CHAPTER 1– Literature Review

1. 1. *Fusarium* and *Pythium* spp. as pathogens of sorghum

One of the major concerns to agricultural food production worldwide is diseases caused by phytopathogenic fungi of which *Fusarium* and *Pythium* attack most of the economically important crop plants (Gohel *et al.,* 2006). Sorghum (*Sorghum bicolour* (L) Moench) is ranked second among the five most important cereal crops in Eastern Africa (FAO, 1999). It is believed that sorghum was probably initially domesticated in central Africa in the region of Ethiopia and Sudan. From its initial cultivation in Africa, the crop was introduced into Asia, America and Australia (Forbes *et al*., 1986). Sorghum is an economically important crop providing food and fodder in the semi arid tropics of the World. In Ethiopia, it is widely grown in the Southern part of the country especially in the dry land areas with high temperature and low rainfall. The grain yield currently estimated in the continent is relatively lower than those in other parts of the World (FAO, 1999). Low sorghum yields are mainly due to insect pests and diseases caused by phytopathogenic fungi and bacteria. Most of the fungal pathogens reported on sorghum worldwide occur in Eastern Africa including Ethiopia (Huluka and Esele, 1992).

Several species of fungi are known to cause various types of diseases in sorghum. *Pythium* and *Fusarium* spp. are among the most common phytopathogenic fungi that cause seedling and root rot diseases in sorghum (Forbes *et al*., 1986; Horne and Frederickson, 2003). *Pythium* spp. survives in the soil as oospores and germinates in response to seed and root exudates in wet soil. They germinate either directly by producing germ tubes or indirectly by producing zoospores. The pathogens can then rapidly penetrate host cells and tissues that lack secondary wall thickenings (Forbes *et al.,* 1986). Evidence has been presented that *Pythium ultimum* Trow var. *ultimum* causes a chronic root and seed rot of grain sorghum and negatively affects grain yield in a continuous sorghum production system (Davis and Bockus, 2001). *Pythium graminicola* Subramanian is also reported to cause root rot in sorghum (Horne and Frederickson, 2003). *Pythium* root rot in sorghum is characterized by various symptoms including necrosis of seedling leaf tip and blade, collar rot root rot and streaking of the vascular system leading to the death of the plant (McLean and Lawrence, 2001).

Sorghum is also attacked by root and stalk diseases caused by a number of *Fusarium* spp. The primary inoculum of *Fusarium* spp. consists of conidia and mycelia that have over seasoned in crop debris and the propagules are not capable of surviving more than three months in the absence of

plant debris (Claffin, 1986). The fungi are widely distributed in host root tissues under field conditions and respond to stress in the plant by taking advantage of preferential growth conditions to incite diseases (Leslie, 1990). In one experiment for example (Leslie, 1990), *F. moniliforme* and *F. proliferatum* have been recovered from root tissues in 71 % sorghum samples.

In tropical and temperate regions, *F. moniliforme* is the major causative agent of seed rot; seedling blight; root and stalk rot (Claffin, 1986; Horne and Frederickson, 2003). *F. moniliforme* is also involved in causing grain mold of sorghum, a serious disease which became a major constraint to sorghum improvement and production worldwide (Navi *et al.,* 2005). Prominent in the root and stalk rot of sorghum are also other *Fusarium* spp. such as *F. oxysporum*, *F. graminearum*, *F. tricinctum*, *F.solani* and *F. equiseti* (Claffin, 1986). Mahalinga *et al.* (1988), reported that *F. oxysporum* and *F. pallidoroseum* are potentially pathogenic on some sorghum genotypes causing a negative effect on seed germination and seedling growth. *Fusarium* spp. from sorghum, millet and maize have recently become the subject of in depth research due to serious production losses in these crops as a result of stalk rot, ear rots and grain mold infections by these fungi (Leslie *et al.,* 2005). According to this report, *Fusarium* isolates recovered from sorghum and millet are identified as *F. moniliforme.* This is an indication that sorghum is attacked by a wide spectrum of *Fusarium* spp. leading to a serious loss in yield. Losses due to seedling blight and root and stalk rot caused by *Fusarium* spp. vary from 5-10% and may approach 100% in localized areas (Claffin, 1986).

Although many fungicides can be applied to sorghum seeds, they do not provide effective control of seedling and root diseases caused by *Pythium* and *Fusarium* spp. In the past, seed treatment with systemic oomycetes fungicide metalaxyl to prevent *Pythium* spp. has been found effective. However, as these fungicides are specific to oomycetes only and with the reported development of fungicide resistant species, the use of fungicides has become restrictive. In the light of global chemophobia, alternative disease control strategies such as biological control have become important.

1. 2. Plant growth promoting rhizobacteria (PGPR)

Bacteria that can improve plant growth through various mechanisms have been known for decades and have been introduced into soil, on seeds or roots to improve plant growth and health (Raaijmakers *et al.,* 2002). The Genus *Rhizobium*, an example of a growth-promoting organism, is the most widely known group. It has been successfully commercialized with many practical applications in agriculture by developing symbiosis with plants.

Early in this century, many bacterial species associated with plants but without symbiotic association were discovered (Bashan and Holguin, 1998). Although many of these bacteria were able to promote plant growth, they were not widely recognized until the mid 1970's, with the discovery that some bacteria mainly *Pseudomonads* are capable of controlling soil borne pathogens and indirectly enhance plant growth (Kloepper *et al.,* 1980). The discovery of *Azosprillum* species, a diazotrophic free living bacterium that proliferates in the rhizosphere of many tropical grasses (Maria *et al.,* 2002), is the other break through in the study of plant- microbe- interaction in the rhizosphere.

The term plant growth promoting rhizobacteria (PGPR) was originally used to describe this unique biocontrol group (Kloepper *et al.,* 1980). As this term does not encompass all the beneficial bacteria associated with plants in the rhizosphere, generally the plant growth promoting rhizobacteria are classified into two major group viz. Biocontrol Plant Growth Promoting Bacteria (Biocontrol PGPR) and Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998).

1. 2. 1. Biocontrol PGPR

The application of chemical inputs such as fertilizers and pesticides has long been used to improve productivity in conventional agriculture. However, there is now a growing desire for alternatives to this system (Mark et al., 2006). The use of bacteria as biocontrol agents of soilborne plant pathogens has been investigated for several decades (Landa *et al.,* 2004). However, suppression of soilborne root pathogens in soil has drawn considerable attention only recently as alternative farming method to maintain the productivity of agro ecosystems (Hu *et al.,* 1997). Rhizobacteria designated as biocontrol PGPB are those that suppress plant pathogens by producing various types of inhibitory substances, or by increasing the natural resistance of the plant (Jetiyanun and Kloepper, 2002; Gardner *et al.,* 2001; Bashan and de Bashan, 2002) or by displacing (out competing) the pathogen (O'Sullivan and O'Gara, 1992). Such biocontrol PGPBs have the capacity to rapidly colonize the rhizosphere, and compete with deleterious microorganisms as well as soilborne pathogens at the root surface (Rangarajan *et al.,* 2003). Some of these modes of action used by biocontrol plant growth promoting bacteria are discussed below.

