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Induction of systemic resistance by endophytic bacteria against Sclerospora graminicola in pearl millet
INTRODUCTION

In recent years of plant disease management, the process of immunization or induced systemic resistance (ISR) against disease is receiving increased importance as it is promising strategy in the era of organic farming to minimize the use of synthetic pesticides. Induced systemic resistance is well demonstrated using biotic and abiotic agents in many crop systems (Hammerschmidt et al., 2001). Among biotic agents, the concern over endophytic bacteria is realized for the benefit in various cropping systems to manage the diseases (Seghers et al., 2004). Endophytes microflora is an important living thing, seated in the inner tissue of plants, which generally enhances the competitive ability of host plants with metabolites released during the interaction. The designation of ‘endophyte’ can be used restrictively to refer only to those microorganisms, which reside in vascular tissues of the plant and hence move freely inside the plant or it can be used more broadly to refer to any microorganism, which resides inside the plant without regard to the specific tissue colonized. The endophytic bacteria can broadly defined as those bacteria, which can be isolated from inside surface-disinfested plants. It was originally reasoned that endophytic bacteria, which could colonise vascular tissues of plants, would be potential antagonists of vascular-invading pathogens, such as Fusarium oxysporum and Verticillium spp. (Hallmann et al., 1997). The endophytic microorganisms colonization in internal tissues of wide variety of plants and this association can be grouped as detrimental, neutral and beneficial. Endophytic microbes during the interaction with host produce phytohormones, toxins and metabolites attributed to the enhanced competitiveness of host plants. These substances being considered as of biotechnological interest to exploit for pharmaceutical drugs and agriculture crop protection. Rare and important microbial compounds like taxol, cryptocin, cryptocandin, jesterone, oocydin, isopestacin, the pseudomycins, ambuic acid etc., were purified from the several endophytic microbes such as fungi, bacteria and actinomycetes for medicine and agriculture. (Hallman, 2001; Strobel, 2002). General conceptions of microbial symbionts present in soil and roots can be exploited for the agriculture is in transition to endophytic microflora as it is most promising area for agriculture profit. In the last decade, a diverse endophytic microflora, including fungi (Redlin and Carris, 1996; Saikkenen et al., 1998; Girlanda et al., 2002; Arnold et al., 2004; Bacon et al., 2006) and bacteria was isolated and identified by various biochemical and cultural
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morphology, further in advancement in identification, DNA-based methods employed to identify the endophytes isolated from various symptomless parts of plants (Barac et al., 2004).

Endophytic beneficial bacteria are the major form in total endophytes, play an important role in the sustainability of agro ecosystems are used for plant growth promotion and disease management (Benhamou et al., 2000; Hallman, 2001; Iniguez et al., 2005). Whilst endophytic bacterial colonization enhances the competitive ability of the plants to resist pest and diseases of plants by inducing the systemic resistance known to release and enhance the pathogenesis related proteins, enzymes and antimicrobial compounds (Tuzun and Kloeper, 1995; Chen et al., 1995; Benhamou et al., 1996; Van Loon, 1997; Benhamou et al., 1998; Manjula et al., 2002; Ongena et al., 2004). The effect of colonizing and its useful effect on host have made endophytic bacteria valuable for agriculture as a tool to improve crop performance in the era of organic farming, especially for those bacteria having commercial features such as plant growth promotion and activation of plant defense mechanisms. Growing awareness of the environmental damage caused by the use of the chemical substances for plant disease control in agriculture has raised the need to study biological alternatives, such as activating the defense responses of the plant crops by inducers that are not toxic to the environment and host as well. The rationale of the present study is to explore the newer bacterial populations for enhancing the competitiveness of pearl millet against downy mildew disease of pearl millet.
Introduction

The word endophyte is derived from Greek: Endo- meaning within, and Phyte- meaning plant. Since the first report (de Bary, 1866) endophytes have been discovered in high numbers within different tissues of various plants, besides the mycorrhizal roots, fungal endophytes have been most studied in grasses and tree leaves (Redlin and Carris, 1996). Endophytic bacteria have been defined variously by several groups of researchers. Kado, 1992 defined as bacteria that reside within the plant tissues without doing substantive harm or gaining benefit other than securing residency. Quispel, 1992 considered as endophytic bacteria that establish an endosymbiosis with the plant, whereby the plant receives an ecological benefit from the presence of symbiont, such as increased stress tolerance or plant growth promotion. From the functional point of view, Hallmann et al. (1997) considered a bacterium as an endophyte if it can be isolated from surface disinfected plant tissue or extracted from inside the host and if it does not visibly harm the plant.

Endophytic microorganisms are to be found in virtually every plant organs on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Bacterial endophytes have most frequently been detected in the nonsymbiotic root and vascular tissues of several non-leguminous plants (Hallman et al., 1997; James and Olivares, 1998). Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture and industry. Novel antibiotics, antimycotics, immunosuppressants and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products. The prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great. Other applications in industry and agriculture may also be discovered among the novel products produced by endophytic microbes (Strobel et al., 2004).

Major traits of how endophytic bacteria can affect plant health include: 1) direct antagonism or niche exclusion of the pathogen, 2) induction of systemic
resistance, and 3) increasing plant tolerance towards biotic stresses. Besides these obvious effects, plants are often colonized by endophytic bacteria not showing any effect at all. The bacteria form either a neutral association with the plant or they reside latent until becoming active in later stages of plant development. What makes a bacterium an endophyte and why are plant defense mechanisms not activated? Why does the plant tolerate these intruders? These are all questions not yet been sufficed and in the future research might help to understand plant and endophyte interactions. Endophytic and epiphytic bacteria may increase the fitness of the plant host by increasing resistance to stress conditions, alterations in the physiologic conditions, fixation of atmospheric nitrogen, nutrient supplying and plant growth regulators production. The major genera of endophytic bacteria were identified as belonging to the genera *Pseudomonas, Ralstonia, Enterobacter, Pantoea, Bacillus* and *Acinetobacter* (Hallamnn, 2001; Kuklinsky-Sobral et al., 2004).

**Isolation of endophytic bacteria**

As the endophytic bacteria resides within the plant tissues several techniques and methods have been employed and successfully isolated numerous endophytic bacteria belongs to various classes. Sharma et al. (2005) isolated the endophytic bacterial species related to *Rhizobium, Agrobacterium* from wheat roots. A total of 150 bacterial isolates were recorded from different cultivar of wheat roots and characterized based specific media and 16r DNA. Colonization of wheat roots was established by inoculating externally proves the versatility of the commercial exploitation.

Of 102 rhizoplane and endophytic bacteria isolated from rice roots and stems in California, 37% significantly inhibited the growth in vitro of two pathogens, *Achlyya klebsiana* and *Pythium spinosum*, causing seedling disease of rice. Four endophytic strains were highly effective against seedling disease in growth pouch assays, and these were identified as *Pseudomonas fluorescens* (S3), *Pseudomonas tolaasii* (S20), *Pseudomonas veronii* (S21), and *Sphingomonas trueperi* (S12) by sequencing of amplified 16S rRNA genes. Strains S12, S20, and S21 contained the nitrogen fixation gene, nifD, but only S12 was able to reduce acetylene in pure culture. The four strains significantly enhanced plant growth in the absence of pathogens, as evidenced by increases in plant height and dry weight of inoculated rice seedlings relative to noninoculated rice. Three bacterial strains (S3, S20, and S21) were evaluated in pot bioassays and reduced disease incidence by 50-73%. Strain S3 was as effective at
suppressing disease at the lowest inoculum density \((10^6 \text{ cfu/mL})\) as at higher density \((10^8 \text{ cfu/mL})\) or undiluted suspension. This study indicates that selected endophytic bacterial strains have potential for control of seedling disease of rice and for plant growth promotion (Adhikari et al., 2001).

One hundred and thirty-three bacterial strains were isolated from inner tissue of potato tubers collected from Datong, Taiyuan and Inner Mongolia Autonomous regions. On the basis of antagonistic examination \textit{in vitro}, greenhouse and field tests, five strains named as 069, 085, 110, 116 and 118 were chosen for their suppression of bacterial ring rot or their growth promotion. Strain 118 is an endophytic bacterium with three effects of colonization, growth promotion and suppression of the pathogenic bacteria, showing good prospects for commercial use (Yuan et al., 2002).

