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. raistricki*) 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 (662ˉg P mlˉ1) 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 l, 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 l8 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 *Trichoderna* 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 viren*s 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* worldwide 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.