1. 2. 1. 1. Antibiosis

Recent advances in the understanding of genetics and the regulation of synthesis of bacterial metabolites especially antibiotics have contributed significantly to the advancement of plant protection. The biocontrol PGPBs are mainly endowed with the capacity to produce antibiotics

against a number of phytopathogenic fungi and bacteria. Such biocontrol PGPB produce one or more of the antibiotics 2, 4-diacetylphloroglucinol (2, 4-DAPG), phenazine compounds (*Phz*), pyrrolnutrine (*Prn*), and pyluteorin (*Plt*) (Mazzola *et al.,* 1992; Raaijmakers *et al.,* 1997). These antibiotics are currently the major focus of research in biological control in soil ecosystems (Raaijmakers *et al.,* 1997). Hydrogen cyanide (HCN) is also reported to be one of the anti-fungal secondary metabolites produced by such biocontrol plant growth promoting bacteria (Cheryl *et al.,* 1998). More recently the production of new antifungal metabolites belonging to the class of cyclic lipopeptides such as visconsinamide and tensin has been reported (Bloemberg and Lugtenberg, 2001).

Screening of microorganisms to identify biocontrol agents, which are active against many phytopathogenic fungi and bacteria, has been carried out in the past. The mode of action of many bacteria has been ascribed to the antibiotics they produce. Among the potential biocontrol agents, which are active in the rhizosphere, *Pseudomonas* and *Bacillus* spp. have been widely investigated (Williams and Asher, 1996). Several strains of *Pseudomonas* spp. are used to control diseases in a variety of crops and other non-crop plants (Commare *et al.,* 2002). During their stationary growth phase, biocontrol strains of *Pseudomonas* synthesize the antibiotics phenazine carboxylic acid (PCA), 2, 4-DAPG, pyoluterin and pyrrolnitrin (Schnider *et al.,* 1995). Many of these antibiotics produced by *Pseudomona*s spp. *in-situ* contributed to the suppression of many plant diseases. Such antibiotic producing *Pseudomonas* spp. have been isolated from the rhizosphere soils that are naturally suppressive to diseases (Keel *et al.,* 1996). Plant diseases caused by the fungal pathogens *P. ultimum* and *Rhizoctonia solani* Kühn are, for instance, suppressed by different strain of *Pseudomonas fluorescens* (Cheryl *et al.,* 1998). *P. ultimum* mediated damping-off in sugar beet has been inhibited due to the production of 2, 4-diacetylphloroglucinol (2, 4-DAPG) by *P. fluorescens* F111 biocontrol strain. This product is also produced by other *P. fluorescens* strains and has been found effective against *Fusarium oxysporum*. attacking tomatoes. Recently, it has been demonstrated that fluorescent *Pseudomonas* spp. producing the antibiotic 2, 4-DAPG play a key role in the suppressiveness of take-all decline (TAD) in soils (de Souza *et al.,* 2003a) and the amount of 2, 4-DAPG produced *in-vitro* by these strains correlated with disease inhibition.

Phenazine antibiotics are another group of secondary metabolites effective against phytopathogenic fungi. *Pseudomonas* strains which produce phenazine antibiotics are reported for their suppression of take all of wheat caused by *Gaeumanomyces graminis* var. *tritici* (Mazzola *et al.,* 1992). To determine the importance of this antibiotic in suppression of take all, experiment with phenazine deficient mutants (*Phz*$^{-}$) generated by Tn 5 mutagenesis failed to inhibit *G. graminis* var tritici on media supportive of antibiotic production (Thomashow and Weller, 1990).

Antibiotics of the group cyclic lipopeptides such as visconsinamide produced by *P. fluorescens* have been shown to have an impact on the control of *Pythium* spp. and *R. solani* (de Souza *et al.,* 2003b). These cyclic lipopeptides induce encystment of *Pythium* zoospores and adversely affect the mycelia of *R. solani* and *P. ultimum* by causing reduced growth and intracellular activity, hyphal swelling and increased branching (Thrane *et al.,* 2000; de Souza *et al.,* 2003b). Nielson and Sorensen (2003), screened *P. fluorescens* strains capable of antagonizing *P. ultimum* and *R. solani* on agar plates. Further investigation during the early seed germination and root development of sugar beet revealed that the cyclic lipopeptide antibiotics were responsible for the antagonistic activity *in-vitro* (Nielson *et al.,* 1999; Nielson *et al.,* 2000; Nielson *et al.,* 2002).

The next most widely researched and commercialised bacteria for biocontrol activity in soil ecosystems are the endospore forming genus *Bacillus*. Most of the antibiotics produced by *Bacillus* spp. *in-vitro* were found to be peptide antibiotics and are responsible for biocontrol *in-vivo* (Leiferat *et al.,* 1995). *Bacillus cereus* UW85 that produce the antibiotics zwittermycin A and antibiotic B tend to suppress damping off disease more effectively than do *Bacillus* strains that do not produce antibiotics (Stabb *et al.,* 1994). This strain was initially identified from a collection of rhizosphere isolates by its ability to suppress alfalfa damping off consistently (Handelsman *et al.,* 1990). Since then, *B. cereus* UW85 has proven an effective biocontrol agent against Phytophthora damping-off and root rot of soy beans (Emmert and Handelsman, 1999). In general, the antibiotic zwittermycin A produced by this strain has been reported to adversely affect the growth and activity of a wide range of plant pathogenic fungi (Silo-Suh *et al.,* 1998).

Several other members of the genus have been shown to produce antibiotics of which the most important species is *Bacillus subtilis* (Foldes *et al.,* 2000). *B. subtilis* is one of the most widely distributed bacterial species in agricultural systems. The most commercially successful strains among this group is *B. subtilis* GBO3. This strain which effectively colonizes plant roots and produce antifungal compounds is the active ingredient in one of the widely distributed biofungicide (Kodiac, Guftafson LLC) (McSpadden and Fravel, 2002). Another best known biocontrol strain of this species isolated 25 years ago in Australia is *B. subtilis A13* (Kim *et al.,* 1997). This strain, in addition to inhibiting all the nine pathogens tested in an *in-vitro* test, subsequently promoted the growth of cereals, sweet corn and carrots when applied as seed inoculants (Kim *et al.,* 1997). *Bacillus* spp. are therefore considered ideal candidates for use as biocontrol agents in seed treatment programs against soilborne pathogens (Walker *et al.,* 1998).

1. 2. 1. 2. Siderophore production

Iron is one of the most abundant minerals on earth, yet in the soil, it is unavailable for direct assimilation by plants or microorganisms. This is because ferric iron (Fe^{+3}), the most common form of iron in nature, is only sparingly soluble (10^{-18} M at pH 7). Therefore, the amount of soluble iron in the soil barely supports microbial growth (Glick and Bashan, 1997). To overcome this problem, soil microorganisms secrete siderophores, iron-binding proteins of low molecular mass (400-1000 daltons) which bind Fe^{+3} with a very high affinity (KD = 10^{-20} to 10^{-15}). Most aerobic and facultative anaerobic microorganisms produce Fe^{+3} chelating siderophores which bind and transport ferric iron back to the microbial cells, where it is taken up by means of cellular receptors (Brait, 1992; Glick and Bashan, 1997; Bultreys *et al.,* 2001).