Endophytic and epiphytic bacteria were isolated from two soybean cultivars. Significant differences were observed in bacterial population densities in relation to season of isolation, soybean growth phase and the tissues from which the isolates were obtained. The isolates were identified by partial 16S rDNA sequence analysis, with most of the isolates belonging to the \textit{Pseudomonaceae}, \textit{Burkholderiaceae} and \textit{Enterobacteriaceae} groups. The potential of the isolates for plant growth promotion was evaluated by screening for indoleacetic acid (IAA) production and mineral phosphate solubilization; 34% of endophytic bacteria produced IAA and 49% were able to solubilize mineral phosphate. A high frequency of IAA producing isolates occurred in the early ripening Foscarin cultivar whereas a high percentage of phosphate solubilizing isolates were obtained from plants in the initial development stage (V6). Furthermore, authors also found that 60% of endophytic and 69% of epiphytic isolates that produced IAA and solubilized mineral phosphate were also able to fix nitrogen \textit{in vitro}. The soybean-associated bacteria showing characteristics related to plant growth promotion were identified as belonging to the genera \textit{Pseudomonas}, \textit{Ralstonia}, \textit{Enterobacter}, \textit{Pantoea} and \textit{Acinetobacter} (Kuklinsky-Sobral et al., 2004).

Three hundred and ninety-three groundnut-associated bacteria, representing the geocarposphere, phylloplane and rhizosphere and endophytes were applied as seed treatment in greenhouse. Maximum increase in plant biomass (up to 26%) was observed following treatment with a rhizosphere isolate identified as \textit{Bacillus firmis} GRS 123, and two phylloplane isolates \textit{Bacillus megaterium} GPS 55 and \textit{Pseudomonas aeruginosa} GPS 21. Actively growing cells and peat formulations of
GRS 123 and GPS 55, and actively growing cells of GPS 21, significantly increased the plant growth and pod yield (up to 19%) in field. Rifampicin-resistant mutants of GRS 123 and GPS 21 colonized the ecto- and endorhizospheres of groundnut, respectively, up to 100 days after sowing, whereas GPS 55 was recovered from both the habitats at 100 DAS. Seed bacterization with phylloplane isolates promoted groundnut growth indicating the possibility of isolating rhizosphere beneficial bacteria from different habitats (Kishore et al., 2005).

**Growth promotion by Endophytic bacteria**

A major factor influencing plant growth and health is the microbial population living in the rhizosphere and within the healthy plant tissues. The endophytes are able to colonise the internal tissue of the plant and play a beneficial role directly within the plant, where conditions are optimum for its survival and effectiveness. Several endophytic bacteria have been shown to promote plant growth in different plant species.

Under glasshouse conditions, endophytic bacteria *Herbaspirilla* sp., inoculation after 30 days resulted in increased growth in rice varieties cvs IR45 and Moroberekan and also showed significantly greater nitrogenase activity. In a long-term experiment, by 120 d, cv. Moroberekan showed a significant increase in N content after inoculation. *Herbaspirilla* sp., were localized on and within roots and aerial parts of cvs Moroberekan and IR45 under growth conditions (Gyaneshwar et al., 2002).

Bacterial Endophytes reside within the interior of plants without causing disease or forming symbiotic structures. Some endophytes, such as *Klebsiella pneumoniae* 342 (Kp342), enhance plant growth and nutrition and data are presented to support the hypothesis that plant defense response pathways regulate colonization by endophytic bacteria. (Boddey et al., 2003).

A bacterial strain *Burkholderia* sp. MSSP was isolated from surface-sterilized root nodules of *Mimosa pudica*. MSSP was Gram-negative, capsulated, motile, non-endospore forming rod with free nitrogen (N) fixation ability. Unlike N-fixing bacteria forming symbiotic relationship with legumes that largely exist in α-subclass of proteobacteria, MSSP belongs to β-class of proteobacteria. Phylogenetic analysis of 16 S rDNA demonstrated that MSSP belongs to the genus *Burkholderia*. This isolate secretes phytohormone, ACC deaminase, solubilizes phosphate and is antagonistic against phytopathogens. *Burkholderia* sp. MSSP was positive for almost all these
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characteristics, indicating its role in promotion of growth of host plant while colonizing the roots (Pandey et al., 2005).

Three *Bacillus* strains, *B. subtilis* NEB4 and NEB5 and *B. thuringiensis* NEB17, plant growth promoting bacteria were isolated from inside the nodules of vigorous field-grown soybean plants and were shown to have plant growth promoting activity on pouch-grown soybean plants under greenhouse conditions and also help to overcome the deleterious problems. To test their ability to improve soybean nodulation and growth under low RZTs, these strains were co-inoculated onto soybean plants, with *Bradyrhizobium japonicum*, under greenhouse conditions at root zone temperatures (RZTs) of 25, 17, and 15°C, and under field conditions in a short growing season area. In all cases, the experiments were conducted with soybean cultivar OAC Bayfield. All the three *Bacillus* strains enhanced soybean nodulation and growth in greenhouse and field experiments. Co-inoculation with NEB17 provided the largest and most consistent increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen, and grain yield. The other two strains provided positive responses in only one of the two year of field-testing. Thus, *B. thuringiensis* NEB17 would be suitable for use as a plant growth promoting bacterial strain in soybean production systems in short growing season regions (Rita et al., 2005).

Chi et al., 2005 studied the effect of root nodule endosymbionts of leguminous plants and also form natural endophytic associations with roots of important cereal plants. Examined the infection, dissemination, and colonization of healthy rice plant tissues by four species of *gfp*-tagged rhizobia and their influence on the growth physiology of rice and their movement from root to leaves and found to be high population. Rice plants inoculated with certain test strains of *gfp*-tagged rhizobia produced significantly higher root and shoot biomass, increased their photosynthetic rate, stomatal conductance, transpiration velocity, water utilization efficiency, and flag leaf area and accumulated higher levels of indoleacetic acid and gibberellin growth-regulating phytohormones

**Mechanism of growth promotion and disease protection**

**Production of phytohormones**

Some of the Endophytic bacteria are known for their growth promotion and these groups of bacteria are capable of producing auxins, cytokinins, gibberellins, and
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Ethylene or abscisic acid. Cytokinins and gibberellins produced by several bacteria promote root formation and cytokinins are signals involved in mediating environmental stresses from roots to shoots (Jackson, 1993). Ethylene, mediates several responses to developmental and environmental signals in plants and involvement in plant growth when excreted around the roots has been shown (Arshad and Frankenberger, 1998). Auxin production results in lateral root emergence, increases the density and length of root hairs as well as the elongation rates of lateral roots, increasing the root surface area. IAA may stimulate plant cell proliferation or elongation and results in plant production of ACC (Dobbelaere et al., 2001).

Production of enzymes

Genetic and biochemical experiments provide strong evidence that phytase activity of Bacillus species is important for plant growth stimulation under phosphate limitation. Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributed to its plant-growth-promoting effect. Diluted culture filtrates of B. amyloliquefaciens FZB45 stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. The amino acid sequence deduced from the phytase phyA gene cloned from FZB45 displayed a high degree of similarity to known Bacillus phytases (Idriss et al., 2002).

ACC deaminase (1-aminocyclopropane-1-carboxylate) activity

Recent work has shown that the inhibitory effect of ethylene on plant root elongation can be reduced by the activity of ACC deaminase, an enzyme produced by some rhizosphere soil microorganisms, including rhizobacteria. This enzyme catalyzes the cleavage of ACC to ammonia and \( \alpha \)-ketobutyrate. A model describing the function of ACC deaminase in plant growth-promoting rhizobacteria was suggested by Glick et al. (1998). *Pseudomonas* and *Azospirillum* strains into which ACC deaminase genes have been introduced also gained the ability to promote the elongation of roots of canola seedlings (Shah et al., 1998; Glick et al., 1998; Holguin and Glick 2001; Ma et al., 2002).

