Molecular Diversity, Temporal Dynamics and Colonization Behavior of Cultivable Diazotrophic Endophytic Bacteria Recovered from Pearl Millet (*Pennisetum glaucum*)

THESIS

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By

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Under the supervision of

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Twameva Mata Cha Pita Twameva

Twameva Bandhuscha Sakha Twameva

Twameva Vidya Dravinam Twameva

Twameva Sarvam Mama Deva Deva

I dedicate

this piece of

work to

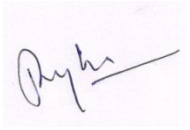
my lovely parents.

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI, RAJASTHAN, INDIA

CERTIFICATE

This is to certify that the thesis entitled "**Molecular Diversity, Temporal Dynamics and Colonization Behavior of Cultivable Diazotrophic Endophytic Bacteria Recovered from Pearl Millet (*Pennisetum glaucum*)**" submitted by **Garima Gupta, I.D. No. 2008PHXF411P** for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.



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Abstract

***Introduction*:** Endophytic bacteria reside in the interior of plants and may benefit host plant through one or more plant growth promoting properties including nitrogen-fixation, production of phytohormones, induced systemic tolerance, induced systemic resistance and antagonistic activities. Nitrogen fixing (diazotrophic) endophytic bacterial community of crop plants can be used for efficient land-use management to attain enhanced crop yield. Thus, understanding of biology of suitable endophytic biofertilizer agent and host plant relationship will have immense importance in sustainable agriculture. Therefore, the aims of present study were: to study temporal dynamics of endophytic diazotrophic bacteria during various growth stages of pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants; exploration of the potential PGPR isolates for the development of efficient bio-fertilizer/biocontrol agents; evaluation of effects of selected biofertilizer isolates on plant growth in laboratory, green-house and field conditions; understanding possible mechanism of endophytic colonization of selected bacteria and; comparative study of immune response of the host plant towards endophytic bacteria and pathogenic bacteria.

***Methodology*:** Diazotrophic endophytic bacteria were isolated from pearl millet at regular time intervals during the entire cultivation period. ERIC-PCR was used to type bacteria as well as a biomarker to track the identity of various isolates during different time intervals. Representative isolates of each ERIC types were identified by 16S rRNA gene sequence analysis. Data of each ERIC types corresponding to various isolates obtained at different growth stages were used to estimate diversity of diazotrophic bacteria. All isolates were screened for certain plant growth promoting (PGP) activities such as nitrogen fixation, mineral phosphate solubilization and production of IAA and siderophore. Based on PGP traits, fourteen best isolates were screened for cross-infection studies and, for their effect on plant growth in laboratory, green house and field conditions. The most dominant isolates obtained in this study (*Pseudomonas aeruginosa* PM389) was further characterized in detail for the various characteristics of endophytic colonization (e.g., motility, biofilm formation, exopolysaccharide production, enzymatic activity) and antagonistic activities (HCN, degradative enzymes, siderophore, antifungal protein production and

development of induced systemic resistance). To use it as a biocontrol agent, *P. aeruginosa* PM389 was further assessed for its effect on plant growth in presence of pathogenic bacteria and fungi, under *in vitro* and pot conditions. In addition, qualitative and quantitative changes in the host plant immune responses to endophytic (*P. aeruginosa* PM389) and pathogenic (*Erwinia carotovora*) bacteria were compared by measuring level of certain defence compounds produced in plants. Further, changes in PR gene expression on endophytic bacterial inoculation were quantified using Real-time qPCR approach.

