually maximize the band separation on DGGE gel, and ensure that all related soil *Trichoderma* species have been Liu *et al.* (2008) suggested that the combination of soil dilution plating, DGGE and DNA sequence analysis are effective approaches to facilitate extensive examinations of the propagule numbers of *Trichoderma*, to reliably identify *Trichoderma* species in soils with different management practices.

Summary

A review of literature has been presented focusing on the isolation and characterization of agriculturally important microorganisms (fungi, bacteria and actinomycetes), evaluation of plant growth promotion activity by phosphate solubilizing microorganisms and disease control by biological agents and their diversity analysis.

Materials used and methods followed have been presented in Materials and Methods.

Brief description of sampling area from forest soil, riverine soil and rhizosphere soil of different crops of Terai-Dooars regions has been provided. 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 and proper records like soil pH, Texture of the samples were taken.

Microorganisms (fungi, bacteria and actinomycetes) were isolated from soil sample of forest, riverine beds and rhizosphere of different crops using various selective medium and isolation soil plating technique. 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. On the basis of their morphological characters it was found that most of the fungal isolates belonged to the genera *Fusarium, Aspergillus, Curvularia, Penicillium, Alternaria, Sclerotia, Talaromyces, Paecilomyces, Sporotrichum, Acremonium, Drechslera, Rhizopus, Bipolaris, Rhizoctonia, Absidia, Emenicella, Noesertoria, Colletotrichum, Trichoderma* and *Macrophomina.*

Bacterial identification was performed on the basis of morphological and biochemical studies following Bergey's Manual of Systematic Bacteriology. Isolates were characterized for H_2S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, protease production, urease production, cellulase production and indole production. Out of 87 bacterial isolates 75 showed gram positive reaction and rest negative. Thirty bacterial isolates showed phosphate solubilizing activity, 45 were siderophore producers, all isolates showed positive result in catalase tests and 45 bacterial isolates were cellulase producers. The bacterial genera like *Micrococcus sp., Bacillus sp., Coryneform sp. and Staphylococcus sp.* were more abundant.

Identification of 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, urease production, cellulase production and indole production. Ten actinomycetes isolates showed phosphate solubilizing activity, 24 showed chitin degrading activity and 24 isolates showed cellulase production. Eight isolates produced indole. Generally *Streptomyces griseorubens* and *Streptomyces griseus* occurred profusely in the soil.

Fungal isolates were screened for phosphate solubilizing activity in Pikovskaya's agar medium. A total of 70 fungal isolates showed phosphate solubilizing activity. Among them *Aspergillus niger* showed maximum phosphate solubilization on PVK medium while *A. clavatus* showed minimum. 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* (RS/P 14) showed maximum solubilization of phosphorous while isolate *A. melleus* showed minimum of phosphorous solubilization when medium was supplemented with tricalcium phosphate.

One of the potential bacterial isolate *Bacillus pumilus* (B/RHS/P22) obtained from paddy rhizosphere and *Streptomyces griseus* (A/RHS/P026) isolated from potato rhizosphere showed phosphate solubilization, starch hydrolysis, protease and chitinase activities in preliminary screening experiments in *in vitro* conditions and was also found to inhibit fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani*. On the basis of these positive activities the isolates were evaluated for the growth of *Vigna radiata* and *Cicer arietinum* both *in vitro* and *in vivo* conditions. 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* and *S. griseus* on biochemical components of *Vigna radiata* and *Cicer arietinum*. Biochemical tests like peroxidase, ß-1, 3- glucanase and chitinase activities were conducted following standard protocols and methods in both *in vivo* and *in vitro* conditions. Significant results were obtained.

Trichoderma harzianum, *T. viride*, *Aspergillus niger*, *A. melleus*, *A. clavatus*, and *A. fumigatus* were grown in solid medium (PDA) and tested for their antagonistic activity against certain phytopathogens – *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* by dual pairing tests. *Aspergillus* group of fungi showed antagonistic activity moderately. *Aspergillus niger* had overgrown against phytopathogen. *Trichoderma viride* isolates successfully inhibited the pathogens. The group of *T. harzianum* isolates like

RHS/AC 480, AG/S476 showed highly antagonistic activity against *Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum.*

In order to select cellulase producing 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 isolates of *Aspergillus melleus, A. niger, A. clavatus* and *A. fumigatus* showed moderate cellulase activities while isolates of *Trichoderma* showed good celluase activities

Light microscopic and scanning electron microscopic studies of nineteen *Trichoderma* isolates were carried out. Assay of both endo and exo chitinase activities of the different isolates of *T. harzianum* and *T. viride* was carried out. The isolates of *T. viride* and *T. harzianum* were deposited to National Agriculturally Important Microbial Culture Collection (NAIMCC) to get the accession number. Antibodies raised against antigens from mycelia of *T. harziamum* were tested by the various immunological tests and showed positive result.

Reduction of scleorotial blight incidence of *Phaseolus vulgaris* by eight *Trichoderma harzianum* isolates were tested in potted conditions. Experiments were conducted to assess the effect of *T. harzianum* (Ag/S476) and *T. harzianum* (RHS/AC480) on biochemical components of *Phaseolus vulgaris*. Biochemical tests like peroxidase, ß-1, 3-glucanase and chitinase activities were conducted following standard protocols and methods in *in vivo* conditions.

RAPD analyses of ten potential phosphate solubilizing fungi were conducted by using decamer primer. UPGMA cluster analysis was carried out following similarity coefficient matrix of reproducible bands using PC software NTSYSPc software. Cluster 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%)

The genetic relatedness among thirty phosphate solubilizing bacterial isolates were analysed by random primers 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. Relationships among the isolates were 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. Similarity co-efficient ranged from 0.65-1.00. Based on the results obtained all nine isolates can be grouped into 8 main clusters.

PCR RAPD among actinomycetes isolates was carried out 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). Relationships among the isolates were 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. Similarity co-efficient ranged from 0.65-1.00. Based on the results obtained all isolates can be grouped into 4 main clusters.

The genetic relatedness among isolates of eight *Trichoderma viride* and isolates of eleven *Trichoderma harzianum* 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 genetic 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. 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. The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using BIO Profil 1D image software for each primer and NTSYSpc software. 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 clusters contains four different isolates.

All nineteen isolates of *Trichoderma* were taken up for ITS-PCR amplification. Amplified products of size in the range of 600bp was produced by the primers.
In the present investigation PCR products of *Trichoderma harzianum* were sequenced bidirectionally (Genei, Bangalore India). The sequence information was then analysed through BLASTn program which indicated that the sequences (604, 611, 608, 479, 605, 521, 254 bp) contains the genetic information of internal transcribed spacer region of rDNA gene of *Trichoderma harzianum* (Accession no. GU187914, HM107420, HM107421, HM117840, GQ454925.1, GU564469, GU564470).

Further analysis of the ITS sequences of seven isolates of *Trichoderma harzianum* obtained from Terai-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. Further, hundred ex -type sequences of potential biocontrol agent *Trichoderma harzianum* obtained from NCBI Gene bank database were aligned with these seven sequences for their phylogenetic placemat and a phenogram was developed using UPGMA method by Mega4 software. Next combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website
A second level of variation was achieved with both morphological characters and sequence data from the ITSI region of the ribosomal gene complex. In general, sequence data from the ITSI region of the seven isolates tested were able to distinguish by DGGE analysis. The primer pair NS1 and GCFung, was used. DGGE was performed with "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimize a suitable concentration and finally 20 to 60% denaturant was found optimal for the best result in 110V for 6h. The isolates of *Trichoderma harzianum* was further confirmed by DGGE.