In addition, by lowering ethylene levels, ACC deaminase-containing bacteria protect plants from the deleterious effects of numerous environmental stresses, including phytopathogens (Wang et al., 2000), flooding (Grichko and Glick, 2001), metals (Burd et al., 1998, 2000; Grichko et al., 2000; Nie et al., 2002), drought and salt (Mayak et al., 2004).
Gene expression studies over time in canola roots treated with the ACC deaminase containing bacteria *Enterobacter cloacae* indicated that roots isolated from canola seeds treated with the ACC deaminase-producing *E. cloacae* upregulated genes involved in cell division and proliferation but down-regulate stress genes. This data is in agreement with a model in which ACC deaminase-containing PGPR reduce plant stress and induce root elongation and proliferation in plants, largely by lowering ethylene levels (Hontzeas *et al.*, 2004). The down regulated genes included one encoding a glycine-rich RNA binding protein with a function in RNA processing or binding during ethylene-induced stress, which is expressed only in roots, and another gene thought to be involved in a defense signaling pathway.

Fifty-three out of the 435 selected bacterial isolates were found to be antagonistic against *V. dahliae* and several other soil borne pathogens. Significant biocontrol activity against *V. dahliae* in glasshouse trials was demonstrated in three of 18 evaluated antagonistic isolates, identified as *Bacillus* sp. Finally, two of the most effective bacterial isolates, designated as K-165 and 5-127, were shown to be rhizosphere colonizers, very efficient in inhibiting mycelial growth of *V. dahliae* in dual cultures and successfully controlling *Verticillium* wilt of *solanaceous* hosts. In glasshouse experiments, root dipping or soil drenching of eggplants with bacterial suspension of $10^7$ cfu ml$^{-1}$ resulted in reduced disease severity (40–70%) compared to controls under high *V. dahliae* inoculum level. In heavily *Verticillium* infested potato fields, experiments with potato seeds dusted with a bacterial talc formulation ($10^8$ cfu g$^{-1}$ formulations), showed significant reduction in symptom development of diseased potato plants and a 25% increase in yield over the untreated controls. As for their effectiveness in increasing plant height, both bacterial isolates K-165 and 5-127 produced indolebutyric, indolepyruvic and indole propionic acids (Tjamos *et al.*, 2004).

**Parasitism and production of extracellular enzymes**

The ability of bacteria, especially actinomycetes, to parasitize and degrade spores of fungal plant pathogens is well established (El-Tarabily *et al.*, 1997). Assuming that nutrients pass from the plant pathogen to bacteria, and that fungal growth is inhibited, the spectrum of parasitism could range from simple attachment of cells to hyphae, as with the *Enterobacter cloacae* (Jordan) Hormaeche & Edwards–*Pythium ultimum* interaction to complete lysis and degradation of hyphae as found with the *Arthrobacter–Pythium debaryanum* interaction (Mitchell and Hurwitz, 1965).
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If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort have been done to identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. For example, biocontrol of Phytophthora cinnamomi Rands root rot of Banksia grandis Willd. was obtained using a cellulase-producing isolate of Micromonospora carbonacea Luedemann & Brodsky (El-Tarabily et al., 1996) and control of Phytophthora fragariae var. rubi Hickman causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of β-1,3-, β-1,4- and β-1,6-glucanases (Valois et al., 1996). Chitinolytic enzymes produced by both Bacillus cereus Frankland and Pantoea (Enterobacter) agglomerans (Beijerinck) Gavini et al. also appear to be involved in biocontrol of Rhizoctonia solani Kühn (Cherin et al., 1995; Pleban et al., 1997). Tn5 mutants of E. agglomerans (Beijerinck) Gavini et al. deficient in chitinolytic activity were unable to protect cotton (Gossypium barbadense L.) and expression of the chiA gene for endochitinase in Escherichia coli (Migula) Castellani & Chalmers allowed the transformed strain to inhibit R. solani on cotton seedlings. Similar techniques involving Tn5 insertion mutants and subsequent complementation demonstrated that biocontrol of Pythium ultimum in the rhizosphere of sugar beet by Stenotrophomonas maltophila (Hugh) Palleroni and Bradbury W81 was due to the production of extracellular protease (Dunne et al., 2000).

Induced resistance

Perhaps the greatest growth area in biocontrol in the last few years has been concerned with induced resistance defined as the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (Kloepper et al., 1992; 2004). The effect had previously often been overlooked through inadequate techniques or controls as well as the biocontrol agent exhibiting other modes of action at the same time. Most work has focused on the systemic resistance induced by non-pathogenic rhizosphere-colonizing Bacillus and Pseudomonas species in systems where the inducing bacteria and the challenging pathogen remained spatially separate for the duration of the experiment, and no direct interaction between the bacteria and pathogen was possible (Sticher et al., 1997; van Loon, 1997). Such split root or spatial root inoculation experiments were used to
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demonstrate the phenomenon in radish (*Raphanus sativus* L.) and *Arabidopsis* against *Fusarium oxysporum* (Leeman *et al.*, 1996; van Wees *et al.*, 1997) and in cucumber (*Cucumis sativus* L.) against *Pythium aphanidermatum* (Edson) Fitzp. (Chen *et al.*, 1999). Various combinations of timing and position have indicated that induced resistance also occurs in carnation (*Dianthus caryophyllus* L.) (van Peer *et al.*, 1991), tobacco (*Nicotiana tabacum* L.) (Maurhaufer *et al.*, 1994) and tomato (*Lycopersicon esculentum* Mill); (Duijff *et al.*, 1997). Bacteria differ in ability to induce resistance, with some being active on some plant species and not others; variation in inducibility also exists within plant species (van Loon, 1997). The full range of inducing moieties produced by bacteria is probably not yet known, but lipopolysaccharides (Leeman *et al.*, 1996) and siderophores (Metraux *et al.*, 1990; Leeman *et al.*, 1996) are clearly indicated.

The definition of induced resistance suggested by Kloeppper group covered both biotic and abiotic inducers (Kloepper *et al.*, 1992). Although the phenotypic effects of root inoculation with bacteria may be similar to treatment with abiotic agents or microorganisms that cause localized damage, the biochemical and mechanistic changes appear to be subtly different. This has resulted in the term induced systemic resistance (ISR) for bacterially induced resistance and systemic acquired resistance for the other forms (Pieterse *et al.*, 2000). The major differences are that pathogenesis-related (PR) proteins such as chitinases, β-1, 3-glucanases, proteinase inhibitors and one or two other rarer types, are not universally associated with bacterially induced resistance (Hoffland *et al.*, 1995) and salicylic acid (a known inducer of SAR) is not always involved in expression of ISR, but this is dependent on bacterial strain and host plant involved (Pieterse *et al.*, 1999; de Meyer *et al.*, 1999; Chen *et al.*, 1999). Ethylene responsiveness may also be required at the site of inoculation of the inducing bacteria for ISR to occur (Knoester *et al.*, 1999).

Changes that have been observed in plant roots exhibiting ISR include: (a) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (Benhamou *et al.*, 1996; 2000; Duijff *et al.*, 1997; Jetiyanon *et al.*, 1997; M'Piga *et al.*, 1997) (b) increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase (M'Piga *et al.*, 1997; Chen *et al.*, 2000) (c) enhanced phytoalexin production (van Peer *et al.*, 1991; Ongena *et al.*, 1999) (d) enhanced expression of stress-related genes (Timmusk and Wagner, 1999). However, not all
these biochemical changes are found in all bacterial–plant combinations (Steij et al., 1999). Similarly, the ability of bacteria to colonize the internal tissue of the roots has been considered to be an important feature in many of the bacterial–root interactions involving ISR, but is not a constant feature of them all.

**Bioactive molecules**

The microbes inhabiting in biosphere valued as repository of important biocatalysts and bioactive compounds for industrial, agricultural and pharmaceutical applications. Hence, microbial diversity constitutes an infinite pool of novel chemistry, making up a valuable source for innovative biotechnology. The most recent estimates evinced approximately 5% of the total species of fungi and may be as little as 0.1 % of the bacteria have been studied and few of them exploited for mankind usage. Amongst, few were examined for metabolite profile. The microbial secondary metabolites can be subjugated in three different ways: the bioactive molecule can be produced directly by fermentation; or the fermentation product can be used as starting material for subsequent chemical modification or thirdly the molecules can be used as lead compounds for a chemical synthesis (Moncheva et al., 2001). However, the chemical composition of microbialy produced bioactive compounds are often intriguingly complicated, including stereo-chemical diversities which in many instances will make chemical synthesis close to impossible at least seen from an economical perspective.