Biocontrol PGPBs prevent the proliferation of soilborne pathogens and facilitate plant growth through the production and secretion of such siderophores. The siderophores bind most of the Fe^{+3} available in the rhizosphere thereby effectively preventing any fungal pathogen in the immediate vicinity from proliferating due to a lack of iron (O'Sullivan and O'Gara, 1992). Siderophores produced by fungal pathogens have a much lower affinity for iron than those of biocontrol PGPB. Thus, biocontrol PGPB out-compete fungal pathogens for the available iron in the rhizosphere (Glick and Bashan, 1997). Siderophores also indirectly stimulate the biosynthesis of other anti-microbial compounds by making these minerals easily available to the bacteria (Duffy and Defago, 1999).

The major types of siderophores produced by biocontrol PGPB include pyoverdin, pyochelin and salicylic acid (Lemanceau *et al.,* 1992; Duffy and Defago, 1999; Bultreys and Gheysen, 2000). Numerous studies indicate that among the biocontrol PGPB in the rhizosphere, the fluorescent *Pseudomonas* species are efficient competitors for ferric iron (Fe^{+3}). The most commonly detected siderophores in these species are called pyoverdins or pseudobactins (Lemanceau *et al.,* 1993). Many potential biocontrol strains of this species produce pyoverdines. They are generally peptide siderophores all containing the same quinoline chromophore which is responsible for the colour of the molecule, a peptide chain and a dicarboxylic acid connected to the chromophore (Bultreys and Gheyson, 2002; Bultreys *et al*., 2003). The characteristic fluorescent pigments of fluorescent *Pseudomonas* are due to the pyoverdins (Budzikiwicz, 1993). Apart from this taxonomic importance and most importantly, pyoverdins produced *in-situ* chelate iron and make iron unavailable to pathogens in the rhizosphere (Looper and Henkels, 1999). Some fluorescent *Pseudomonas* species

also produce a non-fluorescent siderophore called pyochelin, a salicylic substitute cystein peptide (Leeman *et al.,* 1996).

Many workers have reported the suppression of disease development of several soilborne pathogens by different strains of biocontrol *Pseudomonas* spp. producing siderphores. A pyoverdin siderophore called pseudobactin 358, for instance, produced by a strain of *Pseudomonas putida* was reported as an effective biocontrol agent against *Fusarium* wilt (Lemanceau *et al.,* 1993.). *Fusarium* wilt diseases are currently responsible for important yield loses on a variety of crops (de-Boer *et al.,* 1999). Many strains belonging to the *Fusarium* genus often cause severe diseases such as vascular wilt, root rot and abnormal growth in various agricultural crops (Kurek and Jaroszuk-Scisel, 2003). Although many inputs of agrochemicals are used to protect the crops against this pathogen, they are adversely affecting the quality of the food product and that of the environment (Lemanceau *et al.,* 1992).

More sophisticated techniques are currently being used to evaluate the importance of siderophore mediated competition for iron by biocontrol rhizobacteria. Recent studies using a well defined mutant (Pvd^-) has indicated the involvement of pyoverdin siderophores in the control of *Fusarium* wilt of radish and carnations (Lemanceau *et al.,* 1992; Lemanceau *et al.,* 1993; Raaijmakers *et al*., 1995; Thomashow, 1996). Similarly, *Pythium* induced post emergence damping-off has also been suppressed in hydroponically grown tomato using strains which produce pyoverdins and pyochelins (Buysens *et al.,* 1996).

In many other studies, the efficacy of siderophores of biocontrol PGPB strains of *Pseudomonas* spp. has been proven to be very promising. In this respect, for instance, a mutant strain of *Pseudomonas aerogenosa* that lacks the ability to produce siderophore no longer had the ability to protect tomato plants from damping off (Glick and Bashan, 1997). Normally, siderophores are produced by bacteria under iron limiting conditions in the rhizosphere. In an effort to prove this, researchers (Elsheriff and Grosmen, 1994) conducted an experiment in which the amount of iron present in the soil was increased to 40 μmol Fe^{+3} / lit. The result obtained indicated a concomitant decrease in both the amount of siderophores produced and the inhibitory effect against the wheat pathogen *G. graminis* var *tritici*.

Whether biocontrol PGPB in the rhizosphere actually synthesizes siderophores in response to iron limiting conditions can be detected by means of a more advanced technique, namely an ELISA assay

using monoclonal antibodies. With this method it is possible to quantify the amount of siderophores produced in an ecosystem (Buyer *et al.*, 1993).

1. 2. 1. 3. Induction of Systemic Resistance

Under normal conditions all plants possess active defence mechanisms against pathogens' attack, which sometimes fails upon infection by a virulent pathogen. This happens as a result of the pathogen suppressing the resistance reactions (van Loon *et al.*,1998). If, however, defence mechanisms are triggered by stimulus before infection by the pathogen, the disease can be minimized i.e. the plants will have enhanced defensive capacity. This systemic protection of a plant by an inducing agent when applied to a single part of the plant is known as *Induced Systemic Resistance* (ISR). (Liu *et al.,* 1995; Nandakumar *et al.,* 2001; Ramamoorthy *et al.,* 2002). In nature induced resistance occurs as a result of limited infection by a pathogen and the subsequent development of a hypersensitive reaction (van Loon *et al.,* 1998).

Induced resistance brought about by the inducing agent is systemic as the defensive capacity is increased not only in the primary infected tissue, but also in the non-infected tissue. According to Sticher *et al.* (1997), induced resistance is commonly referred to as *Systemic Acquired Resistance* (SAR) due to its systemic character. In some cases however, localized acquired resistance occur when only those tissue exposed to the primary invader become more resistant (van Loon *et al.,* 1988).

Different biotic and abiotic inducers are involved in induction of systemic induced resistance in plants against various pathogens. These include pathogens, chemical plant products and PGPR (Liu *et al*., 1995; Leeman *et al*., 1995; Nandakumar *et al.,* 2001). The mechanism by which these inducing agents stimulate resistance is that they activate defence genes encoding chitinase, peroxidase, β-1, 4-glucanase and enzymes involved in the synthesis of phytoalexins (van Per *et al*., 1991; Maurhoef *et al*., 1994).

Induced systemic resistance against plant pathogens by biocontrol PGPR is a relatively new topic in disease suppression. It is mediated by effective biocontrol agents such as *Pseudomonas* spp. (Leeman *et al,* 1996). In most of the investigations so far conducted, several strains of *P. fluorescens* are rendering promising results by ISR in many crops. Biocontrol PGPRs elicit ISR in plants through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological

and biochemical reaction of the host plant. This leads to the synthesis of defence chemicals against the challenge pathogen (Ramamoorthy *et al.,* 2001).

Physical and mechanical strength of the cell wall was induced for instance by a biocontrol PGPR in peas (Benhamou *et al.,* 1996a). According to these researchers, treatment of pea plants with a strain of *P. fluorescens* resulted in the formation of structural barriers, i.e. cell wall papillae and deposition of phenolic compounds at the site of the penetration of the invading hyphae of *P. ultimum* and *F. oxysporum*. Similar experiments in potato resulted in the deposition of phenolic compounds, which inhibited the growth of *F. oxysporum* f. sp.radicis Lycopersici in the epidermal cell wall and outer cortex of the root system (Ramamoorthy *et al.,* 2001).