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Published Papers

Screening of Phosphorus Solubilizing Aspergilli Isolates from Soils of North Bengal and Their Effects on Soybean

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Abstract

Three hundred and fifty four Aspergilli isolates obtained from soils of agricultural fields, forests and river basin of north Bengal were evaluated for phosphate solubilizing ability in Pikovskaya's agar medium. A total of 70 fungal isolates showed phosphate solubilizing activity. Further, quantitative evaluation of phosphate solubilization in liquid medium supplemented with two phosphate sources (tricalcium phosphate and rock phosphate) was done. Three isolates of *Aspergillus niger* (FS/L-04, RS/P-05 and FS/L 40) showed high levels of activity. The next best were five isolates of *A. melleus* (RHS/R-12, FS/L-13, RS/P-14, FS/L-17 and FS/L-18). One isolate of *A. clavatus* (RHS/P-38) showed a minimum phosphate solubilization activity. Nine phosphorus solubilizing Aspergilli isolates were tested for their effect on growth and nodulation in soybean plants. *A. niger* was the most effective in plant growth promotion and increase in nodulation under glass house condition.

Key words: Phosphorous solubilizing fungi, *Aspergillus niger, A. melleus, A. clavatus*, soybean

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Microorganisms are ubiquitous in nature and form vital components of all known ecosystems on earth. Soil bacteria and fungi play pivotal roles in various biogeochemical cycles (BGC) and are responsible for the recycling of organic compounds (Molin and Molin 1997). Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (George et al 1995) plant health (Smith and Goodman 1999), soil structure (Wright and Upadhya 1998) and soil fertility (Yao et al 2000). It has been widely recognized, particularly in the last two decades, that majority of harsh environments are inhabited by surprisingly diverse microbial communities. Bacteria, actinomycetes, and fungi are three major groups of soil inhabiting microorganisms. An estimated 1,500,000 species of fungi exist in the world (Giller et al 1997). Fungi are important components of soil microbiota, typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Hawksworth et al 1995). Microfungi are responsible for considerable part of microbial activity in the soil (Karaglu and Ulker 2006). About 25,000 different fungal species have been isolated from agricultural soils all over the world and since fungi interact with the plant community, it is expected that farming and forestry will change the soil fungus flora (Carlile et al 2001). The saprobic fungi represent the largest proportion of the fungal species in the soil and they perform a crucial role in decomposition of plant structural polymers such as cellulose, hemicellulose, and lignin, thus contributing to the maintenance of the global carbon cycle. Thien and Myers (1992) indicated that by increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced. The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds (Asea et al 1988; Nahas et al 1990; Bojinova et al 1997) has opened the possibility of inducing microbial solubilization of phosphates in the soil. Among the phosphate solubilizing microorganisms, fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas 1996). Considering the importance of fungi as phosphate solubilizers, we screened phosphate solubilizing fungi from soils of agricultural fields, forests and river basins of north Bengal and explored the possibility of exploiting them for plant growth improvement.

Materials and Methods

Isolation and identification of microorganisms. Soil samples were collected from three districts viz,

Darjeeling, Jalpaiguri and Cooch Behar of north Bengal. Soil samples were collected from forests at Sukna, Lohagarh, Cinchona, Mongpong and Gorumara; riverine soil from river basin of Balasan, Mahananda, Dhorola, Torsa and Raidak; from agricultural land (paddy and bamboo); from rhizosphere of tea, rubber and mandarin (plantation crops) and *Cryptomeria.* Fungi from these soil samples were isolated using different techniques like soil plate method (Warcup 1950) and direct soil plating technique (Thomas et al 1965). Various types of media were used which include potato dextrose agar (PDA), PDA– rose Bengal, peptone dextrose agar, Elliott's agar , acid media, RM media, Czapek's Dox agar, Asthana Howker's media and Pikovskaya's agar (PVK).

Primary phosphorus solubilizing activity. Screening for primary phosphate solublizing activity of the isolates was carried out by allowing the fungi to grow in selective media, i.e., Pikovskaya's agar (Pikovskaya 1948) for 7 to 10 d at 25C. The appearance of a transparent halo zone around the fungal colony indicated the activity of phosphate solubilizing fungus (PSF). For further quantification of the phosphorous solubilizing activity, 70 fungal isolates obtained on the basis of primary screening on PVK agar medium, were grown in two sets of PVK liquid medium (yeast extract, 0.50 g/l, dextrose, 10 g/l, calcium phosphate/rock phosphate, 5 g/l, ammonium phosphate, 0.5 g/l, potassium chloride, 0.2 g/l, magnesium sulphate, 0.1 g/l, manganese sulphate, 0.0001g/l, ferrous sulphate, 0.0001 g/l, pH,·6.5) amended with 0.5% tricalcium phosphate (P=997 mg/l) and 0.5 % rock phosphate (P=500 mg/l) separately over a period of 10 d. The media were inoculated with spore suspension (5 % v/v) prepared from 7 d old culture grown on PDA slants and incubated at room temp for 4-10 d on a shaking incubator (28C), (100 rpm). The mycelia were harvested later 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. Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Kundsen and Beegle (1988). Amount of phosphate utilized or solubilized by the isolates were expressed as mg/l phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified PVK liquid medium. Initial and final pH was noted in each case.

Extraction of soil and root phosphate. Soil and root phosphate were extracted in Mehlich 1 extracting solution (0.025 N H_2SO_4, 0.05 N HCl) (Mehlich 1953). Soil sample (5 g) was air dried and suspended in 25 ml of the extracting solution to which of activated charcoal (0.01 g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No.2 filter paper. The filtrate was collected and analyzed for P content. In case of plant samples, oven dried plant material was crushed with extracting solution.

Mass multiplication and application of PSF. PSF isolates were grown separately in the PDA for sporulation for 4-5 d The harvested spore mass was suspended in sterile distilled water. For mass multiplication of the PSF, well decomposed FYM heaps were used. Spore suspension (100 ml) containing 10^6 spores/ml was used to inoculate FYM heaps (5 kg). The FYM was first moistened slightly to optimize the PSF growth and kept in polythene bags in shade for 10 d. The mixture was regularly raked every 3 d during the total of this 10 d incubation period. Soybean (*Glycine max*) cultivar JS-335 obtained from National Research Center for Soybean, Indore was selected for trial with nine selected phosphate solubilizers to assess their effect in glass house on growth, leaf area and number of nodules. Surface sterilized soybean seeds were sown in pots filled with the amended PSF. After an interval of every 20 d plant ht, leaf area, nodulation and phosphate content in the roots as well as soil was measured. For each treatment, 15 replicate plants were maintained. FYM without any amendments served as appropriate controls for all the treatments.

Results and Discussion

A total of 354 fungal isolates were obtained from soil samples collected from Darjeeling, Jalpaiguri and Cooch Behar districts of north Bengal. Soil samples from forest, riverine and agricultural land (rhizosphere of plantation and agricultural crops) yielded 117, 52 and 185 fungal isolates, respectively. Fungal isolates were screened for phosphate solubilizing activity in PVK medium; formation of halo zones around the fungal colony indicated positive results (Fig. 1 a-c). A total of 70 fungal isolates showed phosphate solubilizing activity (Table 1).

