Endophytic Nitrogen-Fixing Bacteria as Biofertilizer

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Abstract Nitrogen is the most limiting nutritional factor for the growth of plants. Since plants cannot reduce atmospheric N_2, they require exogenously fixed nitrogen for growth and development. Atmospheric N_2 must be first reduced to ammonia to be used by plants. In practice, chemical N fertilizers are used to provide nitrogen nutrition to plants. However, manufacture and use of N fertilizers are associated with environmental hazards that include release of greenhouse gases at the time of manufacture, as well as contamination of underground and surface water due to leaching out of nitrates. Moreover, manufacture of chemical fertilizers requires non-renewable resources like coal and petroleum products. Excess and continuous use of chemical fertilizers to improve the yield of commercial crops has negative effect on soil fertility and reduces their agricultural sustainability. All these concerns necessitate the search for an alternative strategy that can provide nitrogen nutrition to the plants in an efficient and sustainable manner. Here biological nitrogen fixation has immense potential and can be used as an alternate to chemical fertilizers. Biological nitrogen fixation has been reported to be exclusively carried out by few members of the prokaryotic organisms. Biological nitrogen fixation is a process

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where atmospheric N_2 is reduced to NH_3. This process is catalyzed by microbial enzyme nitrogenase. Microorganisms having the capacity to fix atmospheric N_2 can be used as efficient biofertilizer.

In this chapter, we review application, properties, ecology, and advances in biology of nitrogen fixing bacteria with reference to endophytic bacteria that colonize the interior of plant without exerting any substantive harm to their host plant. Nitrogen-fixing endophytic bacteria have edge over its rhizospheric counterparts because, being sheltered inside plant tissues, they face less competition and can make available the fixed nitrogen directly to plants. Moreover, the partial pressure of oxygen inside the plant tissue is more acquiescent for efficient nitrogen fixation. Nitrogen fixing endophytic bacteria have been isolated from several plant species and found to contribute upto 47% of nitrogen derived from air, which in turn enhance plant growth. Nitrogen fixing ability of bacteria can be evaluated by total nitrogen difference method, acetylene reduction assay, analysis of nitrogen solutes in xylem and other plant parts and N-Labeling Methods. Furthermore, molecular approaches such as amplification, analysis of nitrogen-fixing genes (*nif* genes), and qualitative and quantitative estimation of their products can be used for evaluation of nitrogen fixing ability of the bacteria.

In addition to nitrogen-fixation ability, these bacteria can influence plant growth through one or more properties. These include production of phytohormones, siderophores, induced systemic tolerance through production of 1-aminocyclopropane-1-carboxylase deaminase, induced systemic resistance and antagonistic activities. The make-up of endophytic bacterial communities depends on various factors such as soil type, soil composition, soil environment, plant genotype and physiological status, bacterial colonization traits, and agricultural management regimes. Colonization and abundance of different bacterial species varies widely with host plants. Endophytic bacterial community can be analyzed employing stable isotope probing as well as various modern molecular approaches which are based on analysis of 16S ribosomal deoxyribonucleic acid (DNA), gene encoding products for nitrogen fixation and repetitive DNAs. Moreover, metagenomic approaches allow estimation and analysis of unculturable bacteria at genomic as well as functional genomic level. Colonization process of an endophytic bacterium involves various steps which include migration towards root surface, attachment and microcolony formation on plant surface, distribution along root and growth and survival of the population inside plant tissue. Ongoing progress towards in-depth analysis of genomic and whole protein profile of some of the potential endophytic bacteria such as *Azoarcus* sp., *Gluconoacetobacter diazotrophicus, Herbaspirillum seropedicae, Serratia marcesens* can help understand mechanism involved in plant-endophyte interaction which in turn will be deterministic in use of suitable formulations of endophytic bacteria to be used as biofertilizer for sustainable agriculture.

Keywords 1-aminocyclopropane-1-carboxylase deaminase • Biofertilizer • Diazotrophic • Endophytic • Nitrogen • Reverse transcription-polymerase chain reaction

Abbreviations

CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
gfp	*gfp* is a gene which encodes for green fluorescent protein
gus	*gus* is a gene which encodes for β-glucuronidase
HCN	Hydrogen cyanide
mRNA	messenger RNA which is used as template for protein synthesis.
N	Nitrogen
N_2	Atmospheric Nitrogen
NO_2	Nitric oxide
nifHDK	These are set of genes which encodes structural part of nitrogenase, an enzyme which catalyzes nitrogen fixation.
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
r DNA	ribosomal DNA encodes for rRNA, a structural component of ribosome

1 Introduction

Nitrogen is an important limiting factor for plant growth in various environmental conditions. Despite abundance of atmospheric nitrogen (78%), it cannot be utilized for growth and metabolism. It must be reduced to ammonia for use by any organisms by a process called nitrogen fixation. Application of industrially manufactured nitrogen fertilizer has been one of the most popular ways to provide nitrogen nutrition to the plants to attain high crop productivity. However, excessive and continuous use of chemically synthesized fertilizer can lead to several consequences which include: (i) ground water contamination of nitrate due to leaching and denitrification which is detrimental for human and animal health, (ii) surface water contamination by eutrophication which may arise due to leaching of nitrogen in water and affects growth of aquatic organisms and (iii) production of greenhouse gases CO_2 and NO_2 during manufacture of nitrogen fertilizer using non-renewable resources like natural gas and coal, thus contributing to global warming (Bhattacharjee et al. 2008). Moreover, increase in prices of petroleum products has led to an upsurge in the cost of chemical fertilizer. Therefore, use of alternative fertilizers, which are cost effective and environmental friendly, must be sought.

Biological nitrogen fixation is considered to be the most potential way to provide fixed form of nitrogen to the plants. However, nitrogen fixation is performed solely by prokaryotes (bacteria and cyanobacteria) and archeans. The diazotrophic (N_2-fixing) bacteria are involved in the fixation process, in which these bacteria either in the free living form or in symbiosis can covert the atmospheric nitrogen into NH_3 with the help of nitrogenase enzyme. Nodulated legumes with endosymbiosis with rhizobia are among the most prominent nitrogen fixing system in agriculture.

Although most of the biologically fixed nitrogen made available to the plants is contributed by *Rhizobium* sp. and cyanobacteria, their use is restricted only to certain plant species. Applications of plant growth promoting endophytic bacteria are being considered as a potential biofertilizer in recent years (Bhattacharjee et al. 2008; Akhtar and Siddiqui 2010). This has driven intensive research towards in-depth characterization and better understanding of endophytic diazotrophic bacteria isolated from various plant species.

Any bacterium could be considered as an endophytic diazotroph if (i) it can be isolated from the surface of disinfected plant tissue or extracted inside the plants (ii) it proves to be located inside the plant, either intra- or inter-cellularly by *in-situ* identification and (iii) it fix nitrogen, as demonstrated by acetylene reduction and/or ^{15}N-enrichment. This definition includes internal colonists with apparently neutral or saprophytic behavior as well as symbionts (Hartmann et al. 2000). Endophytic bacteria are better than their rhizospheric and rhizoplanic counterparts in terms of benefiting their host through nitrogen fixation as they can provide fixed nitrogen directly to their host (Cocking 2003). As low partial oxygen pressure is necessary for the expression of the O_2 sensitive enzyme, nitrogenase, endosphere of plant root is more amenable for N_2-fixation reaction. Moreover, endophytic bacteria are less vulnerable to competition with other soil microbes for scarce resources and remain protected to various abiotic and biotic stresses (Reinhold-Hurek and Hurek 1998). In addition to diazotrophy, endophytic bacteria may enhance plant growth through one or more mechanisms which include phytohormone production, siderophore production, induced systemic tolerance and biocontrol potential. The applications of diazotrophic endophytes in various fields have been depicted in the Fig. 1. The intimate relationship of endophytic bacteria with plant can be utilized in developing efficient biofertilizer and biocontrol agents for attaining sustainable agriculture (Sevilla and Kennedy 2000). The present chapter unveils the importance of diazotrophic endophytic bacteria to exploit its properties for the development of sustainable agriculture.

