Chapter 2

Isolation and characterization of diazotrophic endophytic plant growth promoting bacteria from indigenous Poaceae plants

"Faith is of no avail in the absence of strength. Faith and strength, both are essential to accomplish any great work."

- Sardar Vallabhbai Patel

2.1 Introduction

Many rhizosphere microbes are helpful in improving plant health, reducing susceptibility towards disease and relieving abiotic stress (Compant et al. 2010). Endophytes are organisms which colonize and thrive inside the plant tissues during their life cycle and their community varies with plant type, age and stress. From the complex microbial community associated with the exterior surfaces of plants, only a small group is able to enter in to the plant parts and successfully thrive in the interior of the plants without inducing a defense response. The study of endophytic bacteria is important not only for understanding the ecological role of such bacteria in their interaction with plants but also for the biotechnological application of these bacteria for the promotion of plant growth and agricultural yields (Kuklinsky-Sobral et al. 2004). Plant growth promoting endophytes are important as inoculants for enhancing plant growth and decreasing the severity of disease incidence in crops (Kang et al. 2007). Endophytic bacteria entering in plants and seeds are more important for site restoration compared to the rhizobacteria which require application every year to maintain their effects (Chanway et al. 2000).

Diazotrophic (nitrogen fixing) endophytic bacteria have tremendous potential in increasing nitrogen availability to plants particularly in the case of cereals which unlike legumes do not form symbiotic nitrogen fixing organs like nodules with the bacterial partner. Several studies carried out in rice, sorghum, pearl millet, wheat and maize showed that diazotrophic endophytic colonization improves plant growth (Carvalho et al. 2014). *In planta* nitrogen assimilation was studied by incorporating $^{15}N_2$ in sugarcane plants by endophytic *Acetobacter diazotrophicus* which is found to be higher in shoot compared to the roots of the plants. Further studies conducted with the wild type and *nif* mutant of *A. diazotrophicus* under N limiting conditions in sugarcane plants showed the reduction in plant growth in plants inoculated with the mutant strain as compared to the plants inoculated with the WT strains. The plants inoculated with *nif* mutant showed the almost similar plant growth as compared to the uninoculated controls (Sevilla et al. 2001).

The present study was aimed at studying the diversity of diazotrophic endophytic bacteria from various *Poaceae* plants by culture-independent as well as culturing methods. The cultured bacteria were studied for their ability to reenter inside another host plant apart from which they were isolated and assayed for their effect on plant growth.

2.2 Materials and Methods

2.2.1 Community DNA extraction

Total bacterial endophytic community DNA was extracted from wheat, sorghum, rice, pearl millet and maize (Garbeva et al. 2001). Root, stem, and leaves were separated, washed with sterile distilled water and surface-disinfected. Plants parts (100 mg) were minced and resuspended in 1ml of 120 mM sodium phosphate buffers (pH 7.1) which was incubated for 2 hours under shaking conditions after which 1.5 ml of the content was centrifuged at 6708 xg for 5 min. The following procedure for DNA isolation was followed as mentioned in Sambrook and Russell (2001). The cell pellets were suspended in 100 mM TE buffer (100 mM Tris-cl and 1 mM EDTA) and 5 µl Proteinase K (10 mg ml^{-1}) was added and contents were mixed gently. Subsequently, 30 µl of 10% (w/v) sodium dodecyl sulfate (SDS) was added followed by incubation at 37 °C for 1 h. To the cell lysate 100 µl of 5 M NaCl was added along with 80 µl of 0.7 M NaCl containing 10% (w/v) cetyltrimethylammonium bromide (CTAB) for 10 min at 65 °C. This was followed by the addition of 250 µl of chloroform:isoamyl alcohol:phenol (24:1:25), and the contents were mixed a few times and centrifuged 9660 xg for 5 min. Upper phase was taken out in a fresh centrifuge tube and equal volume of chloroform:isoamyl alcohol (24:1) was added again and centrifuged 9660 xg for 5 min. The upper phase was transferred into a fresh tube and 0.6 volume of isopropanol was added and incubated at -20 °C for 1 h. Whole content was centrifuged at 6708 xg for 5 min and the supernatant was discarded followed by resuspension of the pellet in 95% (v/v) ethanol. Again the whole contents were centrifuged at 6708 xg for 5 min, air dried and finally dissolved in 10 mM TE buffer (10 mM Tris-Cl and 1 mM EDTA).

For isolation of community DNA of culturable nitrogen fixing endophytes, macerated tissue samples as prepared above were inoculated into fresh semi-solid nitrogen-free base (NFb) medium (Appendix II). The bacterial mass obtained after growth from each tube was transferred to new semi-solid NFb medium tube after 7 d of incubation. This process was repeated several times and finally transferred to NFb broth and incubated under static conditions, from which community DNA was extracted using a similar protocol as for total bacterial endophytic community DNA extraction mentioned above.

2.2.2 PCR amplification of 16S rRNA gene fragments

Polymerase chain reaction (PCR) has been used in this study for 16S RNA amplification for strain identification and for denaturing gradient gel electrophoresis (DGGE). For DGGE analysis, 16S rRNA gene was amplified from total as well as enriched culturable diazotrophic community using 341F forward primer having a GC clamp and 534R reverse primer as described by Subrahmanyam et al. (2011). (Table 2.1) The polymerase chain reactions (PCR) (were carried out in 50 µl systems consisting of 50 ng DNA template, 1 µl of 10 µM each primer, 2 µl of 2.5 mM dNTPs each, 0.5 U Taq DNA polymerase and 2.5 µl 10X Taq buffer. Amplification was carried out with a Applied Biosystems, USA thermocycler with initial denaturation at 95 ºC for 5 min, followed by 20 cycles consisting of initial denaturation at 94 ºC for 1 min, annealing temperature of 65 °C (reduced by 0.5 °C with each cycle) for 45 s, elongation at 72 ºC for 1 min; 15 cycles of 94 °C for 1 min, 55 °C for 45 s, elongation at 72 °C for 1 min, final elongation carried out at 72 ºC for 10 min.

Table 2.1 PCR Primers used in this study

Gene target	Primer[a]	Sequence (5'-3')[b]	Expected amplicon size (bp)	Reference
Universal eubacterial primer for 16S rRNA gene amplification	27F 1107R	AGAGTTTGATCCTGGCTCCAG GCTCG TTGCGGGACTTAACC	1080	Rajendran et al. (2008)
Universal eubacterial primer for 16S rRNA gene V3 region amplification for DGGE analysis	GC-341F 534R	CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG ATTACCGCG GCTGCTGG	~200	Muyzer et al. (1993)
nifH gene amplification	PolF AQER	TGCGAYCCSAARGCBGACTC GACGATGTAGATYTCCTG	321	Poly et al. (2001)

NifHF	AAAGGYGGWATCGGYAARTC CACCAC	435	Gaby and Buckley (2012)
NifHR	TTGTTSGCSGCRTACATSGCCA TCAT		

[a]F- forward, R- Reverse; GC- GC clamp; [b]Degenerate oligonucleotide: Y = C/T; S = G/C; R = A/G; B = C/G/T; W = A/T

2.2.3 DGGE analysis

DGGE analysis was carried out in duplicates on two independent gels of 10% polyacrylamide gel with a gradient of 40 to 70% denaturant (100% denaturant contained 7 M urea and 40% formamide) using D-code system (Bio-Rad, Hercules, CA, USA), in 1X TAE buffer, at constant voltage 50 V for 16 h at 65 °C (Sharaff and Archana 2015). One of the two gels was stained by silver staining and other with SYBR green (Invitrogen, UK).

Silver staining protocol was as follows. Gels were washed with distilled water and were kept in 150 ml of 10% methanol for 12 min under shaking, then the methanol was discarded and 100 ml of 0.7% nitric acid was added for 6 min under shaking. This was followed by washing with distilled water. After washing 100 ml of 0.2% (w/v) silver nitrate was added and incubated in dark for 30 min. Gels were further washed with distilled water and 150 ml developer (2.29 g sodium carbonate, 150 µl Formaldehyde, 2 ml of 0.2% sodium thiosulfate and made up to 100 ml with distilled water) was added and gently mixed till clear bands appeared. In order to prevent the excessive background staining, 50 ml of glacial acetic acid was added to stop further reaction. Finally, gels were washed with distilled water and kept in 1XTAE buffer (Sambrook and Russell et al. 2001).

For SYBR staining, SYBR gold was diluted to 1X concentration in 1XTAE buffer (pH 8.0) and enough amount of SYBR solution was added to cover the whole gel, incubated under shaking conditions in the dark for 15 min and observed under UV transilluminator (Alpha Innotech, CA). Imaging was done by AlphaEase 4.0 software (Alpha innotech, USA). The diversity indices Shannon-Wiener, Species Richness (Margalef), Species Evenness (Pielou) were calculated using the GelCompar II (Applied Maths NV) to analyze the bacterial diversity. Selected DNA bands were carefully cut out from the gel stained with SYBR green (for ease of reamplification) and placed in 100 µl sterile distilled water at 4 °C for overnight. Re-PCR

amplification of the eluted bands was carried out using 341F without the GC clamp and 534R primers. Amplified PCR products were purified and subjected to Sanger sequencing (SciGenom Labs, India). The resulting sequence was analyzed by BLASTn in NCBI database (Altschul et al. 1990).

2.2.4 Isolation of diazotrophic endophytic bacteria

Isolation of diazotrophic endophytic bacteria was carried from stems and leaves of various *Poaceae* family plants (maize, wheat, pearl millet, sorghum, and rice) of Gujarat (India) region. Epiphytic bacteria were removed by washing plant parts under running tap water followed by surface sterilization process with 70% ethanol for 1 min, 2% sodium hypochlorite for 1 min and followed by five times rinsing with sterile distilled water. The effectiveness of surface sterilization was checked by inoculating the final rinse water onto Luria Bertani (LB) agar (Appendix II). One gram of surface sterilized sample was macerated in 1 ml of 0.9% NaCl (N saline) solution. Tissue extract was serially diluted in N saline and aliquots of various dilutions (undiluted, 10^{-1} to 10^{-5}) were added into semi-solid NFb medium tubes and also spread on NFb solid medium and incubated at 3 °C for 7 d. Bacterial colonies were purified on JNFb solid medium by repeated subculturing (Döbereiner et al. 1995).

2.2.5 Bacterial identification

2.2.5.1 Biochemical characterization

Biochemical characteristics of endophytic bacteria were determined for each colony type. They included gram staining, catalase, Hugh Leifson oxidase test, MR-VP, indole test etc. performed as mentioned in the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The isolates were tested for carbohydrate fermentation with 10 sugars (Himedia, India): glucose, galactose, fructose, xylose, mannitol, maltose, meso-erythritol, sucrose, inositol, lactose. In this test, 1% protease peptone water with Andrade's indicator was used for detection of carbohydrate fermentation and inverted Durham tubes were added for the detection of gas production. A 1% sugar solution was sterilized at 10 psi for 20 min and added separately into the medium. Overnight grown cultures were used as inoculum and test result recorded after incubation at 35 °C for 24-48 h (Appendix II for reagent preparation).

