CHAPTER 2
LITERATURE REVIEW

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

Rhizosphere Microflora

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

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

Agriculturally Important Microorganisms (AIMs)

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

(i) Microorganisms with direct effects on plant growth promotion [plant growth promoting microorganisms (PGPM)] and

(ii) Biological control agents (BCA) that indirectly assist with plant productivity through the control of plant pathogens.

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

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

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

Phosphate solubilizing microorganisms (PSMs)

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

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

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

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

Mechanisms of Phosphorus Solubilization

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

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

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

Phosphorus mobilization by soil microorganisms

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

Solubilization of Ca-bound P

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

agronomic levels of plant growth (Goldstein, 2000). Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. Their solubility increases with a decrease of soil pH. Phosphate solubilization is the result of combined effect of pH decrease and organic acids production (Fankem *et al.*, 2006). Microorganisms through secretion of different types of organic acids e.g. carboxylic acid and rhizospheric pH lowering mechanisms dissociate the bound forms of phosphate like Ca3(PO4)2. Nevertheless, buffering capacity of the medium reduce the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha, 2009). Acidification of the microbial cell surroundings releases P from apatite by proton substitution / excretion of H+ (accompanying greater absorption of cations than anions) or release of Ca2+ (Goldstein, 1994; Illmer and Schinner1995; Villegas and Fortin 2002). While, the reverse occurs when uptake of anions exceeds that of cations, with excretion of OH^- / HCO3 exceeding that of H+ (Tang and Rengel, 2003). Carboxylic anions produced by PSB, have high affinity to calcium, solubilize more phosphorus than acidification alone (Staunton and Leprince 1996). Complexing of cations is an important mechanism in P solubilization if the organic acid structure favors complexation (Fox *et al.*, 1990). It is controlled by nutritional, physiological and growth conditions of the microbial culture (Reyes *et al.*, 2007), but it is mostly due to the lowering of pH alone by organic acids or production of microbial metabolites (Abd-Alla, 1994). Organic anions and associated protons are effective in solubilizing precipitated forms of soil P (e.g. Fe - and Al - P in acid soils, Ca - P in alkaline soils), chelating metal ions that may be associated with complexed forms of P or may facilitate the release of adsorbed P through ligand exchange reactions (Jones, 1998). Calcium phosphate (Ca-P) release results from the combined effects of pH decrease and carboxylic acids synthesis, but proton release cannot be the single mechanism (Deubel *et al.*, 2000).

Solubilization of Al- / Fe-bound P

Solubilization of Fe and Al occurs via proton release by PSB by decreasing the negative charge of adsorbing surfaces to facilitate the sorption of negatively charged P ions. Proton release can also decrease P sorption upon acidification which increases $H2PO4^-$ in relation to HPO_4^{-2} having higher affinity to reactive soil surfaces (Whitelaw, 2000). Carboxylic acids mainly solubilize Al-P and Fe-P (Henri *et al.*,

2008; Khan *et al.,* 2007) through direct dissolution of mineral phosphate as a result of anion exchange of PO43- by acid anion, or by chelation of both Fe and Al ions associated with phosphate (Omar, 1998). It is through root colonizing pseudomonads with high-affinity iron uptake system based on the release of Fe3+- chelating molecules i.e. siderophores (Altomare, 1999). Moreover, carboxylic anions replace phosphate from sorption complexes by ligand exchange (Otani *et al.*, 1996; Whitelaw, 2000) and chelate both Fe and Al ions associated with phosphate, releasing phosphate available for plant uptake after transformation. Ability of organic acids to chelate metal cations is greatly influenced by its molecular structure, particularly by the number of carboxyl and hydroxyl groups. Type and position of the ligand in addition to acid strength determine its effectiveness in the solubilization process (Kpomblekou and Tabatabai, 1994). Phosphorus desorption potential of differentcarboxylic anions lowers with decrease in stability constants of Fe - or Al - organic acid complexes (log *K*Al or log *K*Fe) in the order: citrate > oxalate > malonate / malate > tartrate >lactate > gluconate > acetate > formiate (Ryan *et al.* 2001).

Mineralization of organic P

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

Effect of PSMs on Crop Production

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

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

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

Biological control agents (BCA)

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

Mechanisms of biological control

Direct antagonism

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Induced Systemic Resistance (ISR)

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

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

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

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

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

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

Plant immune responses triggered by beneficial microbes

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

Resistance-inducing traits of beneficial microbes

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

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

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

Induced defense signaling pathway

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

degree of coordination and a continuous molecular dialog between the plant and the beneficial organism. The local and systemic defense responses that are triggered by beneficial and parasitic microorganisms are controlled by a signaling network in which the plant hormones SA, jasmonic acid (JA), and ethylene (ET) play important roles (Glazebrook 2007). There is ample evidence that SA, JA, and ET pathways crosscommunicate, allowing the plant to finely tune its defense response depending on the invader encountered (Koornneef and Pieterse 2008). Well-studied examples of systemically induced resistance are SAR, which is triggered upon infection by necrosis-inducing pathogens and is dependent on SA signaling and ISR, which is triggered by beneficial rhizobacteria, such as *P. fluorescens* WCS417 and requires components of the JA and ET signaling pathway (Pieterse *et al*.,1998). Both pathogen induced SAR and *P. fluorescens* WCS417-triggered ISR are controlled by the transcriptional regulator NPR1 (Pieterse and Van Loon 2004). Because SAR is associated with NPR1-dependent PR gene expression and ISR is not, NPR1 must differentially regulate gene expression, depending on the signaling pathway that is activated upstream of it. Hence, the NPR1 protein is integrating and responding to different hormone-dependent defense pathways. Not only several other rhizobacterial strains but also some beneficial fungi have been shown to induce systemic resistance in a JA-dependent, ET-dependent, and/or NPR1-dependent manner (Ahn *et al*. 2007) while there are also some reports about dependency on SA signaling or requirement of both ISR and SAR c0mponents (Conn *et al*.,2008).

Local immune responses triggered by beneficial microbes

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

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

Multiple functions of soil microbes

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

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

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

Properties of Successful Microbial Inoculum

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

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

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

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

Rhizosphere colonization by AIMs

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

Factors Affecting Root Colonization and Efficacy of AIMs

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

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

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

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

Monitoring of Microbial Inoculants

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

Microbiological Monitoring Methods

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

Direct Monitoring

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

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

Molecular Monitoring Methods

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

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

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

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

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

Diversity analysis of AIMs

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

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

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

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

Analysis of amplified genes

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

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

Controlling the Soil Microflora for Optimum Crop Production and Protection

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

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

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

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

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

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

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

New Dimensions for Sustainable Agriculture

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

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

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