Chapter 1

Review of literature

"Define success on your own terms, achieve it by your own rules, and build a life you are proud to live."

-Anne Sweeney

1.1 Introduction

Enhanced agricultural production is necessary to provide not only sufficient food for the increasing human population but also to provide crops for renewable energy, as well as provide basic compounds in industrial processes. To meet this demand, the farming industry has increased the usage of chemical fertilizers leading to adverse effects on the environment and human health. For instance, contaminated drinking water is the major cause of exposure to nitrogen fertilizer. The presence of nitrate is much higher in farming land compared to the naturally vegetative land. Nitrate converts into the N-nitroso compounds which are proven potent carcinogens and teratogens in animal studies (Ward 2009). To reduce the health and environmental issues, we need to improve the crop production technology by use of biological alternatives to fertilizers and pesticides, which need a major attention for sustainable crop production (Berg 2009) Adding biofertilizers to the soil helps in the maintenance of the nutrient cycle and the natural ecosystem. In addition, long term benefits of biofertilizers include maintenance of soil health, improving productivity, resilience against the environmental stress and development of plant disease resistance against the various pathogens. There are several biofertilizer and biocontrol strains currently commercially marketed. These biofertilizers promote the plant growth mainly by secretion of a phytohormone and improving the nutrient uptake while biocontrol agents suppress the soil pathogens. Most well studied plant beneficial microbes are rhizospheric, however, microbes residing in the interior of plant tissues known as endophytes are of recent interest.

1.2 Bacterial endophytes and their diversity

The plant provides several unique niches for the colonization of specific sets of microorganisms. The rhizosphere, soil surrounding the roots, is host to a number of microorganisms that are attracted by the rhizodeposition in this zone (Hartman et al. 2009). The immediate surface of the root, the rhizoplane, harbours microorganism that forms more intimate interaction with the roots and is exposed to the highest concentration of exudates. A fraction of the microbes associated with the exterior of the plant surfaces is able to enter into the internal tissues. Earlier scientists believed the inner parts of the healthy plants especially stele sterile but

recent studies proved the colonization takes place in every part of plants which was demonstrated with the isolation of diazotrophic endophytic bacteria *Azoarcus* sp. BH72 from non-diseased stele of grasses (Reinhold-Hurek and Hurek 2011). Endophytes are defined as microbes that reside inside the plant tissue and can be isolated from surface disinfected plant parts (Schulz and Boyle, 2006) and are confirmed as endophytes by their reisolation from plant tissues after inoculation or by the direct visualization of fluorescently tagged strains. Recently, using molecular approaches many uncultured endophytic bacteria are sequenced but not isolated (Muller et al. 2016). Therefore, a more appropriate definition of endophytes is the set of endophyte genomes located inside the plant organs. Different plant parts provide unique ecological habitats for the bacterial communities (Beckers et al. 2017). Endophytes are classified into three categories- firstly facultative endophytes which colonize the plants when the opportunity arises with coordinated infection, in the second group of endophytes which have obligatory plant endophyte transfer possible via seeds and third group passive endophytes colonize the plants when wounding along the root hair. Generally, plant growth promoting bacterial (PGPB) endophytes belong to facultative and obligatory lifestyle for plant colonization (Gaiero et al. 2013). Majority of the microbes are neutral to plants but they utilize the organic compounds produced by plants and participate in neutral cycling in the environment in plant vicinity (Schenk et al. 2012). However, many endophytes play a beneficial role towards the plant by various mechanisms which can be broadly classified as plant growth enhancement (Santoyo et al. 2016) and plant disease suppression (Backman and Sikora 2008) (Fig 1.1). Besides this, endophytes are also exploited for the production of novel metabolites that have application as antibiotics and anticancer compounds (Yu et al 2010) and for microbe-assisted phytoremediation (Li et al. 2012; Afzal et al, 2014). During phytoremediation, process plant often does not able to completely eliminate contaminant and causes volatilization in the environment. This problem was addressed by introducing toluene degrading plasmid in endophytic *Burkholderia cepacia* which strongly degrade the toluene and reduce the phytotoxicity and improve the soil quality (Barac et al. 2004; Taghavi et al. 2005) (Fig. 1.1).

A large number of bacteria invade the plant and reside intercellular spaces of plants and mainly belong to alpha, beta and gamma *Proteobacteria*, *Firmicutes, Actinobacteria* and *Bacteroidetes* (Rosenblueth and Martinez-Romero 2006). The important genera include

Azospirillum, *Gluconoacetobacter*, *Methylobacterium* (belonging to α-*Proteobacteria*), *Azoarcus*, *Burkholderia*, *Herbaspirillum* (belonging to β-*Proteobacteria*), *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia* (exemplifying γ-*Proteobacteria*), *Bacillus*, *Paenibacillus* (exemplifying *Firmicutes*), *Arthrobacter*, *Microbacterium*, *Nocardia* and *Streptomyces* (representing *Actinobacteria*) and *Sphingobacterium* belonging to *Bacteroidetes.* Plants in which endophytes have been reported include cereals such as rice, wheat, maize; other crops such as sugarcane, banana, sweet potato; legumes such as soya bean; vegetable plants such as lettuce, radish, tomato as well as trees and grasses (Rosenblueth and Martinez-Romero 2006). However, it is generally believed that no plant is devoid of endophytes. Along with this legume colonizing *Rhizobium* is reported to colonize the non-legume plants of *Poaceae* family by several workers in greenhouse well as in field (Bhattacharjee et al. 2008). Endophytic *Actinobacteria* are reported in various plant systems such as agronomical crops, horticulture crops, medicinal plants, perennial tree, mangrove ecosystem, lichens, mosses and aquatic ecosystem (Govindasamy et al. 2014).

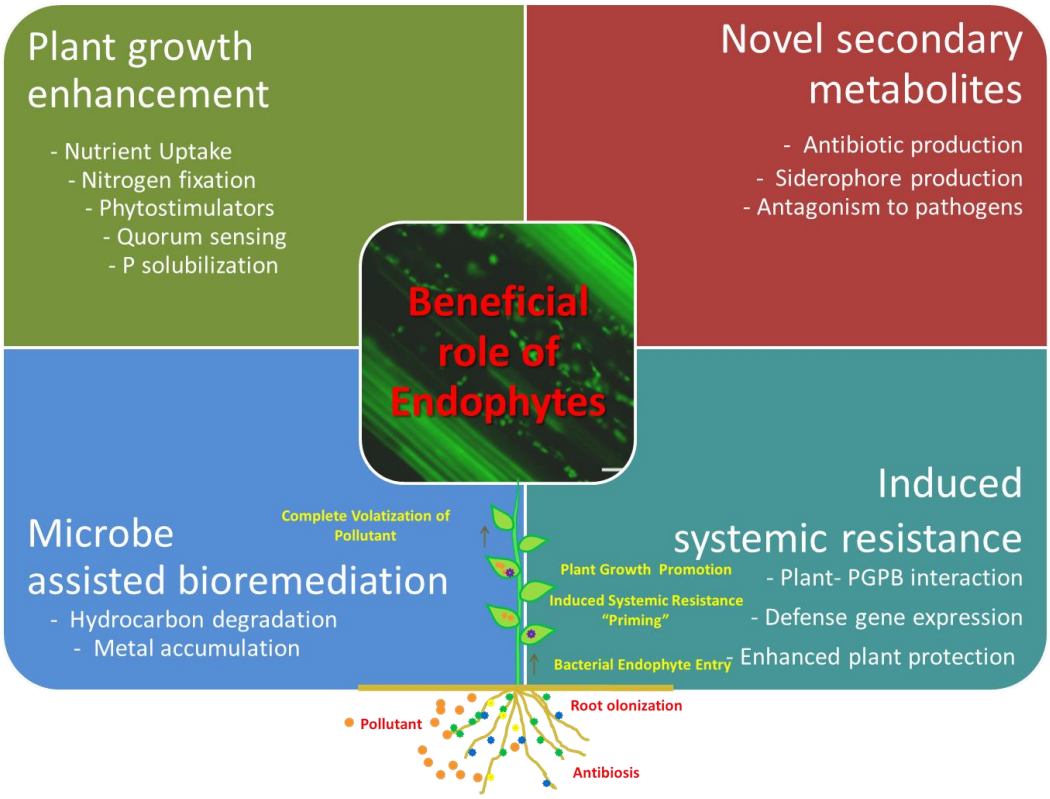


Fig. 1.1 Schematic overview of beneficial role endophytic microbes in plant growth promotion, disease suppression and its biotechnological potential.