2 Nitrogen Fixation by Endophytic Bacteria

In recent years, application of endophytic bacterial inoculants supplying N requirement have drawn attention for increasing plant yield in sustainable manner efficiently to the various crop plants. Percent contribution of plant nitrogen as a result of biological N_2-fixation by endophytic bacteria has been summarized in Table 1. Some of the promising endophytic biofertilizers include the members of *Azoarcus*, *Achromobacter*, *Burkholderia*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella* and *Serratia* (Rothballer et al. 2008; Franche et al. 2009). The efficient N supply by endophytic diazotrophic bacteria in sugarcane and kallar grass suggests the possible avenues of biological nitrogen fixation in interior niches of plants. In addition, bacteria isolated from non-leguminous plants like rice, wheat, maize, sorghum also fix the

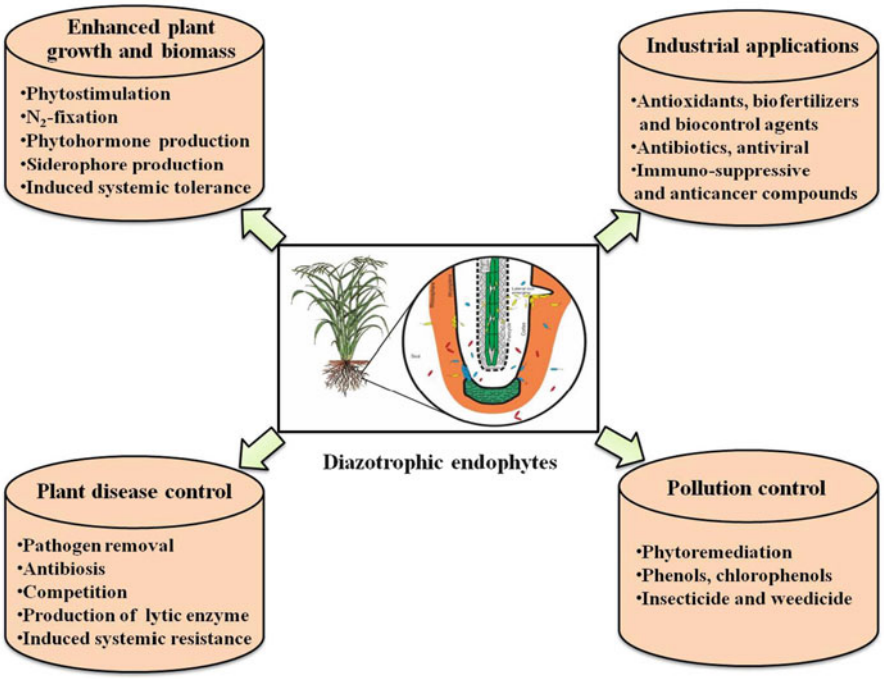

Fig. 1 Multiple applications of diazotrophic (N_2 fixing) endophytic bacteria in various fields including agricultural practices, industries and environment (Modified from Hardoim et al. 2008)

N in endophytic manner. It is evident from the reports that the *Gluconoacetobacter diazotrophicus* (*Acetobacter diazotrophicus*) is the main contributor of endophytic biological nitrogen fixation in sugarcane, and it has the ability to fix the N approximately 150 Kg N ha^{-1} $year^{-1}$ (Dobereiner et al. 1993; Muthukumarasamy et al. 2005). *Azoarcus* is recognized as another potential N_2-fixing obligate endophytic diazotroph. It dwell in the roots of kallar grass, and increased the hay yield upto 20–40 t ha^{-1} $year^{-1}$ without the addition of any N fertilizer in saline sodic, alkaline soils (Hurek and Reinhold-Hurek 2003). In addition, many energy plants (C_4 plants) like *Miscanthus sacchariflorus, Spartina pectinata* and *Penisettum purpureum* have been found to harbour bacterial population, which have the potential to support the N nutrition of the plant (Kirchhof et al. 1997). In a study, *Herbaspirillum* sp., inoculated into rice seedlings maintained in N-free Hoagland solution containing ^{15}N-labelled N, showed ^{15}N dilution amounting upto 40% increase in total N of plant (Baldani et al. 2000). Growth stimulation of wheat, corn, radish, mustard and certain varieties of rice shoots following seed inoculation with a strain of *Rhizobium leguminosarum* bv *trifolii* in pot experiment has also been reported (Hoflich et al. 1995; Webster et al. 1997). These investigations suggest that endophytic diazotrophs have a considerable potential to increase the productivity of non-legumes including important cash crop plants.

Table 1 Biological nitrogen fixation by diazotrophic endophytic bacteria

Endophytic bacteria	Associated plant	N derived from air (%)	References
Burkholderia	Rice	31	Baldani et al. (2000)
Herbaspirillum	Rice	19–47	Mirza et al. (2000)
Rhizobium leguminosarum bv. *trifolii*	Rice	19–28	Biswas et al. (2000) and Yanni et al. (2001)
K. pneumoniae 324	Rice	42	Iniguez et al. (2004)
B. vietnamiensis	Rice	40–42	Govindarajan et al. (2008)
Beijerinckia, Bacillus, Klebsiella, Enterobacter, Erwinia, Azospirillum, Herbaspirillum and *Gluconaacetobacter*	Sugarcane	18	Abeysingha and Weerarathne (2010)
Azospirillum	Rice	9.2–27.7	de Salamone et al. (2010)
Pseudomonas, Stenotrophomonas, Xanthomonas, Acinetobacter, Rhanella, Enterobacter, Pantoea, Shinella, Agrobacterium and *Achromobacter*	Sugarcane	41.2–50.3	Taule et al. (2012)
G. diazotrophicus, H. serpedicae and *H. frisingense*	Elephant grass	5.4–5.5	de Morais et al. (2012)
Microbacterium sp.	Sugarcane	5.4–6	Lin et al. (2012)
G. diazotrophicus, H. seropedicae, H. rubrisubalbicans Burkholderia sp.	Sugarcane	29–74	Urquiaga et al. (2012)

2.1 Quantification of Nitrogen Fixation

Quantification of fixed nitrogen made available to the plants by diazotrophic bacteria can be estimated by the following methods.

2.1.1 Total N Difference Method

This method measures biological nitrogen fixation on the basis of difference between the total N content of crop grown in presence of diazotrophic bacteria and their counterpart grown without bacterial inoculation. It is the oldest and simplest method which is based on an assumption that the control plant and the infected plants absorb same amount of N from soil.

2.1.2 Acetylene Reduction Assay

This technique is based on the fact that the nitrogenase enzyme involved in N_2-fixation can also reduce acetylene into ethylene as both nitrogen and acetylene are triple bonded structures. The assay is done by incubating bacterial culture with acetylene (0.03–0.1 v/v) in an air-tight vessel. Finally, the gas phase of sample is analyzed for ethylene generated as a result of reduction of acetylene by nitrogenase using a gas chromatograph. The calculated ethylene amount can either be directly used to quantify the amount of nitrogen fixed or can be converted into amount of nitrogen fixed by directly multiplying it with a factor 3. It is because in conversion of N_2 to NH_3, three pairs of electron are used while in acetylene to ethylene conversion only single pair of electrons is used. Therefore, this technique measures N_2-fixation indirectly on the basis of electron flux through nitrogenase. The acetylene reduction assay is simple, cheap and sensitive technique.

2.1.3 Analysis of N Solutes in Xylem and Other Plant Parts

In this method, the composition of nitrogenous compounds present in plant the xylem sap is analyzed. The basic idea is to differentiate between the nitrogen fixed and the soil derived nitrate N in the plant. As the uptake from soil increase, it inhibit the N_2-fixation, which results in the change of composition of nitrogenous compounds in xylem sap and by monitoring such changes, the N_2-fixation can be quantitatively analyzed.

2.1.4 N-Labeling Methods

There are three different methods for labeling viz. ^{15}N gas labeling, isotope dilution and A-value method. The principle behind this method is based on the difference of $^{15}N/^{14}N$ present in soil or plant system with that of atmosphere (0.33%). So, if the

plants are incubated with ^{15}N and then, the nitrogen fixation is evaluated in plants, the ratio would differ in plants than that of present in atmosphere. And the change occurring in the ratio can be monitored and analyzed for quantifying the nitrogen fixing ability of microbes.

2.2 Molecular Analysis of Nitrogen Fixation

Bacterial communities show an immense phenotypic and genetic diversity (Ovreas and Torsvik 1998). Since, the majority of microorganisms cannot be cultured on media, estimation and analysis of natural diazotrophic bacterial communities is quite challenging (Borneman et al. 1996). However, this problem can be overcome by employing cultivation independent techniques using universal primers for amplification of gene encoding the key enzyme nitrogenase (Kirk et al. 2004).

There are three types of nitrogenases based on the presence of core metal (molybdenum (Mo), vanadium (V) and iron (Fe)) which bridge two units of this enzyme (Zehr et al. 2003; Raymond et al. 2004). Out of these three types, Mo-nitrogenase is most prevalent. There are three genes namely *nifHDK* which encodes for the structural part of nitrogenase complex. Apart from *nifHDK*, nitrogenase expression and function depends on several other genes (20 in case of *K. pneumoniae*). The genes *nifHDK* encodes for α and β fragments respectively of larger segment of nitrogenase complex called dinitrogenase ($\alpha_2\beta_2$), while *nifH* encodes smaller segment Fe protein (dinitrogenase reductase). Nitrogenase activity can be estimated by following procedures.

2.2.1 Polymerase Chain Reaction (PCR)

Amplification of *nifH*, *nifD*, and *nifK* by PCR or reverse transcriptase-PCR has been frequently employed in detection of N_2-fixing ability of bacterial and cyanobacterial isolates taken either from laboratory grown culture or directly from environmental samples (Ueda et al. 1995a, b; Chowdhury et al. 2007; Bothe et al. 2010). Sequence of *nifH* encoding Fe protein of different species has been reported to be one of the most conserved sequences, except for short species-specific sequence discrepancies, which can be used for species determination (Izquierdo and Nusslein 2006). Therefore, gene sequence of *nifH* is used for probing of nitrogenase among diazotrophic bacteria as well as analysis of diazotrophic communities growing in diverse environmental conditions (Diallo et al. 2008; Jha and Kumar 2009; Bothe et al. 2010). Based on the sequence of *nifH*, a variety of primers have been designed for analysis of both culturable and non-culturable bacteria (Zehr et al. 1998; Widmer et al. 1999; Deslippe and Egger 2006; Izquierdo and Nusslein 2006). However, some of these primers can be biased in terms of amplification efficiency (Diallo et al. 2008). In a very recent study, Islam et al. (2010) have demonstrated significant contribution of diazotrophic bacteria in paddy plants growing in natural condition, using acetylene reduction assay and *nifH* sequence analysis. Similarly, possible

nitrogen contribution by *Azospirillum* sp., *Rhizobium* sp., and *P. pseudoalcaligenes* was indicated on the basis of *nifH* amplification of culturable isolates obtained from *Lasiurus sindicus*, a perennial grass growing in desert (Chowdhury et al. 2007).

