Abstract

Introduction:

A sustainable means of providing the essential macro-element nitrogen (N) to plants is through biological nitrogen fixation (BNF) carried out by a select group of prokaryotes known as diazotrophs. A well-orchestrated symbiotic relationship exists between diazotrophs and legumes, but other groups of plants rely on free-living, associative nitrogen fixing organisms. Associative N fixing bacteria are often applied as biofertilizer to increase N availability to non-legume plants. These however have lesser ability to provide N as compared to the symbiotic diazotrophs (Carvalho et al. 2014). Recent interest is in endophytic diazotrophic bacteria that are able to colonize the internal tissues of the plants such as root and aerial parts and fix nitrogen *in planta*. Endophytic bacteria are defined as those that reside inside the plant tissue and can be isolated from surface disinfected plant parts (Schulz and Boyle 2006). Endophytes live and multiply within the intracellular spaces of plants as a part of their lifecycle or whole life. Many endophytic diazotrophs are known and have promising application in increasing agricultural yields. The endophytic community residing in the plants varies with plant genotype, its age, edaphic factors, soil microflora and various abiotic factors. 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 BNF potential to a wider spectrum of plants. The *Poaceae* family, also known as cereal grasses such as rice, wheat and maize constitutes the most economically important monocot plant family. Diazotrophic endophytic bacteria is an attractive strategy to provide N to cereal plants. Since endophyte populations are determined by environmental and climatic zone parameters, it is important to isolate them from indigenous plants with the idea to develop more robust systems that could also survive well in free-living state in the specific geographic location.

Other 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 production of anti-metabolic 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 but rather presence of many endophytes results in priming of the plants, providing a foundation for a more intense and rapid defense against pathogen attack. Genetic engineering of endophytes for enhancing their beneficial role to plants is envisaged as a future possibility. To date 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 presence of the 2,4 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 following objectives

1. Isolation and characterization of diazotrophic endophytic plant growth-promoting bacteria from various indigenous *Poaceae* family plants
2. Plant growth promotion and disease suppression by antibiotic producing diazotrophic endophytic *Streptomyces* spp.
3. Cloning and expression of 2,4-Diacetylphloroglucinol gene cluster in diazotrophic endophytic bacterial system and evaluation of its ability for disease suppression

Methodology:

- Total endophytic bacterial and nitrogen fixing culturable endophytic diversity were studied in maize, wheat, sorghum, rice and pearl millet from root, stem and leaves using denaturing gradient gel electrophoresis (DGGE) analysis followed by sequencing.
- Isolation of diazotrophic endophytic bacteria was carried out on nitrogen free medium from surface disinfected plant parts. Identification of isolates was performed using the 16S rRNA sequencing.
- Plant growth promoting (PGP) traits in endophytic bacterial isolates such as presence *nifH* gene, production of indole acetic acid (IAA) and siderophores, phosphate solubilization, secretion of hydrolytic enzymes and antifungal activity were monitored by standard methods. The antagonistic molecules produced by *Streptomyces* spp. were identified by GC-MS.

- Cloning and expression of 2,4-diacetylphloroglucinol (2,4-DAPG) gene cluster comprising of *phlDABC* operon was carried out using the broad host range plasmid pUCPM18. 2,4-DAPG extracted from cell-free supernatant was quantified using HPLC.
- Endophytic bacterial isolates were tagged with appropriate *gfp* containing plasmids their colonization upon re-inoculation on seedlings of different plants was monitored by confocal laser scanning microscopy (CLSM).
- Evaluation of the effect of inoculation of either native or genetically modified strains on plant growth parameters was carried out plant-soil system in green house. Disease suppression was monitored by phytopathogen challenge to the aerial parts of the bacterized plants and compared with appropriate controls.
- Defense related gene expression was analyzed in rice plants bacterized with either native or recombinant strains by qPCR using gene specific primers. Defense "priming" was monitored by gene expression under fungal challenge

Results:

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

DGGE band patterns of different plants showed considerable variation with respect to both total as well as diazotrophic communities. Shannon-Wiener diversity indices of total endophytic bacterial community were found to be high in rice, maize and pearl millet whereas enriched diazotrophic communities of rice and maize showed higher diversity as compared to the other plants. Sequence analysis of selected bands from the total community showed best matches with uncultured *Bacillus* and *Proteobacteria* clones, and *Serratia* strains while the selected DGGE bands of the diazotrophic community enriched in NFb medium showed best correspondence with uncultured *Brevundimonas* and *Rhizobium* clones, *Sphingomonas*, *Pseudomonas* and *Agrobacterium* strains.

A total of 31 diazotrophic endophytic bacteria were isolated as pure cultures from five *Poaceae* family plants on the basis of the differences in phenotype on nitrogen free medium. Analysis of their 16S rRNA gene sequences showed them to belong to phyla *Proteobacteria* (53%) [α- *Proteobacteria* (20%), β- *Proteobacteria* (23%), γ- *Proteobacteria* (10%)], *Actinobacteria* (37%) and *Firmicutes* (10%). Interestingly among the 13 gram negative isolates tagged with *gfp*,