Results and Discussion: Overall, 210 diazotrophic endophytic bacteria were isolated from pearl millet plants during the entire cultivation period. Diversity of diazotrophic endophytic bacteria was higher during the early and late stages than the middle stage of pearl millet plant growth. Out of 210 morphotypes obtained in this study, 76 different ERIC types were identified. Based on 16S rDNA sequence, these bacteria were identified as a member of different genera including *Acinetobacter, Agrobacterium, Arthrobacter, Bacillus, Curtobacterium, Enterobacter, Microbacterium, Mycobacterium, Nocardioides, Ochrobactrum, Pantoea, Pseudomonas, Rhizobium, Rhodococcus,* and *Stenotrophomonas*. *Pseudomonas aeruginosa* was noted as the most dominant species during entire growth period of plant. *P. aeruginosa* PM389 emerged as the most dominant isolate during the vegetative growth period of plant. Out of 14 selected diazotrophic bacterial isolates, inoculation of most of the isolates stimulated plant growth. Isolate *Rhizobium* sp. PM10461 was found to be the best followed by *Nocardiodes* sp. PM9404 and *Arthrobacter woluvensis* PM9426 in terms of promoting plant growth under field conditions. Based on the results of lab, green-house and field experiment, *Rhizobium* sp. PM10461 was observed as the most consistent PGPR agent in different conditions. Thus, it can serve as a potential biofertilizer agent in the future. Since, plant growth promoting *P. aeruginosa* PM389 was obtained as the most dominant/efficient endophytic colonizer in pearl millet plants and showed efficient antagonistic activity against bacteria and pathogenic fungi, it was further characterized for mechanistic details of its successful colonization ability and biocontrol activities. It showed various properties of endophytic colonization such as different types of motility, biofilm formation and exopolysaccharide production. In addition to PGP properties, it also showed several mechanisms of antagonistic activities including HCN, lipase, siderophore,

antifungal protein and ISR for inhibition of pathogenic organisms. Significant antagonistic activity of *P. aeruginosa* PM389 against fungal and bacterial pathogens under pot conditions indicated that *P. aeruginosa* PM389 can be used as an efficient biocontrol agent. In order to understand the mechanism of successful colonization, immune response of host plant to endophytic and pathogenic bacteria was compared. Results of biochemical tests for defense enzymes produced in plants following bacterial inoculation suggested attenuated defense response in host plants towards endophytic bacteria. It is inferred that endophytic bacteria enter inside the plant by suppressing plant immune responses. To correlate plant immune response with bacterial colonization, bacterial population was counted at the regular interval. *P. aeruginosa* PM389 showed colonization trend in which level of bacterial colonization was initially higher and then reduced to attain a stabilized population. However, population of bacterial pathogen kept fluctuating during sampling period, which indicated a constant fight between pathogen and plants. There was inverse relationship between population count of bacteria and production of defense enzymes in plants. The qPCR results of different PR-genes (pathogen-related) further supported the hypothesis of immune suppression for establishment of endophytic bacteria inside the plant.

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List of Abbreviation

A_{260}	Absorbance at 260 nm
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ANOVA	Analysis of variance
AprA	Alakaline protease
ARA	Acetylene reduction assay
ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
B_{sup}	Broth supernatant
bZIP	Basic leucine zipper
CARD	Catalyzed reporter deposition
CAS	Chrome-azurol-S
CC-NB-LRR	Coiled coil- NB-LRR
cDNA	Complementary Deoxyribonucleic acid
CFU	Colony forming unit
CKs	Cytokinins
CMC	Carboxymethyl cellulose
COR	Coronatine-interferring JA signaling
CTAB	N-cetyl trimethyl ammonium bromide
CTR1	Constitutive triple response1
DAS	Days after sowing
DEB	Diazotrophic endophytic bacteria
DGGE	Denaturing gradient gel electrophoresis
DIG	Digoxigenin
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphate
EC	Electrical conductivity
EIN2	Ethylene insensitive 2
e-LRR	Extracellular-Leucine rich repeat
EPS	Exopolysaccharide
ERF	Ethylene response factor
ERIC-PCR	Enterobacterial repetitive intergenic consensus sequences-PCR
ET	Ethylene
ETI	Effector triggered immunity
ETR1	Ethylene resistant
FHB	Fusarium head blight
FISH	Fluorescent in-situ hybridization
FTIR	Fourier transform infrared spectroscopy
FY	Financial year