In addition to the detection of *nif* gene, assessment of N_2-fixation in diazotrophic bacteria can also be studied by evaluating expression level of *nifH* gene (Terakado-Tonooka et al. 2008). Evaluation of diazotrophy by estimating the level of *nifH* expression is based on the fact that there is tight relationship between nitrogenase activity and *nifH* expression (Egener et al. 2001). Moreover, the advancement in metagenomic approaches using reverse transcription of *nifH* mRNA (messenger ribonucleic acid) has allowed identification of active diazotrophic bacteria in plants. It also facilitates the identification of bacteria which are not culturable but contributes significant nitrogen nutrition to the host plant. Based on the difference in *nifH* mRNA and deoxyribonucleic acid (DNA) profile obtained from same root extract of rice, Knauth et al. (2005) stated that presence of diazotrophs does not necessarily coincide with active diazotrophs inside the plants growing in environmental condition and reported that active diazotrophs were not related to cultured strains. Recently, metagenomic analysis of *nifH* transcript identified *R. rosttiformans* as active diazotroph of sugarcane and spruce from different locations. It was surprising as none of the known diazotrophs associated with sugarcane such as *G.diazotrophicus, H. seropedicae* or *H. rubrisubalbicans* were found to be active in sugarcane plant (Burbano et al. 2010).

2.2.2 Fluorescent In-Situ Hybridization

Detection of diazotrophic bacteria and estimation of nitrogenase activity based on expression of *nifH* mRNA employing Fluorescent in-situ hybridization is an effective approach. However, use of Fluorescent in-situ hybridization has been not used frequently due to the instability of bacterial mRNA. Use of transcript polynucleotide probes can improve the sensitivity of signal as well as reduce signal to noise ratio. Hurek et al. (1997) detected *in-planta* mRNA expression by *Azoarcus* sp. using transcript oligonucleotide probe. Further, to improve the sensitivity and reliability of the technique, Pilhofer et al. (2009) detected mRNA of *nifH* using digoxigenin-labeled transcript probe. The resultant hybrid was detected by horse-radish peroxidase marked anti-digoxigenin antibody. Subsequently the signal was amplified using catalyzed reporter deposition where tyramide molecules preconjugated with flurochrome were deposited in close proximity of horse-radish peroxidase binding site and intensifies the signal.

2.2.3 Immunoblot Analysis

Nitrogenase activity can also be assessed by detecting nitrogenase complex expressed by bacteria. Detection is based on localizing ca. 27–35 kDa of protein band of dinitrogenase either by radiolabeling or immunoblotting using antibody against Fe protein (Eckert et al. 2001; Jha and Kumar 2007). In a separate study,

endophytic bacteria were localized in intercortical region of plant tissue by immunostaining of dinitrogenase reductase (Chelius and Triplett 2000).

2.2.4 Microarray or DNA-Chip

Hybridization levels can be detected by fluorescence. Microarrays with oligonucleotides of *nifH* gene can be used to determine diazotrophic communities. For example, the microarray developed from Zhang et al. (2006) compares 194 oligonucleotide probes, which covers more than 90% of all *nifH* sequences present in the *nifH* database. It is a highly reproducible and semiquantitative method of mapping.

Conclusively, the nitrogen fixing ability of bacteria can be evaluated by total nitrogen difference method, acetylene reduction assay, analysis of nitrogen solutes in xylem and other plant parts and N-Labeling Methods. Furthermore, molecular approaches such as amplification, analysis of nitrogen-fixing genes (*nif* genes), and qualitative and quantitative estimation of their products can be used for evaluation of nitrogen fixing ability of the bacteria.

3 Plant Growth Promoting Properties of Endophytic Bacteria

Apart from N_2-fixation, endophytic bacteria can benefit their host through various growth promoting effects which includes production of phytohormones (auxin and cytokinin), synthesis of siderophore, 1-aminocyclopropane-1-carboxylate-deaminase activity and antagonistic activity. Several endophytic bacteria have been reported to have ability to solubilize mineral phosphate. However, this ability may not be useful for plants as endophytes reside in the interior of plant issue where insoluble mineral phosphates are not available. The characteristics of some beneficial endophytic bacteria are discussed below.

3.1 Phytostimulatory Compounds

Plant growth promoting bacteria produce phytohormones namely auxins, cytokinins, gibberellins, certain volatiles and the co-factor pyrroquinoline quinine. Many plant associated bacteria have been shown to produce auxins chiefly indole-3-acetic acid, which enhances lateral root growth formation and thus, nutrient uptake and root exudation by plants (Spaepen et al. 2007; Ali et al. 2009; Reinhold-Hurek and Hurek 2011). Most of the beneficial bacteria synthesize indole-3-acetic acid through indole-3-pyruvate pathway. In this pathway, tryptophan to indole-3-pyruvic acid conversion occurs by an aminotransferase, which in turn gets decarboxylated by indole-3-pyruvate decarboxylase to indole-3-acetaldehyde and the oxidation of indole-3-acetaldehyde converts it to indole-3-acetic acid

(Spaepen et al. 2007). Indole-3-acetic acid synthesis occurs in stationary phase of growth and positively regulated by aromatic amino acid through a regulatory protein *Tyr*R (Ryu and Patten 2008).

Cytokinins are also involved in plant growth promotion as *Bacillus megatarium* UMCV1, a rhizospheric bacterium, can promote biomass production in *Arabidopsis thaliana* through the inhibition of primary root growth followed by increased lateral root formation and root hair length of host plant (Lopez-Bucio et al. 2007). *Methylobacterium* sp. strain NPFM-SB3, isolated from *Sesbania rostrata* was also found to produce cytokinin (Schwab et al. 2007). *Azospirillum* sp., *G. diazotrophicus*, *H. seropedicae* have also been reported to enhance plant growth by producing gibberellin (Bottini et al. 2004). Even some isolates are capable of producing more than one phytohormone. Feng et al. (2006) isolated *Pantoea agglomerans*, which produce four major plant hormones viz. abscisic acid, gibberellic acid cytokinin and indole-3-acetic acid.

Zhang et al. (2008) identified the role of bacterial volatile organic compounds by analyzing microarray results and histochemical data of *Arabidopsis* seedlings exposed with *B. subtilis* and stated that the volatile organic compounds may influence the plant growth by regulating auxin homeostasis in plants which was evident from induction of genes encoding enzymes of metabolism of indole-3-acetic acid.

3.2 Induced Systemic Tolerance

Some of the plant growth promoting bacteria (PGPB) help the associated plants to counter biotic and abiotic stresses such as drought, salt, nutrient deficiency or excess, extremes of temperature, presence of toxic metals etc. PGPB-induced physical and chemical changes in plants in response to biotic and abiotic stresses are termed as 'induced systemic tolerance' (Yang et al. 2009). Induced systemic tolerance results from the production of bacterial 1-aminocyclopropane-1-carboxylate deaminase activity, antioxidants, cytokinin or volatile organic compounds.

In response to the various biotic or abiotic stresses, plant produces ethylene to regulate plant homeostasis. Beyond a threshold level, production of ethylene is inhibitory as it reduces root and shoot development and hence described as "stress ethylene". Some of the endophytes have property to synthesize 1-aminocyclopropane-1-carboxylate-deaminase, which can degrade the immediate precursor of ethylene from root exudates and convert it to α-ketobutyrate and ammonia and thus, can promote growth of plant in the vicinity (Glick et al. 2007). In addition to 1-aminocyclopropane-1-carboxylate deaminase mediated induced systemic tolerance, there are various other mechanism through which induced systemic tolerance is generated in response to stresses. It includes volatile organic compounds mediated salt tolerance (Zhang et al. 2008), affecting abscisic acid signaling of plants during stress through production of cytokinin (Figueiredo et al. 2008) and through production of antioxidant catalase (Kohler et al. 2008). The role of phytohormone produced by associative bacteria during salinity or drought stress in the promotion

of plant growth has been well described (Egamberdieva 2009). Indole-3-acetic acid producing bacteria in drought condition can stimulate formation of well developed roots enough for providing sufficient water from soil (Marulanda et al. 2009).

3.3 Biocontrol Agent

The application of microorganism for the control of diseases seems to be one of the most promising ways, as it is eco-friendly and cost-effective. To become an efficient biocontrol agent, it should be stable under varying condition of pH, temperature and concentrations of different ions. Several endophytic bacteria are known to benefit host plant by reducing the growth of pathogenic organisms at laboratory, greenhouse or field level in various studies (Compant et al. 2005; Bhatia et al. 2008; Kannan and Sureendar 2009). An efficient biocontrol agent must also possess certain traits like (a) efficient colonizer of root to deliver antibiotic along the whole root system, (b) to protect itself from predators (protozoans) in the rhizosphere and (c) release antibiotic in right microniche (Lugtenberg and Kamilova 2009). Bacteria can limit pathogen directly through antagonistic property, competition for iron, detoxification or degradation of virulence factors or indirectly by inducing systemic resistance in plants against certain diseases (Lugtenberg and Kamilova 2009). Endophytic bacterial biocontrol agents can inhibit the growth of fungal or bacterial pathogens by one or more of the several mechanisms, some of which are described below.