The other mechanism of ISR mediated by biocontrol PGPR is through development of biochemical or physiological changes in the plant. These include the production of PR-proteins (Pathogenesis related proteins) such as chitinase, peroxidase, sysnthesis of phytoalexins and other secondary metabolites (Zdor and Anderson, 1992; van Per *et al.,* 1999). Increased expression of plant peroxidase and chitinase enzymes in rice using strains of *P. fluorescens* was efficient enough to inhibit mycelial growth of the sheath blight fungus *R. solani* (Nandakumar *et al.,* 2001). In another experiment, seed treatment of pea by one strain of *P. fluorescens* resulted in the production of hydrolytic enzymes such as β-1, 4- glucanase, and chitinase (Benhamou *et al.,* 1996b). In all cases the host lytic enzymes accumulate at the site of penetration of the fungus.

Induction of systemic resistance by biocontrol PGPB is not confined to the aforementioned plant species. *Pseudomonas* spp. mediated ISR was also observed in carnation against *F.* oxysporum f.sp.dianth (van Per *et al.,* 1991), in cucumber against *Colletotrichum orbiculare* (Berk. and Mont.) (Wei *et al.,* 1996) and *Pythium aphanidermatum* (Edson) Fitzp (Chen *et al.,* 2000).

Elicitation of ISR in plants has been reported to be mediated by specific strains *Bacillus* spp. such as *B. amyloliquifaciens, B. subtilis*, *B. cereus*, *B. mycoides*, and *B. pumilis* with significant reductions in the incidence or severity of various diseases (Kloepper *et al.,* 2004). Certain strains of *Bacillus pumilus* have been reported to be involved in SIR in plants by inducing the accumulation of phenolic compounds in the newly formed wall appositions in pea roosts in response to attack by *F.oxysporum* f. sp. *pisi* (Benhamou *et al.,* 1998; Jetiyanun and Kloepper, 2002). The phenolic compounds contribute to enhance mechanical strength of the host cell wall and may also inhibit fungal growth as phenolics are toxic to fungi in nature (Ramamoorthy, 2002). This is an indication for the potential of *Bacillus* spp. to be used as bio-control PGPR similar to *Pseudomonas* spp. in the soil rhizosphere.

The major bacterial determinants that are claimed to produce ISR in plants by the aforementioned mechanisms are the O' antigen of cell wall lipopolysacharides, siderophores and salicylic acid (Leeman *et al.,* 1996; Bloemberg and Lugtenberg, 2001). For instance, the development of ISR in carnation against *Fusarium* wilts by *F. oxysporum*. f. sp. *dianthi* is associated with the lipopolysacharide present in the outer membrane of PGPR *P. fluorescens* strain (Van Per and Shippers, 1992). Similarly in rice, the increased activity of chitinase peroxidase has been reported to be due to the release of these signal molecules by *P. fluorescens* (Ramamoorthy *et al.,* 2001).

The fact that bacterial cell wall lipopolysacharides are involved in ISR was proved in one experiment conducted using a mutant strain of *P. fluorescens* lacking the O' antigen side chain of lipopolysacharides (Leeman *et al.,* 1995). This mutant, unlike the wild strain, failed to induce resistance in radish, showing that the O' antigen side chain of LPS serves as a signal in the induction of systemic resistance. Lipopolysacchride is not the only trait in determining the ISR, because in another study a mutant strain lacking the O' antigen side chain has been reported to elicit defence mechanisms in Arabidopsis (Van Wees *et al.,* 1997). In this respect, while LPS of some *P. fluorescens* strains are the major determinants of ISR under iron replete conditions, siderophores of the same bacterial strain are responsible for ISR in radish against Fusarium wilt under iron limited conditions (Van Loon *et al.,* 1998). How these siderophores trigger ISR is however unclear.

As mentioned before, salicylic acid is also involved in the induction of ISR in plants. Treatment of plants with salicylic acid decreased disease development in tobacco due to tobacco mosaic virus (Kessmann *et al.,* 1994). According to these researchers, certain PGPR strains are endowed with the capacity to produce this compound and induce systemic resistance in plants. Mutant strains lacking the ability to produce salicylic acid production lost their ability to induce systemic resistance in bean as opposed to the wild strain (De Meyer and Hofte, 1997).

Various experiments indicated that ISR by bacterial determinants varies with many factors. These factors include iron limiting conditions, bacterial strains, host plants and their cultivars (Leeman *et al.,* 1996; Van Loon *et al.,* 1998). Although ISR has been studied mainly under laboratory and green house conditions, reports indicate that ISR can protect plants under field conditions (Tuzun *et al.,* 1992; Zhang *et al.,* 2002).

Advantages of ISR over other mechanisms of biological control systems include, once expressed, ISR activate multiple potential defence mechanisms such as increasing the activities of chitinase, β-

1,3 glucanase, peroxidase, pathogenesis related proteins and accumulation of phytoalexins (Wei *et al.,* 1996). Another important aspect of ISR is that, apart from protecting plants against a wide spectrum of pathogens once induced, it also protects plants systematically following application of an inducing agent. Contrary to this, other mechanisms of biological control are generally not systemic (Wei *et al.,* 1996; Zhang *et al.,* 2002).

In conclusion, although plants have their own defence genes, these are quiescent in normal healthy plants i.e. they are inducible genes (Nandakumar *et al.,* 2001). When these endogenous defence mechanisms are induced by appropriate stimuli or signals, the plants own defence mechanisms will be activated. The use of biocontrol plant growth promoting *Pseudomonas* spp. and more recently the application of *Bacillus* spp. to develop ISR in plants is now becoming a novel plant protection strategy.

1.2. 1. 4. Competition in the rhizosphere.

Biocontrol plant growth promoting bacteria also inhibit phytopathogens by other mechanisms than those mentioned before. An example is competition for nutrients and suitable niche on the root surface (O'Sullivan and O'Gara, 1992). The ability to compete for nutrients with indigenous microbial populations within the rhizosphere is an important trait for effective bio-control of soilborne pathogens (Walsh *et al.,* 2001). Strains of *Pseudomonas* spp. have been reported to have the ability to metabolize the constituents of seed exudates in order to produce compounds inhibitory to *Pythium ultimum* (Glick and Bashan, 1997). There is no relationship observed between the ability of these bacteria to inhibit the fungal pathogen by the production of siderophores or antibiotics (Stephens *et al.,* 1993). This was detected by growing the bacterium on a medium that favoured the production of either antibiotics or siderophores.

Due to competition, biocontrol agents have the ability displace some bacterial plant pathogens. The pathogenic *Pseudomonas syringae*, which increases frost susceptibility in tomato and soybean and causes ice nucleation, is reported to have been out competed by an antagonistic ice nucleation deficient medium (Wilson and Lindow, 1994). In one greenhouse experiment (Cooksey, 1990), a non-pathogenic copper resistant Tn 5 mutant of *P. syringae* pv. tomato, the casual agent of bacterial speck of tomato, was co-inoculated with a pathogenic strain. The result was that the non-pathogenic strain decreased the disease incidence significantly by competing with the pathogen for the same niche

In another experiment, protection of tomato seedlings against infection by *P. syringae* pv. tomato was made possible using the plant growth promoting bacterium *Azosprillum brasilense* (Bashan and de-Bashan, 2002). *Azosprillum* spp. are not known as typical biocontrol PGPBs as they lack the ability to produce significant amounts of antimicrobial substances, nor do they induce systemic resistance in plants (Shah *et al.,* 1992). However, because of their rhizo-competent ability and the capacity to form large populations on leaves, *A. brasilense* displaces leaf pathogens and in the process reduce disease severity. The displacement of *P. syringae* pv.tomato by *A.brasilense* was demonstrated by the reduced colonization of the pathogen in the rhizosphere and on the leaf surfaces in the presence of *A. brasilense* (Shah *et al.,* 1992).