GA	Gibberellic acid
GDP	Gross domestic product
gfp	Green fluorescent protein
gus	β-glucosidase
HAI	Hours after inoculation
HR	Hypersensitive response
IAA	Indole-3-acetic acid
ICS/SID2	Isochorismate synthase/Salicylic acid induction
IMViC	"I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test
ISR	Induced systemic resistance
IST	Induced systemic tolerance
JA	Jasmonate
JAR1	Jasmonate resistance 1
LB	Luria Bertini
LOX1	Lipoxygenase
LPS	Lipopolysaccharides
LS	Lower shoot
LSCM	Laser scanning confocal microscopy
LSD	Least significant difference
MA	MacConkey agar
MAMP	Microbe associated molecular pattern
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
M-MuLV	Moloney murine leukemia virus
mRNA	Messenger-ribonucleic acid
MS	Middle shoot
MTI	MAMP-triggered immunity
MUC	4-methylumbelliferyl-β-cellobioside
MUG	4-methyllumbelliferyl-β-D-glucoside
NA	Nutrient agar
NB-LRR	Nucleotide binding-Leucine rich repeat
NBRIP	National botanical research institute-phosphate
NCBI	National center for biotechnology information
NIMIN1	NPR1-interacting proteins
NO	Nitric oxide
NPDB	National project on development and use of biofertilizers
NPR1	Non-expressor of PR1
OC	Organic carbon
OD	Optical density
PAL	Phenylalanine ammonia lyase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PDF1.2	Protein disulphide isomerase 1.2
PGPB	Plant growth promoting bacteria
PDI	Protein disulphide isomerase
PGPF	Plant growth promoting fungi
PGPR	Plant growth promoting rhizobacteria
PO	Peroxidase
P_o	Precipitated protein
PPO	Polyphenol oxidases
PQQ	Pyrroquinoline quinine
PR	Pathogenesis related
PRB	Population reference bureau
PRRs	Pattern recognition receptors
P_{sup}	Protein supernatant
PTI	Pattern triggered immunity
qtls/ha	(Quintals/Hectare)
qPCR	Quantitative-Polymerase chain reaction
rDNA	Ribosomal DNA
rep-PCR	Repetitive-Polymerase chain reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
Rpm	Revolution per minute
R-protein	Resistance-protein
RSA	Relative species abundance
RT-PCR	Real time-Polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIP	Stable isotope probing
SNI1	Suppressor of *npr1* inducible1
SPSs	Surface polysaccharides
SRI	Systematic rice intensification
SSCP	Single strand conformation polymorphism
SSIII	Type III secretion system
SSU	Small subunit
TCA	Trichloroacetic acid
T-RFLP	Terminal restriction fragment length polymorphism
US	Upper shoot
USDA	U.S. department of agriculture

CHAPTER I

General Introduction

"There are people in the world so hungry, that God cannot appear to them except in the form of bread."

- Mohandas Karamchand Gandhi

Part of this chapter has been published in the following research articles:

1. Jha P.N., **Gupta G.**, Jha P., Mehrotra R. Association of rhizospheric/endophytic bacteria with plants: A potential gateway to sustainable agriculture. Greener journal of agricultural sciences 2013, 3, 73-84.
2. **Gupta G.**, Panwar J., Akhtar M.S., Jha P.N. *In*: Endophytic nitrogen-fixing bacteria as biofertilizer. In: E Lichtfouse (ed) Sustainable agriculture reviews. Springer Netherlands, pp183-221.

1.1. Introduction

Nitrogen is the most important limiting factor for plant growth in various environmental conditions. Therefore, sufficient utilizable form of nitrogen must be provided to the plants for optimum growth and yield. Since the inception of green revolution use of chemical fertilizers has gained immense attention to attain the perpetual targets of increasing agriculture productivity. However, excessive and continuous use of chemically synthesized fertilizer can lead to several consequences which include: (i) ground water contamination due to leaching and denitrification of nitrogen fertilizer (ii) surface water contamination by eutrophication caused by leaching of nitrogen in water (iii) depletion of soil matter content (iv) production of other forms of toxic NO (NO_X) and (v) production of green house gases viz. CO_2 and NO_2 during manufacture of nitrogen fertilizer using non-renewable resources like natural gas and coal. These issues have serious health and environmental concerns (Bhattacharjee et al. 2008). Apart from that, increase in prices of petroleum products has led to an upsurge in the cost of chemical fertilizers. Therefore, it is high time to minimize the use of industrially manufactured fertilizers and search for alternative fertilizers, which are cost effective, sustainable and environmental friendly.