2.2.5.2 16S rRNA gene sequence analysis

For identification of bacteria based on 16S rRNA gene sequence, genomic DNA extraction from pure cultures was carried out as described Sambrook and Russell (2001). Universal primers of eubacteria 27F and 1107R (Table 2.1) were used for amplification of 16S rRNA gene. The polymerase chain reactions (PCR) were carried out in 25 µl systems consisting of 50 ng DNA template, 0.5 µl of 10 µM each primer, 1 µl of 2.5 mM dNTPs each, 0.5 U Taq DNA polymerase and 2.5 µl 10X Taq buffer. Amplification was carried out at initial denaturation at 95 ºC for 5 min, followed by 30 cycles consisting of initial denaturation 94 ºC for 45 s, annealing temperature of 58 ºC for 30 s, elongation at 72 ºC for 1 min, final elongation was carried out at 72 ºC for 10 min (Chaturvedi and Archana 2012). Amplified PCR products were analyzed on 1% agarose gel. Sequencing of 16S rRNA amplicons was carried out using the services of Xcelris Labs Ltd (Ahmedabad, India). The sequences were analyzed by the Sequence Match tool at Ribosomal Database Project (RDP) (Wang et al. 2007) and Nucleotide BLASTn in NCBI database (Altschul et al. 1990). The phylogenetic tree was constructed by neighbor-joining algorithm with Jukes-Cantor model method of MEGA 6 (Tamura et al. 2013).

2.2.6 Characterization of plant growth promoting (PGP) traits

2.2.6.1 Amplification of nifH gene

Diazotrophic nature of endophytic bacterial isolates was confirmed by detecting the presence of *nifH* gene which encodes the dinitrogenase reductase enzyme by PCR primer mentioned (Table 2.1). *NifH* gene amplification with only primer difference was carried out with PolF and AQER primers (Poly et al. 2001). The PCR reaction mixture was made as described earlier for 16S rRNA amplification and amplification carried out as follows: an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 45 s and 72 °C for 1 min with a final extension at 72 °C for 5 min. Amplified PCR product was analyzed on 2 % agarose gel and stained with ethidium bromide. The positive control taken was *Herbaspirillum seropedicae* Z67 and negative control *E. coli* DH5α. *nifH* genes from selected endophytic isolates were amplified with NifHF and NifHR (Gaby and Buckley 2012) primers (Table 2.1). PCR cycle similar to the above was used where annealing temperature was changed to 65 °C for 45 s.

2.2.6.2 Phosphate solubilization

Phosphate solubilization was examined on Pikovskaya's agar (Himedia, India) (Appendix II) plates by spot inoculation with overnight grown cultures and incubated at 28 °C for 72 h. Isolates showing positive (zone of clearance) results were further studied for acidification of Tris-Rock phosphate (TRP) using buffered minimal media containing 100 mM glucose and 50 mM Tris-Cl pH 8.0 (Gyaneshwar et al. 1999). Senegal RP (Sharma et al. 2005) was used as sole phosphate source and methyl red was used as a pH indicator. Overnight grown cultures were spot inoculated on the plates and incubated at 28 °C for 96 h. Acidification was observed by a change in color from yellow to red. For quantitative analysis of RP solubilization, a similar medium was used except that pH indicator and agar were absent; P released in supernatant was measured at regular time intervals by ascorbate method described by Gyaneshwar et al. (1999).

2.2.6.3 Indole Acetic Acid (IAA) production

All bacterial isolates were screened for the IAA production by growing in 50 ml of LB broth amended with 5 mM L- tryptophan for 48 h at 28 °C under shaking conditions. IAA production was quantitated spectrophotometrically by using Salkowski's reagent as described by Ahmad et al. (2008). Briefly after incubation culture was centrifuged at 21,690 xg for 5 min., supernatant was collected and 2 ml of supernatant was mixed with 2-3 drops of orthophosphoric acid followed by addition of 4 ml Salkowski reagent (12 g of $FeCl_3$ per liter in 7.9 M H_2SO_4) mixed well and incubated in dark for 30 min. IAA production was recorded by measuring optical density 530 nm. Pure IAA (Himedia, India) was taken as standard for calibration.

2.2.6.4 Siderophore production

All endophytic bacteria were screened for the siderophore production by using Chrome Azurol S (CAS) agar plate (Schwyn and Neilands 1987). Overnight grown culture was inoculated on CAS agar incubated at 28 °C for 7 d and observed for orange to yellow halo formation around the colonies.

Detection of siderophore type was carried out as described by Khan et al. (2006), Endophytic bacterial isolates were grown in deferrated LB broth and incubated for 48 h under shaking condition at 180 rpm. Identification of hydroxamate siderophore was carried by adding 0.5 ml of 6 N H_2SO_4 to 0.5 ml culture supernatant and autoclaving the mixture at 15 psi for 30

min. After reaction mixture cooled down, 1 ml sulphanilic acid (1% w/v in 30% acetic acid v/v) was added followed by 0.5 ml of iodine (1.3% iodine in 30% acetic acid). Excess of iodine was removed by addition of 2% (w/v) 1 ml sodium arsenate (Na_3AsO_4). Then α-naphthylamine (0.3% w/v in 30% acetic acid) was added and the reaction mixture was incubated for 30 min. Absorbance was taken at 526 nm. Hydroxylamine hydrochloride was used as standard (0.1-1 µg ml^{-1}) for calibration. Catechol-type of siderophore was measured by Arnow test. Isolates were grown in similar manner as above, culture supernatant (1 ml) was mixed with 1 ml of 0.5 N HCl followed by 1 ml of nitrite molybdate reagent (10% each sodium nitrite and sodium molybdate in distilled water), allowed to stand for 5 min and then 1 ml 1N NaOH added. The absorbance of the pink color developed was recorded at 510 nm and calibrated against 2, 3-dihydroxybenzoic acid as standard (10-100 µg ml^{-1}).

2.2.6.5 HCN production

Bacterial isolates were analyzed for hydrogen cyanide production by the method of Lorck (1948). LB agar was amended with glycine 4.4 g l^{-1} and the bacterial cultures were inoculated on its surface in top agar. A Whatman filter paper No. 1 saturated in a solution containing sodium carbonate (2% w/v) and picric acid (0.5% v/v) was attached to the lid of the plate, which was then sealed with parafilm and incubated at 35 °C for 4-5 d. The development of orange to brown color on the filter paper indicated HCN production.

2.2.6.6 Dual culture test for antifungal activity

Dual culture test was performed to analyze the antagonistic activity of endophytic isolates against plant pathogenic fungus *Rhizoctonia solani*. The endophytic isolates were grown for 12-16 h (except *Streptomyces* spp. which were grown for 48 h) in LB broth at 28 °C. *R. solani* was grown for 5 d on potato dextrose agar (PDA) and an agar plug with the mycelia was transferred on to one side of a fresh PDA plate while test cultures were streaked on the opposite side. Plates were incubated at 30 °C for 5 d and observed for zone of inhibition of the fungal mycelia around the bacterial growth.

2.2.6.7 Lytic enzyme activity

Many endophytic organisms produce extracellular enzymes which may help the bacteria in enter inside the host plant, derive the nutrition or help in inhibition of the pathogens. Cellulase,

chitinase, pectinase and protease activities were determined qualitatively on appropriate media plates spot inoculated with overnight grown cultures and incubated at 28 °C.

Cellulase production was detected by congo red according to Teather and Wood (1982) on carboxy methylcellulose (CMC) (Sigma-Aldrich, India) agar (0.5% CMC, 1% yeast extract, and 0.05% peptone) plates after incubation for 60 h. Detection of chitinase activity was carried out by flooding with congo red solution (0.1%) for 15 min, subsequently overlayed with 1M NaCl, incubated 15 min and observed for the zone of clearance around the colony. Chitinase production (Nagpure and Gupta 2012) was determined on Monreal Reese medium (Appendix II) containing colloidal chitin (0.5%) [prepared from chitin (Himedia, India), Appendix II] after incubation for 7 d and observed as a zone of clearance. Protease (Pillai and Archana 2008) and pectinase (Verma et al. 2001) activities were determined in LB agar containing 2% skim milk powder and 0.5% pectin respectively. Positive protease production was seen by zone of clearance. Pectinase positivity was detected by overlaying the plates after the incubation with 2% CTAB solution for 30 min followed by a washing with 1M NaCl solution to analyze the clear zone of pectin degradation.

The quantitative enzyme assays for cellulase, chitinase, protease and pectinase activity were performed using culture supernatants of cells grown in dilute LB (0.3X) containing individually CMC (1 g l^{-1}), colloidal chitin (10 g l^{-1}), skim milk powder (10 g l^{-1}), or pectin (5 g l^{-1}) respectively. After incubation at 28 °C for 5 d under shaking conditions culture supernatants were centrifuged at 21690 xg for 15 min. The enzyme assays were carried out by methods described for cellulase (Saha et al. 2006), chitinase (Nagpure and Gupta 2012), protease (Pillai and Archana 2008), and pectinase (Khatri et al. 2015) using cell-free supernatant as the source of enzyme.

Cellulase unit enzyme activity was determined from 0.5 ml of culture supernatant; 0.5 ml CMC (1%) dissolved in 100 mM citrate buffer (pH 5.0) and incubated at 37 °C for 30 min. The reactions were terminated by adding 1 ml 3,5-dinitrosalicylic acid (DNS) and heating in boiling water-bath for 10 min, followed by the measurement of absorbance at 540 nm (Miller). Glucose was taken as standard.

Chitinase unit enzyme activity was determined using a reaction system consisting of 1 ml supernatant and 1 ml colloidal chitin and incubated 37 °C for 30 min. The reactions were stopped by centrifuged at 9279 xg for 2 min and 1 ml of the supernatant was mixed with 1 ml of DNS, placed in boiling water bath for 5 min, followed by measurement of absorbance at 540 nm. Glucose was taken as standard.

Protease unit enzyme activity was determined by a reaction system consisting of 1 ml culture supernatant and 1 ml casein (1%) prepared in 50 mM Tris-Cl buffer of pH 8.0 and incubated at 37 °C for 30 min. The reaction was stopped by adding 2 ml 10% trichloroacetic acid and centrifugation on 9279 xg for 15 min. The absorbance was measured using clear supernatant at 280 nm (Kembhavi et al. 1993). Tyrosine (SRL, India) was taken as standard.

Pectinase unit enzyme activity was determined by a reaction system consisting of 1 ml of 0.5% pectin, 0.5 ml of sodium acetate buffer (0.1 M, pH 5.0), with 0.5 ml of supernatant and incubated 30 °C for 10 min. The reaction was stopped by adding 2 ml DNS and the mixture was placed in boiling water-bath for 10 min, followed by measurement of absorbance at 575 nm. D-Galacturonic acid was taken as standard.

2.2.7 Transformation of plasmid pHC60

Plasmid pHC60 (Cheng and Walker 1998) (Fig. 2.1) (GenBank FJ151627.1) is a spontaneous mutant of the pHC41 plasmid that constitutively expresses the *gfp* gene. In addition it contains the stabilization fragment from plasmid RK2. The *in planta* study showed the stability of *gfp* expressing pHC60 plasmid in absence of selective pressure made it possible for us to examine the endophytic entry of different isolates in wheat plant. Positive selection marker is tetracycline (40 $\mu g\ ml^{-1}$).