Bioactive molecules from microbes as products, which are produced by biotechnological applications used with and/or without chemical modification in agriculture. Some of the microbial products such as Avermectins, Blasticidins, Kasugamycins, Validamycins can be used directly from fermentation technology without modification. On otherhand, some of the products cannot be used directly as fermentation products such as Strobilurins and Pyrrolnitrine molecules which need to be stabilized with chemical modifications to avoid the unacceptable residue. In case of endophytic bacterial group it is established that Bacillus and Pseudomonas species are common producers of peptides or modified peptides, which can be used for development of agrochemical formulations (Strobel and Daisey, 2003; Cooper, 2004) accentuate the importance on endophytic microbes for beneficial exploitation.

In the era of modern biotechnology, after surpassing the traditional isolation and cultivation of microbes to the technology of high throughput, automated, recombinant screening systems of enzyme or drug discovery efforts have yielded over
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700 unique enzymes and a collection of novel bioactive molecules. This would be the important platform for combinatorial approach for biocatalyst development and recombinant natural product discovery (Moncheva et al., 2001). Discovery of novel lead compounds of microbial origin considered to have great potentials for further industrial applications. However, realization of these potentials will require ingenuity and intensive research at both basic and applied levels, particularly progress in basic science on microbial diversity seems to be a key to future innovations under the current circumstances. Novel natural products and the organisms that make them offer opportunities for innovation in drug and agrochemical discovery paving way for exciting possibilities for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology, and their potential usefulness.

Endophytes currently considered to a wellspring of novel secondary metabolites offering the potential for medical, agricultural, and industrial exploitation. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it appears that these bio-typical factors can be important in plant selection for studying the endophytes as they govern the novelty and biological activity of the products associated with endophytic microbes (Strobel and Daisey, 2003).

Commercialization of endophytic bacteria

In recent years it has become evident from public opinion and environmental concerns, that new and safer alternative to traditional synthetic pesticides are both desirable and mandated. Due to prolonged use of pesticides, there is significant interest in finding alternative control strategies for use in integrated pest management approach for plant diseases.

Although endophytes were already described in the past century, endophytic microorganisms only received substantial attention in the last two decades, because of their capability to protect their hosts against insects-pests, pathogens. As endophytic fungi and bacteria started to be better analysed, it became clear that they could confer other important characteristics to plants, such as greater resistance to stress conditions alteration in physiological properties, production of phytohormones and other compounds of biotechnological interest (i.e. enzymes and pharmaceutical drugs). In addition to the economical aspects, the study of endophytic microorganisms has
strong academic interests, concerning the discovery of new microbial species, mainly when tropical hosts are investigated (Azevedo et al., 2000).

These are different commercially available microbial formulation and plant growth stimulants and production companies namely: Agracetus, Inc. (Middleton, WI), Gold Coat (tm) microbial seed treatment for soybeans, CelPril (Manteca, CA), Rhizo-Kote XL forage legume seed inoculants (rhizobia), LiphaTech, Inc. (Milwaukee, WI), Nitratin (tm) nitrogen-fixing soil bacterial soil/seed inoculants (rhizobia), Plant Genetics, Inc. (Saskatoon, SK, Canada), Gel-Coat (tm) for woody plants and ornamentals, R&A Plant-Soil,Inc.(Pasco,WA)-Alga-Tilth® microalgal soil conditioners (http://www.nal.usda.gov/bic/Misc_pubs/bioprod.html).

To develop a biocontrol strategy against *Rhizoctonia solani* which causes yield losses in numerous economically important crop, three potato-associated ecto and endophytically living bacterial strains *Pseudomonas fluorescens* B1, *Pseudomonas fluorescens* B2, and *Serratia plymuthica* B4 were evaluated against *R. solani* in potato and in lettuce. The disease-suppression effect of the 3 biocontrol agents (BCAs) was tested in a growth chamber and in the field. In growth chamber experiments, all 3 BCAs completely or significantly limited the dry mass (DM) losses on lettuce and the disease severity (DS) caused by *R. solani* on potato sprouts. Strain B1 showed the highest suppression effect (52 % on average) on potato. Under field conditions, the DS on both crops, which were bacterized, decreased significantly, and the biomass losses on lettuce decreased significantly as well. The greatest disease-suppression effect on potato was achieved by strain B1 (37%), followed by B2 (33%) and then B4 (31%), whereas the marketable tuber yield increased up to 12% (B1), 6% (B2), and 17% (B4) compared with the pathogen control at higher disease pressure. Furthermore, in all experiments, B1 proved to be the most effective BCA against *R. solani*. Therefore, this BCA could be a candidate for developing a commercial product against *Rhizoctonia* diseases (Rita et al., 2005).

The addition of nutritional supplements in combination with biocontrol agents has been reported to improve disease control. This improvement in control may be the result of improved growth of the BCA, stimulation of host defense mechanisms, inhibition of the pathogen by the supplement, or a combination of these and other factors. Bacterial biocontrol agents such as Serenade™ (*Bacillus subtilis*) (Walton, 2002) are registered for control of grape botrytis and powdery mildew, and Blossom
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Bless® (Pantoea agglomerans) (Vanneste et al., 2002) for control of fire blight (Erwinia amylovora), a bacterial disease of pears and apples.

Stephan et al., 2005 screened 22 biocontrol products and plant extracts against late blight of potato caused by Phytophthora infestans using detached leaf bioassay and on potted plants. The 10 most active treatments were selected for further investigation. In the detached leaf assays the commercial preparations Elot-Vis, Serenade and Trichodex, and plant extracts of Rheum rhabarbarum and Solidago canadensis showed a significant effect on the level of infestation by P. infestans. In the case of Serenade, the metabolites produced by its active microorganism, Bacillus subtilis were demonstrated to be the effective component of the formulation, and not the microorganism itself. In order to take curative and protective modes of action into account, the test substances were applied 24 h before, or 90 min after inoculation with P. infestans. Generally, better effects were obtained when the applications were made 24 h before inoculation. For defining the optimum time of application, potted plants were treated 72 or 24 h before, and 1 and 24 h after inoculation with P. infestans. In these tests, Trichodex showed no activity, while Elot-Vis gave best results when applied 1 day before inoculation. Serenade and the extracts of R. rhabarbarum and S. canadensis (at all 5% concentration) however, were effective when applied up to 3 days before and just after inoculation with P. infestans. The results of the experiments on potted plants indicated direct effects on the pathogen for all agents except the extract of S. canadensis, but other mode of actions, induced resistance, could not be ruled out.
Future prospects

To reduce the dependence on chemical crop protectants for disease control in agriculture, biological agents are receiving increasing attention. Endophytic bacteria mediated biocontrol can be extended to foliar and systemic diseases, even when the endophytes are applied only to seeds and roots, if the mechanism for control involves induction of host defenses. Conceptually, it should also be possible to induce resistance via the use of some of the techniques described above to enhance antagonism of indigenous soil bacteria. For example, use of certain organic amendments such as chitin such as furfural could act, as elicitors and further work should be aimed at testing this hypothesis. As described, plant associated bacteria can have diverse interactions with plant roots. We are beginning to be able to direct these interactions into practical benefits for crops by the introduction of specific endophytic or PGPR bacteria or by the targeted enhancement of components of the indigenous soil bacterial community. The future challenge will be to integrate these approaches to provide an economically significant level of induced suppressiveness against crop.

The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and spend time isolating microbial components. This may facilitate the product discovery processes.

Finally, perhaps the greatest interest in the future lies with the application of modern molecular techniques and their integration with conventional experimental procedures to understand and utilize soil plant microbe interactions. The significance of these techniques has already been described with the monitoring of endophytic bacterial agents and their impact as microbial populations in understanding the modes of actions of endophytic bacteria particularly with induced resistance in plants.