3.3.1 Antagonism

Endophytic bacteria can exhibit biocontrol activity (antifungal and antibacterial) through production of allelochemicals or antibiotics. Gram negative biocontrol agents like *Pseudomonas* produce HCN, pyoleutorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and phenazines chiefly phenazine-1-carboxylic acid and phenazine-1-carboxamide (Lugtenberg and Kamilova 2009). In *Pseudomonas fluorescens* CHA0, all these above mentioned metabolites are required for biocontrol. However, the expression of their genes may change with different plant cultivars (Rochat et al. 2010; Jousset et al. 2011). Few other compound including gluconic acid and 2-hexyl-5-propyl resorcinol produced by the antagonistic bacterial strains have also been demonstrated recently (Cazorla et al. 2006; Kaur et al. 2006). Munumbicin, an antibiotic produced by endophytic bacteria inhibits growth of phytopathogenic fungi *P. ultimum* and *F. oxysporum* (Castillo et al. 2002). Certain volatile organic compounds like 2,3-butanediol, or blends of volatiles produced by *Bacillus* sp. also act as biocontrol agents (Strobel 2006). Level of antibiotic synthesis depends upon the nutritional factors viz. type of carbon source utilized, trace elements and availability of other nutrients as well as non-nutritional factors like environmental influences (Compant et al. 2005).

Bacteria can restrict the growth of pathogens by producing hydrolytic enzymes such as chitinase, β-1,3-glucanase, protease, laminarinase etc. (Ordentlich et al. 1988). *Bacillus cepacia* has been reported to destroy *Rhizoctonia solani*, *R. rolfsii*, and *Pythium ultimum* by producing β-1,3-glucanase (Fridlender et al. 1993). Addition of endophytic bacteria *B. cereus* 65 directly to soil has been reported to protect cotton seedlings from root rot disease caused by *Rhizoctonia solani* (Pleban et al. 1997). Secretion of protease and chitinase by endophytic *Enterobacter* and *Pantoea* species isolated from cotton were found to protect the plants against fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (Li et al. 2010).

3.3.2 Siderophore Production

Under iron-limiting condition, some biocontrollers produce small molecular weight compound, known as siderophore, which has the capability to chelate unavailable iron and make it available to plants and cohabiting microorganism, and thus, deprive pathogen (Compant et al. 2005). An array of siderophores is produced but majority of biocontrollers are known to produce catacholate, hydroxymate and/or phenolate type (Rajkumar et al. 2010). In addition to biocontrol, siderophores are known to play multiple roles in diazotrophic bacterial species. As the diazotrophic bacteria require both iron and molybdenum for the activity of nitrogenase, the role of siderophore seems pivotal under iron deficient conditions (Kraepiel et al. 2009).

3.3.3 Induced Systemic Resistance

During their interaction with plants, endophytic bacteria results in improving the immune response of plants for future attack by pathogens, a phenomenon called as induced systemic resistance (Compant et al. 2005; van Loon 2007). In contrast to biocontrol mechanisms, extensive colonization of root system is not required for induced systemic resistance (Lugtenberg and Kamilova 2009).

The bacterial products that elicit induction of induced systemic resistance are of diverse category and show their induction in plants which possibly possess receptors for the respective ligands. These inducers may be lipopolysaccharides, flagella, siderophores, antibiotics, volatile organic compounds and quorum-sensing signals (van Loon 2007). It has been reported that both siderophore (pyochelin) and antibiotic (pyocyanin) are needed for induced systemic resistance by *P. aeruginosa* 7NSK2 (Audenaert et al. 2002). Role of volatile organic compounds such as 2,3-butanediol produced by *Bacillus* sp. in induced systemic resistance has been reported by Ryu et al. (2004). Mostly induced systemic resistance activated by plant growth promoting bacteria are jasmonate or ethylene mediated (van Loon 2007). In *Arabidopsis thaliana,* jasmonate or ethylene mediated induced systemic resistance by *Bradyrhizobium* sp. (Cartieaux et al. 2008) strain, ORS278 in *Arabidopsis thaliana* by transcriptome analysis and SA mediated by endophytic bacteria *Paenibacillus alvei* (Tjamos et al. 2005) have been reported. The level of salicylic acid production and intensity of fungal

growth inhibition was found to vary with different bacterial isolates (Forchetti et al. 2010). In a recent study, *Bacillus cereus* AR156 was reported to trigger induced systemic resistance in *A. thaliana* through salicylic acid and jasmonic acid/ethylene-signaling pathways in an NPR1-dependent manner (Niu et al. 2011).

Induced systemic resistance may induce various genes to immunize the host plant mechanically or metabolically by increasing cell wall strength, alteration of host physiology or metabolic responses, enhanced synthesis of plant defense chemicals such as phenolic compounds, pathogenicity related protein (PR-1, PR-2, PR-5), chitinases, peroxidases, phenyl alanine ammonia lyase, phytoalexins, oxidase and/or chalcone synthase. These metabolic products shield the host plant from future attacks from pathogens (Duijff et al. 1997; Compant et al. 2005).

Local immune response induced by plant growth promoting bacteria has also been demonstrated in few studies. However, it is genotype specific and depends on bacterial species. *Burkholderia phytofirmans* PsJN induces local immune response by ion fluxes, salicylic acid production and defense gene activation in grapevine, while production of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase activities have been observed in cucumber plant in response to *Pseudomonas s*p. (Chen et al. 2000). Moreover, production of phytoalexins such as resveratrol and viniferin in host plant have also been reported recently (Verhagen et al. 2010).

In addition to nitrogen-fixation ability, these bacteria can influence plant growth through one or more properties. These include production of phytohormones, siderophores, induced systemic tolerance through production of 1-aminocyclopropane-1-carboxylase deaminase, induced systemic resistance and antagonistic activities.

4 Ecology and Diversity of Endophytic Bacteria

The make-up of endophytic bacterial communities is very likely affected by deterministic factors as well as stochastic events (Hardoim et al. 2008). Other than soil factors, plants also offer a selective environment to microorganisms, 'filtering out' specific microbial groups from the diversity found at plant roots (Rosenblueth and Martinez-Romero 2006). Thus, various factors like plant genotype and physiological status, bacterial colonization traits, abiotic conditions and agricultural management regimes can affect the diversity of bacterial communities in root tissues (Hardoim et al. 2008). Out of these factors, plant genotype may play a key role in the selection of distinct bacterial communities that associate with plants (Andreote et al. 2010).

Endophytes can colonize more aggressively and displace others when inoculated with other bacteria in a competition experiment. This opinion is based on the reports where *Pantoea* sp. was found to be outcompeting *Ochrobactrum* sp. in rice (Verma et al. 2004) and with different *R. etli* strains in maize (Rosenblueth and Martinez-Romero 2006). Many endophytes have a broad host range. However, it has not been studied in a systematic and quantitative manner. Recently, *Klebsiella oxytoca* and

Achromobacter xylosoxidans originally isolated from *Typha australis* and wheat respectively were reported to colonize rice plants (Jha and Kumar 2007, 2009). However, colonization ability of different bacterial strains varies widely. Dong et al. (2003) studied the colonization pattern of *gfp*-tagged *Escherichia coli* K-12, *Salmonella enterica* serotype *Typhimurium* strain ATCC 14028, and *K. pneumoniae* 342 and concluded that significant strain specificity exists for plant entry and for most strains. They also studied the kinetics of invasion by endophytic bacteria and found a strong correlation between rhizosphere colonization and interior colonization for all strains. Despite being best colonizer in the interior, *K. pneumoniae* 342 showed the lowest correlation between rhizospheric colonization and endophytic colonization among the other six strains which showed a strong positive correlation. Based on their experiment, they deduced that single colony forming unit in the inoculum was sufficient to cause invasion of the plant interior for certain isolates while higher number of cells was required for effective colonization for other strains. They reported that endophytic colonization is an active process controlled by genetic determinants from both partners (Dong et al. 2003).

A proper understanding of interactions and resulting exchange of signals between microbial communities would facilitate the development of new strategies to promote beneficial interaction between the microorganisms and plants. The genetic diversity of bacteria can be evaluated using amplified ribosomal DNA (rDNA) restriction analysis, rep-Polymerase chain reaction genomic fingerprinting and small subunit ribosomal DNA sequencing etc. (Grange and Hungria 2004). Sequence analysis of amplified *nifH* has also been used to study the diversity of diazotrophic bacteria isolated from plants (Rosado et al. 1998; Zhang et al. 2006; Chowdhury et al. 2007). The *nifH* sequence of several known diazotroph families cluster were similar to that of 16S ribosomal RNA (rRNA) analysis. Therefore, *nifH* analysis is also used for study of diversity among diazotrophic bacteria (Ueda et al. 1995a; Zhang et al. 2006; Venieraki et al. 2011).