2.2.7.1 Plasmid transformation of E. coli by competent cell preparation

E. coli S17.1 were transformed with pHC60 plasmid by $CaCl_2$ mediated competent cell preparation followed by plasmid transfer by heat shock as given in Sambrook and Russell (2001).

2.2.7.2 Plasmid transfer into endophytes by biparental mating by conjugation

Endophytic bacterial isolates and *E. coli* S17.1 (pHC60) were inoculated separately in LB broth and incubated at 30 °C and 37 °C respectively for 12 h. Tetracyclin (40 $\mu g\ ml^{-1}$) was added only

to *E. coli* S17.1 (pHC60). Overnight grown cultures were centrifuged at 1677 xg for 5 min and the supernatant was discarded, the pellet resuspended in N-saline and again centrifuged the contents at 1677 xg for 5 min. Supernatant was discarded and pellet resuspended in 100 µl of saline. In bi-parental mating recipient (Endophytic bacteria) and donor (*E. coli* S17.1 carrying pHC60) were both mixed at 1:1 ratio and inoculated on LB agar plate without any antibiotic. Incubation was carried out at 30 °C for 14-16 h. Growth obtained on the plate was scraped and resuspended in fresh LB broth, and 30 µl was spread on NFb medium containing 40 µg ml^{-1} tetracycline (Tanaka et al. 2006).

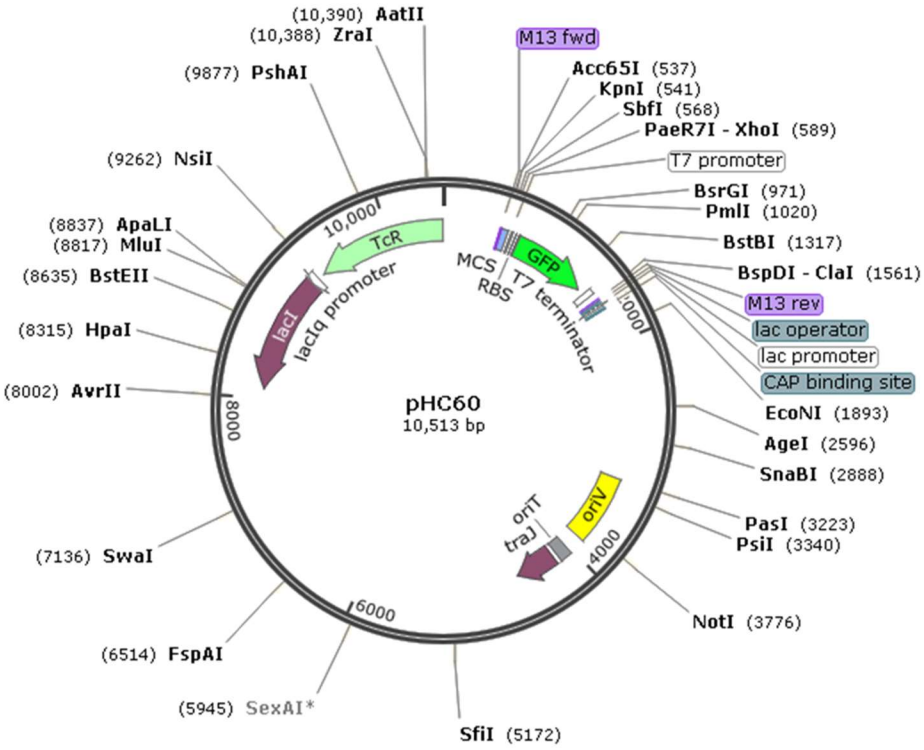


Fig. 2.1Plasmid map of plasmid pHC60 containing GFP gene (Cheng and Walker 1998)

2.2.8 Detection of endophytic presence of isolates by confocal laser scanning microscopy

The endophytic nature of isolates was confirmed by inoculating in wheat plants with *gfp* tagged endophytes and growth of plants under hydroponics system in Murashige-Skoog medium (Himedia, India) (Appendix II) and observing for the presence of bacteria in plant parts was detected by confocal laser scanning microscopy (CLSM). Wheat seeds were surface sterilized by first washing under running tap water and then transferred to 1% $HgCl_2$ for 5 min, treated with

70% ethanol for 5 min followed by repeated washes with sterile distilled water. Seeds were then transferred on 0.8% agar containing petri-plate and germinated under dark at 25 °C for 2-4 d. Germinated seedlings with similar lengths of radicle were soaked for 30 min with overnight grown pure cultures of endophytic bacteria grown individually and diluted appropriately (in N saline) to give 10^8 CFU ml^{-1}. Inoculated seedlings were transferred for growth into the hydroponics system and cultivated at 25 °C under greenhouse conditions with natural daylight. On 7^{th} d after inoculation plants were harvested and different plants parts were processed immediately for CLSM (LSM 700 Carl Zeiss, GmbH). Bacterial counts (CFU g^{-1} fresh weight) were determined from surface sterilized plants by plating extracts on NFb medium. The endophytic bacteria that were not tagged with *gfp* were also processed similarly for CFU ml^{-1} determination for confirming their endophytic nature.

2.2.9 Pot inoculation experiments

The diazotrophic endophyte bacterial isolates were analyzed for their plant growth promotion ability in gnotobiotic condition (sterilized soil and surface sterilized seeds). Bacterized wheat seedlings (dipped in culture suspension of approximately 10^8-10^9 CFU ml^{-1}) were prepared for pot inoculation as mentioned above for hydroponics. Each pot received 2 kg of sterile soil (obtained from Pulse Research Station, Anand Agricultural University, Model Farm, Vadodara, Gujarat) and four bacterized seedlings (two pots per treatment); the uninoculated plants were taken as negative control and seedlings inoculated *Herbaspirillum seropedicae* Z67 were taken as positive control. Plants were maintained in a totally random arrangement in greenhouse at 25 °C, under natural daylight (approximately 12 h photoperiod) and relative humidity of 75%. After 30 d of growth, plants were uprooted, surface sterilized and processed for CFU g^{-1} of fresh weight of tissue for detection endophytic presence of bacteria. Effect of various strains on the following different parameters of wheat plants was determined: chlorophyll content (Arnon 1949), root length, and shoot length, the dry and wet weight of root, stem and leaves. Nitrogen content of the plants was measured using the micro-Kjeldahl method (Villegas et al. 1984). Pot experiments were repeated twice and results compiled from plants of both the experiments.

2.2.9.1 Chlorophyll estimation

Chlorophyll was extracted from samples by mincing 100 mg of leaf tissue and transferring into preheated 7 ml of dimethyl sulphoxide (DMSO) followed by incubation 65 °C for 30 min after which 3 ml DMSO was added to make up the 10 ml total volume. Absorbance was measured at 645 nm and 665 nm, DMSO used as blank (Richardson et al. 2002). Arnon (1949) equations for measurement of chlorophyll are as given below

$$\text{Chla (g l}^{-1}) = 0.0127\ A663 - 0.00269\ A645$$

$$\text{Chlb (g l}^{-1}) = 0.0029\ A663 - 0.00468\ A645$$

$$\text{Total Chl (g l}^{-1}) = 0.0202\ A663 + 0.00802\ A645$$

2.2.9.2 Determination of total nitrogen content in plants

Total nitrogen content was measured with 30 d old plants to determine the influence of diazotrophic microbial colonization on the nitrogen content of the plants. Above ground parts of the plant was used for analysis. Stem and leaf parts were dried in an oven at 100 °C and crushed to fine powder to avoid error due to uneven distribution of stem and leaf area. The weighed 40 mg of sample was transferred into a digestion flask containing 1 g of catalyst mixture (consisting of 99.0 g of K_2SO_4, 4.1 g of HgO and 0.8 g of $CuSO_4$ ground in a mortar for uniform mixing) and 2 ml H_2SO_4. Digestion of sample was carried out at 370 °C for 40 min, allowed cool at room temperature till it became colorless. Minimum amount of distilled water was added to dissolve solids formed in flask. To this mixture 10 ml of sodium hydroxide sodium thiosulfate solution (50 g NaOH and 5 g of Na_2SO_4 .$5H_2O$ and in a volume 100 ml) was added and transferred to a to distillation unit. Along with this flask containing 10 ml boric acid (4g of boric acid dissolved in 100 ml distilled water) with 1-2 drops indicator solution (one part of 0.2% of methyl red indicator prepared in ethanol mixed with 5 parts 0.2% Bromo cresol green prepared in ethanol) also transferred to a distillation unit. The distilled nitrogen was collected in boric acid containing flask which allows the colour change dark brown to violet colour. After distillation was over, remaining boric acid was titrated with 0.02N H_2SO_4 till the original color of indicator is produced (a colour which produces when boric acid and indicator mix). For dominating the nitrogen content already present in reagents a blank was prepared, which received the entire reagent except for the sample and processed similarly as for sample.

Calculation of nitrogen content was done using the following formula:

$$\%N = \frac{(\text{ml } H_2SO_4 \text{ used for titration-ml of } H_2SO_{4\ used} \text{ for blank}) \text{ X Normality X100X14.007}}{\text{mg sample}}$$

2.2.10 Data analysis

Data are expressed as a mean along with standard deviation and the number of replicates mentioned at each experiment. Statistical analyses for plant inoculation experiments were performed in Microsoft Excel. The variance in data was analyzed using Levene's test which was then followed by the Student's t-test to determine significant differences between the means. Principal component analysis was carried out using PAST software (Hammer et al. 2001).

2.3 Results

2.3.1 Total and diazotrophic endophytic bacterial community profiling of *Poaceae* plants by DGGE

The diversity of diazotrophic community residing in the different plants (analyzed using 16S rRNA DGGE of bacterial population enriched in NFb medium) was compared with total endophytic bacterial community (directly obtained from plant tissues without any enrichment) (Fig. 2.2). 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 (root-1.04 and stem-0.99), maize (root-0.76, stem-0.92 and leaves-0.77) and pearl millet (root-0.82) whereas enriched diazotrophic communities of rice (root-1.13) and maize (stem-0.70 and leaves-0.63) showed higher diversity as compared to the other plants (Table 2.2). 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 (Table 2.3).