With this background the proposed work is aiming to investigate the importance of endophytic bacteria in improving the seed quality parameters such as seed germination, seedling vigor and their role in eliciting the defense reactions in pearl millet plants against downy mildew disease.
MATERIALS AND METHODS

Isolation and identification of endophytes

Isolation: Five medicinal plants Cymbopogon citratus, Boerhaavia diffusa, Azadirachta indica, Phyllanthus emblica, Tinospora cardifolia two agricultural crop plants Pisum sativum, Sorghum bicolor and one weed plant Parthenium hysterophorus were selected in the present study to isolate the endophytic bacteria and to study their efficacy to induce the systemic resistance in pearl millet against downy mildew disease (Table 2.1). The plants were washed thoroughly in running tap water. The plant parts viz., stem, root and leaf regions were separated and cut into 1cm pieces and surface sterilized with sodium hypochlorite (2%) containing 0.1% Tween 20 for 3 minutes. The disinfectant was removed by rinsing plant parts using sterile distilled water for five times, dried on sterile paper towels (Hallmann et al., 1997; Zinniel et al., 2002) and then pressed onto nutrient agar. The plant parts were also macerated separately (leaf, stem and root) with sterile mortar and pestle and tissue extracts were then serially diluted in sterile water and plated in triplicate to recover bacterial endophytes present in the plant tissue. Inoculated plates were incubated at 27 ± 2 °C for 24 to 48h. Endophytic isolates were isolated and sub cultured using nutrient agar and identified using specific media. Recovered isolates of bacteria were stored at – 40 °C for further experiments.

Identification: Isolated Endophytic bacteria were identified based on gram staining and others characters such as color, form, elevation, margin, diameter, surface, opacity, texture, endospore formation. Biochemical tests like catalase test, KOH solubility test and oxidase test (Sneath et al., 1986) were also used for further identification. After identifying the isolates, they were individually tested in replicates for their effect on germination and vigour index and their ability to protect the pearl millet plants upon pathogen inoculation in greenhouse condition. Subsequently the effective bacterial isolates were selected for further studies in field condition and growth promotion studies.

Host: Seeds of pearl millet cv. 7042S (highly susceptible) to the downy mildew pathogen S. graminicola
Source of pathogen and inoculum preparation as described in chapter 1

Effect of endophytic bacterial isolates on asexual spores of *S. graminicola* to study the fungitoxicity of the inducers

To study the effect of the endophytic bacterial isolates on sporulation of *S. graminicola*, infected leaves were collected, washed with running tap water to remove the existing sporulation and cut into pieces. The leaf bits were smeared on the abaxial side with the test bacterial suspension and were incubated inside a moist petriplate and observed for the sporulation the next morning. Leaf bits treated with sterile distilled water served as control and those treated with metalaxyl (Apron 35SD) served as chemical control. A sporangial suspension of *S. graminicola* was mixed (1:1 v/v) with all the test endophytic bacterial isolates solutions in different concentrations like 10^6 and 10^8 cfu/ml and incubated in dark. During the incubation period the solutions were observed under the microscope for release of zoospore. Sporulation and mobility of zoospores is a factor of infectivity and was rated as 100% (+ + +), ≥50% (+ +), ≥25% (+) and immobile (-) based on proportion of spores showing sign of motility. A minimum of five microscopic fields was observed in three independent experiments for each treatment.

Preparation of bacterial isolate and effect of seed treatment on seed quality parameters

Bacterial cultures grown in nutrient agar were inoculated to nutrient broth for 48 h to obtain spore/cells for use as seed treatment. Bacterial suspensions were centrifuged at 6,000 revolutions per min (rpm) for 15 min. The pellet obtained was resuspended in sterile distilled water and the optical density of the suspension was spectrophotometrically adjusted at 610 nm (Hitachi U-2000, Japan) to obtain density of 1 x 10^8 cfu ml⁻¹ (Umesha *et al.*, 1998; Niranjanraj *et al.*, 2003). The suspension was used for pearl millet seed treatment. Seeds of pearl millet were surface sterilized with 2% sodium hypochlorite (2 min) and washed in sterile distilled water, dried on sterile blotter paper and soaked in the bacterial suspension (1 x 10^8 cfu mL⁻¹) at 25 ± 2 °C for 6 h at 150 rpm in rotary shaker. Seeds treated with endophytic bacteria (1 x 10^8 cfu mL⁻¹) were dried under shade overnight and placed on moist germination paper and incubated at 26 ± 2 °C. Seeds treated with sterile distilled water served as control. Seeds treated with Apron 35SD @ 6g/kg seeds served as positive controls.
Chapter 2

Endophytic bacteria

Germination test was carried out according to International seed testing association (ISTA, 2003) and vigor index (VI = Mean Root Length + Mean Shoot Length x (% Germination) was calculated at the end of 7 days (Abdulbaki and Anderson, 1973). Four replicates of 100 seeds were used per treatment.

Efficiency of bacterial isolates in inducing resistance against downy mildew disease in greenhouse and field conditions

Greenhouse conditions: Seeds treatment was carried out as described previously. Seeds treated with sterile distilled water served as the non-treated control. Seeds treated with the systemic fungicide metalaxyl formulation Apron 35 SD at 6g/kg concentration served as a positive control. The treated seeds were sown in earthen pots filled with autoclaved soil, sand and manure at the ratio of 2:1:1. Each treatment consisted of 4 replications, 20 pots per replication with 20 seedlings per pot. Three-day-old seedlings were challenge-inoculated by the whorl inoculation method (Singh and Gopinath, 1985) with the zoospore suspension of *S. graminicola* at a concentration of 40,000 zoospores/ml prepared as described earlier. Plants were maintained under greenhouse conditions (90-95% RH, 20-27°C temperature) and observed for disease development. The plants were rated diseased when they showed any one of the typical downy mildew symptoms such as sporulation on the abaxial leaf surface, chlorosis, stunted growth, or malformation of the earheads. Downy mildew disease incidence was recorded at 30 days after sowing (DAS). The experiment was repeated three times. For studying the colonization ability of the tested bacterial isolates pearl millet tissues like stem, leaf and roots were separately analyzed in 15 days interval up to 60 days.

Field conditions: Field trials were designed to test the resistance reaction elicited by bacterial isolates, which were effective in greenhouse conditions. The trials were conducted at the Mysore University Downy Mildew Nursery (in an area with soil that was heavily loaded with oospores of *S. graminicola*). Treatments and the controls were the same as previously described. Soil-borne oospores of *S. graminicola*, served as the source of primary inoculum. Additional inoculum was provided by infector rows that were raised 21 days prior to the raising of the test rows as described by Williams (1984). Each treatment consisted of four replications. Each replicated row was manually seeded with 100-150 seeds per row. The experiment was a randomized complete block design. The plants were rated diseased when they showed any one of the typical downy mildew symptoms described above. Downy mildew disease
incidence was recorded at 30 DAS, and final counts were made at 60 DAS. The experiment was repeated three times.

**Effect of seed treatment with inducers on growth promotion of pearl millet**

Seed treatment was done as described above. The seeds were sown in earthen pots and maintained in greenhouse as described earlier. At 30 days after sowing (DAS), vegetative growth parameters such as height, fresh weight, dry weight and number of basal tillers per plant were recorded. The treated pearl millet plants were also observed for (a) Total number of productive tillers (b) Number of days required to 50% flowering (c) height of the plant during flowering (d) Length and girth of the ear head and (e) 1000 seed weight and in comparison with untreated plants (Shailashree *et al.*, 2001).

**Study of the nature of resistance induction**

Systemic nature of protection was demonstrated by maintaining spatio-temporal separation of the inducer treatment and the pathogen inoculation as described earlier (Shailashree *et al.*, 2001). Pearl Millet seeds were plated on moist blotters and were incubated at $25\pm2^\circ C$ in an incubator. 36 h later the roots of the seedlings were treated with the inducer by soaking the roots in the suspension of inducers for 6 h and the seedlings were transplanted into earthen pots filled with soil, sand and manure in the ratio 2:1:1. Seeds treated with distilled water served as control. The seedlings were then inoculated with zoospore suspension of *S. graminicola* (40 000 zoospores /ml) following the whorl inoculation procedure with a time gap of 1, 2, 3, 4, and 5 days in different sets of plants. Plants were maintained under greenhouse conditions and were observed for the downy mildew disease reaction and downy mildew disease data recorded as described earlier. The experiment was repeated three times.

**Data analysis:** All the experimental results were subjected to Duncan's multiple range test (DMRT). The means were compared for significance using DMRT ($P \leq 0.05$). All the results are based on two independent experiments.
Figure 2.5: Endophytic *Pseudomonas fluorescens* isolate ISR-36 on Kings B medium isolated from *Azadirachta indica*.