For studying diversity of diazotrophic bacteria, combined use of rDNA and rRNA analysis has been proposed to give more detailed understanding of bacterial community (Nogales et al. 2001). Community diversity in terms of desired metabolic activity or rRNA-based approach can also be studied comprehensively by stable isotope probing method (Manefield et al. 2002; Kiely et al. 2006). Active endophytic bacterial community has been studied recently by analyzing 16S rRNA sequences of density resolved DNA using stable isotope probing (Rasche et al. 2009). Denaturing gradient gel electrophoresis of amplified rDNA and *nifH* of culturable or unculturable rhizospheric and endophytic bacteria has also been used for studying molecular diversity (Lovell et al. 2000; Araujo et al. 2002; Abreu-Tarazi et al. 2010; Burbano et al. 2010; West et al. 2010). In addition, other methods like single strand conformation polymorphism, Terminal restriction fragment length polymorphism of rDNA can also be helpful in elucidating the prevalence of molecular diversity and studying phylogenetic relationship among bacteria. Metagenomic approach has been useful in understanding community structure of both cultivable and uncultivable bacteria. Metagenomics is the genomic analysis of uncultured microorganisms, which is of two types: a function-driven approach, in which metagenomic libraries are initially

screened for an expressed trait, and a sequence-driven approach, in which libraries are initially screened for particular DNA sequences (Schloss and Handelsman 2003; Zeyaullah et al. 2009).

The make-up of endophytic bacterial communities depends on various factors such as soil type, soil composition, soil environment, plant genotype and physiological status, bacterial colonization traits, and agricultural management regimes. Colonization and abundance of different bacterial species varies widely with host plants. Endophytic bacterial community can be analyzed employing stable isotope probing as well as various modern molecular approaches which are based on analysis of 16S ribosomal deoxyribonucleic acid (DNA), gene encoding products for nitrogen fixation and repetitive DNAs. Moreover, metagenomic approaches allow estimation and analysis of unculturable bacteria at genomic as well as functional genomic level.

5 Colonization of Endophytic Bacteria

Colonization of bacteria in the plant is a complex process, which involve interplay between several bacterial traits and genes, and plant responses. The colonization is an orchestra of number of steps: (a) migration towards root surface i.e. chemotaxis, (b) attachment and microcolony formation, (c) distribution along root and, (d) growth and survival of the population. Colonization pattern of bacteria can be obtained by tagging the putative colonizing bacteria with a molecular marker such as auto-fluorescent marker such as green fluorescent protein (*gfp*) or β-glucuronidase (*gus*) followed by electron or confocal laser scanning microscopy (Singh et al. 2011). The presence of various reporter genes on the colonization of diazotrophic endophytic bacteria was summarized in Table 2. Although, molecular mechanism involved in the endophytic colonization process is not well understood, recent reports based on genomic data suggest resemblance of colonization process between pathogenic and endophytic bacteria (Krause et al. 2006; Hardoim et al. 2008).

5.1 Chemotaxis and Electrotaxis

Root colonization is the first and critical step in the establishment of plant-microbe association. Microorganisms move towards rhizosphere in response to root exudates which are rich in amino acids, organic acids, sugars, vitamins, purines/pyrimidines and other metabolic products. Thus, motility and chemotaxis play a key role in the root colonization. At the same time, in addition to providing nutritional substances, plants start cross-talk with microorganisms by secreting some signals which cause colonization by some bacteria while inhibit the others (Bais et al. 2006; Compant et al. 2010b). Like plant-rhizobia interaction, plant root exudates do influence the expression of genes in associating bacteria. Stimulation of colonization of wheat and *Brassica napus* by *A. brasilense* and *A. caulinodans* in response to flavonoids

Table 2 Effect of reporter genes on the colonization of diazotrophic endophytic bacteria

Gene	Gene product/ function	Advantages/disadvantages	References
*tfd*A	2,4-dichlorophe noxyacetate monooxygenase	Low resolution	King et al. (1991)
*pho*A	Alkaline phosphatase	Soluble end product	Reuber et al. (1991)
*xyl*E	Catechol 2,3-dioxygenase	Amplification or photographic exposure for detection	Winstanley et al. (1991)
Heavy metal resistance	Heavy metal resistance	Requires plate counting	de Lorenzo (1994)
l*ac*Z	β-galactosidase	High background in most plant and bacteria	Kovach et al. (1994)
Antibiotic resistance	Antibiotic Resistance	Requires plate counting	Kovach et al. (1995)
*cel*B	β-glucosidase	Detection after denaturation of endogenous enzymes	Voorhorst et al. (1995)
*lux*A, *luc*	Luciferase	Low resolution	Ladha and Reddy (2000)
gfp, bfp, yfp, cfp, rfp	Autoflourescent protein	High resolution. Real time application. Requires oxygen for proper folding	Godfrey et al. (2010)
*gus*A	β-glucuronidase	No background in bacteria and plants, requires substrate	Singh et al. (2011)

from host exudates indicates that flavonoid may be determinant for endophytic colonization (O'Callaghan et al. 2000). In a more recent report, naringenin, a flavonoid present in exudates of plants has been reported to modulate the expression of genes in *H. seropedicae* and this alteration in gene expression were the decisive for endophytic colonization (Tadra-Sfeir et al. 2011). In addition to chemotaxis, electrotaxis (electrogenic ion transport at the root surface) has also been considered as a possible mechanism for initiating rhizobacterial colonization (van West et al. 2002). Sloughed up root cap cells also have large impact on plant-microbe interaction (Hawes et al. 1998). Root hair regions and emergence points are preferred site for colonization (Lugtenberg and Kamilova 2009).

Colonization of root by microorganism may further induce release of exudates which can create 'biased' rhizosphere with exudation of specific metabolic products, which in turn induce flagellar motility that directs their colonization on plant surfaces. Motility of endophytic bacteria is considered as one of the most important aspect for successful colonization event. *P. fluorescence* defective in chemotaxis-driven flagellar motility was found to have reduced colonization efficiency (Lugtenberg et al. 2001). Similarly, type IV pili mediated twitching motility has been found to be instrumental in the establishment of successful endophytic colonization by *P. stutzeri, Azoarcus sp.* and *H. seropedicae* (Bohm et al. 2007; Yan et al. 2008; Pedrosa et al. 2011).

5.2 Attachment on Root Surface

Chemotaxis or electrotaxis driven migration of bacteria to roots is followed by adhesion of bacteria on root surface to get entry into the plant tissue. Adherence of these bacterial cells depends on various cell surface molecules which includes cell appendages (flagella or pili), major outer membrane proteins and secretion system of bacteria which play major role for invasion. Bacterial flagella and pili also play an important role in the colonization process by adhering on plant surfaces. Glycosylated polar flagellum is thought to act directly as a root adhesion (Croes et al. 1993). Role of flagella in colonization has evidenced recently where FliC3 (product of flagellin genes) which was considered previously as microbe associated molecular pattern, has been found to be required for endophytic colonization in the *Azoarcus*-rice interaction, most likely for spreading inside the plant (Reinhold-Hurek and Hurek 2011). Involvement of type IV pili in adherence to plant surface in *Azoarcus* has also been demonstrated (Dorr et al. 1998). Additional mechanisms can also be operative for initial plant-microbe interaction. Bilal et al. (1993) suggested that cellulose fibrils, a cell-surface protein and Ca^{2+} dependent adhesion may be implicated in the specific interaction with plants. Moreover, chemical composition of lipopolysaccharides present on the surface of bacteria might be determinative for successful colonization in host plants (Serrato et al. 2010).

Role of bacterial major outer membrane protein in early host recognition has been recognized in earlier report, where major outer membrane proteins from *Azospirillum brasilense* showed stronger adhesion to extracts of cereals than extracts of legumes and tomatoes. It suggests the involvement of major outer membrane proteins in adhesion, root adsorption and cell aggregation of bacterium (Burdman et al. 2000). Bacterial cells are equipped with various secretion systems which enable them to interact successfully with the host plant. Preston et al. (2001) identified secretion system type III (hrp) in *P. fluorescens* SBW25 by *in-vitro* expression technology, a promoter trapping technique used to identify genes expressed *in-vivo* during colonization process.

5.3 Entry and Distribution Along Root

Entry of endophytic bacteria in plant roots is known to occur through (a) wounds particularly where lateral or adventitious roots occur; (b) root hairs and (c) space between undamaged epidermal cells (Sprent and de Faria 1988). Chi et al. (2005) demonstrated that the colonization of *gfp*-tagged rhizobia in crop plants begin with surface colonization of the rhizoplane at lateral root emergence, followed by endophytic colonization within roots, and then ascending endophytic migration into the stem base, leaf sheath, and leaves where they develop high populations. *Azospirillum* may also colonize endophytically through wounds and cracks of the plant root. Spreading of *Azospirillum* from the lateral root emergence to other part of root depends on the status of the nitrogen and carbon source present in the vicinity

(Ramos et al. 2002). *G. diazotrophicus* gains its entry into micropropagated sugarcane plantlet through wounds caused by emerging lateral roots (Sevilla and Kennedy 2000). James et al. (2001) reported that *G. diazotrophicus* enters in sugarcane and other gramineous plant roots via lateral root junctions and/or root tips and subsequently colonizes the root vascular system from where it is translocated to the lower stem in the xylem. Similarly, massive colonization of *P. fluorescens* PICF7 on root hairs and site of differentiation of olive plant was noticed in a very recent report (Prieto et al. 2011). Crack entry of *A. caulinodans* ORS571 in response to release of flavonoids such as naringenin from host plant and subsequent intercellular colonization of the cortex of root systems of rice, wheat and *Arabidopsis thaliana* have earlier been observed (Gough et al. 1997; Webster et al. 1997). Compant et al. (2008) reported the chronological detection of endophytic *B. phytofirmans* PsJN on the root surfaces, in the endorhiza and inside inflorescence stalks of *Vitis vinifera* (Fig. 2).