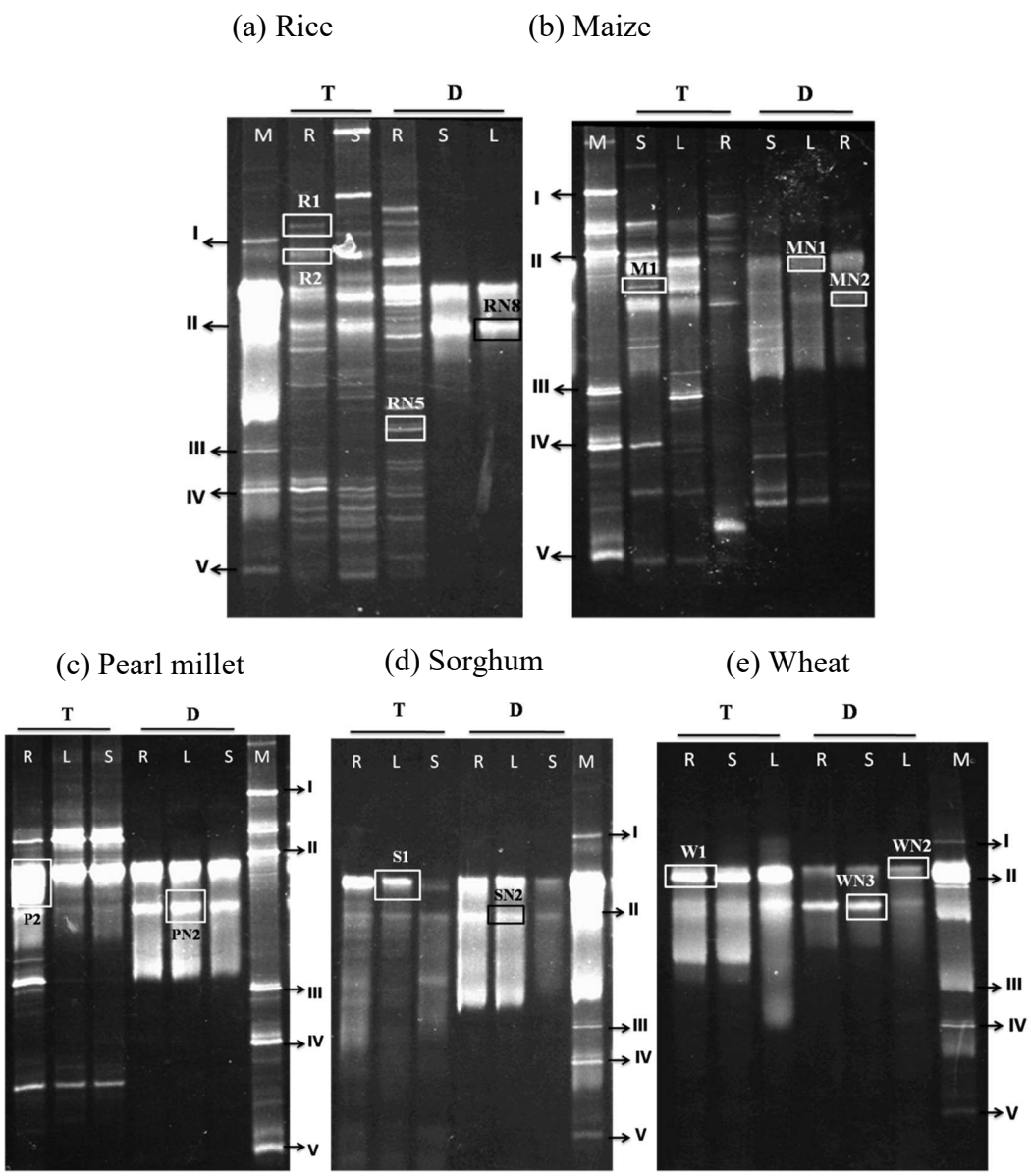


Fig. 2.2 16S rRNA DGGE community fingerprints of endophytic bacteria from *Poaceae* plants detected by SYBR green staining. T represents total eubacterial community and D represents diazotrophic endophytic eubacterial populations. R- root; S- stem; L- leaves. Lane M- Marker consisting of mixture consisted of a 16S rRNA amplicons from *Staphylococcus* sp. (I), *Pseudomonas* sp. (II), *Ralstonia* sp. (III), *Brevundimonas* sp .(IV), *Streptomyces* sp. (V)

Table 2.2 Bacterial diversity indices of total (uncultured) and diazotrophic (enriched in nitrogen free medium) endophytic communities in *Poaceae* plants

Endophyte Community	Plant part	Sample	Diversity index (Shannon-Wiener)	Species Richness (Margalef)	Species Evenness (Pielou)
Total	Root	Rice	1.04	3.19	0.93
		Maize	0.76	1.11	0.97
		Pearl-millet	0.82	1.07	0.93
		Sorghum	0.30	0.29	0.81
		Wheat	0.48	0.76	0.80
	Stem	Rice	0.99	2.94	0.84
		Maize	0.92	1.59	0.94
		Pearl millet	0.38	0.33	0.79
		Sorghum	0.30	1.44	1.00
		Wheat	0.46	0.78	0.77
	Leaves	Rice	ND	ND	ND
		Maize	0.77	1.64	0.81
		Pearl millet	0.38	0.33	0.79
		Sorghum	0.21	0.36	0.69
		Wheat	0.39	0.57	0.81
Diazotrophic	Root	Rice	1.13	3.79	0.90
		Maize	0.42	0.80	0.87
		Pearl millet	0.56	0.55	0.92
		Sorghum	0.35	0.67	0.74
		Wheat	0.41	0.83	0.85
	Stem	Rice	0.37	0.74	0.78
		Maize	0.70	1.49	0.90
		Pearl millet	0.52	0.62	0.87
		Sorghum	0.24	0.72	0.81
		Wheat	0.43	0.76	0.90
	Leaves	Rice	0.43	0.64	0.90
		Maize	0.63	1.36	0.89
		Pearl-millet	0.56	0.55	0.92
		Sorghum	0.21	0.62	0.84
		Wheat	0.16	0.46	0.50

ND-not determined

Table 2.3 Sequence analysis of selected DGGE 16S rRNA bands from total endophytic bacterial community and diazotrophic community enriched in nitrogen free medium from *Poaceae* plants

Endophyte community	Plant part	DGGE band (Accession number)	Closely related sequence in NCBI (Accession number)	% Sequence identity (number of bases)[a]	Isolation source[b]	Taxonomic group
Total endophytes	Rice root	R1 (KT304781)	Various uncultured bacterial clones including uncultured bacterium clone OTU8560 (KT791495)	99% (114)	*Solanum muricatum* rhizosphere soil	α-*Proteobacteria*
	Rice root	R2 (KT304782)	Various uncultured bacterial clones including uncultured *Bacillus* sp. DGGE band (KR089377)	89% (122)	mushroom compost	*Firmicutes*
	Maize stem	M1 (KT304794)	*Serratia marcescens* RS-1 (DQ182326)	96% (166)	Arsenic contaminated mine area	γ-*Proteobacteria*
	Pearl millet root	P2 (KT304785)	*Serratia marcescens* RS-1 (DQ182326)	99% (144)	Arsenic contaminated mine area	γ-*Proteobacteria*
	sorghum stem	S1 (KT304787)	Uncultured bacterium clone Otu01309 (KX991574)	78% (126)	Fruit fly ovary	γ-*Proteobacteria*
	Wheat root	W1 (KT304789)	Uncultured *Bacillus* sp. clone QNSW24 (FJ384500)	80% (118)	Yellow Sea sediment	*Firmicutes*
Diazotrophic endophytes	Rice leaves	RN8 (KT304784)	Uncultured *Brevundimonas* sp. clone MWWTP-g09 (JN625544)	100% (101)	Activated sludge	α-*Proteobacteria*
	Rice root	RN5 (KT304783)	*Sphingobacterium bambusae* strain IBFC2009 (NR_117296)	99% (136)	Bamboo plantation soil	*Bacteroidetes*

	Maize root	MN2 (KT304793)	*Pseudomonas* sp. G24-13 (HQ333028)	89% (160)	Plant root from intertidal zone of the South China Sea	γ-*Proteobacteria*
	Pearl millet leaves	PN2 (KT304786)	*Pseudomonas aeruginosa* strain BRPO3 (KX664101)	100% (141)	Saline soil	γ-*Proteobacteria*
	Sorghum leaves	SN2 (KT304788)	*Pseudomonas putida* strain P4 (KY548817)	100% (140)	Oil refinery	γ-*Proteobacteria*
	Wheat leaves	WN3 (KT304790)	Various *Agrobacterium* and *Rhizobium* clones/strains including *Agrobacterium tumefaciens* strain ISSDS-369 (EF620429)	89% (156)	Agriculture soil	α-*Proteobacteria*
	Wheat leaves	WN2 (KT304791)	Various *Rhizobium* and *Agrobacterium* clones including uncultured *Rhizobium* sp. clone S13 (KF003171)	100% (142)	Grass carp gut mucus	α-*Proteobacteria*

[a]Numbers in parentheses correspond to the number of bases used for sequence identity; [b]The ecological niche from where the clone/organism that best matches was obtained.

2.3.2 Isolation, characterization and identification of diazotrophic endophytic bacteria

Upon plating plant extracts on NFb medium, it was noticed that the cultivable bacteria able to grow on this medium were in a range of 10^3-10^5 CFU g^{-1} of leaves and stem samples of the plants. A total of 31 endophytic diazotrophic bacteria were isolated as pure cultures from five *Poaceae* family cereal plants. Isolates were selected on the basis of the differences in phenotype on NFb medium as well as their ability to be repeatedly cultured on NFb medium indicating their diazotrophic nature. Biochemical characterization of all isolates is given in Table A1 (Appendix I). Analysis of 16S rRNA gene sequence in NCBI and RDP databases showed affiliation of the isolates to phyla *Proteobacteria* (53%), α-*Proteobacteria* (20%), β- *Proteobacteria* (23%), γ-*Proteobacteria* (10%)], *Actinobacteria* (37%) and *Firmicutes* (10%) (Figs. 2.3, 2.4).The 16S rRNA gene sequences obtained from the bacterial isolates are deposited in GenBank (Accession numbers KR921473- KR921502, KT123190).