Table 1. Phosphate solubilizing Aspergilli isolates obtained from different soil types of north Bengal, India

Soil	Phosphate solubilizing fungi	Isolates
Forest	*Aspergillus niger*	17
	Aspergillus melleus	7
Riverine	*Aspergillus niger*	5
	Aspergillus nidulans	2
	Aspergillus melleus	4
Rhizosphere	*Aspergillus melleus*	17
	Aspergillus niger	5
	Aspergillus clavatus	9
	Aspergillus fumigatus	4

Figure 1. a-c = Screening of Aspergilli isolates for phosphate solubilizing activity in Pikovskaya's agar, formation of halo zone indicated phosphate solubilizing potential of isolates; d-g = growth of soybean plants in a green house; d = in unamended soil; e = in soil amended with *A.niger*; f = in soil amended with *A.melleus*; g = in soil amended with *A. clavatus*

Isolate of *A.niger* (RS/P 14) showed a max (852 mg/l) while isolate of *A.melleus* (FS/S 262) showed min (795 mg/l) of phosphorous solubilization when medium was supplemented with tricalcium phosphate. When the medium was supplemented with rock phosphate, isolate of *A.melleus* (FS/L 13) showed a max (381 mg/l) and isolate of *A. niger* (FS/S 64) showed min (211 mg/l) phosphorous solubilization. (Table 2). In a similar study, it was reported that *Aspergillus* and *Penicillium* isolated from agricultural soil showed a max level of phosphate solubilization activity *in vitro* when liquid medium was supplemented with both tricalcium phosphate and rock phosphate separately (Pradhan and Sukla 2005). Acid production and drop in the pH of the medium have been reported in earlier studies (Alla 1994; Whitelaw 2000). Though the avg drop in pH was from 7 to 3.5 in our study, no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium.

Table 2 Evaluation of phosphphorus solubilization by Aspergilli isolates obtained from forest soil, riverine soil and rhizosphere soil of agricultural crops in liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP)

Aspergillus sp.	Isolates	TCP (mg/l)	RP (mg/l)	*Aspergillus* sp.	Isolates	TCP (mg/l)	RP (mg/l)
Forest soil				**Rhizosphere soil**			
A. niger	FS/L04	856	366	*A. niger*	RHS/R-12	810	385
	FS/L-13	817	381		RHS/P-37	807	345
	FS/L-17	820	379		RHS/P-51	849	374
	FS/L-18	821	376		RHS/P-105	807	349
	FS/L-40	847	370		RHS/P-106	813	344
	FS/C-140	824	344		RHS/P-107	807	355
	FS/C143	821	345		RHS/P-45	842	287
	FS/C-160	824	346		RHS/P-48	841	342
	FS/S-165	830	352		RHS/P-117	837	360
	FS/S-173	802	343		RHS/D-280	806	336
	FS/S-177	843	341		RHS/D-281	807	362
	FS/S-108	808	350		RHS/D-282	817	355
	FS/S-109	802	355		RHS/D-283	816	334
	FS/S110	842	367		RHS/D-284	840	375
	FS/S-112	842	354		RHS/D-285	839	370
	FS/S-113	848	360		RHS/D-286	804	336
	FS/S-262	795	360		RHS/D-287	807	340
A. melleus	FS/L-42	830	360	*A. melleus*	RHS/P-82	838	350
	FS/G-226	847	352		RHS/P-198	841	346
	FS/S-64	842	211		RHS/P-200	838	345
	FS/L-41	843	214		RHS/P-201	836	342
	FS/S -63	839	332		RHS/P-202	829	350
	FS/S-24	810	338	*A. fumigatus*	RHS/P-205	842	340
	FS/S-278	829	339		RHS/P-209	827	331
Riverine soil					RHS/B-220	837	344
A. niger	RS/P05	854	370		RHS/P-114	838	335
	RS/P/14	852	360	*A. clavatus*	RHS/P-38	799	288
	RS/D-288	830	350		RHS/P-114	829	340
	RS/T-57	809	352		RHS/T-99	832	341
	RS/T-58	802	354		RHS/T-190	825	350
A. nidulans	RS/T-59	830	350		RHS/T-191	827	351
	RS/P -60	840	340		RHS/P -50	850	342
A. melleus	RS/R-115	836	338		RHS/P -54	839	350
	RS/T-182	810	309		RHS/P-43	812	350
	RS/T-183	850	317		RHS/P-47	811	348
	RS/P -61	847	343				

PSF = Phosphate solubilizing fungi; TCP = tricalcium phosphate (P = 997 mg/l); RP = rockphosphate (P=500 mg/l)

Table 3. Effect of amendment of soil with phosphphorus solubilizing Aspergilli isolates on growth and nodulation of soybean plant

Soil amended with	Isolates	Leaf area (cm2)	Nodules /plant
A.niger			
	FS/L 04	41.4 ± 1.42	21±1.42
	RS/P-14	38.0±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

Values are mean of 15 replicate plants following 80 days of each treatment; difference between untreated control and treated plants significant at $P=0.01$ in *t-test*

A greater part of soil phosphorous (95-99%) is present in the form of insoluble phosphates and cannot be utilized by the plants (Vassileva et al 2001). However, many soil fungi and bacteria are known to solubilize these inorganic phosphates (Illmer and Schinner 1992). In the present study, of the 70 PSF isolates, nine were further selected as potential phosphate solubilizers on the basis of the earlier data obtained from the quantitative estimation of all the isolates in the liquid medium, which included three of *Aspergillus niger*, five of *A.melleus* and one isolate of *A.clavatus*. Finally after two months of treatment of soybean with PSF isolates, differences were observed in leaf areas, number of root nodules counted in uprooted plants. 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. However, isolates of *A. melleus* also showed an increase in leaf area (Fig.1 d-g). Differences in both leaf area and nodulation in all treatments were significantly greater than that in control (Table 3).

Leaf area index of plants are known to be affected by the availability of nutrients in soil. Increased content of soluble phosphate in soil due to the activity of PSF increases the leaf area of these plants (Yinsuo et al 2004). Nodulation of leguminous plants is directly affected by the availability of the nutrients in the soil (Zaidi and Khan 2006). Increased content of soluble phosphate in soil due to the activity of PSF accelerates the rate of nodulation on these plants. Results of the present study also indicated more nodules in plants treated with the three isolates of *A.niger* (Table 3). Increased nodulation in the roots of these plants could be taken as an indication of the role of PSF in improving the plant health status of the soybean plants.

All the tested PSF isolates increased growth in relation to control of which three isolates of *A. niger* were most effective (Fig. 2).