1.3 Factors influencing the colonization of endophytes in various plants

Endophytic strain colonization and their diversity are influenced by many plant and environmental factors which decide the fate of the endophyte (Fig. 1.2). Besides microbial communities in plants are constantly shifting with a change in the age of plants due to changes in root exudate production.

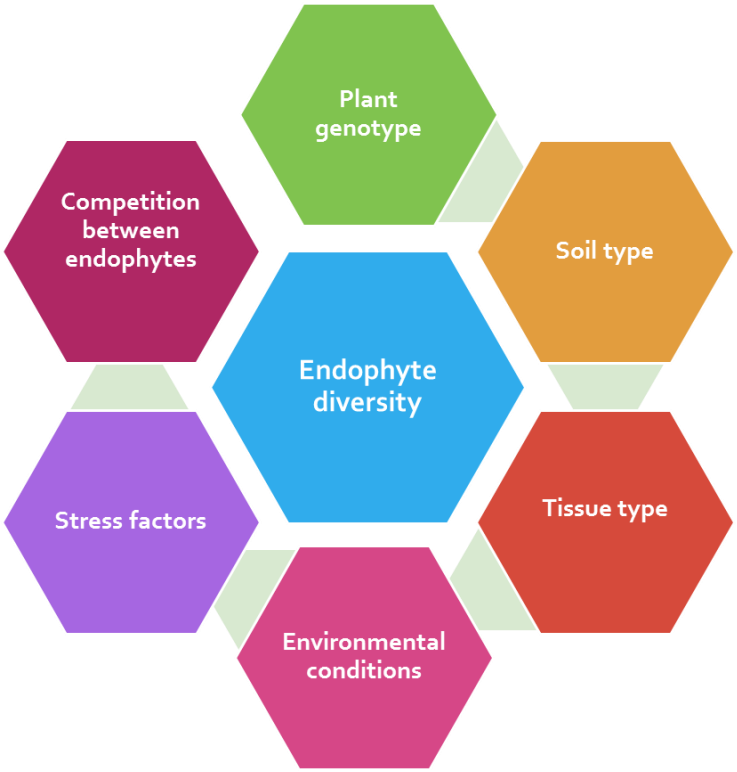


Fig. 1.2 Factors influencing endophyte diversity in plants

1.3.1 Environmental factors

The endophytic bacterial distribution in plants may affect by the soil management, geographical location and genotype of the plant. The effect of agricultural practices on culturable endophytic bacterial diversity was demonstrated through collecting various plants from the organically and conventional farming (Xia et al. 2015). In this study comprising of 336 endophytic bacterial isolates, authors were able to discern higher endophyte diversity in organically grown plants as compared to mineral fertilized plants. The comparison of culturable bacteria from different pants and their parts showed considerable variation based on plant species as well as tissue type (Xia et al. 2015). Another study has also shown that variation in fertilization (mineral and organic) in

soil affects not only the bulk soil microbial community but also the endophytic bacterial community composition (Seghers et al. 2004). There were lower methanotrophs observed in the mineral fertilized soil as compared to organically fertilized soil. Ren et al. (2015) found that plant growth stage affects endophytic bacterial communities to a greater extent than other factors such as elevated CO_2 level and variation in nitrogen fertilization. The effect of changes in environmental conditions on the endophytic community was studied by transferring the Mexican breed maize seeds to Canadian soil. The young maize plants were harvested and checked whether the original endophytic community was replaced with the native strains due to change in geographical location and environmental condition. They observed the partial buffering of endophytes which were found originally present in maize grown in Mexican soil. This study demonstrates that true endophytes of plants were passed into the seed and can be transported to one place to other in the world (Johnston-Monje et al. 2014). Genotypically different maize and rice plants were analyzed for the endophytic bacterial community using the PCR-DGGE method showed the variation in community with the change in host genotype. But along with this there was also observed the presence of specific bands of DGGE in all the cultivars commonly. This study suggests some specific endophytic bacteria remain consistently present in plants without affected by host genotype as a native colonizer. Genotypically different maize and rice plants analysis also suggests the presence of specific strains of *Burkholderia*, *Achromobacer* and *Stenotrophomonas* in all the cultivars (da Silva et al. 2014, Hardoim et al. 2011). The plant genotype was not only factor that affected the bacterial community composition but the age of the plant also plays role in selective colonization of endophytes inside the plant tissue (Ren et al. 2015, Van Overbeek and Van Elsas 2008). These reports suggest the endophytic bacterial structure and successful colonization and establishment inside the plant tissue influenced by many different factors.

1.3.2 Role of quorum sensing in endophytic lifestyle

Endophytes display cell-to-cell communication signals i.e., quorum sensing (QS) signals that allow bacteria to monitor their density and to coordinate gene expression only when a certain threshold cell density is achieved (Fuqua et al. 1994). Quorum sensing (QS) mechanism was evaluated in several endophytic bacterial systems for their role in physiological activities and colonization. *Burkholderia phytofirmans* PsJN, known for its plant growth promotion ability in

Arabidopsis thaliana was studied to analyze the effect on rhizosphere and endophytic colonization due to impaired QS system. The mutant strain that produced the significantly lower level of 3-hydroxy-C8-homoserine lactone compare to the wild-type of strain showed a reduction in rhizospheric colonization and null colonization of endophytes inside the plant tissue. This study was further extended to understand the effect on biofilm formation and root surface adherence which showed a considerably lower colonization possibly due to the reduction in swimming motility of the mutant strain. This result supports the importance of QS system in rhizospheric and endophytic colonization in plants (Zúñiga et al. 2013). QS inactivation of the rice endophyte *Azospirillum lipoferum* B518 by quorum quenching showed a reduction in pectinase activity and IAA production (in stationary phase) but an increase in the siderophore synthesis and no effect is observed on cellulase activity and on the phytostimulatory effect (Boyer et al., 2008). In the endophytic strain *Serratia plymuthica* G3, QS regulates antifungal activity and production of exoenzymes positively, but negatively regulates IAA production (Liu et al. 2011). Interestingly, diazotrophic endophytic *Azoarcus* sp. BH72 does not show the presence of QS genes in the genome (Suarez-Moreno et al. 2008, Krause et al. 2006).

1.3.3 Effect of root exudates in colonization

Rhizosphere area have a highest diversity of bacteria compared to the area which away from plant vicinity. As plants secrete different root exudates which plays the important role in attracting microbes towards the roots by chemotaxis mechanism and since endophytes are usually a subset of rhizosphere microbes, the composition of root exudates influences endophytic flora. The effect exerted by root exudates on endophytic and rhizosphere bacteria was studied in sterile hydroponic system with rice plants which showed that major root exudates compounds involved were carbohydrates and amino acids (Bacilio-Jiménez et al. 2003). High concentrations of glucose, arabinose, mannose, galactose and glucuronic acid were found in this study along with amino acids such as histidine, proline, valine, alanine and glycine. Endophytic bacteria showed the higher chemotactic response towards root exudates as compared to rhizosphere isolates.

1.4 Mechanism of endophytic colonization

1.4.1 Attachment to root surfaces

Endophytic entry inside the plant tissue requires the first efficient attachment to the root surface of the plant. The role of various components was studied by constructing mutant strain of diazotrophic endophyte *Gluconobacter diazotrophicus* defective in exopolysaccharide (EPS) production. The EPS mutant strain was applied rice seedlings to understand the role in biofilm and colonization. The mutant strain does colonize the root even when the most favorable conditions were provided. The ability to colonize was partially restored when purified EPS from wild-type strain culture supernatant was applied to mutant strain (Meneses et al. 2011). Similarly, lipopolysaccharide mutant strain of *H. seropedicae* also showed reduction in maize root attachment capability (Balsanelli et al. 2010). Role of superoxide dismutase (SOD) and glutathione reductase (GR) in colonization was studied in *G. diazotrophicus* using rice as host plant. The mutant strain of SOD and GR were less efficient in colonization as observed by cell count and fluorescence in situ hybridization analysis (Alquéres et al. 2013). Along with this, plant defense pathways control the endophytic colonization inside the plant tissue. Hyper colonization of endophytic *Klebsiella pneumoniae* 342 was observed in ethylene insensitive mutant of *Medicago truncatula* compared to the parent genotype (Iniguez et al. 2005).