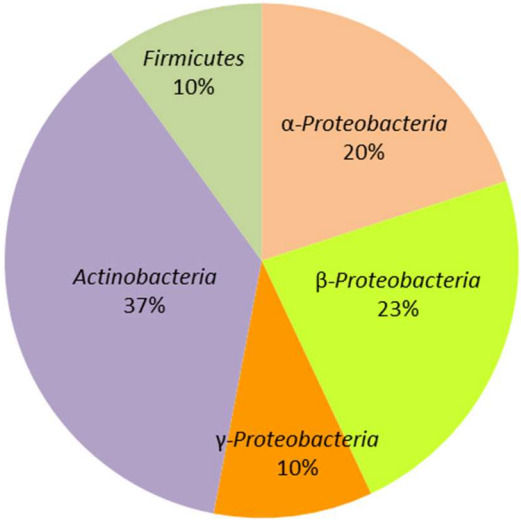


Fig. 2.3 Phylogenetic distribution of endophytic bacterial isolates

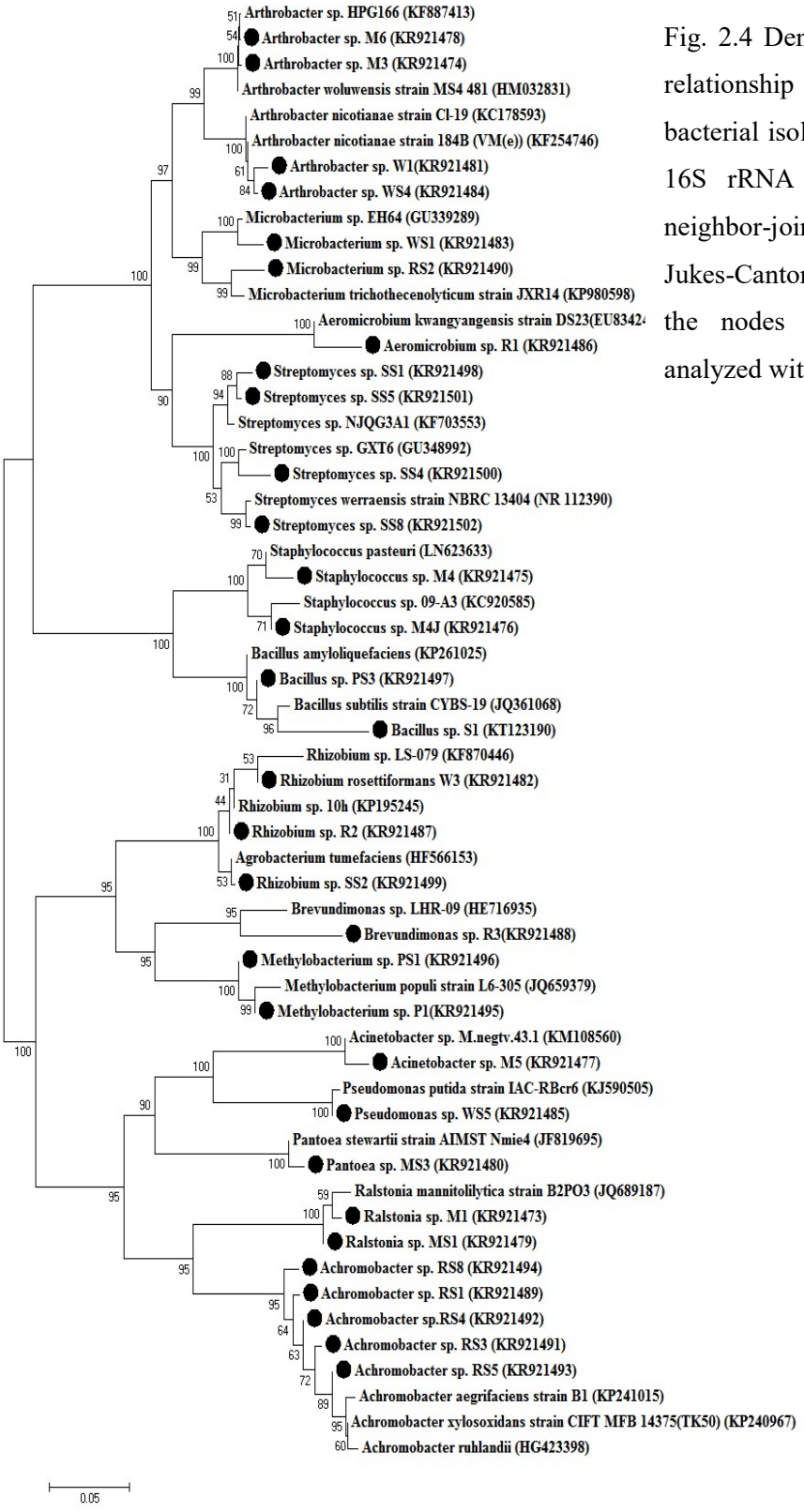


Fig. 2.4 Dendrogram showing phylogenetic relationship of diazotrophic endophytic bacterial isolates from *Poaceae* plants using 16S rRNA gene partial sequences. The neighbor-joining method was used with Jukes-Cantor model algorithm. Numbers at the nodes indicate the bootstrap value analyzed with 1000 replicates

2.3.3 Plant Growth-promoting traits of endophytic diazotrophic bacterial isolates

2.3.3.1 Detection of nif genes in endophytic bacterial isolates

All the endophytic isolates used in this study showed the growth on nitrogen-free medium even after multiple transfers on this medium. Amplification of *nifH* gene was performed with the gene specific primer and all the isolates used in this study shown the amplicon of expected size of~**320 bp** (Fig. 2.5). The *nifH* gene of endophytic strains (*Pantoea* sp. MS3, *Arthrobacter* sp. W1, *Bacillus* sp. S1, and *Rhizobium* sp. R2) was confirmed by sequencing and analyzed using BLASTn which showed it to match with the dinitrogenase reductase gene of *Pseudomonas stutzeri* (CP002622) (Appendix I, Fig. A6).

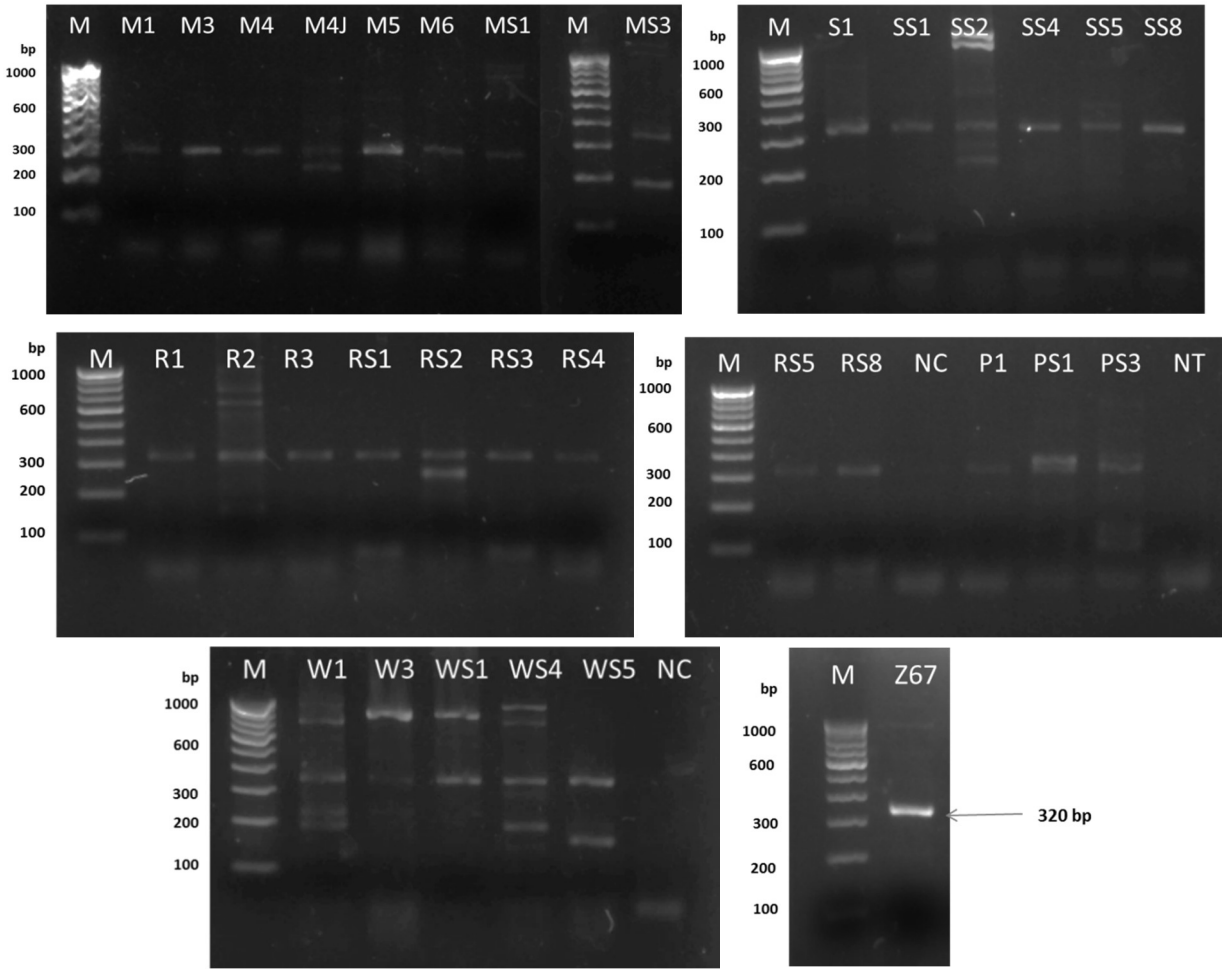


Fig. 2.5 Diazotrophic nature of endophytic bacterial isolates confirmed by *NifH* gene amplification. Z67- *Herbasprillum seropedicae* Z67; NC- negative control of *E. coli* DH5α; NT- No template control; M- Marker 100 bp. Endophytic strains nomenclature as given in (Appendix I, Table A2)

2.3.3.2 Antifungal activity of endophytic bacterial isolates

Among all isolates, only *Streptomyces* spp. (SS1, SS5, and SS8) showed the inhibition of *R. solani*. Antagonistic effect of *Streptomyces* spp. was observed by inhibition zone > 30 mm after 7 d (Fig. 2.6).

Fig. 2.6 Antagonistic effect of *Streptomyces* spp. against *R. solani*

2.2.3.3 HCN production by endophytic bacteria

All the diazotrophic endophytic isolates were negative for HCN production.

2.3.3.4 Phosphate solubilization by endophytic bacterial isolates

Of 31 isolates, fifteen endophytic bacterial strains showed the zone of clearance on Pikovskaya's medium for phosphate solubilization (Appendix I, Table A2). When examined on rock phosphate containing medium, only four isolates showed positive result (*Staphylococcus* sp. M4J, *Staphylococcus* sp. M4, *Acinetobacter* sp. M5, and *Ralstonia* sp. MS1) and *Acinetobacter* sp. M5 alone showed the detectable level of Pi to be released in buffered minimal medium with 0.294 µmole (pH 4.3) of P after 96 h and also showed the pink color formation on rock phosphate medium.

2.3.3.4 IAA production by endophytic bacterial isolates

A large number of (81%) of endophytic bacterial isolates produced IAA. High IAA production was seen in many strains showing above 100 µg ml^{-1} IAA. High IAA producing strains included *Ralstonia* sp. M1, *Staphylococcus* sp. M4, *Staphylococcus* sp. M4J, *Arthrobacter* sp. M6, *Pantoae* sp. MS3, *Arthrobacter* sp. W1, *Rhizobium* sp. W3, *Curtobacterium* sp. WS1, *Pseudomonas* sp. WS5 and *Aeromicrobium* sp. R1 (Appendix I, Table A2).