Biological nitrogen fixation is considered to be the most potential way to provide the fixed form of nitrogen to the plants. Nitrogen fixation is performed solely by prokaryotes (bacteria and cyanobacteria) and archeans. The N_2-fixing (diazotrophic) bacteria are involved in the fixation process, in which these bacteria, either in the free-living form or in symbiosis, can convert the atmospheric nitrogen into NH_3 with the help of nitrogenase enzyme. However, most of the biologically fixed nitrogen made available to the plants is contributed by *Rhizobium* sp. and cyanobacteria, which form association with specific plant species. It restricts their use in agriculture to certain plants only. Discovery and characterization of other associative and endophytic diazotrophic bacteria obtained from diverse plant species in the last couple of decades have raised prospects of their use as biofertilizer (Bhattacharjee et al. 2008; Akhtar and Siddiqui 2011). In particular, endophytic bacteria, which colonize plants interior without adversely affecting plant growth, are considered to be better plant growth promoting agents in terms of benefiting host plants. This has driven intensive research towards in-depth studies and better understanding of endophytic diazotrophic bacteria isolated from various plant species.

Application of endophytic bacteria as an effective agent for sufficing nitrogen requirement and other plant growth promoting properties to associated host plant appears to be one of the most promising approaches in the sustainable agriculture system.

1.2. Nitrogen fixation by endophytic bacteria

In recent years, potential of endophytic bacterial inoculants to meet N requirement of the various crop plants have drawn attention for increasing plant yield in the sustainable manner. Some of the promising endophytic biofertilizer include the members of *Azoarcus*, *Achromobacter*, *Burkholderia*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella* and *Serratia* (Franche et al. 2009). An efficient N supply by endophytic diazotrophic bacteria in sugarcane and kallar grass suggests the possible avenues of biological N_2-fixation (Dobereiner et al. 1993; Muthukumarasamy et al. 2005). In addition, several bacteria isolated from non-leguminous plants like rice, wheat, maize and sorghum are also known to fix N in the endophytic manner. Endophytic *Gluconoacetobacter diazotrophicus* (previously known as *Acetobacter diazotrophicus*) is considered to be one of the main contributors in biological N_2-fixation in sugarcane. It was reported to fix the N approximately 150 Kg N $ha^{-1}yr^{-1}$ (Muthukumarasamy et al. 2005). *Azoarcus* is recognized as another potential N_2-fixing obligate endophytic diazotroph. It dwells in the roots of kallar grass, and is known to increase the hay yield up to 20-40 t ha^{-1} yr^{-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 harbor bacterial population, which have the potential to provide 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 up to 40% increase in total N of the plants (Baldani et al. 2000). These investigations suggest that endophytic diazotrophs have a considerable potential to increase the productivity of non-legumes including important cash crop plants. Percent contribution of plant nitrogen as a result of biological N_2-fixation by endophytic bacteria has been summarized in Table 1.1. Quantitative estimation of fixed nitrogen made available to the plants by diazotrophic bacteria can be made by: a) Total N difference method, b) ARA (Acetylene reduction assay), c) Analysis of N solutes in xylem and other plant parts and d) N-

Table 1.1. Contribution of BNF by endophytic bacteria.