1.4.2 Bacterial entry inside the plant tissue

After colonizing the rhizosphere area, endophytes invade the interior parts of the root by passing through the cracks and gaps present during lateral root development and enter to the cortex region of the plant. For entry to the aerial part of the plant, they require to surpass the obstacle of cortex and endodermis. The central cylinder of the plant is protected with the endodermis which can be cross by the active or passive mechanism of endophytes (Fig. 1.3). In the active mechanism, endophytes secrete the cell wall degrading enzymes which help them for further colonization. In passive mechanism, they enter by using the gaps present during secondary root development. After entering in xylem part of the plant, they are transported further to aerial parts of the plants e.g. *Herbaspirillum seropedicae* Z67, *B. phytofirmans* strain PsJN (James et al. 2002; Compant et al. 2008; Compant et al. 2010).

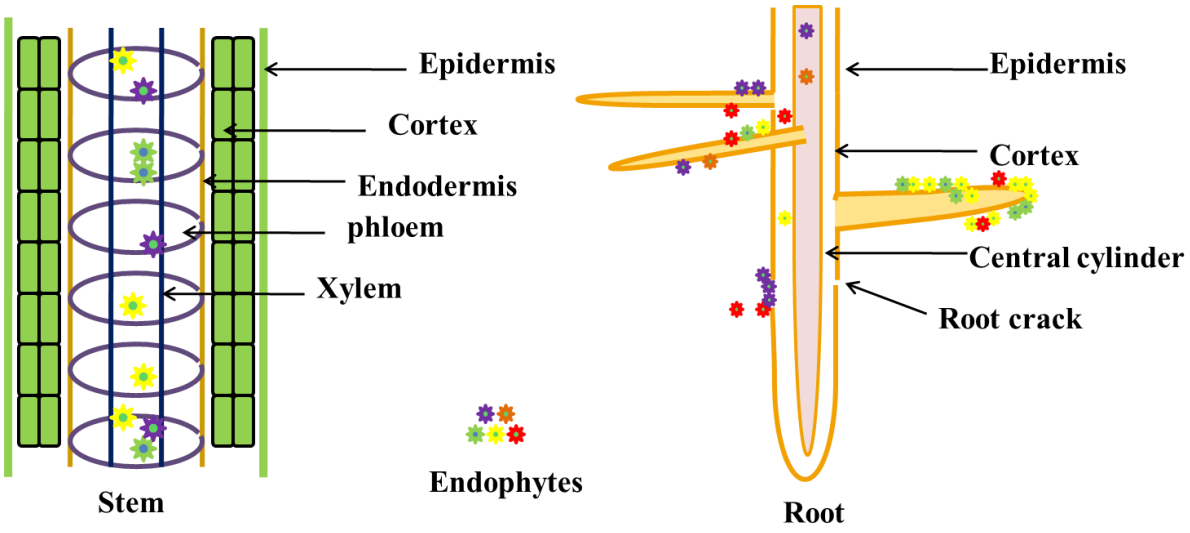


Fig. 1.3 Barriers to be crossed by endophytes for colonization plant stem and root

Along with the cell wall degrading enzymes, lipopolysaccharide, flagella, pili, and twitching motility help in mobility and colonization in host plants (Compant et al. 2010; Muller et al. 2016). Entry into the host tissue always does not require the active penetration which suggests that all rhizosphere bacteria can potentially act as endophytes. Passive penetration takes place through natural openings near root hair emergence and through wounds. The organisms enter the interior parts of the plants ranging from 10^5-10^7 CFU g^{-1} fresh weight (Compant et al. 2010). Colonization in xylem vessels by bacteria leads to their spread in stem and leaves, where the range of cultivable population is 10^3 -10^4 CFU g^{-1} fresh weight of tissue. There are also reports of endophytic bacterial colonization in fruits and flowers but under natural conditions bacterial density is very low in the range of 10^2-10^3 CFU g^{-1} fresh weight of tissue. Strains of *Pseudomonas* and *Bacillus* are found in the interior of flowers, fruits and seeds of grapevine The endophytic bacterial presence was detected in intracellular spaces of plants by different methods such as tagging the endophytic bacteria with green fluorescent protein (GFP) and β-glucuronidase (GUS) which allowed their detection interior plant tissue (Compant et al. 2010).

1.5 Plant growth promotion by endophytic bacteria

Plant growth promoting bacteria (PGPB) affect the plant growth by direct or indirect ways such as improving the plant health by nitrogen fixation, increasing availability of minerals to plants, repression of plant pathogens by production of siderophores, antibiotics, inducing the resistance against the pathogens, competition for available nutrients, improving plant stress tolerance

towards drought, salinity, and metal toxicity and production of phytohormone such as indole acetic acid (IAA). Along with this production of lytic enzymes reduce the growth of phytopathogens and assist in entry inside the plant tissue. The colonization of endophyte can lead to ability to control plant pathogens by the production of antimetabolites such as 2,4-diacetyl phloroglucinol (2,4-DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, thiotropocin, tropolone, as well as many others such as cyclic lipopeptides, rhamnolipids, oligomycin A, kanosamine, zwittermicin A, and xanthobaccin which help in competing with other microbes.

1.5.1 Nitrogen fixation by endophyte

Nitrogen is an essential macroelement for plant growth and plants usually absorb the nitrogen from soil in the form of nitrate or ammonium ion from the root. A first nitrogen-fixing bacterium was discovered in 1888 by Beijerinck, a Dutch microbiologist, who isolated from root nodule the organism *Rhizobium leguminosarum*. The earlier view was that *in planta* biological nitrogen fixation (BNF) is largely contributed by *Rhizobium* spp. and is limited only to the legume plants. However, now it is known that diazotrophic endophytic bacteria colonize the legumes and non-legumes plants (Franche et al. 2009).

Earlier nitrogen fixing ability was restricted to very few genera but recent advances in technology and understanding allowed the isolation of many new diazotrophic endophytic bacteria from various plant systems (Table 1.1). Diazotrophic endophytic bacterial (*Azoarcus* sp. BH72 and *Gluconobacter diazotrophicus* Pal5) genome sequence showed presence similar nitrogen fixing machinery which present in other diazotrophs of rhizosphere and legume (Bertalan et al. 2009, Krause et al. 2006). Diazotrophic endophytic bacteria are isolated by growth on the N limiting media. *In planta,* nitrogen assimilation is studied by incorporating $^{15}N_2$ in plant biomass. Nitrogen fixed in rice plants by endophytic *Herbaspirillum*, *Burkholderia,* and *Azospirillum* may reach up to 31% of the total plant N (Carvalho et al. 2014). In sugarcane plants, N contributed by biological nitrogen fixation (BNF) by *Acetobacter diazotrophicus w*as found to be higher in the shoot as compared to the root of the plants. Studies conducted with the wild type and *nif* mutant strains in N limiting condition using sugarcane plants showed the reduction in plant growth in *nif* mutant strain inoculated plants compare to the plants inoculated with the wild type strain. The *nif* mutant inoculated plants showed almost similar growth to the

uninoculated controls (Sevilla et al. 2001). A similar result was observed with *Klebsiella pneumonia* 342 *nifH* mutant strains in the wheat plant (Iniguez et al. 2004). In another study of *in planta* nitrogen fixation in endophytic *Azoarcus* sp. the *nifH* gene expression was monitored using *gfp* and *gus* reporter gene constructs and nitrogenase activity was localized and detected i*n situ*. The expression of nitrogen fixing genes of *Azoarcus* sp. BH72 in planta was analyzed by extracting total endophytic bacterial RNA from rice root and quantified gene expression by qPCR (Hurek et al. 2002).