2.3.3.5 Siderophore production by endophytic bacterial isolates

All endophytic isolates showed siderophore production (Appendix I, Fig. A1). Majority of the endophytes showed mixed type of siderophore production except *Ralstonia* sp. M1, *Staphylococcus* sp. M4J, *Microbacterium* sp. WS1, *Methylobacterium* sp. P1, *Aeromicrobium* sp. R1 which showed the presence of only catecholate type of siderophore while isolate *Achromobacter* sp. RS3 displayed only hydroxamate type of siderophore. Quantitative analysis of siderophore showed the highest production in *Rhizobium* sp. R2 isolates from rice and *Methylobacterium* sp. PS1 from pearl millet (Appendix I, Table A2)

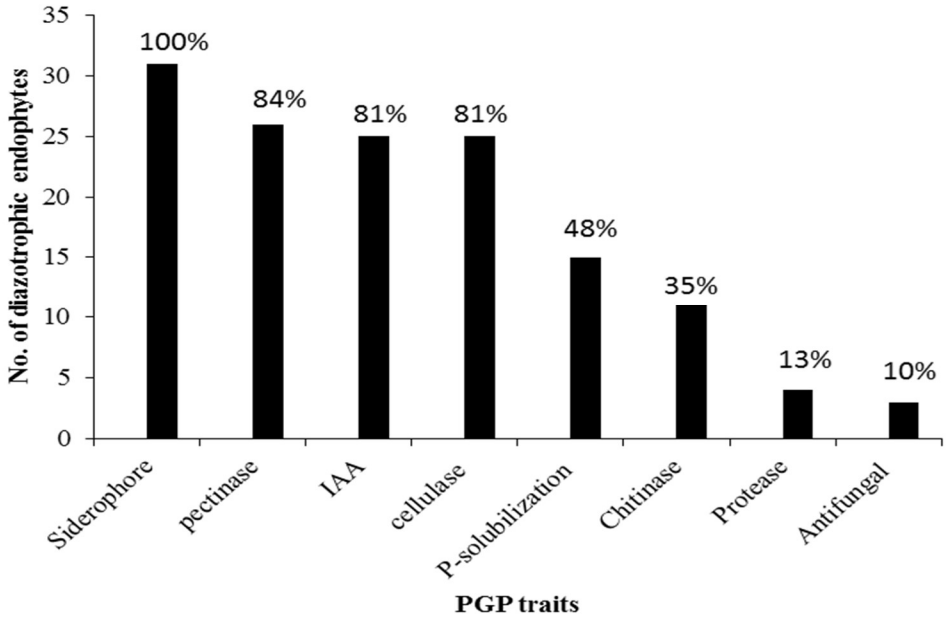
2.3.3.6 Hydrolytic enzyme activities of endophytic bacterial isolates

It was observed that 81%, 84%, 35%, and 13% isolates were positive for cellulase, pectinase, chitinase, and protease respectively (Appendix I, Figs. A2, A3, A5, A6 and Fig 2.6a). Highest cellulase activity was detected in case of *Aeromicrobium* sp. R1 (0.05 Unit ml^{-1} min^{-1}). Protease activity was high in *Arthrobacter* sp. M6 (0.08 Unit ml^{-1} min-1) and chitinase activity was found high in *Staphylococcus* sp. M4, *Acinetobacter* sp. M5 and *Pantoea* sp. MS3 (0.01 Unit ml^{-1} min^{-1}) (Appendix I, Table A2).

2.3.4 Different combination of PGP traits present in endophytic bacteria

Diazotrophic endophytic bacterial isolates were analyzed for total 8 PGP traits but none of the isolates was found positive for all the PGP traits. There were total 8 isolates 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 (Fig. 2.7).

(a)



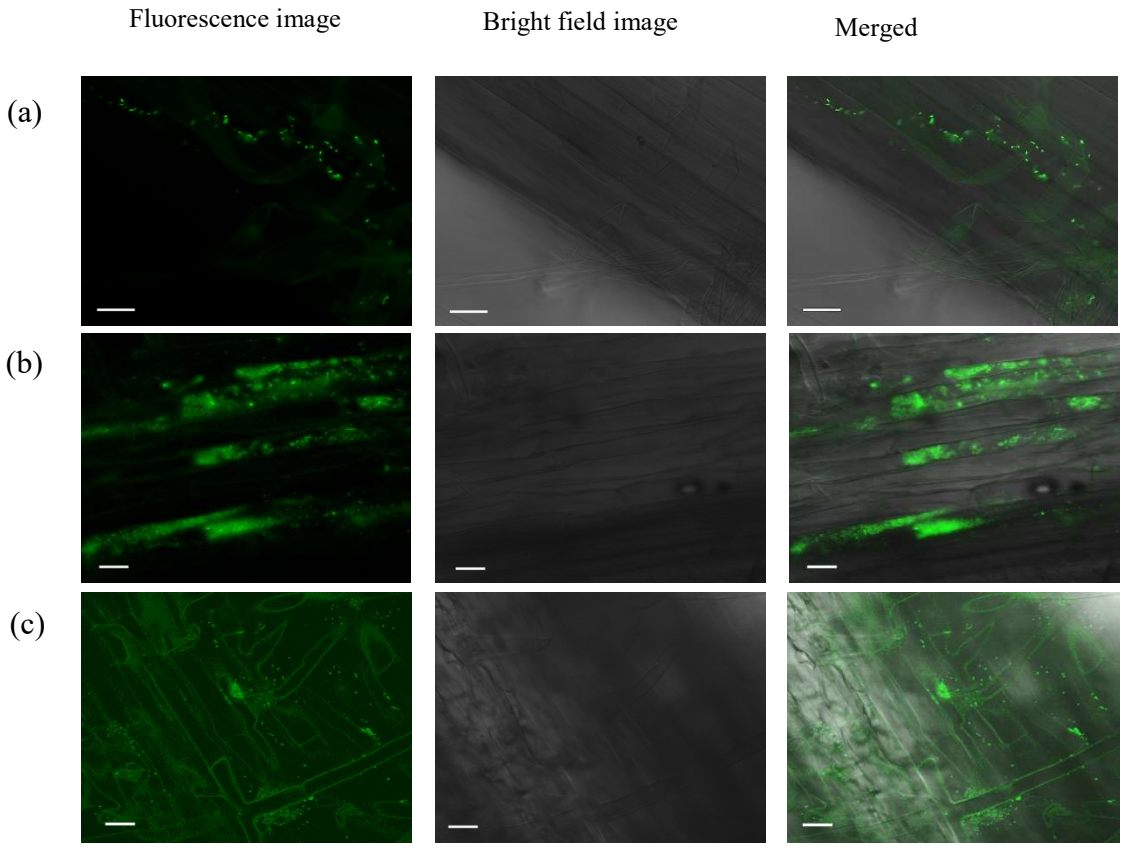
(b)

Diazotrophic endophytic bacterial isolates	Plant Growth Promoting traits
M3, M6, WS1, WS4	Siderophore, IAA, Cellulase, Pectinase, Chitinase, Protease
M4, M5, MS1, MS3	Siderophore, IAA, Cellulase, Pectinase, Chitinase, P-solubilization
W1, WS5	Siderophore, IAA, Cellulase, Pectinase, Chitinase
R1, RS2, RS4	Siderophore, IAA, Cellulase, Pectinase, P- solubilization
SS1, SS5	Siderophore, IAA, Cellulase, P- solubilization, Antifungal
M4J	Siderophore, IAA, Pectinase, Chitinase, P- solubilization
W3, R2, R3, S1, SS2	Siderophore, IAA, Cellulase, Pectinase
RS3, RS5, RS8	Siderophore, Cellulase, Pectinase, P- solubilization
SS4	Siderophore, IAA, Cellulase, P- solubilization
SS8	Siderophore, Cellulase, Pectinase, Antifungal
M1	Siderophore, IAA, Pectinase
RS1	Siderophore, Pectinase, P-Solubilization
P1, PS1	Siderophore, IAA
PS3	Siderophore, Pectinase

Fig. 2.7 Distribution of plant growth promoting (PGP) traits in diazotrophic endophytes isolated from *Poaceae* family plants. (a) Number of diazotrophic isolates positive for a PGP trait and (b) Distribution of PGP traits in individual isolates

2.3.4 Endophytic colonization by *gfp* tagged bacterial isolates in wheat

For the confirmation of the endophytic presence of bacteria, thirteen gram negative isolates (*Ralstonia* sp. M1, *Acinetobacter* sp. M5, *Ralstonia* sp. MS1, *Pantoea* sp. MS3, *Rhizobium* sp. W3, *Rhizobium* sp. SS2, *Rhizobium* sp. R2, *Brevudimonas* sp. R3, *Achromobacter* sp. RS1, RS3, RS4, RS5, RS8) along with the positive control *H. seropedicae* Z67 were tagged with *gfp* containing plasmid pHC60 and inoculated individually on wheat plants. On the 7th d after inoculation, different parts of the plants were found to show the endophytic presence of bacteria by confocal microscopy. Fig. 2.8 shows representative images of root endophytic presence of few isolates (refer Appendix I, Fig. A7 for more confocal images of isolates). Depending on the position of the root observed, its thickness varied from 50 to 130 µm and bacteria were generally detected at 10 to 30 µm depths from root surface indicating their endophytic colonization. This was further confirmed by CFU count (on tetracycline containing NFb medium) which ranged from 10^3 to 10^5 CFU g^{-1} of fresh weight of tissue for all the isolates studied. In control uninoculated plants, no cells were detected by microscopy as well as plating.



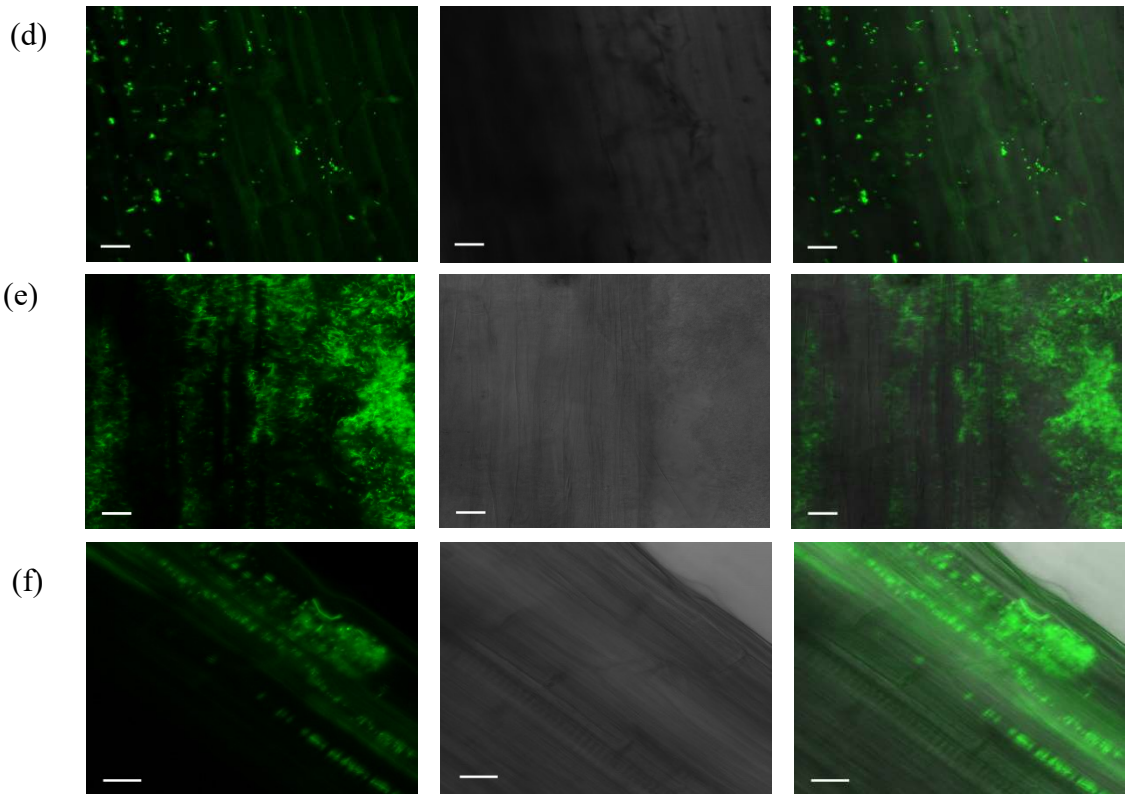


Fig. 2.8 Endophytic colonization of *gfp* tagged bacteria detected by confocal microscopy in roots of 7 d old wheat plants. (a) *Ralstonia* sp. M1, (b) *Acinetobacter* sp. M5, (c) *Rhizobium* sp. W3 (d) *Rhizobium* sp. R2, (e) *Achromobacter* sp. RS4, (f) *Achromobacter* sp. RS5. Scale Bar represents 20 µm