Endophytic bacteria	**Associating plant**	**% Ndfa***	**Reference**
Burkholderia	Rice	31	Baldani et al. 2000
Herbaspirillum	Rice	19-47	Mirza et al. 2000
Rhizobium leguminosarum bv. *Trifolii*	Rice	19 to 28	Biswas et al. 2000, Yanni et al. 2001
Klebsiella pneumoniae 324	Rice	42	Iniguez et al. 2004
Burkholderia vietnamiensis	Rice	40-42	Govindrajan et al. 2008
Azospirillum	Rice	9.2-27.7	de Salamone et al. 2010
Beijerinckia, Bacillus, Klebsiella, Enterobacter, Erwinia, Azospirillum, Herbaspirillum and *Gluconaacetobacter*	Sugarcane	18	Abeysingha and Weerarathne 2010
Pseudomonas, Stenotrophomonas,	Sugarcane	41.2-50.3	Taulé et al. 2011
Xanthomonas, Acinetobacter, Rhanella, Enterobacter, Pantoea, Shinella, Agrobacterium and Achromobacter			
Glucanoacetobacter diazotrophicus, Herbaspirillum seropedicae, Herbaspirillum rubrisubalbicans, Burkholderia sp.	Sugarcane	29-74	Urquiaga et al. 2012
Microbacterium sp.	Sugarcane	5.4-6	Lin et al. 2012

*Nitrogen derived from air

labeling methods (Gupta et al. 2012).

1.2.1. Molecular analysis of nitrogen fixation in endophytic bacteria

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 and metagenomic analysis (Kirk et al. 2004; Sessitch et al. 2012).

There are three types of nitrogenase based on the presence of core metals molybdenum (Mo) vanadium (V) or iron (Fe), which bridge two units of this enzyme (Zehr et al. 2003). Out of these three types, Mo-nitrogenase is most prevalent. There are three genes namely *nifH, nifD, nifK* which encodes for the structural part of Mo-nitrogenase complex. Apart from structural genes *nifHDK*, nitrogenase expression and function depend on several other regulatory genes (vary in different diazotrophic bacterial species, e.g., 20 in case of *Klebsiella pneumoniae*). The genes *nifD and nifK* encode 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). Some of the recent methods used to detect and measure nitrogenase activity are described below.

Amplification of *nifH*, *nifD*, and *nifK* by PCR or RT-PCR has been frequently employed in detection of N_2-fixing ability of bacterial and cyanobacterial isolates (Chowdhury et al. 2007; Bothe et al. 2010). DNA sequence of *nifH* of different diazotrophic bacterial species has been reported to be one of the most conserved sequences, except for short species-specific sequence discrepancies, later of which can be used for identification of species (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 (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 cultivable and non-cultivable bacteria (Zehr et al. 1998; Izquierdo and Nusslein 2006; Poly et al. 2001). Several studies have demonstrated diazotrophic nature of several endophytic bacteria on genotypic and expression level using *nifH*

amplification in various plants (Chowdhury et al. 2007, Terakado-Tonooka et al. 2008, Islam et al. 2010). 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 and use of reverse transcription (RT-PCR) 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 cultivable, but contribute significant nitrogen nutrition to the host plant. Based on the difference in *nifH* mRNA and DNA profile obtained from the 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. Above observation is supported in a recent metagenomic study in which *Rhizoctonia rosttiformans* was observed as an active diazotroph of sugarcane and spruce from different locations. However, it was surprising as none of the known diazotrophs associated with sugarcane such as *Glucanoacetobacter diazotrophicus, Herbaspirillum seropedicae* or *Herbaspirillum rubrisubalbicans* were found to be active in sugarcane plant (Burbano et al. 2010).

Detection of diazotrophic bacteria and estimation of nitrogenase activity based on expression of *nifH* mRNA employing FISH (Fluorescent in-situ hybridization) is an effective approach. However, FISH has not been used frequently due to the instability of bacterial mRNA. Use of transcript polynucleotide probes can improve the sensitivity of signal and reduce signal to noise ratio. Hurek et al. (1997) detected *in-planta* mRNA expression in *Azoarcus* sp. using the transcript oligonucleotide probes. Further, to improve the sensitivity and reliability of the technique, Pilhofer et al. (2009) detected mRNA of *nifH* using digoxigenin (DIG)-labeled transcript probe. The resultant hybrid was detected by horse-radish peroxidase (PO) marked anti-DIG antibody. Subsequently, the signal was amplified using catalyzed reporter deposition (CARD) where tyramide molecules pre-conjugated with flurochrome were deposited in close proximity of horse-radish peroxidase binding site and intensifies the signal. Furthermore, microarrays with oligonucleotide of *nifH* gene can be used to determine diazotrophic communities. For example, the microarray developed by Zhang et al. (2006) compares 194