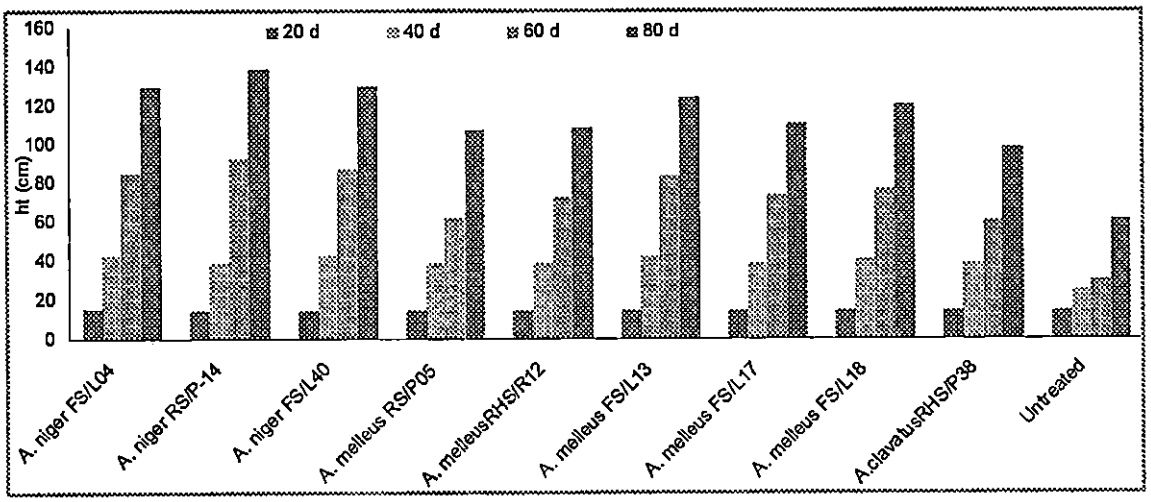

Figure 2. Effect of amendment of soil with phosphphorus solubilizing Aspergilli isolates on growth of soybean plants

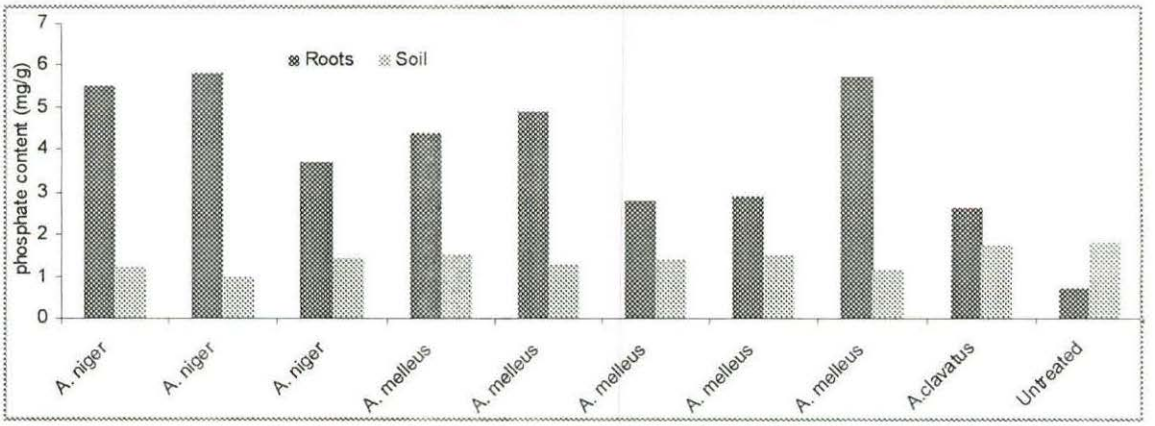

Figure 3. Phosphate content in roots and soil of the soybean plants after 30 days of amendment with phosphphorus solubilizing Aspergilli isolates

Phosphate level in the roots was found to be more in those plants treated with the amendments using isolates of *A. niger* (Fig. 3). In a study carried out to determine the role of dual application of vesicular arbuscular mycorrhiza and PSF on the growth of tea seedlings, it was found that when both were inoculated in soil mixed with rock phosphate a significant increase in growth in terms of plant ht, leaves and dry wt of shoot and root were noted (Bora et al 2003). In another study it was reported that *A.niger* was one of the most efficient phosphatase producing fungi among many PSFs screened for efficiency It was also observed that the reduction of the pH of the medium was max with *A.niger* isolate which efficiently hydrolyzed different compounds (mono- and hexa-) of organic phosphorous (Tarafdar et al 2003).

In conclusion it can be stated that among the various PSF isolates obtained from soil, most efficient phosphorus solubilizers are species of *Aspergillus*. The strains with max phosphate solubilizing activities also showed good plant growth promoting activity. Such microorganisms could be used in the field as efficient biofertilizers.

Acknowledgement

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Evaluation of Phosphate Solubilizers from Soils of North Bengal and Their Diversity Analysis

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Abstract: Four hundred isolates obtained from soil samples collected from forest, river basin, agricultural fields and rhizosphere of plantation crops of North Bengal were screened for phosphate solubilizing activity on Pikovskaya's agar medium. Among the screened isolates, ninety showed phosphate solubilizing activity. Out of these, ten isolates belonging to *Aspergillus niger, A. melleus* and *A. clavatus* were selected for further studies. *In vitro* evaluation of phosphate solubilization by the different isolates using tricalcium phosphate (TCP) and rock phosphate (RP) revealed that the isolates could solubilize TCP better than RP. Selected isolates were mass multiplied using farm-yard manure (FYM) and were tested *in* vivo for their growth promoting activity in soybean. While all the isolates promoted growth, *A. niger* RSP-14 was found to be most effective. While the soil P content decreased due to the activity of the PSFs, root phosphate content showed an increase. These isolates were further analyzed for genetic variability. Genomic DNA from the fungal isolates were obtained and purified. UPGMA cluster analysis divided the ten isolates into two groups with the genetic similarity ranging from 0.35- 0.61. One group consisted of four isolates of *A. niger* and five isolates of *A. melleus*, while the other group had one isolate of *A. clavatus*.

Key words: Phosphate solubilizing fungi • *Aspergillus niger* • *A. melleus* • *A. clavatus*

INTRODUCTION

Fungi are important components of soil microbiota, typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions [1]. A wide range of soil fungi are reported to solubilize insoluble phosphorous. Strains of *Aspergillus niger* and *Penicillium* are the most common fungi capable of phosphate solubilization. Many bacterial, fungal, yeast and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied [2-5]. However, studies on genetic diversity of these important group of phosphate solubilizing fungi (PSF) are limited. Williams, *et al.* [6] and Welsh and McClelland [7] demonstrated the utility of single short oligonucleotide primers of arbitrary sequence for the amplification of DNA segments distributed randomly throughout the genome. Also Welsh and McClelland showed that the pattern of amplified bonds could be used for genome fingerprinting and Williams, *et al.* [6] showed that the differences (polymorphisms) in the pattern of bands amplified from genetically distinct individuals behaved as mendelian genetic markers named Random Amplified Polymorphic DNA (RAPDs). Most of the published studies on genetic characterization, detection of genetic variations and gene mutations were concentrated on the variations in chromosomes, isozyme polymorphism and biochemical diversity. A single set of arbitrary-sequence 10 mers may be used for fingerprinting any species. The many advantages of RAPD markers over RFLDs or isozyme markers accelerated the adoption of RAPD technology for the construction of genetic maps, fingerprinting and population genetic studies. Current reviews of the applications of RAPD technology are available. The utility of DNA markers as RAPD-DNA in detecting genetic variability among many phytopathogenic fungi have been recorded by various authors [8-10].

The present study was undertaken to test selected isolates of PSFs for their *in vitro* and *in vivo* activities and screen the random primers as effective molecular markers for genetic variability analysis among the isolates.