1. 2. 2. Plant growth promoting PGPR

The second important division of beneficial bacteria in the rhizosphere are those referred to as plant growth promoting bacteria (PGPB), which promote growth via production of phytohormones and improvement of plant nutrition status (Bai *et al.,* 2002). Because of these properties, the co-inoculation of these PGPB with the symbiotic rhizobia is currently becoming a valuable technique in the development of sustainable agriculture. Among the major groups of plant growth promoting bacteria, the most widely studied and efficient group include *Azosprillum* spp. (Bertrand *et al.,* 2001), *Pseudomonas* spp. (Amy *et al.,* 2002) and *Bacillus* spp. (Bai *et al.,* 2002).

1. 2. 2. 1. Synthesis of phytohormones

The ability of rhizobacteria, particularly the plant growth promoting bacteria (PGPB) to synthesize various metabolites, influences plants as well as the availability of mineral nutrients for plants and the soil structure. A great proportion of microorganisms capable of producing *in-vitro* phytohormones are found to survive in the rhizosphere (Vancura and Jander, 1986). According to this finding, 20 % of the bacteria produced phytohormones. Moreover, from 50 bacterial strains isolated from the rhizosphere of agriculturally important plants, 43 strains produced auxins (IAA), 29 gibberilins, 45 kinetin like substances and 20 strains all three types of phytohormones. All of these strains were able to solubilize poorly soluble phosphates and thus enable phosphorous up take (Vancura and Jander, 1986). The auxin type phytohormone known as indole-3-acetic acid (IAA) is the main type of phytohormone produced by plant growth promoting bacteria (Patten and Glick, 1996; Gonzalez and Bashan, 2000; Patten and Glick, 2002).

One mechanism by which PGPB affect plant growth in the rhizosphere is by contributing to the host plant endogenous pool of phytohormones such as IAA (Patten and Glick, 1996). Beneficial bacteria synthesize IAA through the indole-pyruvic acid pathway. In this pathway, the amino acid trypthophan is first transformed into indole-3 pyruvic acid by oxidative deamination, which is then decarboxylated to indole-3-acetaldehyde. Indole-3-acetaldehyde is finally oxidized to IAA (Vancura and Jander, 1986; Patten and Glick, 2002).

Among the most efficient PGPB studied for their capacity to produce phytohormones are *P. putida*, *P. fluorescens*, *Azosprillum* spp. and *Bacillus* spp. In all these bacteria the formation of IAA and other auxins has been proved using HPLC and mass spectrometers (Vancura and Jander, 1986).

Recently, the role of many such rhizobacterial IAA in the development of the host plant root system has been studied. In one experiment, canola seeds treated with a wild type of *P. putida* strain that produce IAA and another IAA deficient mutant constructed by insertional mutagenesis responded differently (Patten and Glick, 2002). The canola seeds primary roots from seeds treated with the wild type strain were on the average longer than the roots from seeds treated with the mutant strain and the roots from un-inoculated seeds. It was previously indicated by other studies that, while low levels of IAA stimulate primary root elongation, high levels of IAA stimulates the formation of lateral and adventitious roots (Sawar and Kremer, 1995; Xie *et al.,* 1998).

Bacterial IAA promotes root growth either directly by stimulating plant cell elongation or cell division or indirectly by its influence on the ACC deaminase activity. 1-Amino cyclopropane-1-carboxylic acid deaminase (ACC deaminase) is an enzyme produced by many plant growth promoting bacteria (Glick *et al.,* 1998) and is involved in the stimulation of root elongation in seedlings (Glick and Bashan, 1997; Lie *et al.,* 2000). The ACC deaminase hydrolyses plant ACC, the immediate precursor of the phytohormone ethylene. Ethylene in plants acts as a secondary messenger stimulating leaf or fruit abscission, disease development and inhibition of growth (Glick and Bashan, 1997). Mutants of plant growth promoting bacteria that do not produce ACC deaminase, for instance, have lost the ability to stimulate root elongation (Lie *et al.,* 2000). There are several other reports of the role of IAA produced by plant growth promoting rhizobacteria in enhancement of growth and yield of many crops (Ayyadurai *et al.,* 2006).

Another key member of the plant growth promoting bacteria, *Azosprillum brasilense* promotes the growth of many terrestrial plants upon seed or root inoculation (Bloemberg and Lugtenberg, 2001; Bashan and de Bashan, 2002). All the known *Azosprillum* species produce IAA (Gonzalez and

Bashan, 2000) and it is reported that this is the most abundant phytohormone secreted by *Azosprillum*. It is also generally agreed that in most *Azosprillum* species, it is the production of IAA rather than nitrogen fixation that contributes to stimulation of rooting and enhancement of plant growth (Bloemberg and Lugtenberg, 2001). The auxin type phytohormone produced by *Azosprillum* spp. affect root morphology and thereby improve nutrient uptake from soil (Barea *et al.,* 2005). Apart from increasing the density and length of legume root hairs, IAA secreted by *Azosprillum* increases the amount of flavinoids that are exuded and act as signals for initiations of root nodulation by rhizobial strains (Glick *et al.,* 2001).

To summarize, although IAA does not apparently function as a hormone in the bacterial cells, it is important in the microbial-plant relationship, particularly when it comes to stimulating the development of the host plant root system.

1. 2. 2. 2. Assymbiotic nitrogen fixation

In order to sustain sufficient crop production, a reliable source of nitrogen is vital. Microbial oxidation of soil organic matter may thus provide plants with potentially available nitrogen. However in soils with poor soil organic matter, biological fixation of nitrogen is that which fills the deficiency in the soil organic nitrogen pool (Chote *et al.,* 2002). In the rhizosphere, free living nitrogen-fixing PGPR affect plant growth directly by non-symbiotic nitrogen fixation.

Many non-legume plants have been shown to be associated with the free living diazotrophic nitrogen-fixing bacteria. With the advent and the application of the acetylene reduction assay, it has now become a common practice to screen plants and microorganisms for the presence of the nitrogenase activity (Malik *et al.,* 1997). In ecosystems where legumes are sparse or absent, nitrogen fixation by free living diazotrophic bacteria is the mechanism to meet part of the nitrogen requirement of the plants (Brejda *et al.,* 1994). Most of this nitrogen fixation by free-living diazotrophic bacteria in the rhizosphere is associated with the roots of grasses and is regarded as an important component of the nitrogen cycle in many ecosystems.