Figure 2.6: Endophytic *Bacillus* sp isolate ISR-38 on nutrient agar medium isolated from *Cymbopogon citratus*. 
RESULTS

Isolation and identification of endophytic bacteria

Five medicinal plants, two agricultural crop plants and one weed plant were tested for the presence of endophytic bacteria. The medicinal plants used were C. citratus, A. indica, P. emblica, B. diffusa, T. cardifolia and two cultivars of P. sativum and S. bicolor and P. hysterophorus. C. citratus is a member of Graminae used for scented oil. A. indica is used for wide range of products such as pesticides and human health care. P. emblica is a rich source of antioxidants. B. diffusa and T. cardifolia are used in herbal medicine to correct liver disorders and osmoregulator. P. sativum and S. bicolor are important legume and cereal crop respectively and harbor wide range of bacteria in root and stems. P. hysterophorus is a weed with plenty of allelopathic chemicals secreted in the rhizosphere and known vulnerable to for endophytic infections as rhizosphere is rich in the nutrients.

Sixty endophytic bacterial isolates were recovered from all the plant species tested in the different inoculation technique. Endophytic bacteria isolated from each plant species were different and they were identified based on gram staining and biochemical tests as described above and tabulated in the Table 2.1. Out of sixty isolates, ten isolates showed promising effects on pearl millet growth and were used for further experiments. Endophytic bacterial isolates recovered were named based on the generic character and few were named on species level. Among the different groups of bacteria isolated, P. fluorescens and Bacillus sp. were predominant group (Fig. 2.5 & 2.6). C. citratus isolate was identified as Bacillus sp. ISR 38, A. indica as P. fluorescens ISR 36, P. emblica as Bacillus sp. ISR 42, B. diffusa as Bacillus sp. ISR 40 and T. cardifolia as P. fluorescens ISR 34, was observed. In P. sativum revealed Bacillus sp. ISR 39 and P. fluorescens ISR 33. S. bicolor revealed P. fluorescens ISR 35 and Bacillus sp. ISR 41 respectively. From P. hysterophorus, the Bacillus sp. ISR 37 was isolated. For each bacterial isolate recovered a specific name was designated and deposited in the culture collection of the department (Table 2.2).
Table 2.1: Endophytic bacteria isolated from root and stem regions of different plant species

<table>
<thead>
<tr>
<th>Category of plants</th>
<th>Plant species</th>
<th>Gram reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram positive</td>
</tr>
<tr>
<td>Medicinal plants</td>
<td><em>Cymbopogon citratus</em></td>
<td>04</td>
</tr>
<tr>
<td></td>
<td><em>Azadirachta indica</em></td>
<td>02</td>
</tr>
<tr>
<td></td>
<td><em>Phyllanthus emblica</em></td>
<td>03</td>
</tr>
<tr>
<td></td>
<td><em>Boerhaavia diffusa</em></td>
<td>02</td>
</tr>
<tr>
<td></td>
<td><em>Tinospora cardifolia</em></td>
<td>01</td>
</tr>
<tr>
<td>Crop plants</td>
<td><em>Pisum sativum</em></td>
<td>04</td>
</tr>
<tr>
<td></td>
<td><em>Sorghum bicolor</em></td>
<td>04</td>
</tr>
<tr>
<td>Weed plant</td>
<td><em>Parthenium hysterophorus</em></td>
<td>04</td>
</tr>
</tbody>
</table>

Table 2.2 Promising endophytic bacterial isolates used in the present study

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Endophytic bacteria</th>
<th>Isolate number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cymbopogon citratus</em></td>
<td>Bacillus sp</td>
<td>ISR 38</td>
</tr>
<tr>
<td><em>Azadirachta indica</em></td>
<td>Pseudomonas fluorescens</td>
<td>ISR 36</td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em></td>
<td>Bacillus sp</td>
<td>ISR 42</td>
</tr>
<tr>
<td><em>Boerhaavia diffusa</em></td>
<td>Bacillus sp</td>
<td>ISR 40</td>
</tr>
<tr>
<td><em>Tinospora cardifolia</em></td>
<td>Pseudomonas fluorescens</td>
<td>ISR 34</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>Bacillus sp.</td>
<td>ISR 39</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas fluorescens</td>
<td>ISR 33</td>
</tr>
<tr>
<td><em>Sorghum bicolor</em></td>
<td>Pseudomonas fluorescens</td>
<td>ISR 35</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp</td>
<td>ISR 41</td>
</tr>
<tr>
<td><em>Parthenium hysterophorus</em></td>
<td>Bacillus sp</td>
<td>ISR 37</td>
</tr>
</tbody>
</table>
Effect of seed treatment with endophytic bacteria on seed germination and seedling vigor

Preliminarily, all sixty isolates were tested for germination and seedling vigour at concentration $1 \times 10^8$ cfu/ml. In general, all the endophytic bacterial isolates significantly enhanced the seed germination and seedling vigor of pearl millet. However, the degree of enhancement varied between the bacterial isolates treatment. The highest germination percentage and vigor index was recorded for the *Pseudomonas* isolates. *P. fluorescens* ISR 33 and *P. fluorescens* ISR 36 achieved 96% germination followed by *Bacillus* sp. ISR 37 and *P. fluorescens* ISR 35 with 94% germination, which was significantly higher when compared with the control (87.5%). The lowest percent germination of 87 was observed in *Bacillus* sp. ISR 39 (Fig. 2.1).

The similar trend was also noticed for the seedling vigor in which all the bacterial isolates enhanced the vigor index to varied degrees. The highest seedling vigor 1507 was recorded for the treatment with *P. fluorescens* ISR36, followed by *P. fluorescens* ISR33, *Bacillus* sp ISR37, ISR35, ISR 38, ISR 42, ISR 40, ISR34, ISR41 and ISR39 ranged from 1482-1292 while control showed 1263.

Effect of endophytic bacterial isolates on asexual spores of *S. graminicola* to study the fungitoxicity of the inducers

All the test endophytic bacterial isolates did not have any fungitoxic effect on *S. graminicola* (Table 2.3). There was no inhibition of the asexual sporulation of *S. graminicola* since the leaf bits treated with the test inducers showed profuse sporulation on the abaxial side. Control leaf bits also showed profuse sporulation whereas in leaf bits treated with metalaxyl there was complete inhibition of sporulation. All the tested endophytic bacterial isolates did not exhibit any effect on the release of zoospore from sporangia, and zoospore mobility at tested concentrations. Complete inhibition of zoospore release was recorded in metalaxyl treatment. In control set there was no inhibition of release of zoospores (Fig. 2.7).

Effect of seed treatment with endophytic bacteria on pearl millet downy mildew incidence under greenhouse and field conditions

**Greenhouse conditions:** Among ten endophytic isolates, sixty isolates of *Bacillus*, 4 isolates of *P. fluorescens* isolates were efficient in reducing the downy mildew incidence compared to control under greenhouse and filed conditions as well (Fig. 2.2). However, the degree of disease reduction varied with the isolates tested ranging from 15.9 to 57.9 %. The maximum protection of 57.9% offered by *P. fluorescens*
Figure 2.1 Effect of seed treatment with endophytic bacteria on seed germination and seedling vigor in pearl millet. Vertical bars indicate standard error. VI – Vigor index.
Table 2.3 Effect of endophytic bacterial isolates on asexual spores of *Sclerospora graminicola* to study the fungitoxicity

<table>
<thead>
<tr>
<th>Endophytic bacterial isolates</th>
<th>Sporulation of asexual spores</th>
<th>Zoospores release from sporangia</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus sp</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bacillus sp</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bacillus sp</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bacillus sp</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bacillus sp</em></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Distilled water</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Apron 35SD</td>
<td>No sporulation</td>
<td>No zoospore</td>
</tr>
</tbody>
</table>

+=25%, ++=50%, +++=100% sporulation and zoospore release.