2.3.5 Effect of endophytic bacterial colonization on wheat plants

Diazotrophic endophytic bacteria showed a range of effects on wheat plant growth in 30 d pot inoculation experiments. Majority of endophytes showed the improvement in dry mass of stem, root and leaves and an increase in the chlorophyll content (Table 2.4). The effectiveness of the isolates on the plant growth was compared with positive control *H. seropedicae* Z67 and with uninoculated controls. Total nitrogen content of aerial part of plants was also increased significantly over uninoculated controls. Significant plant growth promotion in above and below ground parts of the wheat was observed with the *Streptomyces* spp. compare to the other isolates (Table 2.4).

Table 2.4 Effect of diazotrophic endophytic bacterial isolates on the growth of the wheat plants in sterile soil under greenhouse condition

Treatment	Root Parameters			Shoot Parameters			Total chlorophyll (mg/g of leaves)	N-content (%)
	Wet weight (g)	Dry weight (mg)	Length (cm)	Wet weight (g)	Dry weight (mg)	Length (cm)		
Control[a]	0.04±0.01	5.50±1.97	6.60±1.08	0.26±0.05	29.17±4.67	32.67±2.16	0.866±0.02	3.68
M1	0.05±0.01	11.33±4.23*	6.63±0.71	0.40±0.09**	40.50±8.53**	35.67±3.83	1.00±0.08**	3.85
M3	0.05±0.01	8.17±0.75*	5.67±0.18	0.31±0.04	32.83±4.75	35.67±2.58	1.06±0.01***	5.15
M4	0.06±0.01*	7.67±1.03*	5.78±0.13	0.45±0.06**	38.50±9.22	42.67±2.80***	0.99±0.03***	5.08
M4J	0.05±0.01	8.17±1.94*	6.30±0.43	0.55±0.06***	44.17±2.71***	39.38±1.02***	1.14±0.17*	5.60
M5	0.05±0.01	11.67±3.67**	6.07±1.11	0.342±0.08	33.83±5.95	35.67±3.83	1.05±0.04***	5.92
M6	0.03±0.00	5.00±1.41	3.92±0.22	0.46±0.03***	35.83±3.82*	38.17±0.68**	1.14±0.18*	5.50
MS1	0.05±0.01	12±2.10***	7.78±0.97	0.51±0.07***	44.83±6.37***	36.52±2.32*	1.01±0.19	6.96
MS3	0.06±0.01	15.33±0.82***	10.33±0.94***	0.29±0.01	28.67±2.25	32.45±1.93	0.97±0.07*	3.50
W1	0.03±0.01	3.17±0.41	5.75±0.99	0.52±0.06***	42.67±5.35***	43.92±2.97***	1.23±0.13**	3.84
W3	0.04±0.01	5.33±1.75	6.77±1.40	0.44±0.07***	47.50±6.41***	43.83±3.71***	1.36±0.02***	3.84
WS1	0.03±0.01	4±0.63	7.75±0.88	0.66±0.04***	55.17±6.68***	45.20±5.05***	1.43±0.16***	3.84
WS4	0.03±0.01	4.33±1.03	7.47±0.52	0.41±0.03***	37.33±2.88***	42.50±1.64***	1.08±0.06***	3.84
WS5	0.03±0.01	8.67±2.50*	9.68±1.37**	0.29±0.05	30.50±8.34	30.68±0.83	1.28±0.13***	4.12
R1	0.15±0.02***	18.83±0.75***	12.23±1.42***	0.36±0.07*	39.67±5.32**	42.35±1.94***	1.30±0.16**	3.84
R2	0.12±0.01***	16±3.58***	13.43±1.05***	0.33±0.12	29.33±7.94	38.25±1.72***	1.50±0.10***	4.82
R3	0.03±0.01	5.67±0.82	5.93±0.16	0.50±0.01***	44.50±4.46***	41.42±0.38***	1.43±0.20**	4.19
RS1	0.11±0.01***	12.17±3.19**	22.42±4.22***	0.28±0.07	33.33±7.42	35.17±1.47*	1.50±0.10***	3.77
RS2	0.04±0.01	6.17±0.75	5.28±0.25	0.43±0.03***	39.50±3.27***	38.62±2.98**	1.37±0.07***	5.31
RS3	0.03±0.01	6.83±0.41	5.13±0.77	0.49±0.08***	45.17±8.11**	40.50±3.08***	1.58±0.07***	4.96
RS4	0.04±0.01	9.83±1.83**	15.80±1.40***	0.29±0.05	38.33±3.01**	34.98±0.85*	1.26±0.09***	3.70
RS5	0.07±0.01**	9.17±0.75**	11.53±2.07***	0.39±0.05**	37.83±4.62**	40.83±1.17***	1.30±0.07***	3.98
RS8	0.06±0.02	9.67±2.07**	12.25±1.00***	0.24±0.03	28.83±3.13	34.67±1.03	1.33±0.08***	3.84
P1	0.05±0.01	6±1.26	5.93±0.18	0.56±0.08***	58.17±3.54***	35.93±2.05*	1.80±0.07***	4.26
PS1	0.05±0.01	5±0.63	5.92±0.60	0.50±0.1***	41±7.29***	37±2.10**	1.55±0.10***	4.68
PS3	0.03±0.03	5.83±1.17	4.65±0.56	0.58±0.11***	40.83±12.12	43.92±2.97**	1.40±0.05***	4.19
S1	0.08±0.03*	11±4.38*	13.03±2.51***	0.29±0.16	23±3.90	33.67±2.88	1.36±0.04***	4.96
SS1	0.04±0.01	4.83±0.41	7.02±0.41	0.58±0.12***	55±7.97***	40.43±4.56**	1.55±0.09***	4.40
SS2	0.10±0.02***	15.50±2.59***	18.50±0.84***	0.37±0.14	36.17±2.79**	36.37±2.38*	1.95±0.12***	4.96
SS4	0.09±0.01***	10.50±0.84***	8.60±1.03**	0.39±0.1***	41.50±5.54***	39.38±1.20***	1.61±0.02***	4.19
SS5	0.10±0.00***	14.50±0.84***	7.92±0.22*	0.58±0.04***	51.33±2.25***	49.33±1.51***	1.57±0.16***	4.99
SS8	0.05±0.01	8.67±0.82**	6.38±1.03	0.52±0.13***	55.17±2.64***	43.33±3.01***	1.72±0.15***	4.96
Z67[b]	0.10±0.01***	8.83±1.94*	10.03±0.59***	0.47±0.04***	42.83±3.82***	41.83±1.82***	1.61±0.21***	4.26

[a] Control plants were not inoculated with any bacteria, [b]*Herbaspirillum seropedicae* Z67 standard strain, * p< 0.05, ** p< 0.01, *** p< 0.001

Endophytic colonization was confirmed in 30 d old plants by counting the CFU from surface sterilized plant parts. Colonization capacity varied in the different parts of the plants. Higher colonization was observed in roots and stems (10^2-10^7 CFU g^{-1} of fresh tissue) as compared to leaves (10^2-10^4 CFU g^{-1} of fresh tissue) (Table 2.5). The uninoculated control plants when plated similarly on NFb medium did not show presence of any diazotrophic endophytic bacteria.

Table 2.5 Endophytic presence of isolates detected after 30 d after planting

Endophyte isolate	Bacterial counts (CFU g^{-1} of fresh tissue)		
	Root	Stem	Leaves
Control[a]	-	-	-
M1	9.2×10^5	1.1×10^5	-
M3	2.2×10^6	2.34×10^5	-
M4	1.56×10^6	3.5×10^7	5.25×10^4
M4J	1.72×10^5	2.1×10^5	4.8×10^2
M5	5.9×10^5	6.6×10^6	-
M6	2.57×10^7	3.3×10^4	-
MS1	1.9×10^6	1.5×10^5	1.95×10^3
MS3	1.98×10^5	5.55×10^4	-
W1	6.7×10^6	5.55×10^4	-
W3	1.47×10^7	7.8×10^5	2.88×10^3
WS1	1.87×10^5	1.4×10^5	-
WS4	5.9×10^5	7×10^4	-
WS5	-	2.3×10^5	1.1×10^3
R1	1.97×10^5	1.8×10^3	-
R2	1.72×10^5	2.9×10^3	7×10^2
R3	-	2.9×10^3	-
RS1	2.37×10^5	1.95×10^2	-
RS2	-	2.85×10^4	7×10^2
RS3	-	2.25×10^3	-
RS4	-	3.45×10^3	-
RS5	1.2×10^5	2.85×10^3	5.8×10^2
RS8	2.3×10^5	6.45×10^4	1.5×10^2
P1	1.98×10^5	3.1×10^5	2.9×10^3
PS1	1.23×10^4	4.9×10^5	-
PS3	2.57×10^5	7×10^3	2.97×10^4
S1	1.97×10^4	5.55×10^2	-

SS1	4.4×10^{2}	7×10^{2}	-
SS2	3.2×10^{2}	-	-
SS4	1.5×10^{3}	1.5×10^{5}	-
SS5	4×10^{4}	2.99×10^{3}	4.8×10^{2}
SS8	4.9×10^{2}	5.7×10^{2}	-
Z67[b]	2×10^{5}	1.8×10^{2}	-

[a]Control- Unbacterized; [b]Z67- *Herbaspirillum seropedicae* Z67; - indicate the no bacteria recovered after plating

2.3.6 PCA analysis of microbial traits contributing to plant growth promotion

The plant growth promotion was analyzed by principal component analysis (PCA) to determine the critical component of the study which influences the overall growth of the wheat plants. PCA was performed using nine variables (mentioned in Table 2.6) 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. The loading score showed the PC1 correlation with the siderophore production, root length, wet and dry weight of root whereas PC2 showed the positive correlation with the shoot length, root length, the wet and dry weight of shoot, the wet and dry weight of root, and total chlorophyll content (Table 2.6). The scatter graph plot is divided into the four groups with correlation with PC1, PC2, both PC1 and PC2 and does not show any correlation with the principal component (Fig. 2.9).