Rhizosphere bacteria commonly known for such non-symbiotic nitrogen fixation include *Azosprillum, Herbasprillum and Beijerinkia* (Anonymous, 2003). These bacteria are commonly microaerophilic and can be best recovered from tissues by growth in semi solid media with malate as energy source. These free living nitrogen fixers in the rhizosphere are nowadays given attention

(Chotte *et al.,* 2002) as they are known for the utilization of plant exudates as a source of energy to support the fixation process.

Azosprillum spp. proliferates in the rhizosphere of many tropical grasses, fixing nitrogen and transferring it to the plant (Maria *et al.,* 2002). Field inoculation with *Azosprillum* in many investigations revealed that these bacteria are capable of promoting the yield of many important agricultural crops (Okon and Gonzalez, 1994). In wheat, for example, a non-tropical cereal, *Azosprillum* has been assayed widely for field inoculation and resulted in significant yield increase (Maria *et al.,* 2002). *Azosprillum* are also involved in pronounced nitrogen fixation in several other crops such as rice (Malik *et al.,* 1997), corn (*Zea mays*), sorghum (*Sorghum bicolor*) and switch grass (*Pinatum virgutum*) (Bredjda *et al.,* 1994). Although the main emphasis in the search for nitrogen fixing plant growth promoting rhizobacteria in the soil rhizosphere focused on the isolation of *Azosprillum* (Berge *et al.,* 1991), other nitrogen fixing strains such as *Bacillus* spp. have also been found in association with grass roots.

1. 2. 2. 3. Solubilization and mineralization of organic and inorganic phosphates

Phosphate is the second most critical plant nutrient after nitrogen. In the soil rhizosphere, although the total phoshorous pool is high, only a part of this is available to plants. Thus it can be inferred that many soils throughout the world are P-deficient as the free P-concentration even in fertile soils is not higher than 10μM at pH 6.5 (Rodriguez and Fraga, 1999; Gyanshewar *et al.,* 2002).

Soluble phosphorous has a high level of reactivity with calcium, iron or aluminium. This leads to phosphorous precipitation resulting in low levels of P (Gyanshewar *et al.,* 2002). The type of the soil and pH affects the fixation and precipitation of 'P' in soil. Thus, in acidic soils, P is fixed by free oxides and hydroxides of 'Al' and 'Fe', while in alkaline soils it is fixed by 'Ca' (Jones *et al.,* 1991). To overcome the problem of P-deficiency, chemical fertilizers are added. However, the production of chemical phosphatic fertilizers is such an energy intensive process that it requires energy worth $4 billion per annum so as to meet the global need (Goldstain *et al.,* 1993). Despite the fact that most agricultural soils contain large reserves of 'P' due to regular application of phosphorous, a large part of this applied inorganic 'P' is rapidly immobilized and become unavailable to plants (Rodriguez and Frag, 1999).

Because of the aforementioned problems of 'P' availability to plants, there is now a growing need in the selection and manipulation of biofertilizers in plant nutrition. In terms of phosphate

solubilization, the arbuscular mycorrhizae belong to the former category. In recent years the ability of different bacterial species to solubilize inorganic phosphate compounds has been detected and proved to be beneficial in agriculture (Rodriguez and Fraga, 1999; Gyanshewar *et al*., 2002). The higher proportion of these phosphate-solubilizing bacteria is commonly found in the rhizosphere (Baya *et al.,* 1981). The mechanism by which these microorganisms solubilze Ca-P complexes is by their ability to reduce the pH of their surroundings either by the release of organic acids or protons (Gyanshewar *et al.,* 2002). Once the organic acids are secreted, they dissolve the mineral phosphate as a result of anion exchange of $PO_4^{=}$ or they chelate both Fe and Al ions associated with phosphates (Gyanshewar *et al.,* 2002).

Pseudomonas and *Bacillus* spp. are reported as the most important phosphate solubilizers among the PGPR (Baya *et al.,* 1981). At first the production of antibiotics, siderophores and phytohormones has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Kloepper *et al.,* 1989). However, at present there is evidence supporting the role of this mechanism in plant growth enhancement. A strain of *P. putida* for example, stimulated the growth of roots and shoots and increased 'P' labelled phosphate uptake in canola (Lifshitz, 1987). Inoculation of crops with *Bacillus firmis* (Datta *et al.,* 1982) and *Bacillus polymyxa* also resulted in phosphate uptake and yield increase. Rice seeds inoculated with *Azosprillum lipoferum* strain 34H, a known rhizobacteria, increased phosphate ion content and resulted in significant improvement in root length and shoot weights (Murty and Ladha, 1988).

A second major source of plant available phosphorous is that derived from the mineralization of organic matter. Soil contains a wide range of organic substances. Particularly in tropical soils a large part of 'P' is found in organic forms (Rodriguez and Fraga, 1999; Kwabiah *et al.,* 2002). This organic phosphate (Po) is so complex that plants can not directly utilize it but only utilize 'P' in its inorganic form. It is therefore necessary that to make the organic phosphate available to plants, it must be first hydrolyzed to inorganic 'P'. This is called mineralization of organic phosphorous and it is achieved by the activity of phosphatase enzyme, which hydrolyses Po to inorganic forms (George *et al.,* 2002). Plant growth promoting bacteria in the rhizosphere show a significant phosphatase activity (Dinkelager and Marshner, 1992). Plants inoculated with PSMs showed growth enhancement and increased 'P' content as a result of mineralization of organic phosphates. Among these, *Bacillus megaterium* is regarded as the most effective PSM in many field experiments releasing 'P' from organic phosphate, but does not solubilze mineral phosphate (Gyanshewar, 2002).

Phosphate solubilizing bacteria are also reported to function as mycorrhizal helper bacteria (Kraus and Loper, 1995). When such bacteria are associated with mycorrhizal fungi, they promote root colonization. The principle is that, their association with mycorrhizal fungi contribute to the biogeochemical cycle of nutrients by more than just providing a greater surface area for scavenging nutrients that may be relatively immobile in soil (Toro *et al.,* 1997). Generally the role of microorganisms, especially of the growth promoting rhizobacteria in 'P' solubilization and mineralization is very crucial to make 'P' easily available to plants.

1. 3. Bio-formulations and application of rhizobacteria as biocontrol agents

The economic feasibility of any given biocontrol agent is affected by many factors of which formulations of these agents and their delivery system are very important. It has been a common practice to use seed treatment with cell suspensions of many PGPR to control several diseases. This methodology is however becoming impractical due to difficulty in handling, transport and storage of bacterial suspensions (Trapero-Cascas, 1990; Parke *et al.,* 1991). It is very difficult to use bacterial cell suspension for large scale field use. Therefore the need arises to device techniques for the development of formulations in which the biocontrol agents can survive in a carrier material for longer periods of time (Rabindran and Vidhyasekaran, 1996).

Formulation can be considered as the industrial art of converting a promising laboratory proven bacterium into a commercial field product (Bashan, 1998). Such microbial inoculum formulations not only overcome loss of viability during storage in the growers' warehouse, they have also longer shelf life and stability over a range of temperatures between -5 - 30 ^{o}C while in the marketing distribution chain (Bashan, 1998). Formulations are in general composed of the active ingredients i. e. microorganisms or spores which are carried by an inert material used to support deliver the active ingredients to the target (Hynes and Boyetchko, 2005).