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MATERIALS AND METHODS

Isolation of Microorganisms: Soil samples were collected from three districts (Darjeeling, Jalpaiguri and Cooch Behar) of North Bengal. Source of soil samples includes forests (Sukhna, Lohagarh, Cinchona, Mongpong, Gorumara), river basin (Balasan, Mahananda, Dhorola, Torsa, Raidak) agricultural field (paddy, bamboo); rhizosphere of tea, rubber, mandarin (plantation crops) and *Cryptomeria*. Fungi from these soil samples were isolated using different techniques like Warcup's soil plate method [11]. Different types of media were used which included Potato Dextrose Agar (PDA), Potato Dextrose Agar-Rose Bengal (PDA- rose Bengal), Peptone dextrose agar, Elliott's Agar, Acid media, Richard's agar, Czapek's Dox agar, Asthana Howker's, Pikovskaya's agar (for screening phosphate solublizing activity).

Screening for Phosphorus Solubilizing Activity: Screening for primary phosphate solublizing activity of the isolates was carried out by allowing the fungi to grow in selective media, i.e., Pikovskaya's agar [12] for 7 to 10 days at 25°C. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solublizing activity of the fungus.

Evaluation of Phosphate Solubilizing Activity of Selected Isolates: Fungal isolates were grown in two sets of Pikovskaya's liquid medium (yeast extract, 0.50 g/L, dextrose, 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 separately over a period of 10 days. Fifty ml of the liquid medium was inoculated with 5 % v/v of the spore suspension prepared from 7 day old cultures grown on PDA slants and incubated at room temperature for 4-10 days with routine shaking at 100 rpm at 28°C in a rotary incubator. The initial pH of the medium was recorded. 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 rpm for 5 min. Quantitative estimation of phosphate was done following ammonium molybdate ascorbic acid method as described [13]. 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 Pikovskaya's liquid medium.

Mass Multiplication of PSF Isolates and Their Trial for Improvement of Plant Health: 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. For mass multiplication of the PSF, well decomposed FYM heaps were used. Spore suspension (100 ml) containing 106 spores / ml was used to inoculate 5 kg of FYM heaps. 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. *Glycine max* was selected for trial with ten selected Phosphate solubilizers to assess their effect on growth in glass house conditions. Surface sterilized soybean seeds were sown in pots filled with the amended PSF. After germination and a few days of growth the physical parameters of the plants were monitored and the observations were noted after specific intervals.

Isolation of Genomic DNA: Fungi 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°C 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 (25:24:1) at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and then the DNA was precipitated with chilled ethanol (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.

Purification of DNA: Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60 µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol as described earlier.

PCR Amplification: Four random decamer primers [OPB-2, OPB-3, OPB-6 and OPD-5] were used for RAPD analysis. 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

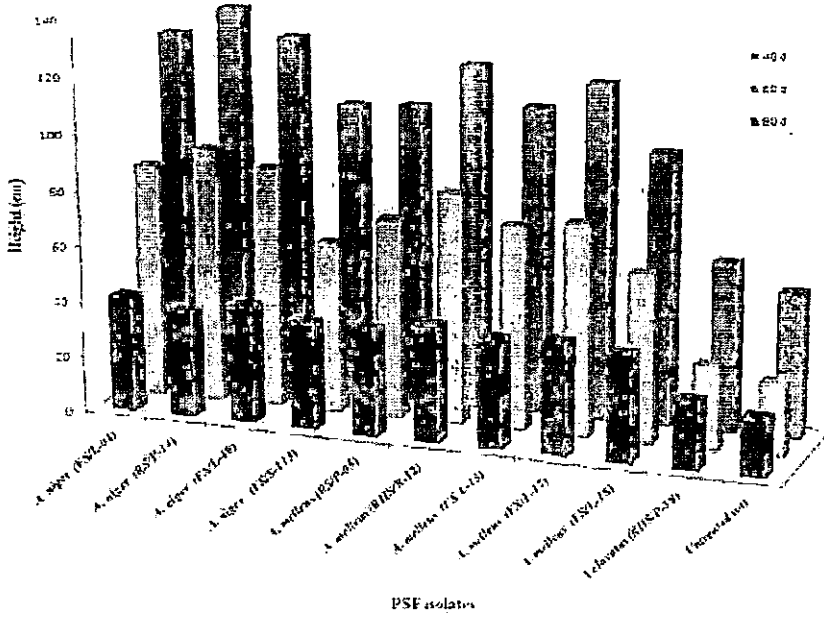

Fig. 1: Effect of amendment of soil with phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* on growth of soybean plants

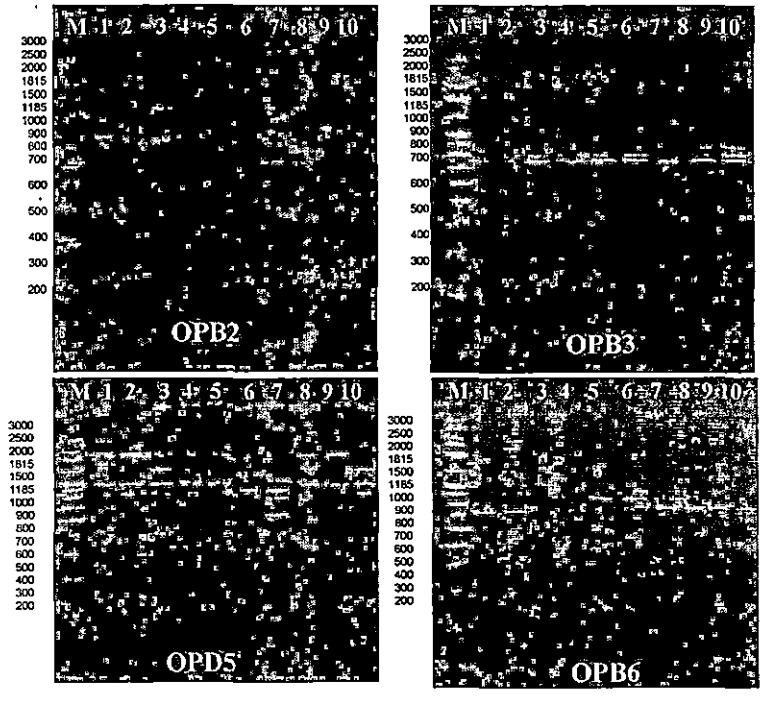

Fig. 2: RAPD amplified products of phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* using four random primers

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)

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.

Gel Electrophoresis: PCR product (20 µl) was mixed with loading buffer (8 µ1) 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.

Analysis of the Obtained Data: 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 absence and presence of RAPD - markers). UPGMA cluster analysis was carried following similarity coefficient matrix of reproducible bands using PC software NTSYS.

RESULTS AND DISCUSSION

Out of isolated fungi from the soil a total of 90 fungal isolates showed phosphate solubilizing activities as detected in Pikovskaya's agar medium by the appearance of halos around the inoculum on the medium. Ten isolates which showed maximum phosphate solubilizing activities in PVK agar medium were further tested for their activities in liquid medium using two types of inorganic phosphates, tricalcium and rock phosphate. *A. niger* (isolate RS/P-14) showed maximum solubilization of phosphorous (856 mg/l) whereas *A. clavatus* (isolate RHS/P-38) showed minimum of (799 mg/l) of phosphorous solubilization when the media were supplemented with tricalcium phosphate. When the medium was supplemented with rock phosphate, *A. melleus* (isolate RHS/R-12) showed maximum of 385 mg /L phosphorous solubilization and *A. clavatus* (isolate RHS/P-38) showed minimum of 288 mg/L phosphorous solubilization (Table 1) All the tested isolates increased growth in relation to control of which three isolates of *A. niger* were most effective (Figure 1). Phosphate level in the roots was found to be more in those plants treated with the amendments using isolates of *A. niger* (Table 2).