Table 2.4 Effect of seed treatment with promising endophytic bacteria on vegetative growth parameters of pearl millet plants

<table>
<thead>
<tr>
<th>Endophytic bacteria isolates number</th>
<th>Height of plants (cms)</th>
<th>Fresh weight (g)(average per plant)</th>
<th>Dry weight (g)(average per plant)</th>
<th>Number of basal tillers (average per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISR37</td>
<td>40.5*</td>
<td>16.75*</td>
<td>6.15*</td>
<td>4.5*</td>
</tr>
<tr>
<td>ISR38</td>
<td>38.0*^</td>
<td>16.20*</td>
<td>5.8*ab</td>
<td>4.0*ab</td>
</tr>
<tr>
<td>ISR39</td>
<td>36.0=</td>
<td>12.45*</td>
<td>4.70*ef</td>
<td>3.0*bd</td>
</tr>
<tr>
<td>ISR33</td>
<td>40.0*</td>
<td>16.40*</td>
<td>6.0*ab</td>
<td>4.5*</td>
</tr>
<tr>
<td>ISR40</td>
<td>40.0*^</td>
<td>16.10*</td>
<td>6.0*ab</td>
<td>4.0*ab</td>
</tr>
<tr>
<td>ISR34</td>
<td>35.5*</td>
<td>14.80*</td>
<td>5.50*cd</td>
<td>2.5*cd</td>
</tr>
<tr>
<td>ISR35</td>
<td>37.5*bc</td>
<td>14.60*</td>
<td>5.55*bcd</td>
<td>3.0*bcd</td>
</tr>
<tr>
<td>ISR41</td>
<td>35.0*</td>
<td>11.70*</td>
<td>4.25*fg</td>
<td>2.5*cd</td>
</tr>
<tr>
<td>ISR36</td>
<td>42.0*</td>
<td>17.00*</td>
<td>6.30*</td>
<td>4.5*</td>
</tr>
<tr>
<td>ISR42</td>
<td>35.5*</td>
<td>14.10*</td>
<td>5.15*de</td>
<td>3.5*abc</td>
</tr>
<tr>
<td>SDW Control</td>
<td>31.0*d</td>
<td>9.95*d</td>
<td>4.0*g</td>
<td>2.0*d</td>
</tr>
</tbody>
</table>

Results were taken 30 days after sowing and are based on the four replicates with 100 plants per treatment. Means followed by the same letter are not significantly different according to DMRT (*P* ≤ 0.05). SDW-sterile distilled water
Figure 2.7: Effect of endophytic bacteria *Pseudomonas fluorescens* ISR - 36 on asexual spores of *Sclerospora graminicola* compared with distilled water control and Apron 35 SD. A - *Pseudomonas fluorescens* ISR 36, B - Distilled water and C - Apron 35 SD.

Figure 2.8: Visual effect of seed treatment with endophytic bacteria *Pseudomonas fluorescens* ISR-36 on vegetative growth of pearl millet plants under greenhouse conditions.
ISR36 followed by *P. fluorescens* ISR33 with disease protection of 53.7%. Among isolates chosen, ISR 41 offers least protection of 17.9% disease. Whereas control treatment showed the diseases incidence of 95%, and Apron treatment offered protection of 88.4% against downy mildew disease (Fig. 2.9).

**Field conditions** Under field conditions similar trend was observed in minimizing the disease incidence. All chosen isolates showed reduced downy mildew incidence. However, the degree of protection varied between isolates (Fig. 2.4). The protection offered due to seed treatment ranged from 18.2 to 61.1%. Maximum protection observed in the *P. fluorescens* isolates ISR36 followed by ISR33 with disease protection of 55.8%. In the isolates of *Bacillus* sp. ISR40 and ISR22 offers the protection of 54.7 and 51.6% respectively. Among the isolates, ISR29 and ISR42 were effective and offer the protection of 46.3%. The lowest protection was recorded in the treatment of ISR41 with disease protection of 18.2%, whilst, Apron treatment recorded the disease protection of 89.4% where as the control recorded 95% disease incidence (Fig. 2.10).

**Effect of seed treatment with endophytic bacteria on growth parameters of pearl millet**

All the tested endophytic bacterial isolates seed treatment were significantly promoting the vegetative growth parameters of pearl millet plants such as height of the plant, fresh weight, dry weight and number of basal tillers. Maximum height of 42 cm was recorded in plants treated with *P. fluorescens* ISR 36 followed by the ISR33 with height of 40cm compared to 31 cm in control. The *Bacillus* isolates enhanced the plant height significantly, in which ISR40 recorded the height of 40cm followed by the ISR22 with 37.5cm height. Similar trend is followed case of fresh weight, dry weight and number of basal tillers enhancement developed (Table 2.4).

Similar to the vegetative growth parameters improvement offered by the endophytic bacterial isolates, reproductive growth parameters such as 50% flowering, length and girth of ear heads, and 1000 seed weight, plant height, tillering of pearl millet plants were also enhanced (Table 2.5). The maximum improvement of all reproductive traits was noticed in plants raised from seeds treated with *P. fluorescens* isolate ISR 36 subsequently followed by the ISR33. Similarly, *Bacillus* isolates ISR40 was effective after the *P. fluorescent* treatment (Fig. 2.8).
Figure 2.4 Effect of seed treatment with endophytic bacteria on downy mildew disease protection under field condition in pearl millet (DMDI - Downy mildew disease incidence, DMDP- Downy mildew disease protection). Vertical bars indicate standard error.
Table 2.5 Effect of seed treatment with promising endophytic bacteria on reproductive growth parameters of pearl millet

<table>
<thead>
<tr>
<th>Endophytic bacterial isolates No.</th>
<th>Height (cms) of plants</th>
<th>No. of Days required for 50% flowering</th>
<th>Length of earhead/Plant</th>
<th>Girth of earhead/Plant</th>
<th>No. of basal tillers/Plant</th>
<th>No. of nodal tillers/Plant</th>
<th>1000 seed weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISR37</td>
<td>128.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISR38</td>
<td>126.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.8&lt;sup&gt;de&lt;/sup&gt;</td>
<td>4.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISR39</td>
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Results were taken 60 days after sowing and based on two replicates with 50 plants per treatment. Means followed by the same letter are not significantly different according to DMRT ($P \leq 0.05$).
Figure 2.9: Effect of seed treatment with endophytic bacteria *Pseudomonas fluorescens* ISR 36 (B) and *Bacillus* spp. ISR 37 (C) on pearl millet downy mildew disease under greenhouse conditions. A - Control

Figure 2.10: Effect of seed treatment with different endophytic bacteria on downy mildew disease incidence of pearl millet under field conditions. A - ISR37, B - ISR38, C - Control, D - ISR36, E - ISR40, F - ISR33
Demonstration of induced resistance by time gap studies in pearl millet

In order to test the nature of resistance offered by endophytic bacterial isolates, induction studies were conducted by treating seeds with the bacteria and inoculating with the pathogen at different time intervals. After seed treatment with endophytic bacteria, protection of 14 – 68% was observed, depending on the time interval between bacteria treatment and inoculation with pathogen. Maximum protection was achieved by the isolate *P. fluorescens* ISR 36, which recorded 68% protection on 4th and 5th day of time gap between seed treatment and pathogen inoculation. On the first day this isolate recorded 44%, which was raised to 66, 65 and 65% on the 2nd and 3rd day followed by *Bacillus* sp. ISR 40, *P. fluorescens* ISR 33. The lowest protection was recorded by *Bacillus* sp. ISR 41, which recorded 14% protection. From all the treatments it is observed that minimum of three days required to develop the resistance systemically after the seed treatment (Fig. 2.3).
Figure 2.2 Effect of seed treatment with endophytic bacteria on downy mildew disease incidence under greenhouse conditions in pearl millet (DMDI - Downy mildew disease incidence, DMDP- Downy mildew disease protection). Vertical bars indicate standard error.

Figure 2.3 Time gap studies between seed treatment with endophytic bacterial isolates and pathogen inoculation on downy mildew protection in pearl millet (DM – downy mildew and DAI – days after pathogen inoculation).
DISCUSSION

Medicinal plants, agriculturally important crops and weed plants harbor different types of microorganisms on the surface as epiphytes or endophytes. Epiphytic microorganisms are associated on the surface of the plants may have beneficial or harmful effects to the plants. However, the endophytic microbes show beneficial effects in harboring host plants and it is identified as an important interaction in medicinal plants, which are known to release many secondary metabolites due to the presence of endophytes (van Peer et al., 1991; Ongena et al., 1999; Steij et al., 1999; Benhamou et al., 1996; 2000). And this interaction of host plant and endophytes has been reported to elicit plant defense responses against a wide range of plant pathogens in many crops, which is being touted as a potential alternative strategy for plant disease management (Metraux et al., 1990; Kloepper et al., 1992; Leeman et al., 1995; Pieterse and van Loon, 1999; Benhamou et al., 1996; Sticher et al., 1997; Duijff et al., 1997; van Loon, 1997; M'Piga et al., 1997; Chen et al., 1998; Ongena et al., 1999; Chen et al., 1999; 2000; Cao et al., 2005).