all showed colonization in the internal parts of wheat regardless of which plant they were isolated from. Presence of *gfp* tagged isolates was detected at 10 to 30 µm depths from root surface indicating their endophytic colonization. All endophytic bacterial isolates used in this study showed *nifH* gene amplification (~320 bp). All the diazotrophic endophytic bacteria isolated in this study showed the siderophore production and 81% of the isolates produced IAA. It was observed that 81%, 84%, 35%, and 13% isolates were positive for hydrolytic enzymes cellulase, pectinase, chitinase, and protease respectively. Diazotrophic endophytic bacterial isolates were analyzed for total 8 PGP traits but none of the isolates was found positive for all the PGP traits. Eight isolates were positive for 6 PGP traits, 8 isolates for 5 PGP traits, 10 isolates for 4 PGP traits, 2 isolates for 3 PGP traits and 3 isolates for 2 PGP traits. Diazotrophic endophytic bacteria inoculated on wheat plant showed significant improvement of plant growth parameters after 30 d of growth period. Majority of endophytes showed the improvement in dry mass of shoot and root and increase the chlorophyll content. PCA analysis was performed using nine variables to extract the principal component (PC). The extracted PC1 - IAA (39% variance) and PC2- siderophore (22% variance) together gave the 61% (eigenvalue >1.5) variance. Results suggested rich diversity of diazotrophic endophytes in *Poaceae* plants from Gujarat. However we did not come across more well reported species such as *Herbaspirillum* and *Glucononacetobacter* spp. The endophytes lacked host specificity and can be used as broad spectrum biofertilizer.

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

Among the 31 diazotrophic endophytic bacterial isolates obtained only three strains of *Streptomyces* showed antagonistic activity against the phytopathogen *Rhizoctonia solani.* They also showed inhibition of the rice blast fungus *Magnaporthe oryzae* B157. Secondary metabolites extracted from *Streptomyces* sp. SS1, SS5 and SS8 were identified based on mass spectrum database and highest REV similarity index as 2-(chloromethyl)-2-cyclopropyloxiran, 2, 4- ditert-Butylphenol, and 1□ethylthio□3□methyl□1, 3□butadiene respectively. The EGFP tagged *Streptomyces* spp. were observed as microcolonies in interior parts of the root, stem and leaves of wheat and sorghum plants. The Z-stack analysis revealed that endophytic colonization of *Streptomyces* spp. was detected from 5 to 31 µm deep inside the root in wheat and sorghum plants while thickness of the roots varied from 45-111 µm. Rice plants inoculated with *Streptomyces* spp. showed enhanced plant growth of aerial parts of the plants whereas in wheat and sorghum plants

were found to be improved in both above and below ground parts of the plant as reflected in root:shoot ratios. Bacterized plants challenged with fungal pathogen showed improved resistance against damage caused by the fungus. The upregulation of *NPR1* gene was observed in rice plants colonized with the *Streptomyces* spp. upon fungal challenge. *Streptomyces* sp. SS1 showed also showed an increase in the expression of genes *PR10a* and *LOX2* by 28.17 and 30.25 fold expression respectively while in *Streptomyces* sp. SS8 showed was 11.08 fold higher *LOX2* gene expression. Thus novel diazotrophic biocontrol strains of *Streptomyces* have been obtained that show disease suppression not only because of direct antagonism but also by triggering induced systemic response (ISR) in plants.

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

Diazotrophic endophytic isolate *Pseudomonas* sp. WS5 was genetically modified by cloning the 2,4-Diacetyl phloroglucinol (2,4-DAPG) gene cluster (*phlDACB*) individually from *Pseudomonas protegens* Pf-5 and *Pseudomonas* sp. G22 in the broad host range plasmid pUCPM18Gm. Quantitative analysis of 2,4-DAPG was carried out by HPLC in which wild type *P. protegens* Pf-5 and lab isolate *Pseudomonas* sp. G22 were also studied to compare their rate of 2,4-DAPG production along with the genetically modified strain. With the natural isolates *P. protegens* Pf-5 and *Pseudomonas* sp. G22 strains, the highest production observed after 120 h of incubation was 1.28 µg ml^{-1} and 1.53 µg ml^{-1} respectively. Similar levels of production were achieved with the genetically modified strains of *Pseudomonas* sp. WS5 carrying Pf-5 and G22 2,4-DAPG gene cluster containing pAJK1.2a and pAJK1.2b plasmids after 24 h of incubation as 1.66 µg ml^{-1} and 1.10 µg ml^{-1} respectively and recombinant strains produced much higher DAPG than the native strains at 120 h of incubation. Colonization of wheat plant by *gfp* tagged bacteria was observed from root hair to aerial parts of the plants. All three plants viz. rice, sorghum and wheat showed enhanced growth of above as well as below ground parts of the plant upon bacterization with the native as well as the genetically modified diazotrophic endophyte. Significant increase in vigour index was also observed in bacterized plants. Plants challenged with the phytopathogens analyzed after 60 d (30 d of healthy growth followed by 30 d of growth after pathogen challenge) showed a significant improved resistance to disease in case of the plants bacterized with the recombinant strain as compared to the native organism. Bacterized rice plants showed the high expression of

NPR1 genes as compared to unbacterized rice plants, with 82.42 and 36.65 fold increase in plants inoculated with the wild type and 2,4-DAPG producing strain respectively. Upon the blast fungal attack, the expression of *NPR1* gene in rice plants was found to be higher by 58.63 and 32.34 fold in wild type and 2,4-DAPG producing strain respectively. Along with this, expression of *PR10a* gene was also observed to be increased 9.10 fold in plants inoculated with the recombinant strains in fungal infected plants compared to other bacterized rice plants infected with *M. oryzae* B157. This study shows that endophytic bacteria can be engineered to produce the antibiotic molecule 2,4-DAPG to make it effective in disease suppression. The biocontrol ability could be attribute to the direct antagonistic effect on the phytopathogen as well as to the induction of ISR by the 2.4-DAPG molecule.