oligonucleotide probes, which covers more than 90% of all *nifH* sequences present in the *nifH* database. Similarly, Steward et al. (2004) developed a DNA macroarray to evaluate its potential to distinguish variants of *nifH* gene. In addition to above-mentioned nucleic acid based approaches, nitrogenase activity can also be assessed by detecting nitrogenase complex based on localizing ca. 27-35 kDa of the protein band of dinitrogenase either by radiolabeling or immunoblotting using an antibody against Fe protein (Jha and Kumar 2007). This approach can also be used effectively for localizing diazotrophic bacteria in plant (Chelius and Triplett 2000).

1.3. Plant growth promoting properties of endophytic bacteria

Apart from N_2-fixation, endophytic bacteria can benefit their host through various other growth promoting properties, which include production of phytohormones (auxin and cytokinin), synthesis of siderophore, induction of induced systemic tolerance through ACC (1-aminocyclopropane-1-carboxylate)-deaminase activity and antagonistic activity against pathogenic miroorganisms (Saharan and Nehra 2011). Several endophytic bacteria have been reported to have ability to solubilize mineral phosphate (Rosenblueth and Martínez-Romero 2006). However, this ability may not be useful for plants as endophytes reside in the interior of plant tissue where insoluble mineral phosphates are not available. The characteristics of some beneficial endophytic bacteria are discussed below.

1.3.1. Phytostimulatory compounds

Plant growth promoting bacteria produce phytohormones, certain volatiles and the co-factor pyrroquinoline quinine (PQQ), which stimulate plant growth under certain conditions. The volatile organic compounds may influence the plant growth by regulating auxin homeostasis in plants (Zhang et al. 2008). Many plant associated bacteria have been shown to produce auxins chiefly indole-3-acetic acid (IAA), which enhances lateral root growth formation and thus, nutrient uptake and root exudation by plants (Reinhold-Hurek and Hurek 2011). Moreover, role of IAA is also important in signaling during plant-microbe communication. Degradation of IAA in *Burkholderia phytofirmans* found to be involved in the plant–PGPR communication process (Zuniga et al. 2013). Other plant hormones produced by some of these bacteria are cytokinins and gibberellins, which are stimulatory to plant growth (Bottini et al. 2004; Lopez-Bucio et al. 2007). 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 (GA_4), cytokinin and IAA.

1.3.2. Induced systemic tolerance

Some of the plant growth promoting bacteria (PGPB) help the associated plants to counter abiotic stressors 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 abiotic stressors are termed as 'induced systemic tolerance' (IST) (Yang et al. 2009). One of the major mechanisms of IST is suppression of 'stress ethylene' in plants produced under biotic and abiotic stress conditions. Some of the plant-associated bacteria including endophytes have property to synthesize ACC-deaminase, which can degrade ACC, the immediate precursor of ethylene (ET), and reduce the level of stress ethylene, which in turn promote growth of associated plants (Glick et al. 2007). In addition to ACC deaminase mediated IST, there are various other mechanisms through which IST is generated in response to stress. It includes induction of salt stress tolerance mediated by volatile organic compounds (VOCs) (Zhang et al. 2008). During stress conditions, certain VOCs affect abscisic acid signaling of host-plants through production of cytokinin (Figueiredo et al. 2008) and catalase (Kohler et al. 2008). Besides, the role of phytohormone produced by associative bacteria in promotion of plant growth during salinity or drought stress conditions has also been demonstrated (Egamberdieva 2009).

1.3.3. Biocontrol agent

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 Sureender 2009). Application of these biocontrol bacteria is one of the most promising approach for minimizing infestation of plant diseases. Use of biocontrol agent is considered to be better alternative of chemical pesticides as it is eco-friendly and cost-effective. Bacteria can limit plant pathogens 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.