Table 1.1 Diazotrophic endophytic bacteria from various plants

Diazotrophic endophyte	Host plant	Colonizing part of the plant	References
α-Proteobacteria			
Gluconobacter diazotrophicus	Carrot, Raddish, Beetroot, Coffee	Root	Madhaiyan et al. 2004
Acetobacter diazotrophicus	Sugarcane	Root, Stem	Fuentes-Ramirez et al. 1993
Azospirillum melinis	Tropical molasses grass	Root	Peng et al. 2006
Methylobacterium sp. L2-4	*Jatropha curcas*	Leaf	Madhaiyan et al. 2015
Sphingomonas sp. S8-608		Stem	
Rhizobium sp. R2-708		Root	
Rhizobium spp.	Rice, Wheat Sorghum	Leaf Stem	Chapter 2
Methylobacterium spp.	Pearl millet	Leaf and Stem	
Brevundimonas sp. R3	Rice	Leaf	
Brevundimonas aurantiaca	Rice	Stem	Prakamhang et al. 2009

β-*Proteobacteria*			
Herbasprillum seropedicae	Rice, Maize, Sorghum	Root	Baldani et al. 1986
Herbaspirillum huttiense	*Jatropha curcas*	Root	Madhaiyan et al. 2015
Burkholderia spp.	Maize	Root, Shoot	Estrada et al. 2002
Achromobacter xylosoxidans	*Catharanthus roseus*	Root	Karthikeyan et al. 2012
Achromobacter spp.	Rice	Stem	Chapter 2
Ralstonia spp.	Maize	Leaf and Stem	
Azoarcus sp. BH72	Kallar grass	Root	Hurek et al. 2002
γ-Proteobacteria			
Serratia marscens	Rice	Root, Stem	Gyaneshwar et al. 2001
Acinetobacter oryazae	Rice	Leaf	Chaudhary et al. 2012
Acinetobacter sp. M5	Maize	Leaf	Chapter 2
Pantoea sp. MS3	Maize	Stem	
Pantoea agglomerans	Rice	Seed, Root, Stem, Leaf	Feng et al.2006
	Sweet potato	Stem	Asis and Adachi 2003
Enterobacter oryzae	Rice	Root	Peng et al. 2009
Enterobacter sp. EN-21	Sugarcane	Root	Kruasuwan and Thamchaipenet 2016
Klebsiella spp.	Maize	Stem	Palus et al. 1996
Klebsiella oxytoca	Sweet potato	Stem	Adachi et al. 2002
Pseudomonas aeruginosa	Pearl millet	Root, shoot	Gupta et al. 2013
Pseudomonas sp. R1-73	*Jatropha curcas*	Root	Madhaiyan et al. 2015

Pseudomonas sp. WS5	Wheat	Stem	Chapter 2
Firmicutes			
Clostridia spp.	*Miscanthus sinensis*, Rice	Root, Stem, Leaf	Minamisawa et al. 2004
Bacillus sp. EN-27	Sugarcane	Root	Kruasuwan and Thamchaipenet et al. 2016
Bacillus spp.	Pearl-millet Sorghum	Stem Leaf	Chapter 2
Staphylococcus spp.	Maize	Leaf	
Paenibacillus sp. R1-312	*Jatropha curcas*	Root	Madhaiyan et al. 2015
Actinobacteria			
Microbacterium spp.	Spontaneous legume	Root nodule	Zakhia et al. 2006
Arthrobacter spp.	Halophyte *Prosopis strombulifera*	Root	Piccoli et al. 2011
Bacteroides			
Sphingomonas spp.	Rice	Root	Videira et al. 2009

The design of primers for specific amplification of *nifH* helps in identification of nitrogen fixing isolates and also total nitrogen fixing bacteria presence in environmental samples. The nitrogen fixation genes were first described in *Klebsiella oxytoca* strain M5a1 carrying the 24 kb nitrogenase gene cluster. The genes involved in nitrogen fixation (*nifH, nifD nifK, nifY, nifB, nifQ, nifE, nifN, nifX, nifU, nifS, nifV, nifW, nifZ*) are highly conserved among the diazotrophic bacteria. This enzyme nitrogenase is highly conserved among the free living and symbiotic diazotrophic bacteria (Franche et al. 2009). Fig. 1.4 shows the nitrogen fixation process in diazotrophic bacteria. The regulation steps is described as follow (1) Diazotrophs increase the host plant internal nitrogen by fixing the atmospheric nitrogen (2) Increase level of the nitrogen was negatively regulated and the machinery may be stopped to add further into the pool available (3) Diazotrophic endophytic bacteria produce the plant growth promoting hormone (4)

Phytohormones level inside the plants is modulated and improve the colonization. Further the level of ethylene is reducing in counter effect to colonization (5) Production of IAA leads to improve root development which increases the microbial colonization on root and ultimately nitrogen level (6) All these aspects increase the endogenous level of the nitrogen (7, 8, 9) and this affects differently the different aspects of physiology (Carvalho et al. 2014).

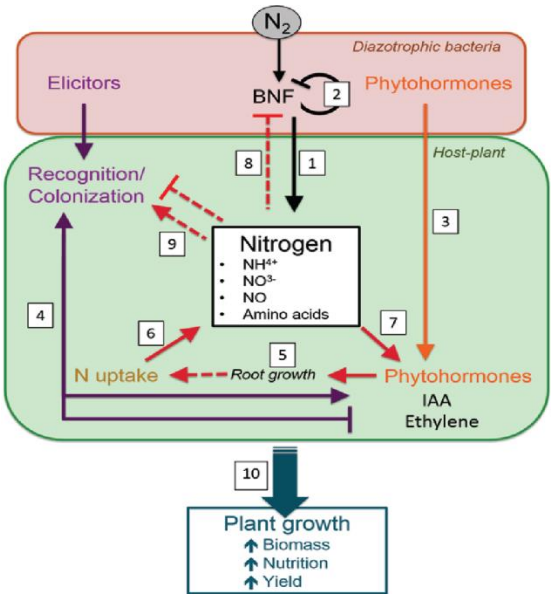


Fig. 1.4 Regulation of nitrogen fixation process between the host plant and bacteria. Regulatory mechanisms can be activating (↓) and/or inhibiting (⊥) metabolic processes (Carvalho et al. 2014)

1.5.2 Biocontrol activity of endophytes

Plant growth promoting bacteria (PGPB) can be classified into two groups: one which solely promotes plant growth and another group that promotes the plant growth as well reduces plant disease. These organisms are denoted as biofertilizers or biocontrol agents. The latter group shows an antagonistic effect against phytopathogens via different mechanisms such as the production of antimetabolite molecules, siderophores, and competition for nutrients and niches or by the induction systemic resistance into the plant (Lugtenberg and Kamilova, 2009). The most widely used biocontrol is *Pseudomonas protegens* used to control the bacterial and fungal pathogens (Berg 2009). Many bacterial genera which are common soil flora producing secondary metabolites are also associated with endosymbiont in plants and include members of *Pseudomonas*, *Burkholderia*, actinomycetes and *Bacillus* (Ryan et al. 2008).

Antibiosis is a well studied mechanism for plant pathogen inhibition and the most reported group of bacteria involved is *Pseudomonas* spp. which produces a wide range of antimicrobial compounds. (Compant et al. 2005). The major groups of antifungal metabolites produced are phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin, viscosinamide and tensin (Bhattacharyya et al. 2012). There are many evidences of secretion of lytic enzymes produced by biocontrol agents which help resist pathogenic attack. Extracellular chitinases are also considered crucial for *Serratia marcescens* to act as an antagonist against *Sclerotium rolfsii*, and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f. sp. *cucumerinum*. The lytic enzyme production is regulated similarly as antibiotic production involves GacA/GacS regulatory system (Compant et al. 2005; Glick 2012).