Bacterial formulations can be prepared either in liquid or dry forms. Liquid formulations may be oil-based, aqueous based, polymer based or combinations while dry formulations include an inert carrier such as fine clay, peat, vermiculite alginate or polyacrilamide beads (Boyetchko *et al.,* 1999). Among the dry formulations, peat based formulations have been widely investigated and used giving significant result in yield increase and bio-control efficiency. Bacteria can survive well in peat-based formulations for longer periods and PGPR have been reported to survive in such types of dry formulations (Vidhyasekeran and Muthamilan, 1995). Peat-based or talc-base dry formulations allow

the antagonists to be supplied to the farmers for seed treatment or to the seed producers to supply treated seeds to the farmers.

Formulations of PGPR are in general used to promote growth and health of crop plants. Treatment with rhizobacterial formulations for instance enhanced the growth of pearl millet plants and reduced the percentage of downy mildew incidence (Nirajan-Raj *et al.,* 2003). Control of rice sheath blight caused by *R. solani* has been achieved using peat-based formulation of *P. fluorescens* (Rabindran and Vidhyasekaran, 1996). In another experiment, field emergence of chickpea plants was improved by seed treatment with talc based *P. fluorescens* formulation (Vidhyasekaran and Muthamilan, 1995). These results were obtained as the formulated products suppressed pre-emergence damping off caused by various pathogens. Powdered formulations of PGPR in an organic carrier mixed into soilless media provide seeding growth promotion and induce systemic disease protection (Reddy *et al.,* 1999). The practical applications of these PGPR formulations were supported due to the fact that the growth promotion detected was highly significant in comparison with the non-treated controls in various experiments.

Peat formulation has been the carrier of choice and the most commonly used in the rhizobia inoculation industry (Bashan, 1998). It has been common to use peat-based formulations to introduce *Azosprillum*, a biofertilizer, into the rhizosphere. There are however some drawbacks of the peat-based formulations. Peat, as it is an undefined complex organic material, affects the final product and causes difficulties in inoculants dosage and storage condition (Bashan, 1998). Moreover, peat formulations are susceptible to contaminations reducing the shelf life of the inoculants. Due to such problems in peat-based formulations, new trends to use unconventional synthetic materials as PGPR formulations are now becoming more practical and proved more advantageous than peat based formulations. These formulations are based on polymers, which encapsulate the living cells thereby protecting the microorganisms against many environmental stresses.

Alginate is the material most commonly used for encapsulation of microorganisms and the resulting inocula are used as biological control agents and in bacterial chemotaxis research (Bashan and Holguin, 1994). It is a naturally occurring polymer composed of β-1, 4 linked D-mannuronic acid and L-glucuronic acid and it is extracted from different microalgae as well as several bacteria (Smidsrod and Skjac-Break, 1990). Compared with peat-based formulations, alginate-based PGPR formulations have such advantages as being non-toxic, biodegradable and slow release of microorganisms into the soil (Kitamikado *et al.,* 1990).

In general, the selection of appropriate formulations not only improves product stability and viability, but also reduces inconsistency of field performance of many potential biocontrol and growth promoting agents (Boyetichko, 1999). Moreover, irrespective of the type of formulation used, it must be born in mind that effective control and yield increase also depends on the method of treatment and the concentration of the microbial inoculum used (Rabindran and Vidhyasekaran, 1996). As formulation of microorganisms or their spores determines efficient delivery, shelf life and stability of its effectiveness against plant pathogens, it can generally be regarded as a key to bio-product success (EL-Hassan and Gowen, 2006).

1. 4. Current status and future prospects of using rhizobacteria as biocontrol and growth-promoting agents

In the preceding sections of this chapter, an overview was given of PGPR in biocontrol, biofertilization and phytostimulation. Plant growth promoting bacteria interact with their biotic environments in a complex pattern. Due to this, substantial advance is being made in understanding the genetic basis of the beneficial effects of these PGPRs on plants (Thomashow, 1996; Bloemberg and Lugtenberg, 2001).

1. 4. 1. Biocontrol PGPR

It has previously been mentioned that most of the biocontrol PGPB such as *Pseudomonas* produce various anti-fungal metabolites (AFMs). The genetic basis of the biosynthesis of the more frequently detected AFMs such as pyoluteorin in *P. fluorescens* Pf5 (Nowak *et al.,* 1999) and 2, 4-DAPG in *P. fluorescens* Q-2-87 (Bangera *et al.,* 1999; Delany *et al.,* 2000) has been elucidated. Various such great advances in the molecular basis of biocontrol agents have been achieved. Recently for instance, the biocontrol efficacy of *P. fluorescens* F113 has been enhanced by altering the regulation and production of 2, 4-DAPG (Delany *et al.,* 2001).

In many studies, it has been demonstrated that antibiotic negative mutants of *Pseudomonas* strains have reduced ability to suppress root diseases compared with the wild strains (Schnider *et al.,* 1995). A phenazine negative mutant of *P. fluorescens* for instance was shown to lack part of its ability to suppress take-all of wheat (Pierson, 1994). Similarly, pyrrolnitrin defective mutant of *P. fluorescens* BL915 failed to suppress *R. solani* induced damping off in cotton (Hill *et al.,* 1994). But when

antibiotic production is restored in these mutants by complementation or recombination, their biocontrol efficiency is also restored.

Promising results are being obtained in improving the biocontrol performance of soilborne *Pseudomonas* by the introduction of antibiotic biosynthetic genes (Dowling and O' Gara, 1994). Vincent *et al*. (1991) transferred a recombinant cosmid expressing the *Phl* structural genes of *P. aurofacience* Q 2-87 to *P. fluorescens* that naturally produces phenazine carboxylic acid. The resulting recombinant strain had increased anti-fungal activity *in-vitro* against *Gaeumannomyces graminis* (Sacc.) var. tritici, *P. ultimum* and *R. solani*. In a related experiment, by introducing the PCA biosynthetic genes of *P. fluorescens* 2-79 into different PCA non-producing strains, it was possible to develop recombinant strains. The recombinant strains proved to be more inhibitory to *G. graminis* var. tritici *in-vitro* and *in-vivo* than the wild types (Schnider *et al.,* 1995). It is thus reasonable to speculate that these and a number of other related advances will lead to more efficient use of these biocontrol strains through their improvement by genetic modifications.

The question still remains, however, why is biological control of soilborne diseases achieved by most biocontrol agents including *Pseudomonas* and *Bacillus* spp. still so inconsistent. One factor particularly associated with this inconsistency is insufficient root colonization by the introduced bacteria (Latour *et al.,* 1996; Bloemberg and Lugtenberg, 2001). Lack of knowledge about the bacterial traits that promote root colonization is another contributing factor. For a biocontrol inoculant to perform effectively, its root colonization ability and hence its rhizosphere competence is very important. In this regard the inoculant bacteria must be able to establish themselves in the rhizosphere at a threshold population density (10^5 cfu/gm) sufficient to produce a beneficial effect (Raaijmarks and Weller, 2001). Rhizobacteria have a superior ability to establish and maintain high rhizosphere population densities over an extended period of time. Saravanan *et al*.(2004), also reported that *Pseudomonas fluorescens* strains that inhibited the growth of *Fusarium oxysporum* f. sp. *cubensis* causing wilt in banana do so by aggressively colonizing roots and establishing themselves at the root environment.