Endophytic bacteria may colonize root tissues and spread actively in aerial parts of plants through expressing moderate amount of degradative enzymes such as pectinases and cellulases. Utilization of aforesaid enzymatic activities for colonization by *A. irakense* (Khammas and Kaiser 1991), *Azoarcus* sp. (Hurek and Reinhold-Hurek 2003) and others, has been demonstrated as one of the efficient methods to get entry into the host plant. Endoglucanase is one of the major determinants for the colonization of endorhizosphere, which was evident from the observation that *Azoarcus* strain lacking endoglucanse was not effective in colonizing the rice plant (Reinhold-Hurek et al. 2006). The endoglucanase preferably attacks oligosaccharides larger than cellobiose and releases larger oligomers from substrates such as carboxymethylcellulose, and is thus likely to loosen larger cellulose fibers, which may help bacteria to enter in the plant tissue (Hurek and Reinhold-Hurek 2003). A homologue of endoglucanase gene has also been identified in *P. stutzeri* A1501, which occasionally colonizes cortex of crop plants (Yan et al. 2008). In addition to endoglucanse, exoglucanases may also help in the colonization process. An exoglucanase having cellobiohydrolase and β-glucosidase activity on wide substrate spectrum including xylosides was identified to be key player in *Azoarcus* sp. BH72 for colonization process (Reinhold-Hurek et al. 2006). *A. irakense* isolates have been found to colonize intracellularly in rice that may be enabled by the expression of pectinolytic, cellulotyic and β-glucosidase enzymes (Somers et al. 2004). In plants like *Elaegnus* and *Mimosa* sp., the endophyte penetrates the root radial walls presumably by digesting the middle lamella and then proceeds between cells and through intercellular spaces. In *Parasponia* plants, the colonizing bacteria stimulate cell division in the outer cortex. In due course these newly formed cells rupture the epidermis resulting in an effective wound infection (Sprent and de Faria 1988). In contrast to above examples, genes encoding plant cell wall degrading enzymes has not been found in the endophytic bacteria *H. seropedicae* strain SmR1 (Pedrosa et al. 2011).

Azoarcus sp., an obligate endophyte of Kallar grass, has been critically studied by using transposon mutant expressing β-glucuronidase constitutively as a reporter gene (Hurek and Reinhold-Hurek 2003). *Azoarcus* sp. BH72 colonize apical region

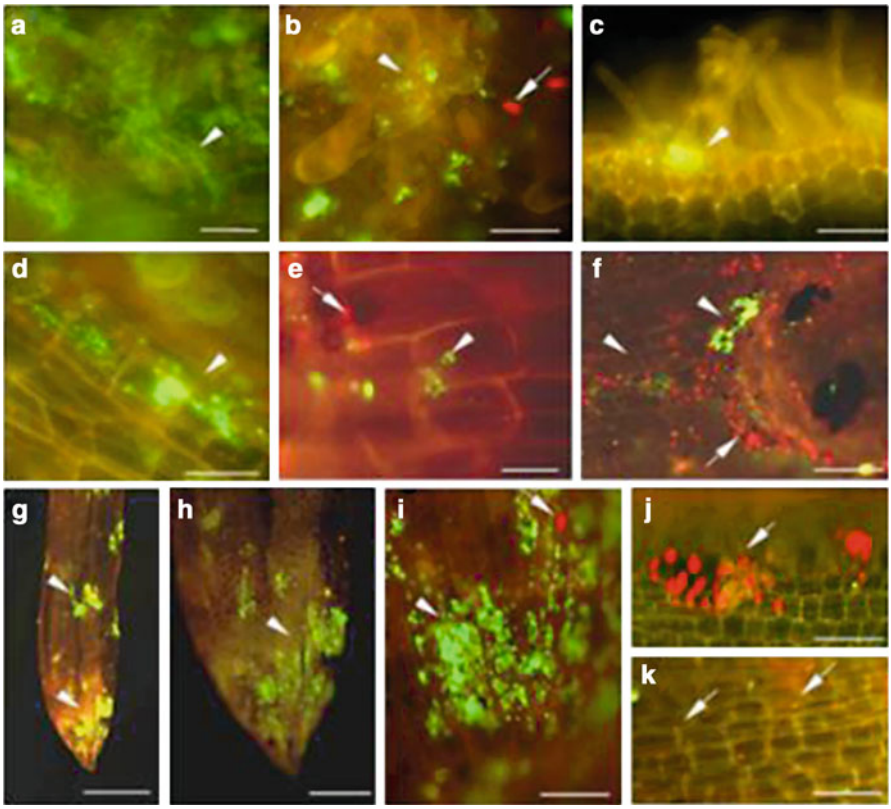

Fig. 2 Epifluorescence microscopic images of roots of grapevine fruiting cuttings inoculated with *Burkholderia phytofirmans* PsJN::*gfp*2 × 1–4 weeks after soil inoculation with 5×10^8 colony forming units g^{-1} of soil. Gfp-tagged bacteria (*arrows*) were visualized at the root hair zone (**a**–**c**) colonized root hairs (**a**–**c**), on other rhizodermal cells (**d** and **e**), at lateral root emergence sites (**f**) and at the root tip (**g**–**i**). A natural epiphytic microbial communities was also detected on the root surface of inoculated plants (*arrows* in **b**, **e**, **f** and **i**) as well as on the roots of control plants (*arrows* in **j** and **k**). Similar rhizoplane colonizations by strain PsJN were found from 1 to 4 weeks postinoculation. *Scale bars*: (**a**) 100 mm, (**b**) 30 mm, (**c**) 75 mm, (**d** and **e**) 30 mm, (**f**) 100 mm, (**g**) 1 mm, (**h**) 500 mm, (**i**) 250 mm and (**j** and **k**) 100 mm (Reproduced from Compant et al. (2008) with permission from Wiley)

of roots behind the meristem intensively and penetrate the rhizoplane preferentially in the zone of elongation and differentiation. It colonize in the cortex region both inter- and intra-cellularly. In older parts of the roots, it also occurs in aerenchymatic air spaces. *Azoarcus* sp. is capable of invading even the xylem vessels suggesting its systemic spreading into shoots through the transport in vessels. However, shoot colonization of Gramineae appears to be more pronounced in *Gluonoacetobacter diazotrophicus* (James and Olivares 1998) and *H. seropedicae* (Gyaneshwar et al. 2002). Intercellular colonization of endophytic bacteria in cortex as well as xylem of root has been reported in recent studies (Prieto et al. 2011; Schmidt et al. 2011).

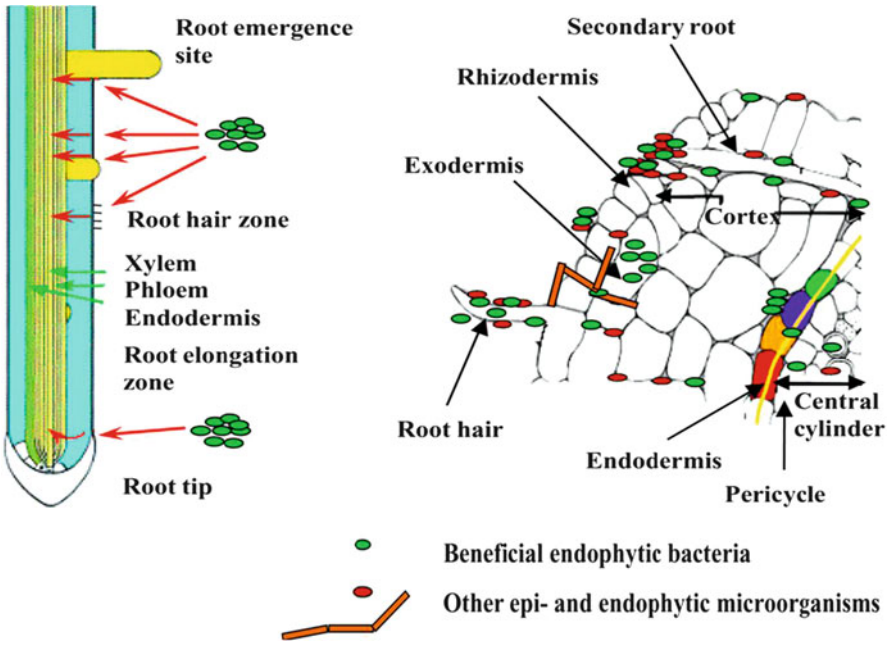

Fig. 3 Schematic presentations for the sites of plant colonization by endophytic bacteria. Figure in the *left panel* represents longitudinal section of root depicting possible sites for entry of endophytic bacteria. Figure in the *right panel* represents transverse section of root showing the distribution and colonization of endophytic bacteria (Reproduced from Compant et al. (2010a) with permission from Elsevier)

Compant et al. (2011) reported the colonization of endophytic bacteria in epidermis and xylem of even reproductive organ of grapevine. Based on the colonization pattern of *P. fluorescens* PICP2 and PICF2 in root hairs of olive plant, Prieto et al. (2011) suggested that endophytic bacteria are confined within an organelle most likely vacuole which arises by narrowing of an internal membranous structure in roots. The possible sites of colonization by diazotrophic endophytic bacteria are depicted in Fig. 3.