Phloroglucinol and is its derivatives are widely used in the different field of industry such as in cosmetic, pharmaceutical, leather industry etc. In bacterial kingdom only *Pseudomonas* spp. is known to produce the phloroglucinol and its derivatives. One of the derivatives of phloroglucinol known as 2, 4- DAPG which widely known to inhibit the bacterial and fungal pathogens such as damping off of sugar beet, a take-all decline of wheat, and black root rots of tobacco. The biosynthesis of DAPG requires the *phl* operon encoded by 8.2 kb region consisting of the ORFs *phlH, phlG, phlF, phlA, phlC, phlB, phlD* and *phlE*. These genes are responsible for the synthesis, transportation outside the bacterial cell and degradation of 2,4-DAPG. The genes responsible for 2,4-DAPG synthesis are *phlA*, *phlC*, *phlB*, and *phlD* (Yang and Cao. 2012). Enzymology studies showed that the *phlD* gene product is unstable with the half-life of 7.2 min at 37 °C and the catalytic activity is 0.56 µM min^{-1} required the large turnover for DAPG production (Zha et al 2008). Several studies on 2, 4- DAPG production in rhizosphere by *P. protegens* CHA0 indicated the modulation of gene expression of 2, 4- DAPG production by plants by the plant hormones such as salicylic acid, jasmonic acid, and methyl jasmonate which reduce the expression of *phlA* gene whereas the IAA induces the *phlA* expression. Studies also showed that pathogen attack or a mechanical injury to plant tissue induces the 2, 4- DAPG production in the root. The production of 2, 4- DAPG was found higher in monocot plants compare to the dicot. Studies with six different variety of maize showed the plant genotype is responsible for the 2, 4- DAPG production. A specific compound in root exudates such as sugar

modulates the various antimicrobial compound productions in *P. protegens* CHA0 (de Werra et al. 2011; Vacheron et al. 2013).

The siderophore producing strains also play an important role in disease control. The studies with the rhizobacteria showed the hydroxamate type of siderophore plays the important role in fungal pathogens inhibition such as *Alternaria* sp. *Fusarium oxysporum*, and *Pyricularia oryzae* (Chaiharn et al. 2009). Besides siderophore, hydrolytic enzymes are also involved in biocontrol. The chitinase activity of *Serratia plymuthica*, *S. marcescens*, *Paenibacillus* sp. 300, *Streptomyces* sp. 385 and *P. stutzeri* showed the antagonistic effect against various pathogens such as *Botrytis cinerea, Sclerotium rolfsii, Fusarium oxysporum* f. sp. *cucumerinum, Fusarium Solani* (Compant et al. 2005). The extracellular proteases produced by the *Stenotrophomonas maltophilia* W81 protect the sugar beet from *Pythium*-mediated damping-off (Dunne et al. 1997).

Additionally, residing microbiome in rhizosphere/internal tissue of the plant can inhibit the invasion of pathogens by competing for nutrient and for the available niche. This phenomenon was confirmed by constructing artificial bacterial strains community with non-virulent *Ralstonia* spp. which is phylogenetically related to the plant pathogen *Ralstonia solanacearum.* The non-virulent strain did not show direct growth inhibition of the virulent organism but prevented the virulent bacteria from establishing disease. This indicates that having a resident community that overlaps with the pathogen in niche colonization was best tool to eliminate the pathogen and higher resident bacterial diversity lessen the chances of pathogen invasion and plant disease incident (Wei et al. 2015).

1.6 Other plant growth promoting traits in endophytes

1.6.1 Indole acetic acid production (IAA)

PGPB (as well as some of the phytopathogens) are known for the production of various phytohormones such as indole acetic acid (IAA), gibberellic acid, abscisic acid and cytokinin etc. These hormones play an important role in root surface architecture modulation. About 80% of rhizospheric bacteria can produce IAA. IAA improves the overall growth of the plant by increasing the root hair, increase root surface area, cell division, cell enlargement, enhance the lateral root development and adventitious roots. Plant root development and nodule formation

are both affected by the level of IAA produced. At low concentrations, exogenous application of IAA stimulates primary root elongation, whereas high IAA levels stimulate the formation of lateral roots, decrease primary root length and increase root hair formation (Spaepen et al. 2007). Several model strains of IAA producing bacteria such as *Agrobacterium tumefaciens*, *Pantoea agglomerans*, *Azospirillum*, and *Enterobacter cloacae* have been used to study the genetics of IAA production via different pathways (Spaepen et al. 2007). IAA biosynthesis involves different pathways such as indole-3-acetamide pathway, indole-3-pyruvate pathway, tryptamine pathway, tryptophan side-chain oxidase pathway and tryptophan-independent pathway. In plants, most of the IAA molecules are conjugated to protect the IAA from degradation also for its transport. Formation these conjugant maintain the levels of IAA in plant and catabolism of IAA (Spaepen et al. 2007). IAA production in plant and bacteria showed the similarity between them. Plant growth promotion by PGPB can also result from indirect stimulation of the plant auxin pathway by nitrite reductase activity which enables them to produce NO during root colonization which modulates the auxin signalling pathway controlling lateral root formation (Vacheron et al. 2013). The antibiotic DAPG can also interfere with the auxin-dependent signalling pathway and thus modify root architecture (Brazelton et al. 2008)

An IAA deficient mutant strain of the IAA producing *Pseudomonas putida* GR-12-2 when inoculated on canola seeds showed the 35-50% lesser root development compared to IAA proficient strain. Overproduction of IAA by a mutant of a mung bean colonizing strain caused shorter root development compared to wild type control. This clearly indicates that different IAA levels show both stimulatory and inhibitory effect on root development. High bacterial IAA, when taken up by plants, stimulates the ACC synthesis which ultimately increases the level of the stress hormone ethylene (Glick 2012). Very high production of IAA by *Azotobacter* spp. up to 7.3-32.8 mg ml^{-1} has been reported (Bhattacharyya et al. 2012).

1.6.2 Siderophore production

Siderophores are organic, low molecular mass compounds that have the ability to chelate ferric iron (Neilands 1995). Microorganisms produce the array of siderophores which are broadly classified as catecholate and hydroxamate. The siderophores transport system varies between gram-positive and gram-negative bacteria. Gram-negative bacteria have the Ton-B dependent outer membrane receptors recognize the Fe (III) and the membrane through an energy-dependent

system consisting of the outer membrane receptor proteins, periplasmic binding proteins, and inner membrane transport proteins. Then the cytoplasmic transport is via ATP-binding cassette (ABC) transport. In gram-positive bacteria there is lack of outer membrane, therefore, the Fe (III) binding receptor anchored in the periplasmic membrane and further transport is similar to the gram-negative bacteria (Scavino et al. 2013). Along with this siderophore production was also reported in plant system belong to *Poaceae* family such as wheat and barley. These plants develop the strategy for chelate the Fe (III) from the environment by producing phytosiderophore. The first identified siderophores produced by the plant is mugieic acid (MA). The phytosiderophore has the lower stability with Fe (III) complex compared to microbial siderophores (Ahmed and Holmström 2014).

1.6.3 Phosphate solubilization

After nitrogen, phosphorous (P) is a second most important element of plant growth. A large amount of P is in the insoluble form which is unavailable to plants. Plants can absorb two forms of P, monobasic ($H_2PO_4^-$) and dibasic ($H_2PO_4^{2-}$). Insoluble form of P can be solubilized by microbes to make it available for plants (Rodriguez and Fraga 1999). The p-solubilization process can be performed by organic acid secretion or proton release. Studies indicate the best P-solubilizing agents are oxalic and citric acid. Many microbes are well known for P-solubilization in agricultural crops like tomato, potato, wheat, radish, pulses etc., are *Azotobacter chroococcum*, *Bacillus circulans* and *Cladosporium herbarum*, *Bradyrhizobium japonicum*, *Enterobacter agglomerans*, *Pseudomonas chlororaphis* and *P. putida* and *Rhizobium leguminosarum.* Organisms showing the P-solubilization on Pikovskaya's agar may not be able to solubilize P in alkaline vertisol or acidic alfisol which is in the form of Ca, Fe and Al complexes. Chemical phosphate fertilizers used in field application at regular intervals also get accumulated in soil insoluble forms due to its high reactivity and only 25% of applied fertilizer is used by the plants. For characterization of organisms able to carry out P-solubilization in conditions that mimic vertisols, a mineral medium with high buffering strength containing rock phosphate as the sole P-source was designed wherein growth and acidification of the medium (detected by the pH indicator) indicated efficient P solubilization (Gyaneshwar et al. 1998). This medium helps in selection superior isolates which can work in alkaline vertisol and able to release P from complex mineral phosphates.