During the last two decades however, the population densities of many *Pseudomonas* strains has declined substantially (Mazzola and Cook, 1991). If a biocontrol agent can not adequately compete within the rhizosphere and colonize the root surface, it will not have an efficient biocontrol activity (Walsh *et al.,* 2001). An efficient inoculum strain must be capable of competing with the indigenous soil bacteria. Among many approaches used to overcome this problem include inoculation at higher concentration than the indigenous population, repeated inoculation and the use of genetically

engineered strain with enhanced competitiveness (Nautiyal, 1997). Thus, the selection of strains that are rhizosphere competent will contribute to improve the efficacy of biocontrol agents.

It is important therefore to investigate bacterial colonization and gene expression *in-situ* in the rhizosphere. In recent years, the green fluorescent protein (GFP) and bioluminescence techniques have been employed to study bacterial root colonization and rhizosphere competence (Walsh *et al.,* 2001; Bloemberg and Lugtenberg, 2001). The GFP technique together with the confocal laser scanning microscopy has facilitated the detection of a single bacterial cell. The technique revealed that *Pseudomonas* biocontrol agent often form microcolonies on the roots of crop plants (Normander *et al.,* 1999; Tombolini *et al.,* 1999).

The identification of genes and traits involved in the process of inoculation and root colonization is therefore an important strategy to improve the inadequate biocontrol activity and inconsistency in field experiments. In this regard, *P. fluorescens* genes that are specifically expressed in the rhizosphere *(rhi* genes) have been identified using the *in-vivo* expression technology (Bloemberg and Lugtenberg, 2001). Many other root colonization genes and traits have been identified from *Pseudomonas* biocontrol species (Lugtenberg *et al.,* 2001).

There are, however, certain instances in which root colonization may play a less significant or even minimal role in determining the level of disease control obtained in response to rhizobacteria that suppress disease via induced systemic resistance mechanisms. Liu *et al*. (1995), for instance demonstrated that ISR activity mediated by PGPR strains did not depend on high root colonization ability and high populations. The study, conducted to determine ISR activities of *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 on cucumber revealed that ISR increased over time whereas the bacterial populations decreased. There was hence no relationship between ISR activity and populations of the two strains on roots.

1. 4. 2. Plant growth promoting PGPR

Although inoculation with PGPR especially with non-symbiotic associative rhizosphere bacteria is not a new technology, many of the attempts failed. Inoculation trials with *Azotobacter* on a large scale in Russia in the late 1930s and an attempt to use *Bacillus megaterium* for phosphate solubilization in the 1930's also failed (Bashan, 1998). It is only in the late 1970's that a major breakthrough in plant inoculation technology was made. One of the major breakthroughs is the finding of the plant growth promoting, free living *Azosprillum spp.* These bacteria enhance the

growth of non-legume plants by directly affecting the metabolism of the plants (Bashan and Holguin, 1997). In later years after the discovery of *Azosprillum* as PGPR, many other bacteria such as *Bacillus*, *Flavobacterium* and *Acetobacter* have been evaluated for their potential in plant growth promotion (Tang, 1994). The biocontrol agents, mainly *P. fluorescens and P. putida* are also regarded as agents of plant growth promotion.

An important feature of these plant growth-promoting bacteria is their ability to colonize roots and promote plant growth (Sharma *et al.,* 2003; Patten and Glick, 2002). The potential of rhizosphere colonization by PGPB is very crucial for what is known as soil biofertilization (Villacieros *et al.,* 2003). The term 'biofertilizer', though misleading is a widely used term to describe bacterial inoculants. It refers to preparation of microorganisms that may be a partial or complete substitute for chemical fertilization like rhizobial inoculants (Bashan, 1998).

Improving plant growth by biofertilization is a crucial mechanism by which iron acquisition in most agricultural crops is achieved. Normally the total iron in the soil is by far much higher than most crops require. However, the concentration of free Fe^{+3} in most soils is far below that required for optimum growth (10^{-9} and 10^{-4}M Fe^{+3}) in the soil solution (Masahla *et al.,* 2000). In the decades before, many studies have indicated that the production of siderophores by plant growth promoting bacteria, particularly by the biocontrol *Pseudomonas* spp. increases plant iron acquisition (Masahla *et al.,* 2000). The high binding affinity and specificity for iron facilitates the transport of iron into the bacterial cells. Plants make use of this ferric-siderophore complex in their systems through the action of enzymes like ferric reductase (Sharma *et al.,* 2003). According to many reports, the possible role of plant growth promoting bacteria in iron uptake by plants in the rhizosphere is indicated by the fact that, under non sterile soil system plants show no iron deficiency symptoms in contrast to plants grown in sterile system (Walter *et al.,* 1994).

Another important aspect of biofertilization is that it accounts for approximately 60 % of the nitrogen supply to crops worldwide. This is achieved both by the symbiotic and free-living nitrogen fixers. To date the genes involved in nitrogen fixation and nitrogen assimilation have been described for *Azosprillum* (Bloemberg and Lugtenberg, 2001).

A promising trend in the field of inoculation technology with plant growth promoting bacteria is, the finding that co-inoculation of growth promoting bacteria with other microorganisms increased growth and yield (Bashan, 1998). Mixed inoculations allow the bacteria to interact synergistically and provide nutrients, remove inhibitory products and enhance some beneficial aspects of their

physiology such as nitrogen fixation. *Azosprillum* spp. co-inoculated with phosphate solubilizing bacteria for instance frequently increased plant growth by providing the plant with more balanced nutrition, improved absorption of nitrogen, phosphorous and iron (Bashan and Holguin, 1997).

At present, the discovery of many traits and genes involved in the beneficial effects of PGPR has resulted in a better understanding of the performance of these growth promoting agents in the field. This also provided the opportunity to enhance the beneficial effects of PGPR strains by genetic modification for future use.

1. 5. Objectives of the study

Sorghum is one of the most important crops and a staple food crop in arid and semi arid areas in Ethiopia. However production is very low in this country because of, amongst other factors, infection by soilborne pathogenic fungi including *F. oxysporum* and *P. ultimum*. Moreover quite a large group of the fungal pathogens reported on sorghum are predominantly recorded in Ethiopia. Repeated attempts to control these pathogens using fungicides were not successful. In addition, it is also believed that the growing cost of chemical pesticides is unaffordable by the poor farmers in the less-affluent countries such as Ethiopia. Attempts to control the disease using biological control are totally lacking. This study was therefore undertaken to asses the biocontrol and growth promoting abilities of rhizobacteria isolated from the rhizosphere of sorghum and the rhizosphere and rhizoplane of several species of grasses.

The main objectives of the current study were to:

- isolate and screen rhizobacteria for *in-vitro* mycelial inhibition of *Fusarium* and *Pythium.*
- evaluate the isolates in terms of biocontrol of *Fusarium* and *Pythium* root rot in sorghum under greenhouse conditions.
- evaluate the isolates in terms of the growth promotion of sorghum in pathogen free soil under greenhouse conditions.
- determine the modes of action used by the most effective antagonistic and PGPR isolates.
- identify the most effective isolates using biochemical and molecular techniques.
- Set out selection criteria for the best performing isolates for future use.

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