5.4 Growth and Survival

Endophytic colonization is not as specific as of *Rhizobia* but successful endophytic colonization does involve a compatible host plant (Ryan et al. 2008). However, endophytic colonization indeed depends upon the physiological changes in plants and is restricted or slowed down by the defense mechanisms (Rosenblueth and Martinez-Romero 2006). Colonization of *G. diazotrophicus* was found to be diminished in plants grown under high nitrogen fertilizer regime. This reduction in colonization was explained as a result of altered plant physiology in the presence of

nitrogen fertilizer, which reduces sucrose concentration to be utilized by endophytic bacteria (Fuentes-Ramirez et al. 1999). Influence of organic amendment on endophytic population has also been demonstrated (Hallmann et al. 1997). Plant defense responses play a critical role in regulating colonization of endophytic bacteria. In dicotyledonous plants, salicylic acid and ethylene restricts the endophytic colonization. Ethylene, a signal molecule of induced systemic resistance in plants decreases endophytic colonization as observed in *Arabidopsis thaliana* inoculated with *K. pneumoniae* 342 (Iniguez et al. 2005). However, proteomic approach used to study the bacterial colonization indicated that instead of ethylene and salicylic acid, it is the jasmonic acid which contributes in restricting endophytic colonization in grasses (Miche et al. 2006). Expression of jasmonic acid induced pathogenesis related proteins (defense proteins) depends upon the compatibility of plant variety and endophytic bacteria. Inoculation of *Azoarcus* sp. to more compatible rice (*Oryza sativa*) culivar IR36 led to the expression of fewer jasmonic acid-induced pathogenesis related proteins than that of less compatible cultivar IR42. Antimicrobial peptides synthesized by some plants like rice and maize may reduce endophytic colonization (Fuentes-Ramirez et al. 1999). Understanding of molecular mechanism and conditions limiting the colonization process need to be elucidated for exploiting the beneficial endophytic or associative interaction with plants.

Colonization process of an endophytic bacterium involves various steps which include migration towards root surface, attachment and microcolony formation on plant surface, distribution along root and growth and survival of the population inside plant tissue.

6 Revamp in Genomic and Proteomic Studies of Endophytic Bacteria

This section critically summarizes the recent advances in the genomic and proteomic aspects of diazotrophic endophytic bacteria.

6.1 Genomic Studies

Prospects of potential application of endophytic bacteria led the exploration of their genomic make up so that these bacteria can be exploited for sustainable and productive agriculture advancement. In recent years, genomic sequences of several endophytic diazotrophs other than rhizobia have been summarized in tabular form (Table 3).

Recently Kaneko et al. (2010) reported the complete genome sequence of *Azospirillum* sp. B510. In this, nitrogenase genes and assembly related protein are located in three separate loci in chromosome. It is also mentioned that it possess genes of 1-aminocyclopropane-1-carboxylate deaminase activity and indole-3-acetic acid

Table 3 Some diazotrophic endophytic bacteria with their genome sizes and annonate genes

Endophytic bacteria	Genome size (bp)	No. of genes annotated	References
Azoarcus sp. EbN1	4,727,255	4,686	Rabus et al. (2005)
Azoarcus sp. BH72	5,376,040	4,073	Krause et al. (2006)
Bacillus amyloliquefaciens FZB42	3,918,589	3,693	Chen et al. (2007)
Klebsiella pneumoniae 342	5,920,257	5,881	Fouts et al. (2008)
Pseudomonas stutzeri A1501	4,567,418	4,237	Yan et al. (2008)
Gluconoacetobacter diazotrophicus Pal5	3,999,591	3,997	Bertalan et al. (2009)
Azotobacter vinelandii DJ, BAA-1303	5,365,318	5,133	Setubal et al. (2009)
Azospirillum sp. B510	3,311,395	2,893	Kaneko et al. (2010)
Enterobacter sp. 638	4,676,467	4,444	Taghavi et al. (2010)
B. subtilis BSn5	4,093,599	4,177	Deng et al. (2011)
Variovorax paradoxus S110	6,754,997	6,279	Han et al. (2011)
Azoarcus sp. strain KH32C	5,081,166	4,531	Nishizawa et al. (2012)
Herbaspirillum seropedicae	5,513,887	4,804	Pedrosa et al. (2011)
Burkholderia phytofirmans PsJN	8,214,658	7,487	Weilharter et al. (2011)
Serratia proteamaculans 568	5,448,853	4,942	Zhu et al. (2012)

production. Other than this, genes related to ion transport, Quorom sensing, secretion system (Type IV), motility genes have been described. The information provided in this study can be exploited to study the role of various genes in the colonization process described above as well as augmentation of genes related to plant growth promoting bacteria activities can provide a potential genetically modified biofertilizer candidate.

The strain *Azoarcus* sp. BH72 consists of gene cluster that encodes cell surface components (Type IV pili) which seems to be important factor for host-microbe interaction as this cluster is absent in related non-endophytic soil bacteria *Azoarcus* sp. EbN1 (Krause et al. 2006). Genes encoding products for exopolysaccharide polymerization, translocation (*gum* operon of *Xanthomonas compestris* and rhizobial *pss* gene cluster) and products required for plant-microbe interaction resemble to that of pathogenic bacteria and rhizobium. It differs from that of pathogenic bacteria in not secreting hydrolases. However, genes for producing low amount of macerating enzyme endoglucanase (*EglA*) is synthesized which is probably required for endophytic colonization (Krause et al. 2006).

Analysis of the *G. diazotrophicus* Pal5 complete genome sequence provides important insights into the endophytic relationship, and suggests many interesting candidate genes for post-genomic experiments (Bertalan et al. 2009). The genome sequence results showed unexpectedly high number of mobile elements for an endophytic bacterium; thus suggest a high number of horizontal gene transfer events. To change niche from rhizosphere to endophytic, the bacteria should penetrate the plant. The putative *gum*-like cluster containing an endoglucanase could be important in this regard. The genome shows various properties such as biological nitrogen fixation, phytohormones and biocontrol genes. Several features for an endophytic lifestyle are found in genome islands, including type IV secretion systems, flagella, pili, chemotaxis, biofilm, capsular polysaccharide and some transport proteins.

Comparison of *G. diazotrophicus* and *Azoarcus sp*. BH72 genome sequences showed that these endophytic diazotrophic bacteria adopted very different strategies to colonize plants. Features like, large number of TonB receptors, *gum*-like and *nif* clusters, and osmotolerance mechanisms are common to both endophytic diazotrophic bacteria. Presence of TonB receptors has been found to be common in almost all endophytic bacteria (Reinhold-Hurek and Hurek 2011). Apart from this, *G. diazotrophicus* has a larger number of transport systems, and it is capable of growing on a wide variety of carbon sources, while *Azoarcus* sp. BH72 has rather complex signaling mechanisms to communicate with its plant host (Bertalan et al. 2009).

Herbaspirillum seropedicae is well characterized diazotrophic endophytic bacteria, which is known to interact with several plant species belonging to family Graminiae. Presence of few mobile elements in most of the endophytic bacteria with exception of *G. diazotrophicus* Pal5 indicates low recombination or rearrangement which might be a reason for their adaptation to endophytic lifestyle (Krause et al. 2006; Pedrosa et al. 2011). It is well equipped with *nif* gene cluster with 46 open reading frames which includes structural and regulatory genes for nitrogenase synthesis and activity, molybdenum uptake, electron transport, metal cluster analysis and other related functions. In addition to diazotrophy, *H. seropedicae* possesses complete machinery for auxin, siderophore and 1-aminocyclopropane-1-carboxylate deaminase synthesis which make it suitable candidate for plant growth promotion. In order to colonize plant, *H. seropedicae* expresses a variety of secretion system including secretion system type II, III, V and type IV pili. Genes for type IV pili encode for proteins which play role in attachment to surfaces, twitching motility, biofilm formation, virulence and protein secretion. Expression of lytic transglycolase help in plant-microbe interaction through its ability to degrade peptidoglycan partially which allow efficient assembly and anchoring of transport complexes (secretions system type II and III) and type IV pili to the cell envelope. However, expression of type III secretion system has not been observed in proteomic analysis which suggest for availability of suitable physiological condition for their synthesis and function. Since, invasion of bacteria into the plant is kind of stress for both microbe and host, genes encoding function to combat osmotic stress, salinity desiccation, nitrogen starvation, ultraviolet radiation, pH and other stresses are present in *H. seropedicae.* These include, synthesis of amylopectin like polysaccharides, trehalose and $Na^+(K^+)/H^+$ antiporter which contribute to defense against osmotic stress. Genes for hemolysin

and hemagglutins have also been found which may be presumably required for surface attachment and biofilm formation during plant tissue colonization. Moreover, availability for metabolic pathways for the degradation of several aromatic compounds indicate that these pathways may be instrumental for given bacteria to thrive on plant tissue owing to their metabolic flexibility and defense against toxic phytochemicals (Pedrosa et al. 2011).