Endophytic bacteria also help in increasing P-availability to the host plants. A recent study on endophyte *Pseudomonas* spp. was conducted in which tricalcium phosphate was added as the P source. The endophytes produced gluconic acid (GA) in the range of 14-169 mM which helped in solubilization of the insoluble form of P (Otieno et al. 2015). P is also reserved in organic form which constitutes 30-50% of total P in soil. The major form of organic P is inositol phosphate synthesized mainly by plants and represents the most stable form and a major component of the organic P. Mineralization of the organic P involves the production of hydrolytic.

1.7 Modulation of plant immune response by pathogens and plant growth promoting bacteria

All plants possess similar defense machinery which is induced by pathogen attack. The first line of defense response is carried out by plants recognizing certain "pathogen-associated molecular patterns" (PAMPs) with the help of "pattern recognition receptors" (PRRs). This information triggers the PAMP-triggered immunity (PTI) response. To overcome this, pathogens have evolved mechanisms to suppress the PTI system or by preventing pattern recognition by the host. In counter response, plants have evolved specialized proteins known as "R-proteins" (resistance proteins), which allow the PTI system to activate the effector-triggered immune (ETI) response which is characterized by localized death of host cells to prevent pathogen spread. Both the PTI and ETI responses highly overlap with each other in activation of systemic defense response which includes the transmission of the signal to undamaged distal tissues of plant, cell wall fortification, antimicrobial agent production, expression of "pathogenesis-related" (PR) proteins such chitinase, glucanase (Pieterse et al. 2009; Pieterse et al. 2014) (Fig. 1.5). The systemic resistance developed by the mounting of a successful defense against the attacking pathogen is known as Systemic Acquired Resistance (SAR) (Van Loon et al. 1998). Downstream to SA in the SAR signalling pathway is an important redox-regulated protein Non-expressor of PR Genes1 (NPR1), which upon activation functions as a transcriptional co-activator of a large set of PR genes.

In addition to the induction of systemic resistance by the attacking pathogen, it was shown in 1991 that certain beneficial organisms can also induce the defense system of plants. This phenomenon was denoted Induced Systemic response (ISR) to distinguish it from SAR. It was

realized that molecular players and the mechanisms of ISR were somewhat different than SAR and do not culminate in activation of PR genes (Fig. 1.3). The induced state of resistance is a latent defense mechanism that potentiates the defense induction upon subsequent challenge by a broad spectrum of attackers. The plant hormones jasmonic acid and ethylene are the important signalling molecules in ISR (Table 1.2). Interestingly, the protein NPR1 is involved in ISR as well as SAR, however, its role is not clearly understood but is different as it does not require nuclear localization and does not result in activation of PR proteins. This phenomenon is also known as priming whereby plants are sensitized for enhanced defense gene expression upon pathogen challenge (Van Loon 2007; Pieterse et al. 2014). The activation of defense-related gene expression also depended on the type of pathogen attack. The three categories of pathogens i.e. necrotrophs, biotrophs, and hemibiotrophs trigger different defense responses in the plant (Pieterse et al. 2009). Defense-related gene expression is well documented in case of *Arabidopsis* plant. These studies showed important JA pathway markers to be *PDF1.2*, *VSP2* and *HEL* and SA pathway marker involved the expression of *PR* genes.

1.7.1 Endophyte induced priming of defense in plants

Bacterial components that induce the systemic resistance in plants while colonizing rhizosphere or entering inside the plant tissues include lipid A, O-antigenic side chain, pseudobactins, pyochelin, flagella, pyocyanin, 2,4-DAPG, N-acylhomoserine lactones, 2,3-butanediol (Van Loon 2007). Most of these molecules are associated with gram-negative cell wall or produced by gram-negative bacteria and well-characterized for rhizosphere bacteria. Most well studied bacteria for ISR response are *Pseudomonas* spp.

On the other hand, many endophytic *Bacillus* spp, reduce the severity of diseases in vegetables such as tomato and cucumber show induction of ISR along with endophytic *Pseudomonas* and *Serratia* strains (Kloepper and Ryu, 2006). ISR has been reported for endophytic *Bacillus pumilus* INR7 which elicited systemic protection against bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* in pepper following application to pepper root. In addition, application of *B. pumilus* INR7 in combination with synthetic chemical elicitor benzothiadiazole (BTH) showed the enhanced plant defense-related gene expression under field condition (Yi et al. 2013). The competency of endophytic actinobacteria to elicit plant defense gene expression against *Fusarium oxysporum* and *Erwinia carotovora* subsp. *carotovora* in

Arabidopsis thaliana was evaluated by Conn et al. (2008). Actinobacterial strains were inoculated at seed stage and fungal pathogens were inoculated on six- to eight-week-old plants. Endophyte treated plants showed the upregulation of SAR and JA/ET related defense gene which strongly upregulated upon pathogen challenge. The priming with the plant growth promoting microbes reduced the effect of pathogen infection and improved the yield. Gond et al. (2015) reported that endophytic antifungal lipopeptide producing *Bacillus* spp. elicits the pathogenesis related (PR) gene expression in maize plants but when alone lipopeptide extract was inoculated on plants it did not elicit the PR gene expression.