Table 1: Evaluation of phosphorus solubilization by fungal isolates in Pikovskaya's liquid medium amended with Tricalcium phosphate and Rock Phosphate

Organisms	Isolate nos.	P-solubilized (mg/L)	TCP* RP**
Aspergillus niger	FS/L-04	856	366
Aspergillus niger	RS/P-14	852	360
Aspergillus niger	FS/L-40	847	370
Aspergillus niger	FS/S-113	848	360
Aspergillus melleus	RS/P-05	854	370
Aspergillus melleus	RHS/R-12	810	385
Aspergillus melleus	FS/L-13	817	381
Aspergillus melleus	FS/L-17	820	379
Aspergillus melleus	FS/L-18	821	376
Aspergillus clavatus	RHS/P-38	799	288

*Initial amount of tricalcium phosphate 997 µg/ml

**Initial amount of rock phosphate 500 µg/ml

Table 2: Phosphate content in root and soil of soybean plants after 20 days of inoculation

	P content (µg/g)	
Soil amended with	Root	Soil
A. niger (FS/L-04)	5.5	1.22
A. niger (RS/P-14)	5.8	0.99
A. niger (FS/L-40)	5.4	1.62
A. niger (FS/S-113)	3.7	1.44
A. melleus (RS/P-05)	4.4	1.54
A. melleus (RHS/R-12)	4.9	1.3
A. melleus (FS/L-13)	2.8	1.4
A. melleus (FS/L-17)	2.9	1.51
A. melleus (FS/L-18)	5.7	1.15
A.clavatus (RHS/P-38)	2.6	1.72
Unamended soil	0.7	1.79

In a similar study it was reported that isolates of *Aspergillus* and *Penicillium* 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 [14]. DNA samples preparation before RAPD-PCR amplification was found crucial for fingerprint. 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 was 1.8 and DNA quantity was evaluated by 0.8% agarose gel electrophoresis. 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

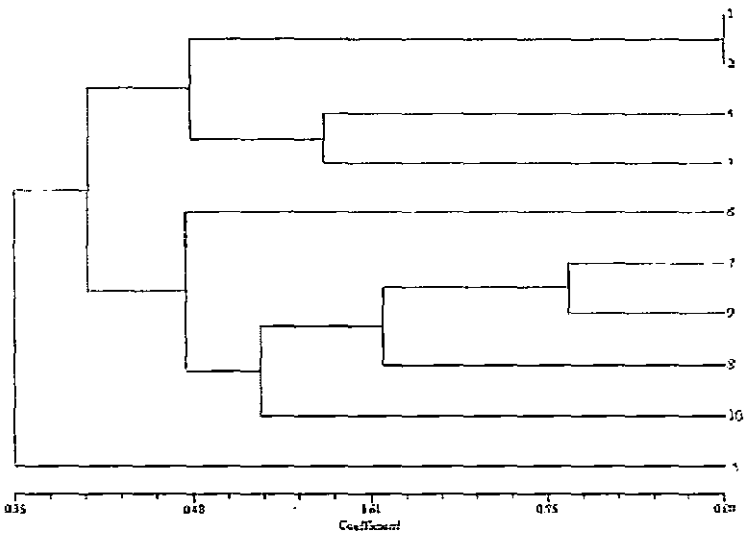

Fig. 3: Dendrogram showing the genetic relationships among 10 Phosphate solubilizing fungal isolates based on RAPD analysis [1: *A. niger* (FS/L-04), 2: *A. niger* (RS/P-14), 3: *A. niger* (FS/L-40), 4: *A. niger* (FS/S-113) , 5: *A. melleus* (RS/P-05), 6: *A. melleus* (RHS/R-12), 7: *A. melleus* (FS/L-13), 8: *A. melleus* (FS/L-17), 9: *A. melleus* (FS/L-18), 10: *A.clavatus* (RHS/P-38)

denaturation time and temperature. 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, OPB-2, OPB-3 and OPB-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. A total of 127 polymorphic bands were obtained with an average of 31.75 bands/ primer (Figure 2). 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%). During the past few years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic diversity among species and within fungi populations and plant populations [15-18]. In studies conducted with the toxin producing *Aspergillus* strains, it was found that *A. flavus* and *A. parasiticus* did not show any relationship between RAPD-based band profile and toxin production [19]. Results of the present study suggest that these random RAPD markers can be used for identification of phosphate solubilizers. The variation obtained in terms of their genetic make-up gives an idea of improving the marker based selection of this beneficial group of organisms.

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Molecular Characterization of *Trichoderma viride* and *Trichoderma harzianum* Isolated from Soils of North Bengal Based on rDNA Markers and Analysis of Their PCR-RAPD Profiles

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Abstract: Nineteen isolates of *Trichoderma viride* and *Trichoderma harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural fields of North Bengal region were studied using RAPD and ITS-PCR. The genetic relatedness among eleven isolates of *T. viride* and eight isolates of *T. harzianum were* analyzed with six random primers. RAPD profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.67 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates. This result indicated the identification patterns of *Trichoderma* isolates.

Key words: *Trichoderma viride* • *Trichoderma harzianum* • rDNAmarkers • RAPD

INTRODUCTION

Soil microorganisms influence ecosystems by contributing to plant nutrition [1], plant health [2], soil structure [3] and soil fertility [4]. It has been widely recognized, particularly in the last two decades, that majority of harsh environments are inhabited by surprisingly diverse microbial communities. Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms. An estimated 1,500,000 species of fungi exist in the world [5].

Trichoderma, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens [6]. Antagonistic microorganisms, such as *Trichoderma,* reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition, the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil. Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media. However, this method does not distinguish between indigenous strains and artificially introduced ones [7]. The *Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores [8]. These can also be distinguished by randomly amplified polymorphic DNA (RAPD)-PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA [9-13]. Molecular characterization of the potential biocontrol agents using Random Amplified Ploymorphic DNA (RAPD) and Internal Transcribe Spacer-Polymerase Chain Reaction (ITS-PCR), helps to determine the diversity and identification. In the present study, genetic variability in nineteen isolates of *Trichoderma* spp. from different ecosystems were evaluated with six different RAPD markers.