Comparative genomic analyses of diazotrophic *K. pneumoniae* 342 with the presumed human pathogen *K. pneumoniae* MGH78578 suggested that the later apparently cannot fix nitrogen, and the distribution of genes essential to surface attachment, secretion, transport, and regulation and signaling varied between both genome, which revealed a critical divergences between the strains that influence their preferred host ranges and lifestyles. Little genome information is available concerning endophytic bacteria. The *K. pneumoniae* 342 genome unveil bacterial-plant host relationships, which could ultimately enhance growth and nutrition of important agricultural crops and development of plant-derived products and biofuels (Fouts et al. 2008).

In addition to above mentioned bacterial species, genome of other endophytic bacteria namely *B. phytofirmans* (strain DSM 17436/PsJN), *Enterobacter* sp. (strain 638), *Methylobacterium populi* (strain ATCC BAA-705/NCIMB 13946/BJ001), *P. putida* (strain W619), *Serratia proteamaculans* (strain 568), *Stenotrophomonas maltophilia* (strain R551-3) have also been sequenced (Table 3). Although, genome sequences of many endophytic bacteria are being compiled, it still needs thoughtful analysis and strategy to develop a potential biofertilizer strain which can be manipulated in different environmental conditions.

6.2 Proteomics Studies

In the last decade, proteomics has been applied for the identification of proteins that are important in plant responses to microorganisms. Several grasses have interaction with plant growth-promoting N_2-fixing bacteria which do not form a specific root structure like a nodule. A large number of proteomic techniques are available for the analysis of various aspects of proteins, including their post-translational modification, expression profile, and interaction network (Pandey and Mann 2000). The two most famous proteomic methods are two-dimensional gel electrophoresis and mass spectrometry. Differential display tool difference gel electrophoresis is an extension of two-dimensional gel electrophoresis technique to compare multiple samples simultaneously. There are numerous other gel-based or gel-free and quantitative or qualitative proteomic methods, which includes isotope-coded affinity tag, isobaric tag for relative and absolute quantification, stable isotope labeling with amino acids in cell culture, label-free comparative Liquid chromatography-Mass Spectrometry, protein phosphorylation identification, and protein microarrays, that can be used to elucidate plant bacterial interactions (Xing et al. 2004; Roe and Griffin 2006; America and Cordewener 2008; Gong et al. 2008).

Proteome analysis of rice roots infected with *Azoarcus* sp. was carried out to characterize the plant responses to these endophytes. Out of 1,000 displayed proteins, 47 responded to inoculation, including salt-stress and pathogenesis-related proteins and a putative receptor kinase. These proteins are involved in the endophyte infection process as they were also induced by jasmonic acid, which inhibits the infection process (Miche et al. 2006)

In *G. diazotrophicus,* 583 proteins were identified by two-dimensional gel electrophoresis /Matrix associated laser desorption/ionization for the establishment of a proteome reference map (Lery et al. 2008). Proteins of various pathways related to nucleotides, amino acids, carbohydrates, lipids, cofactors and energy metabolism have been described for comparative studies with other bacterial species. Various proteins related to Nitrogen-fixation, ion transporting, adaptation and protection related, regulatory and metabolic pathway proteins. When *G. diazotrophicus* colonizes sugarcane present in the cocultivation medium, changes occurs in various metabolic pathways, membrane-associated structure, redox reactions, transcript and translational regulation, and energy metabolism in comparison to control. So, the differentially expressed proteins showing modifications in bacterial metabolism and physiology in *G. diazotrophicus* during cocultivation with sugarcane provided information about the proteins involved in plant-bacterial interaction. The knowledge of metabolic fundamentals and coordination of these pathways are important for studying plant-endophyte interaction for attaining sustainable agriculture (Lery et al. 2008).

Cheng et al. (2009) observed that among the 275 identified proteins, in *P. putida* UW4, 1-aminocyclopropane-1-carboxylate deaminase is noteworthy because of its substantial role in plant growth-promoting activity. Furthermore, majority of the identified proteins were in bacterial cytosol. The identification of periplasmic, membrane-spanning and extracellular proteins indicated that the methodology is capable of providing a degree of representation from all cellular compartments including less soluble membrane fractions. One additional protein was identified as homologous to a *Bradyrhizobium japonicum* protein, which was later confirmed by several independent mass spectrometry analyses. It suggested a possibility that this protein was acquired from *Bradyrhizobium* species via lateral transfer, but it needs further confirmation. The functional diversity of the identified proteins can be used to investigate the responses of *P. putida* UW4 in response to various environmental signals. This data set will be helpful to unveil plant growth-promoting mechanisms present in this and similar bacteria, and in future to characterize bacterial interactions in the environment (Cheng et al. 2009).

Chaves et al. (2007) reported the proteome reference map of *H. seropedicae*. Out of 205 proteins identified during their study, 17 were hypothetical or conserved hypothetical proteins. The annotated proteins were classified in 19 clusters of orthologous groups categories, except proteins involved in defense mechanisms which all were identified. The clusters of orthologous groups categories were grouped in four classes: proteins involved in metabolism, information storage and processing, cellular processes and poorly characterized class (Chaves et al. 2007). Analysis of samples for meta-proteogenomics can be performed following the major steps mentioned as shown in Fig. 4.

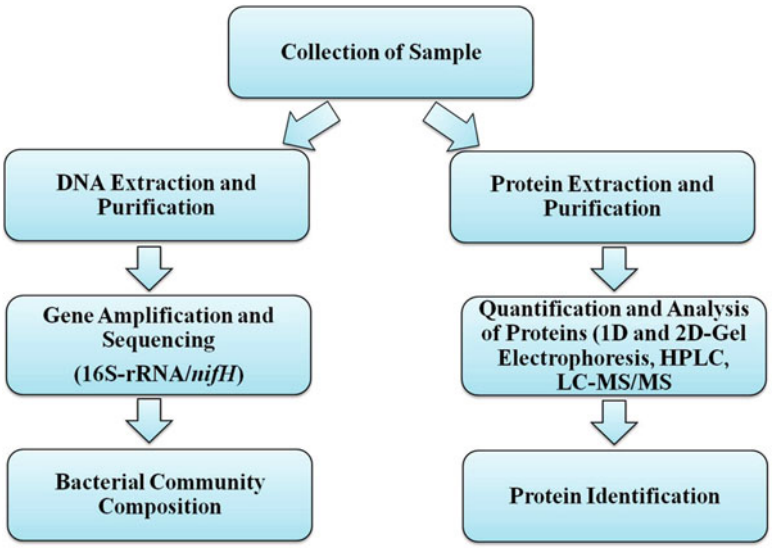

Fig. 4 Diagrammatic representation of meta-proteogenomics sample analysis methods

Ongoing progress towards in-depth analysis of genomic and whole protein profile of some of the potential endophytic bacteria such as *Azoarcus* sp., *Gluconoacetobacter diazotrophicus, Herbaspirillum seropedicae, Serratia marcesens* can help understand mechanism involved in plant-endophyte interaction which in turn will be deterministic in use of suitable formulations of endophytic bacteria to be used as biofertilizer for sustainable agriculture.

7 Conclusion

Exploration of endophytic bacteria and their abilities to enhance plant growth and productivity indeed indicates the existence of their natural associations and beneficial impact which can be exploited to feed burgeoning population of the world. Despite the fact that a large number of associative and endophytic bacteria have shown the potential in laboratory and green house condition but their consistent performance was failed under natural condition (Lucy et al. 2004). The reduction in the efficiency was noted when plants inoculated with endophytic bacteria were shifted from pot to field conditions (Gyaneshwar et al. 2002). These factors that affect colonization and the bacteria derived benefit to plants may be soil type, nutritional status of soil, host plant genotype and age as well as climatic conditions (Muthukumarasamy et al. 2005). It has been also evident from the earlier reports that plant growth and yield can be increased by the combined use of fertilizers and endophytic bacteria. This practice reduced the inputs of chemical fertilizers in the soil (Yanni et al. 1997; Saleh and Glick 2001). However, high amount of available utilizable N results in reduced colonization of endophytic bacteria and also reduce the process of

N_2-fixation due to regulatory mechanism acting in the diazotrophs. As observed in some studies, high nitrogen fertilized soil reduced the colonization of sugarcane by *G. diazotrophicus* and *H. seropedicae* (Fuentes-Ramirez et al. 1999; dos Reis et al. 2000). Therefore, a challenge is posed for systematic optimization for the application of suitable diazotrophs isolates and the amount of fertilizer to be added to obtain maximum output. Use of compost may be useful at some extent which provides utilizable N to support the growth of microorganism and make the plant evade from negative effects of diazotrophs colonization (Muthukumarasamy et al. 2007).

One of the major challenges includes selection of plant genotype and age, and compatible associative bacteria. Understanding of this compatibility would be helpful in enhancing the productivity using specific bacterial strain. Since, the colonization of associative bacteria depends upon seasonal changes and soil hydric stress, multiples field trials are required to optimize the parameters for obtaining the maximum output. Another factor which might be plays a crucial role is the plant defense response because it may limit or reduce the colonization of associative bacteria. In addition, the colonization mechanism is still not well understood. Intelligent analysis of genomic and functional genomic studies can help to manipulate the conditions in order to enhance the bacterial colonization process and increased plant growth attributes and also provides a better way to understand the ecology and behaviours of the endophytic diazotrophs.

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