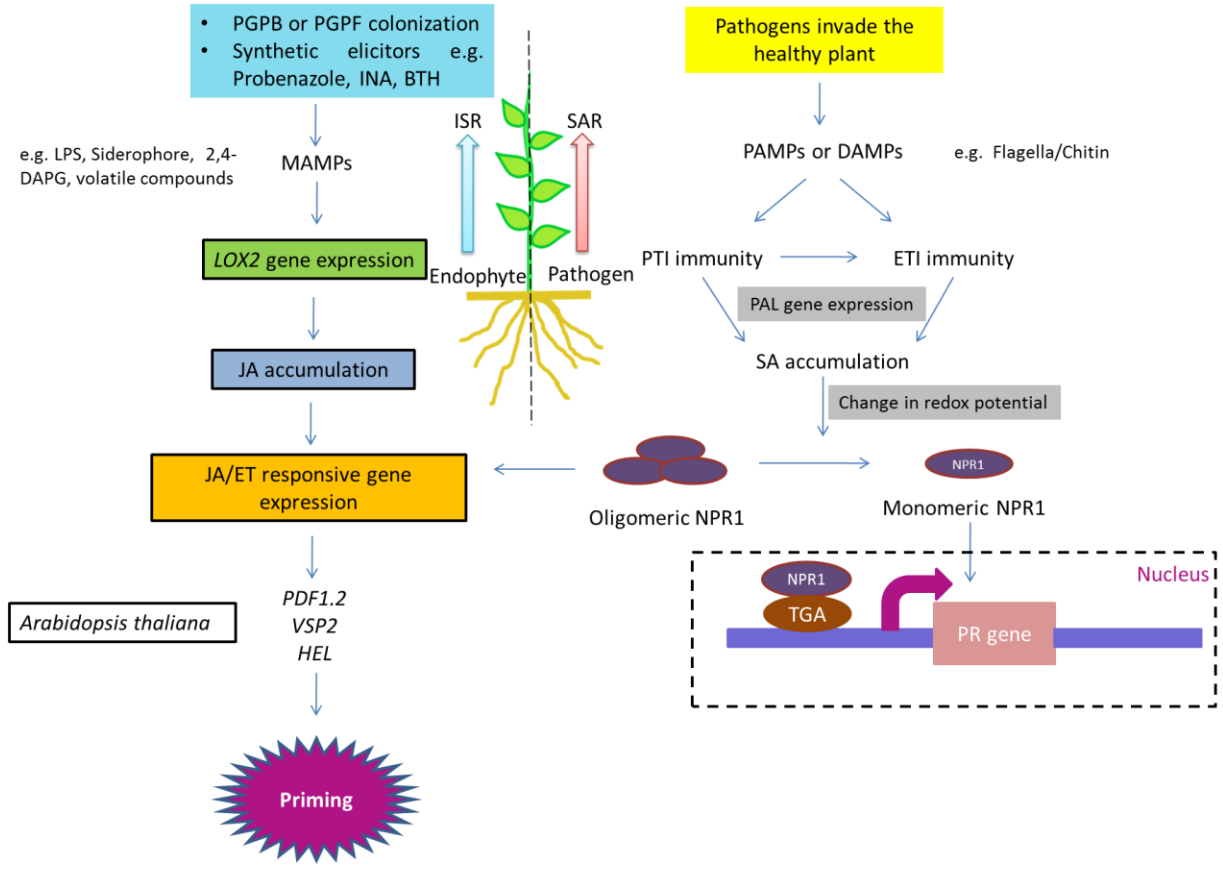


Fig. 1.5 Schematic represent the induction of defense related gene expression in plants upon challenge with PGPB or pathogens. ISR- Induced systemic resistance; SAR-systemic acquired resistance; PAMPs, MAMPs and DAMPs- pathogen- or microbe- or damage-associated molecular patterns; INA- 2,6-Dichloro-isonicotinicacid; BTH- Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester, LPS-Lipopolysaccharide; DAPG- 2,4-diacetyl phloroglucinol; JA- Jasmonic acid; SA- Salicylic acid; ET-Ethylene; *LOX2*- Lipoxygenase; PTI- PAMP-triggered immunity; ETI-Effector-triggered immunity; *NPR1*- Non-expressor of pathogenesis related gene1; PR gene-Pathogenesis related gene; Plant

Defensin1.2 (*PDF1*.2); *VSP2*-Vegetative storage protein2 Hevein-like protein (*HEL*); *TGA*-Transcription factor.

Defense-related pathway elicited by biocontrol agent producing *Pseudomonas fluorescens* PICF7 upon colonization in olive plants were investigated by Cabanás et al. (2014). Three week old olive plants roots were dipped in 10^8 Cell ml^{-1} suspension and aerial part of the plants were collected at different time points showed 26 upregulated transcripts detected in aerial tissues upon PICF7 colonization (i.e. *PAL*, *ACL*, *CAT*, *LOX*, 14-3-3 protein, CaM, thaumatin-like protein, etc.). In another study observed the interaction of endophytic *Azoarcus* sp. strain BH72 in rice plants induced the defense response accompanied by the induction of pathogenesis-related proteins which was also observed to be induced by jasmonate treatment in rice (Miché et al. 2006). Inoculation of rice roots with the endophytic *Herbaspirillum seropedicae* triggered the expression of genes responsive to auxin and ethylene and the repression of the defense-related proteins PBZ1 and thionins (Brusamarello-Santos et al., 2012)

Investigation on plant response to volatile organic compound (VOC) 2,3-butanediol produced by the endophyte *Enterobacter aerogenes* revealed to induce systemic resistance against the northern corn leaf blight fungus *Setosphaeria turcica* in maize plants (D'Alessandro et al. 2014). Overall, studies indicate that endophytic bacteria form an important group of organisms which can be used in priming plant defense and plant growth promotion for sustainable agriculture.

1.8 Genomics and metagenomics of endophytes

The genome analysis of the 56 plant endophytic bacteria showed the presence of various genes that are involved in plant growth promotion such as pyrroloquinoline quinone (PQQ) biosynthesis gene cluster, 1-aminocyclopropane-1-carboxylate (ACC) deaminase genes, genes for nitrogen fixation, nitric oxide synthesis, 2,3-butanediol synthesis, hydrogen cyanide synthesis and auxin biosynthesis in decreasing order in the genomes. Interestingly, in comparison to rhizospheric bacteria, gene cluster for 2, 4-diacetylphloroglucinol (DAPG) biosynthesis was not found in any analyzed endophytic bacterial genome (Bruto et al. 2014).

Molecular approaches have been carried out to rapidly evaluate the composition of endophytes inside the interstitial spaces of various plant systems. With advancement in

technology like next generation sequencing (NGS) helps in the study the complete microbiome from above and below ground parts of the plants by the culture-independent approach. The total endophytic community that resides inside the plant tissue of *Arabidopsis thaliana* and its close relatives, several tree species, and relevant crop plants such as barley, corn, grapevine, lettuce, potato, tomato, rice, sugarcane, and soybean, as well as the salt-excreting *Tamarix* trees is well characterized using metagenomics approach (Muller et al. 2016). Overall analysis of all this study concludes the major endophytic phyla commonly found in most plants are *Proteobacteria*, *Actinobacteria, Bacteroides* and *Firmicutes*. Among these phyla important family of *Pseudomonadaceae* found the highest colonize up to 30-40%, *Rhizobaceae and Streptomyces* found 20-30%, *Bacillaceae* found 10-20% and *Methylobacteriaceae* found 10-20% only in seed and above ground plant parts. Analysis of leaf endophytic bacterial community in *Arabidopsis*, soybean and clover leaves showed that they interestingly share the similar communities with the highest colonization by *Proteobacteria*, *Actinobacteria* and *Bacteroides*. The major genera found were *Methylobacterium, Pseudomonas* and *Sphingomonas* (Muller et al. 2016). The endophytic bacterial diversity was analyzed in the phyllosphere of Amazon *Paullinia cupana* plants by a culture-independent approach using PCR-DGGE and culture dependent using 16s rRNA clone library. The diversity of endophytic bacteria was assessed in asymptomatic and symptomatic plants of *P. cupana* leaves and higher endophytes diversity was found in symptomatic plants compared to the asymptomatic. The phyla found higher to lower order was *Firmicutes*, *Proteobacteria, Actinobacteria* and *Bacteroidates* with the exception of *Bacteroidates* which was found exclusively in symptomatic plants (Bogas et al. 2015). In another approach, T-RFLP of 16S rRNA and cloning were used to study the endophytic diversity in three different rice cultivars. It was seen that a highly similar endophytic bacterial community existed in all the three varieties with only differences in the dominant endophytic strains in each variety (Ferrando et al. 2012). Comparative analysis of endophytic and rhizospheric diversity was studied by 16S rRNA clonal library in *Stellera chamaejasme* L. showed the presence of similar dominated phylum in decreasing order *Proteobacteria, Firmicutes* and *Actinobacteria* in both but found more varied bacteria in the rhizosphere (Jin et al. 2014). The diversity of the endophytes was assessed in copper tolerant plants from copper mine Westland found the predominant phyla *Firmicutes, Actinobacteria* and *Proteobacteria*. These endophytic bacteria were found to increase the dry mass of the plant in vermiculate amended with 4 mg Kg^{-1} Cu (Sun et al. 2010). Analysis of total

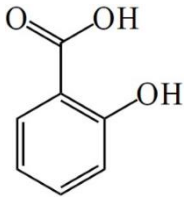
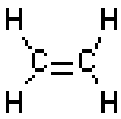
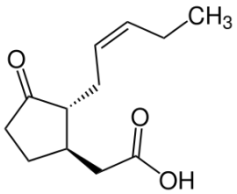
endophytic microbiota associated with the plant system interestingly found the large fraction of it is cultivable due to low complexity in the food web and aerobic environment (Muller et al. 2016).

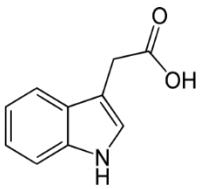
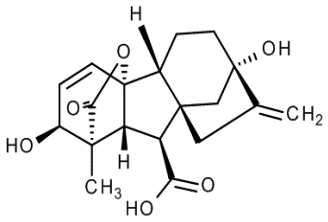
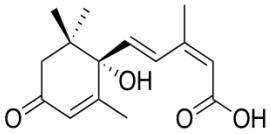
1.9 Genetic modification of endophytes

It has been envisaged that genetically engineered endophytes may be a need for the future (Rosenblueth and Martinez-Romero 2006). Phytoremediation of highly water soluble and volatile organic xenobiotics often does not result in complete elimination of the contaminant and cause volatization in environment through the evapotranspiration through leaves leading to additional environmental pollution. Engineered endophytic *Burkholderia cepacia* L.S.2.4 carrying plasmid pTOM encoding genes for toluene degradation improved phytoremediation, and brought about 50–70% reduction of its evapotranspiration through the leaves and promoted plant's tolerance to toluene (Barac et al. 2004). Andreote et al. (2004) have studied the effect of genetically modified *Enterobacter cloaceae* strain on the endophytic community of citrus seedlings. Various plasmids were transferred in endophytic bacteria and analyzed the impact on their interaction with citrus seed and the endophytic diversity. This study demonstrated that genetically engineered organisms induce the endophytic bacterial community shift according the type of plasmid used in constructing genetically modified strain and also affect the endophytic interaction with the seed.