MATERIALS AND METHODS

Isolation and Identification of *Trichoderma:* Soil samples were collected forests, agricultural field and rhizosphere of plantation crops of three districts of North Bengal. The location of soil samples were recorded through GIS mapping tool (Garmin). *Trichoderma* species were isolated in specific selective medium. TSMC which contained (gm/lit); $MgSO_4.7H_2O$-0.2; K_2HPO_4-0.9;

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Table 1: The nucleotide sequence used for ITS and RAPD PCR

PrinerName	Sequence(5'-3')	Mer	TM	% GC
ITS-Primers pairs				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%
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%

Kcl-0.15; NH_4NO_3-1.0; glucose-3.0, chloramphenicol-0.25, fenaminosulf-0.3, pentachloronitrobenzene-0.2, rose Bengal-0.15, captan-0.02 (post autoclaving), agar-20 as well as in modified TSM (Smith *et al.*, 1990): containing (gm/lit): $Ca(NO_3)_2$-1.0, KNO_3.0.26, $MgSO_4$,$7H_2O$-0.26, KH_2PO_4-0.12, $CaCl_2$, $2H_2O$-1.0, citric acid-0.05, sucrose-2.0, agar-20.0, chlortetracycline-0.05, captan (50% wettable powder)-0.04 [14]. The identification of *Trichoderma* isolates were confirmed by National Center of Fungal Taxonomy (NCFT), New Delhi.

Genomic DNA Extraction from *Trichoderma* Isolates: 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°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water saturated phenol a nd 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).

Qualitative and Quantitative Estimation of DNA: The extraction of total genomic DNA from the *Trichodrma* isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended 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.

Pcr Amplification of its Region of *Trichoderma* Isolates: 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.

RAPD of *Trichoderma* Isolates: For RAPD, six random primers i.e. OPA-1; OPD-6; OPA-4; A-5; AA-04 and AA-11 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 µ1) 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) [15]. 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.

RESULTS AND DISCUSSION

Nineteen isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil, forest soil and agricultural field (Table 2). Among them eleven isolates were identified as *Trichoderma viride* and eight isolates as *Trichodema harzianum.*

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [16]. They also occur in multiple copies with up to 200 copies per haploid genome [17-18] arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [19]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies, In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* spp. 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 (Fig 1). The results are in accordance with Mukherjee [20] who studied the identification and genetic variability of the *Trichoderma* isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in *Trichoderma* [21-24]. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates.

Table: 2 Isolates of *Trichoderma* spp

				GPS location	
Isolates (Code)	Accession No	Type of soil	Source	Latitude	Longitude
Trichoderma viride (FS/L-20)	NCFT2580	A	Lohagarh forest	N26° 45'13.08"	E88°23'28.72
Trichoderma viride (FS/C-90)	NCFT2581	A	Cinchona forest	N26° 47'49.16"	E88° 21.27.75"
Trichoderma viride (FS/S-455)	NCFT2583	A	Sukna forest	N26° 45'11.75"	E88° 23'28.27"
Trichoderma virid e (FS/S-458)	NCFT2584	A	Sukna forest	N 26° 48'18.68"	E88° 21.14.61"
Trichoderma viride (RHS/T-460)	NCFT2585	B	Tea Rhizosphere	N26° 45.11.75"	E88° 23'28.27"
Trichoderma viride (RHS/T-463)	NCFT2586	B	Tea Rhizosphere	N 26° 48'18.68"	E88° 21.14.61
Trichoderma viride (RHS/T-472)	NCFT2588	B	Tea Rhizosphere	N 26° 45'11.75"	E 88° 23'28.27"
Trichoderma viride (FS/S-473)	NCFT2589	A	Sukna forest	N 26° 48'18.68"	E88° 21'14.61"
Trichoderma viride (FS/S-474)	NCFT2590	A	Sukna forest	N 26° 48'18.68"	E88° 21'14.61"
Trichoderma viride (FS/S-475)	NCFT2591	A	Sukna forest	N 26° 48'18.64"	E88° 21'14.61"
Trichoderma viride (FS/S-478)	NCFT2594	A	Sukna forest	N 26° 48'18.68	E88° 21'14.61
Trichoderma harzianum (Ag/S476)	NCFT2592	C	Potato field	N 25° 01'13.13"	E88° 08'19.98"
Trichoderma harzianum (FS/S-477)	NCFT2593	A	Sukna forest	N 26° 48'18.68	E88° 21'14.61
Trichoderma harzianum (Ag/S471)	NCFT2587	C	Rice field	N 25° 01'13.13"	E88° 08'19.98"
Trichoderma harzianum (Ag/S479)	NCFT2595	C	*Brassica* field	N 25° 01'13.13"	E88° 08'19.98"
Trichoderma harzianum (RHS/AC480)	NCFT2596	B	*Accacia* Rhizosphere	N26° 42'42.56"	E 88.° 21'.15.47"
Trichoderma harzianum (RHS/AC481)	NCFT2597	B	*Accacia* Rhizosphere	N26° 42'42.56"	E 88.° 21'.15.47"
Trichoderma harzianum (RHS/AC482)	NCFT2598	B	*Accacia* Rhizosphere	N26° 42'42.56"	E 88.° 21'.15.47"
Trichoderma harzianum (RHS/AC483)	NCFT2599	B	*Accacia* Rhizosphere	N26° 42'42.56"	E 88.° 21'.15.47"

NCFT-National Center of Fungal Taxonomy

A-Forest soil; B-Rhizosphere soil; C-Agricultural soil

Table 3: Analysis of the polymorphism obtained with RAPD markers in 19 *Trichoderma* isolates

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp). Min	Max.	Monomorphic band	Polymorphic bands	Polymorphic (%)
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 4: 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	40.90	0.90	1.00																
4	05.81	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

T.viride (1-11) and *T.harzianum* (12-19) 1-NCFT2580, 2-NCFT2581, 3-NCFT2583, 4-NCFT2584, 5-NCFT2585, 6-NCFT2586, 7-NCFT2588, 8-NCFT-2589, 9-NCFT2590, 10-NCFT2591, 11-NCFT2594, 12-NCFT2592, 13-NCFT2593, 14-NCFT2587, 15-NCFT2595, 16-NCFT2596, 17-NCFT2597, 18-NCFT2598, 19-NCFT2599)

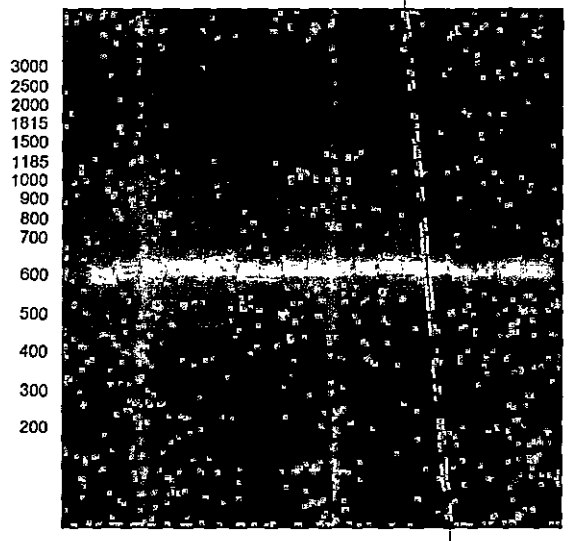
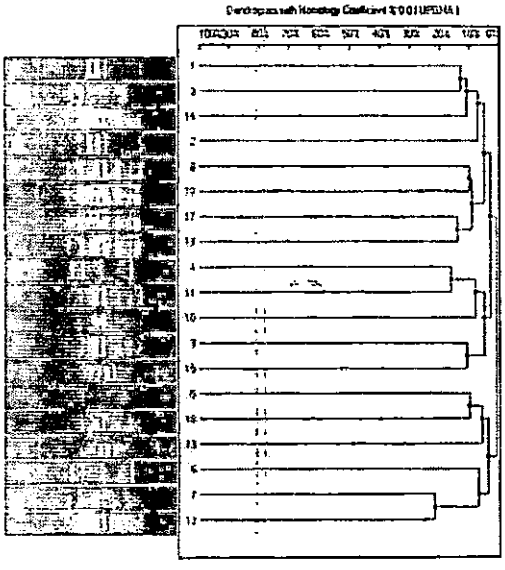
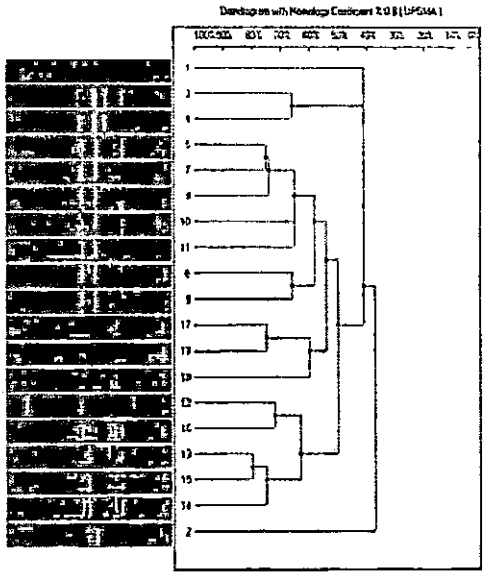