Table 2.6 Principal component analysis of wheat plant growth promotion activity based on PC1 and PC2

	PC 1	PC 2
IAA	0.060205	-0.14653
Siderophore	0.17049	0.29333
Shoot length	-0.29446	0.39489
Root length	0.41186	0.1979
Wet weight of shoot	-0.45882	0.23541
Wet weight of root	0.36755	0.45304
Dry weight of shoot	-0.42493	0.33433
Dry weight of root	0.42618	0.24881
Total Cholorophyll	-0.056333	0.51288

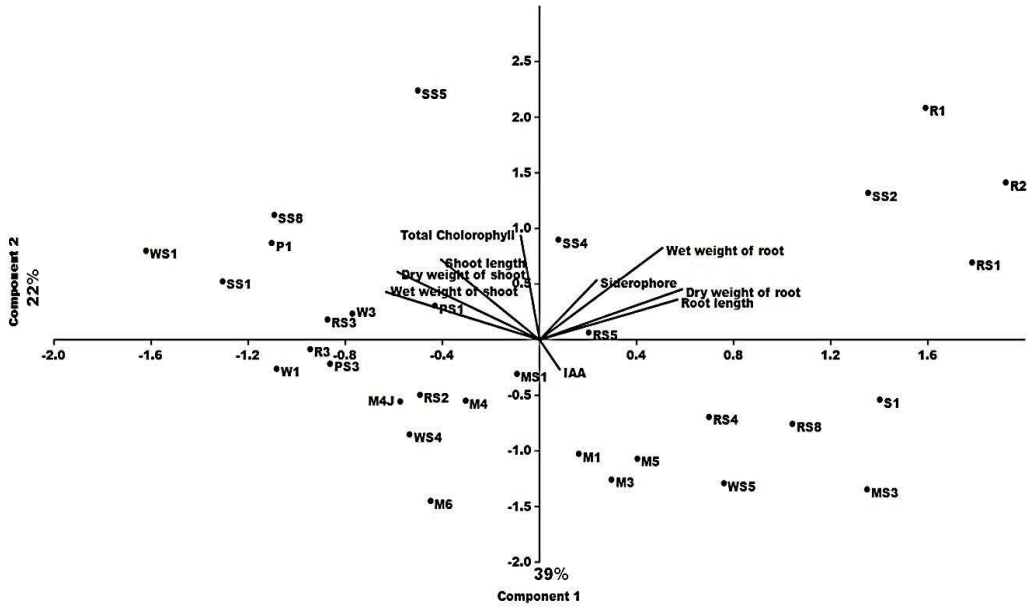


Fig. 2. 9 Principle component analysis of diazotrophic endophytic bacterial isolates on wheat plant growth promotion. Component 1- IAA, Component 2- Siderophore

2.4 Discussion

It is believed that the diversity of endophytic bacterial community varies according to plants species, host environmental condition and geographical location (Zinniel et al. 2002) and successful colonization into the host plant depends on plant genotype and development stage (Andreote et al. 2010). Total endophytic communities have been studied from various plants such as rice (Rangjaroen et al. 2014), maize (Seghers et al. 2004), potato (Garbeva et al. 2000), citrus plants (Araujo et al. 2002) and grasses (Wemheuer et al. 2016). However, endophytic diazotrophic bacterial diversity has not been widely explored. Most reports on endophytic nitrogen fixing populations are available for rice plants (Prakamhang et al. 2009; Sessitsch et al. 2012; Rangjaroen et al. 2014; Ferrando and Scavino 2015). The present work is a systematic study of diazotrophic community residing within above-ground plant parts of five important cereal crops. The study analyzed diazotrophic endophyte diversity by DGGE as well as characterized several diazotrophic endophytic bacteria from the five plant species. In general, the DGGE analysis showed that diazotrophic communities were different as compared to the total

communities of the same plant and also varied with the plant. However, in most cases the diazotrophic community appeared similar in different plant parts. DGGE band sequencing showed majority of endophytes affiliated to *Proteobacteria.* Bands matching with *Pseudomonas* were detected as part of the diazotrophic community in three plant species. It should however be pointed out that the identification based on short reads of the DGGE sequences as well as lower sequence identities might be of relevance only up to the phylum level. The sequences from the data base which best match with the sequences reported here have diverse isolation sources and only *Agrobacterium tumefaciens* strain ISSDS-369 is reported as diazotrophic. Earlier *Bacillus* sp. and *Serratia* sp. have been reported from rice and wheat plants (Gyaneshwar et al. 2001; Larran et al. 2002; Mano et al. 2007; Liu et al. 2010), *Pseudomonas aeruginosa* from pearl millet (Gupta et al. 2013), *Brevundimonas* sp. from rice (Sun et al. 2008), *Sphingomonas* sp. from tomato and rice (Mano et al. 2007; Khan et al. 2014). Interestingly, our study found sequences affiliated to *Rhizobium*/*Agrobacterium* among the bands sequenced from the diazotrophic endophytic population.

The present work was also aimed at isolation and characterization of diazotrophic endophytic bacteria from *Poaceae* family plants. The strategy used here for the isolation of diazotrophic endophytic bacteria was based on repeated subculturing on medium devoid of a combined nitrogen source. The success of this strategy is reflected by the detection of the *nifH* gene in each of the isolate. Pure cultures of 31 diazotrophic endophyte bacteria found to be affiliated to α, β, γ- *Proteobacteria*, *Actinobacteria*, and *Firmicutes*. They belonged to 14 genera including *Achromobacter*, *Arthrobacter*, *Streptomyces* and *Rhizobium* in decreasing order of abundance. Only a few bacteria were found in common between plants, such as *Rhizobium* spp. found in wheat, rice, and sorghum, *Arthrobacter* spp. found in maize and wheat, *Bacillus* spp. found in sorghum and pearl millet. Among the endophytes obtained, only *Ralstonia* spp. are known to cause disease in certain plants. However, there are reports of plant growth promoting *Ralstonia* spp. associated with banana and soybean (Kuklinsky-Sobral et al. 2004; Jimtha et al. 2014). In accordance with the DGGE results, several *Rhizobium/Agrobacterium* spp. were also isolated in as diazotrophs from different *Poaceae* plants. We have not come across any earlier reports showing the direct isolation of free living nitrogen fixing *Rhizobium* spp. growing on nitrogen-free medium. However, Yanni et al. (1997) showed that during crop rotation, the benefit to the cereal crop occurs because of the endophytic colonization of *Rhizobium* spp. on roots of

rice plants. Interestingly, although the standard *H. seropedicae* Z67 showed efficient colonization and plant growth promotion of wheat plants, we did not obtain *Herbaspirillum* spp. in the sequences nor among the isolates.

Our results thus suggest that nitrogen fixation in tissues of cereal plants is dominated by phylum *Proteobacteria*. Many of the bacterial genera reported here such as *Pantoea*, *Staphylococcus, Brevundimonas, Methylobacterium, Pseudomonas, Ralstonia* and *Bacillus* have been previously found as endophytes from banana plants (Thomas et al. 2008) indicating similarity in distribution in cereal and non-cereal plants. Results reported here suggest that they are diazotrophic and have potential to increase N availability to plants. In contrast to endophytes recovered from cereal plants grown in normal agriculture fields, endophytes as well as rhizospheric populations recovered from plants grown in heavy metal contaminated soils showed the predominance of phyla *Firmicutes* and *Actinobacteria* with very few members of *Proteobacteria* (Sharaff and Archana 2015; Román-Ponce et al. 2016). These findings, along with the results presented here, indicate that metal stress might lead to loss of dominant nitrogen fixing endophytic population.

In this work endophytic bacterial entry was confirmed in the wheat plants via *gfp* tagging. This study was restricted to Gram negative isolates. Endophytic bacterial isolates were able to colonize intracellular spaces passing through the epidermis, endodermis and then into the central cylinder of the plant. Many bacterial isolates showed the production of hydrolytic enzymes such as cellulase and pectinase which may assist their entry into the host plant. Due to the problem of high auto-fluorescence in the stem and leaves of the plant it was not possible to detect all *gfp* tagged endophytic bacterial occurrence in these part of the plant. For addressing this problem plate counts were performed which confirmed the presence of endophytes in these plant parts and found them to be present in significant numbers. Interestingly, no matter from which plant the isolate was obtained, all were able to colonize wheat plants indicating low plant specificity.

Phytohormones play an important role in plant growth promotion (Glick 2014). Results show high IAA production by the endophytic isolates ranging from 33–890 μg ml^{-1} with the highest IAA production by *Arthrobacter* sp. M6. Among all the isolates, phosphate solubilization was highest in case of *Acinetobacter* sp. M5 which solubilizes mineral phosphates even in buffered condition. *Acinetobacter* spp. are considered to be effective producers of

pyrroloquinoline quinone (PQQ) which is important for gluconic acid production and P-solubilization (Ogut et al. 2010). All isolates obtained in this study showed siderophore production which is not surprising since many plant associated α, β, γ-*Proteobacteria*, bacilli, actinobacteria demonstrate the ability of siderophore production under low iron availability (Tian et al. 2009). *Rhizobium* spp. isolated in this study showed hydroxamate, catecholate and mixed type of siderophore production. This is interesting since earlier a similar observation has been made with legume nodulating rhizobia (Khan et al. 2006). Among all endophytic bacterial isolates only *Streptomyces* spp. showed the antagonistic activity against the fungal pathogens. These *Streptomyces* spp. do not produce chitinase and protease enzymes, suggesting synthesis of antifungal metabolites production. Different combinations of PGP traits in these diazotrophic endophytic bacterial isolates were observed.

All the isolates obtained in this study promoted the growth of wheat plants. Higher nitrogen content was found in aerial part of the wheat plant as compared to the uninoculated control plants indirectly supporting the *in planta* nitrogen fixation ability of the isolates. The importance of nitrogen fixation ability of bacteria associated with cereal plants is sometimes the main factor promoting the plant growth, as shown for a *nifH* defective mutant of *Klebsiella pneumoniae* which when inoculated on the wheat plant showed stunted growth and yellowing of plant (Iniguez et al. 2004). This may explain why isolates showing lower amount of IAA production were also effective in enhancing plant growth. Interestingly, different endophytic bacterial isolates in this study supported the plant growth by different ways. Some endophytic bacteria showed an increase in the wet and dry weight of shoot and root, while others improved shoot and root length. All the diazotrophs showed an increase in chlorophyll content compared to control. All the isolates showed cross-colonization of wheat plants regardless of their source of isolation. This capacity of endophytes makes them a potential candidate for the broad host range biofertilizer development.

To summarize, the diversity analysis of total bacterial community and nitrogen fixer community residing in plant parts showed the presence of only few nitrogen fixing bacteria inside the plants of Gujarat region, India. This study also indicates the culture dependent and independent methods both very useful for exploring the endophytic bacterial diversity. Several diazotrophic endophytic bacterial isolates have been obtained which show diverse combination of

plant growth promoting traits and have positive effect on plant growth under gnotobiotic conditions. Future studies will be focused on detailed characterization of some of these isolates for their plant protection and growth promotion ability in various plants pathogen systems.