The advantages and obstacles to use bioengineered endophytes have been clearly discussed (van der Lelie et al. 2004; Newman and Reynolds 2005). The major advantage of using genetically modified organism is ease to modify it in comparison to the plant system. Along with this single engineered bacterium can be used in multiple plants. The endophytic bacteria work better than the rhizosphere colonizing bacteria because of bioaugmentation process. Colonization of the plant tissue by endophytic bacteria reduces the competition as compared to the rhizosphere. But to get these advantages of genetically modified endophytic bacteria we need to overcome the obstacles in field application. The major issue is about the stability of modified strain in an environmental condition which requires the constant selection pressure for maintenance at the site of inoculation. It can be expected that the application of genetically engineered endophytic bacteria will become a general strategy to improve the efficiency of phytoremediation, plant growth promotion and inhibition of phytopathogens.

Table1.2 Phytohormones that are directly or indirectly involved in the plant defense pathways

Molecule	Structure	Response	Mechanism	Reference
Salicylic acid		SAR (PR genes ↑)	SA accumulation occurs usually upon pathogen attack. SA mediates the changes in redox potential in cytoplasm that lead to conversion of the oligomeric NPR1 to active monomeric NPR1 that coactivates PR gene expression	Pieterse et al. 2014
Ethylene		ISR (*ERF1*↑)	Ethylene require to induce ISR	Pieterse et al. 2014
Jasmonic acid		ISR (LOX2 ↑)	Defense against pathogens and insects attack	Turner et al. 2002

Indole-3-acetic acid		Modulates JA pathway	Down-regulation of JA responsive gene	Liu and Wang 2006
Gibberellic acid		Degradation of DELLA protein	Gibberellic acid producing pathogens can inhibit the JA/ET pathway	Navarro et al. 2008
Abscisic acid		PR gene ↓	Antagonistic crosstalk between SAR and auxin	Yasuda et al. 2008

Scope of the Thesis

A sustainable means of providing the essential macroelement nitrogen (N) to plants is through biological nitrogen fixation (BNF), carried out by a select group of prokaryotes known as diazotrophs. The most successful application of diazotrophic bacteria to enhance plant growth and yields is that of symbiotic nodule forming bacteria collectively known as rhizobia which provide fixed N to their hosts, the members of *Leguminosae* (or *Fabaceae*) family of plants (with the exception of non-legume *Parasponia*). Since such natural symbiotic relationship with diazotrophs does not exist for other crop plants, free living, plant associated nitrogen fixing bacteria are applied as N biofertilisers to increase N availability to non-legume plants. These however have lesser ability to provide N as compared to the symbiotic diazotrophs. Recent interest is in endophytic bacteria that are able to colonize the internal tissues of the plant such as root and aerial parts and fix nitrogen i*n planta*. Diazotrophic endophytic bacteria provide an attractive strategy to provide N to non-legume plants. The success of this approach is exemplified by three important systems of plant-endophytic N fixation *viz. Gluconacetobacter* mainly with sugarcane; *Herbaspirillum* colonizing mainly rice, maize, sugarcane, and sorghum and *Azoarcus* with Kallar grass and rice.

Many studies have shown that endophytic N fixing bacteria seem to constitute only a small proportion of total endophytic bacteria. Increasing N-fixing endophytic communities is considered as a possibility to increase nitrogen fixation to wider varieties of plants. To this end, it is important to study diazotrophic populations of important crop plants. Since the endophyte population is usually a subset of the soil and rhizosphere flora and is largely dependent on plant variety as well as prevailing environmental and edaphic conditions, it is important to study their populations in site- and climatic-zone dependent manner. Well-characterized diazotrophic endophytes like *Herbaspirillum, Gluconacetobacter* are very scarce or even absent in soil and have problems in surviving in the harsh, competitive soil environment. Thus it is important to isolate newer strains of diazotrophic endophytes with the idea to develop more robust systems that could also survive well in free-living state. Monocot plants constitute the second largest plant group and include the most economically important family among all plant families, the *Poaceae* family, also known as cereal grasses such as rice, wheat, maize etc. Developing robust,

a diazotrophic endophytic strain for this family of plants is very important from the point of view of global food security.

Another important application of endophytes is their use as biocontrol agents to suppress disease in plants. Endophytes are particularly regarded as promising biocontrol agents because they occupy the same niche as the pathogens and could compete with them for nutrients and niche as well as directly antagonize them with the production of antimetabolic compounds. Since endophytes and pathogens use similar machinery to enter and colonize the plant tissue, an emerging aspect of their study involves understanding the mechanism by which they do not evoke a strong defense response from the plants. Moreover, the colonization of many endophytes results in priming of the plants, providing a foundation for a more intense and rapid defense against pathogen attack. Very few studies have been conducted to understand the plant defense system in monocots during the pathogen attack and also the beneficial effect of priming upon pathogen attack.

It has been envisaged that in the near future scientific community may consider the use of genetically engineered endophytes with biological control potential in agricultural crops. At present endophytes have been engineered from the point of view of phytoremediation. 2,4 DAPG, an important antibiotic molecule involved in biocontrol of phytopathogens is produced mainly by rhizospheric strains of *Pseudomonas*. Since endophytes do not show the presence of the DAPG biosynthetic cluster, it would be interesting to study the effect of incorporation of DAPG biosynthetic genes in an endophytic organism.

In light of the above, the present work was focused on the study of diversity and isolation of diazotrophic endophytic bacteria from various *Poaceae* plants. Detailed study with native biocontrol strain and genetically engineered biocontrol strain were designed. Accordingly, the objectives of this work were as follows:

Objectives

1. Isolation and characterization of diazotrophic endophytic plant growth-promoting bacteria from various indigenous *Poaceae* family plants

a) Study the culturable and unculturable diversity of diazotrophic and total endophytes from maize, wheat, pearl millet, sorghum and rice
b) Isolation of diazotrophic endophytic bacteria from different parts of the plants selected viz. maize, wheat, pearl millet, sorghum and rice
c) Identification and characterization of PGPR traits of diazotrophic endophytes from various plants
d) Confirmation of the endophytic nature of isolates by tagging with the *gfp* and reisolation from inoculated plants
e) Study of plant growth promotion in model plant by greenhouse pot experiments

2. Plant growth promotion and disease suppression by antibiotic producing diazotrophic endophytic *Streptomyces* spp.

a) Study of the fungal pathogen inhibition by the *Streptomyces* spp. and analyze the secondary metabolite production and identification by GC-MS
b) Study of the endophytic nature of *Streptomyces* spp. by tagging with the *gfp* plasmid
c) Study of the colonization and plant growth promotion of endophytic bacteria in sorghum plants in greenhouse pot experiment
d) Plant protection and plant growth promotion study in rice, sorghum and wheat under greenhouse condition by pot experiments
e) Quantitative gene expression study of defense genes induced by *Streptomyces* spp. inoculated plants upon fungal pathogen challenge

3. Cloning and expression of 2, 4-Diacetylphloroglucinol, gene cluster in diazotrophic endophytic bacterial system and evaluation of its ability for disease suppression

a) PCR amplification and cloning of the *phlD* gene from *Pseudomonas protegens* Pf-5 and *Pseudomonas* sp. G22 in *E. coli* and diazotrophic endophytic bacteria and to analyze the tolerance for phloroglucinols.

b) Adding the *phlACB* gene cluster gene from *Pseudomonas* spp. into *phlD* containing plasmid and introduce it to endophytic bacterial system

c) Detection and quantification of DAPG production by genetically engineered strains HPLC

d) Study of plant protection and plant growth promotion by the genetically engineered strains in wheat, sorghum and rice

e) Study the ISR induction by genetically engineered strains in rice plants