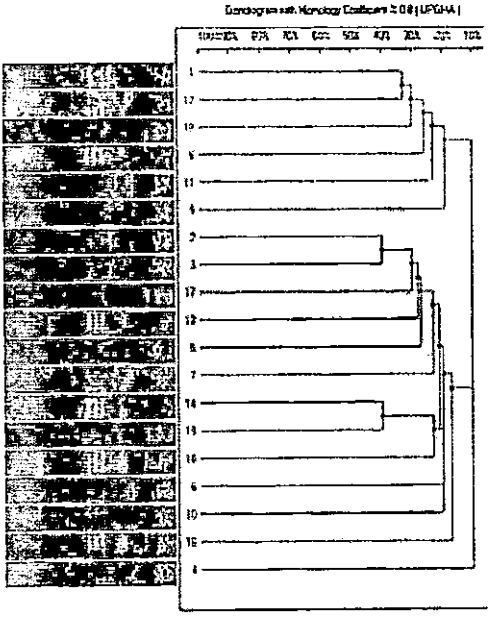
Fig. 1: PCR amplification of ITS region of *Trichoderma* isolates. *T. viride* (Lane 1-11) and *T. harzianum* (Lane12-19) Lane M: Low range DNA Marker, Lane 1-NCFT2580, Lane2-NCFT2581, Lane3-NCFT2583, Lane4-NCFT2584, Lane5-NCFT2585, Lane6-NCFT2586, Lane7-NCFT2588, Lane8-NCFT-2589, Lane9-NCFT2590, Lane10-NCFT2591, Lane11-NCFT2594, Lane12-NCFT2592, Lane13-NCFT2593, Lane14-NCFT2587, Lane15-NCFT2595, Lane16-NCFT2596, Lane17-NCFT2597, Lane18-NCFT2598, Lan19-NCFT2599



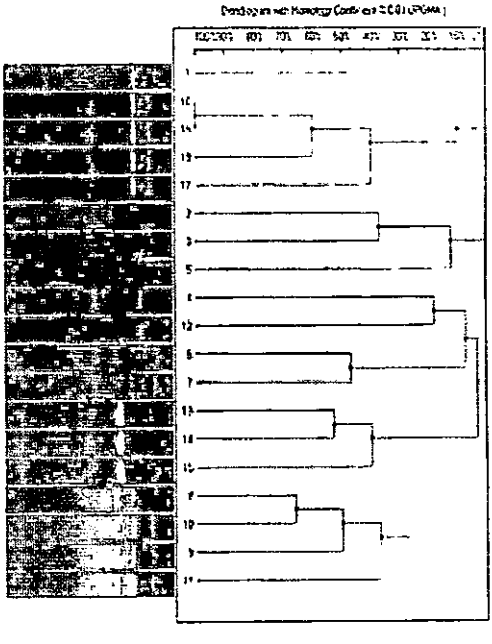
A-04



AA-11



OPD6



A5

Fig. 2: RAPD analysis of isolates of *T.viride* (1-11) and *T.harzianum* (12-19) using BioProfil 1D software with primers (A-04, AA-11, OPD6 and A-5) 1-NCFT2580, 2-NCFT2581, 3-NCFT2583, 4-NCFT2584, 5-NCFT2585, 6-NCFT2586, 7-NCFT2588, 8-NCFT-2589, 9-NCFT2590, 10-NCFT2591, 11-NCFT2594, 12-NCFT2592, 13-NCFT2593, 14-NCFT2587, 15-NCFT2595, 16-NCFT2596, 17-NCFT2597, 18-NCFT2598, 19-NCFT2599).

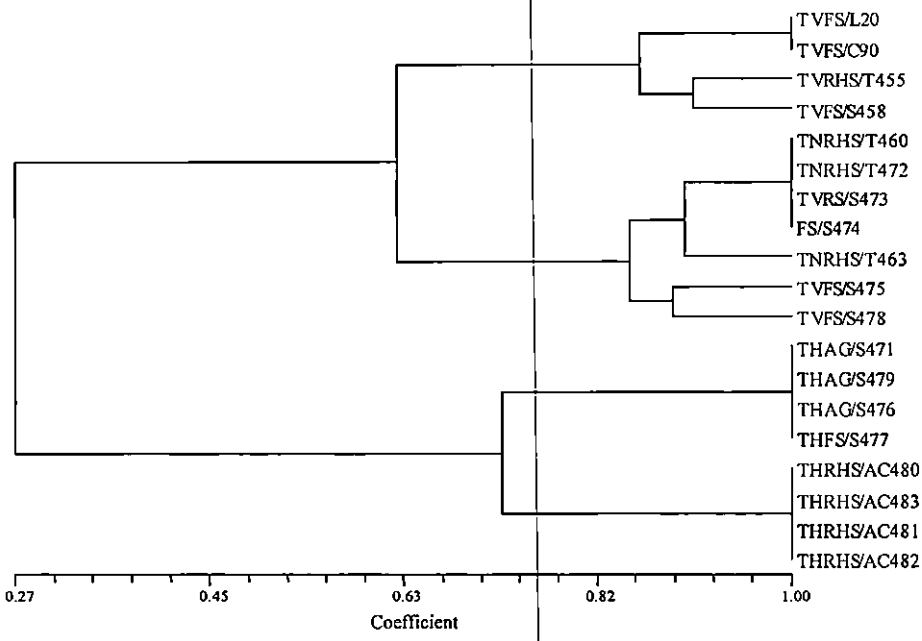


Fig. 3: Dendrogram showing the genetic relationships among 19 *Trichoderma* fungal isolates based on RAPD analysis

The genetic relatedness among eleven isolates of *Trichoderma viride* and eight isolates of *Trichodema harzianum* 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 genetic 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 3). 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 4). The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using BIO Profil 1D image software for each primer (Fig 2) and NTSYSpc software (Fig 3). 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 sub grouped into two. First subgroup with four isolates and second one is with seven isolates of two sub clusters. The cluster of *T. harzianum* divided into two different cluster contains four different isolates.

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