In these study bacterial endophytes isolated from different medicinal plants viz., C. citratus, B. diffusa, A. indica, P. emblica T. cardifolia, P. sativum, S. bicolor and P. hysterophorus were evaluated for their effectiveness in promoting pearl millet growth and downy mildew disease protection. The results showed that ten bacterial isolates from eight plants demonstrated significant protection against downy mildew disease under greenhouse and field conditions when used as seed treatment. Further, the resistance developed was systemic and durable.

Endophytic bacteria isolated and identified based on the methods described earlier are in accordance with the Barac et al. (2004) and it is further brace up by the advanced identification of DNA-based methods. Endophytic bacteria isolated in the present study have shown different beneficial effects to the pearl millet plants, which is grown as important cereal crop of the semi-arid tropics. Most of the endophytic bacteria isolated in the present study belonged to Bacillus and Pseudomonas species. Bacillus and Pseudomonas were particularly selected for further studies, as these two species have been tested and established as potential inducing agents and also as growth promoting agents in various pathosystems. Results of the present study evidenced that seed treatment with endophytic bacteria enhanced seed germination and seedling vigor of pearl millet. The germination percentage of 96 % and seedling
vigor of 1507 was offered by *P. fluorescens* ISR 36. It is interesting to note that in the present study endophytes were able to enhance the seed quality parameters like germination and seeding vigour that led to good crop stand in the field conditions. This may be due the effect of the various growth promoting substances secreted by the endophytic bacteria in the host or different secondary metabolites which in turn may have stimulated the production of growth regulators. Similarly, seedling vigor enhancement has been noticed with seed treatment of other crops by different endophytic bacteria isolated from medicinal, agricultural and weed plants (Xia et al., 1996; M'Piga et al., 1997; Kloeper et al., 1999; Benhamou et al., 2000; Gyaneshwar et al., 2002; Boddey et al., 2003; Niranjanraj et al., 2003; Ryu et al., 2004; Strobel et al., 2004; Pandey et al., 2005; Rita et al., 2005; Compant et al., 2005). Furthermore, tested endophytic bacterial isolates did not inhibit the tested fungal growth. Further, seeds treated with endophytic bacteria showed increased fresh weight and dry weight of the plant, more number of tillers, early flowering and 1000 seed weight. Maximum plant biomass enhancement was recorded in *P. fluorescens* ISR 36. Our results corroborate earlier studies, which *Pseudomonas* treatment has resulted in enhancement of growth parameters of various crop plants. The soybean-associated bacteria showing characteristics related to plant growth promotion were identified as belonging to the genera *Pseudomonas, Ralstonia, Enterobacter, Pantoea* and *Acinetobacter* (Kuklinsky-Sobral et al., 2004). There are a few reports on the increased plant biomass of different crop species such as oilseed rape, tomato, maize, sorghum, wheat and rice when endophytic bacteria used as seed and seedling treatments (Barbieri et al., 1986; Nejad and Johnson, 2000; Sturz, and Nowak, 2000; Gutierrez-Zamora and Martinez-Romero, 2001; Roncato-Maccari, 2003). This phenomenon has been attributed to microbial processes leading to nutrient solubilization by production of phosphorous, siderophores and plant growth hormones such as auxins, cytokinins, gibberlins and abscisic acid (Sturtz et al., 1997). Further, effectiveness in increasing plant height attributed to the effect of indole butyric acid, indole pyruvic acid and indole propionic acid during interaction with host was evidenced by the previous studies (Tjamos et al., 2004). Wherein *Bacillus* isolates K-165 and 5-127 enhanced the growth of potato in field conditions. Whilst endophytic bacterial colonization enhances the competitive ability of the plants to resist pest and diseases of plants by inducing the systemic resistance known to release and enhance the pathogenesis related proteins, enzymes and antimicrobial compounds in many
crop system against plant pathogens (Ongenae et al., 2004). Significant reduction of
downy mildew disease of 53 and 55% were recorded under greenhouse and field
conditions respectively compared to control due to seed treatment with endophytic
bacteria. The nature of protection offered by the endophytes due to seed treatment was
tested in the plant by time gap studies between seed treatment and pathogen
inoculation and it was found to be systemic. In several case studies, an endophytic
bacteria \textit{Bacillus pumilus} SE34 against root rot causing fungus \textit{Fusarium oxysporum}
f. sp. \textit{pisi} in \textit{Pisum sativum} and increased resistance against \textit{Fusarium oxysporum} f.
sp. \textit{lycopersici} in tomato has been recorded (Chen et al., 1995; M'Piga, 1997).
Further, in rice against \textit{Rhizoctonia solani}, cotton against \textit{Verticillium dahliae} and wilt
diseases of oil seed rape and tomato have shown the pretreatment of endophytic
bacteria has resulted in increased host defense responses (Xia, 1996; Krishnamurthy
and Gnanamanickam, 1997; Nejad and Johnson, 2000).

The colonization of the root and the rhizosphere by the endophyte is
influenced by a number of environmental factors such as plant species, soil type and
application technique. Various antibiotics can be produced by \textit{B. subtilis}. It also
produces iturin-like lipopeptides similar to that of fungicidal agents. Besides inducing
resistance, some endophytic rhizobacterial strains have been reported to antagonize
soil borne pathogens directly and to stimulate plant growth (Pieterse and Van Loon,
1999). For testing the durability of the effect of endophytic bacteria inducing
resistance and thus offering the disease protection against the pathogen colonization
effect of each isolates was tested. Among the isolates seeds bacterized, ISR 33, 36
and 38 are recovered in the stems and roots of the pearl millet consistently up to 60
days. But remaining entries were only colonized in the rhizosphere of the treated
plants.

Although, the exact mechanism by which seed treatment with endophytic
bacteria reduces disease incidence are not fully understood, it has been reported that
endophytic bacteria are known to control plant pathogens by induction of resistance
leading to the production of phytoalexins, accumulation of pathogenesis related
proteins, deposition of structural barriers in the cell wall of the host plant and by
production of antimicrobial compounds (Manjula et al., 2002). In addition, a variety
of substances produced by endophytic bacteria have been implicated in the
mechanisms to limit the damage to plants by phytopathogens. These include
siderophores, antibiotics, other small molecules and a number of enzymes (Liu et al.,
1995). Furthermore, plant growth promotion by endophytic bacteria may also be an indirect mechanism of disease control, leading to disease escape when the growth promotion results in shortening the time that a plant is in a susceptible state. In our study the endophytes might have shown multiple actions against downy mildew, viz., endophytic bacteria caused enhanced seedling emergence rate, thereby reducing the susceptible time for establishment of downy mildew pathogen in pearl millet, they might have released growth regulators, induction of resistance and also by effective colonization leading to physical displacement of the pathogens.

Plant growth promoting rhizobacteria (PGPR) and *Psuedomonas* sp. have been well documented to improve the growth promotion and host resistance against the downy mildew pathogen in pearl millet and head mold in sorghum (Umesha et al., 1998; Raju et al., 1999; Niranjanraj et al., 2003). Endophytic bacteria are often closely related to PGPR/ plant health promoting rhizobacteria (PHPR) and these have lately found to colonize the root internally also indicated modes of action described for PGPR/ PHPR also apply for endophytic bacteria. These endophytic bacteria induced resistance can last longer than PGPR, since they establish a much closer relationship with the host and on the other hand, PGPR may be inhibited by competition with other microorganisms on the root surface (Hallmann, 2001). The penetration of endophytic bacteria into the seeds or sprouting seedlings leading to defense elicitation is most likely the possible explanation.

Seed treatment is the only feasible technology in pearl millet due to economic constraints for crop production. Alternative is the exploitation of biotic and abiotic inducers, which have plant growth promoting effects and also induction of resistance against pests and diseases. The use of endophytes may be preferable to reduce the use of chemical fertilizers and pesticides because of cost and contribution to sustainable agricultural systems.