1.3.3.1. Antagonism

Endophytic bacteria can exhibit biocontrol activity (antifungal and antibacterial) through production of allelochemicals (antibiotics) or hydrolytic enzymes. Gram negative biocontrol agents like some members of genus *Pseudomonas* produce antimicrobial compounds such as HCN, pyoleutorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and phenazines chiefly phenazine-1-carboxylic acid and phenazine-1-carboxamide (Lugtenberg and Kamilova 2009). Munumbicin, an antibiotic produced by endophytic bacteria inhibits growth of phytopathogenic fungi *Pythium ultimum* and *Fusarium oxysporum* (Castillo et al. 2002). Certain volatile organic compounds like 2,3-butanediol, or blends of volatiles produced by *Bacillus* sp. also inhibit growth of pathogenic bacteria and fungi (Strobel 2006). Level of antibiotic synthesis in bacteria depends upon the nutritional factors viz. type of carbon source utilized, trace elements, availability of other nutrients and non-nutritional factors like environmental influences (Compant et al. 2005).

Bacteria can restrict the growth of pathogens also by producing hydrolytic enzymes such as chitinase, β-1,3-glucanase, protease, lipase and laminarinase (Ordentlich et al. 1988). *Bacillus cepacia* has been reported to destroy *Rhizoctonia solani*, *Rhizoctonia rolfsii* and *Pythium ultimum* by producing β-1,3-glucanase (Fridlender et al. 1993). Addition of endophytic bacteria *Bacillus cereus* 65 directly to soil to protect cotton seedlings from root rot disease caused by *Rhizoctonia solani* has been reported (Pleban et al. 1997). Similarly, 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).

1.3.3.2. Siderophore production

Under iron-limiting condition, some biocontrollers produce small molecular weight compounds, known as siderophore, which has the capability to chelate iron from the environment and makes it available to themselves and to host plants, and thus, deprive pathogens for available iron (Saha et al. 2012). An array of siderophores is known to be produced by bacteria, but the majority of biocontroller are known to produce catacholate, hydroxymate and/or phenolate type (Rajkumar et al. 2010). In addition to biocontrol, siderophores are also known to play role in diazotrophic bacterial species by making available both iron and molybdenum

required for the activity of nitrogenase and help in N_2-fixation process. Thus, siderophore seems to play a pivotal role in growth, diazotrophy of bacteria under iron deficient conditions (Kraepiel et al. 2009).

1.3.3.3. Induced systemic resistance

During their interaction with plants, some endophytic bacteria improve immune response of plants which protects from future attack of pathogens, a phenomenon called as 'induced systemic resistance' (ISR) (Van Loon 2007). In contrast to biocontrol mechanisms, extensive colonization of root system by endophytic bacteria is not required for development of ISR (Lugtenberg and Kamilova 2009). Diverse types of bacterial products such as lipopolysaccharides, flagella, siderophores, antibiotics, volatile organic compounds and quorum-sensing signals are responsible for eliciting induction of ISR in plants (Van Loon 2007). These elicitors are recognized by specific receptors present on plant cell surface and induce a cascade of events for generation of defense compounds. ISR 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 proteins (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 of pathogens (Duijff et al. 1997; Compant et al. 2005). The understanding of molecular basis of plant immunity can be utilized in enhancing host recognition for potential pathogens, interfering virulence strategies of microbial pathogens and triggering plant defense by biological priming in a non-transgenic manner. Thus, understanding of mechanism behind inducing systemic resistance in plant by endophytic bacteria may open new ways for engineering durable disease resistance in crop plants without the ill-effects of transgenic crops (Gust et al. 2010). All the applications of diazotrophic endophytic bacteria have been depicted in Fig. 1.1.

1.4. Experimental studies on plant growth promotion by endophytic bacteria: Pot and field studies

Non-symbiotic nitrogen fixation by associative and endophytic bacteria has great agronomic significance. However, success of these bacteria is limited due to two major reasons.

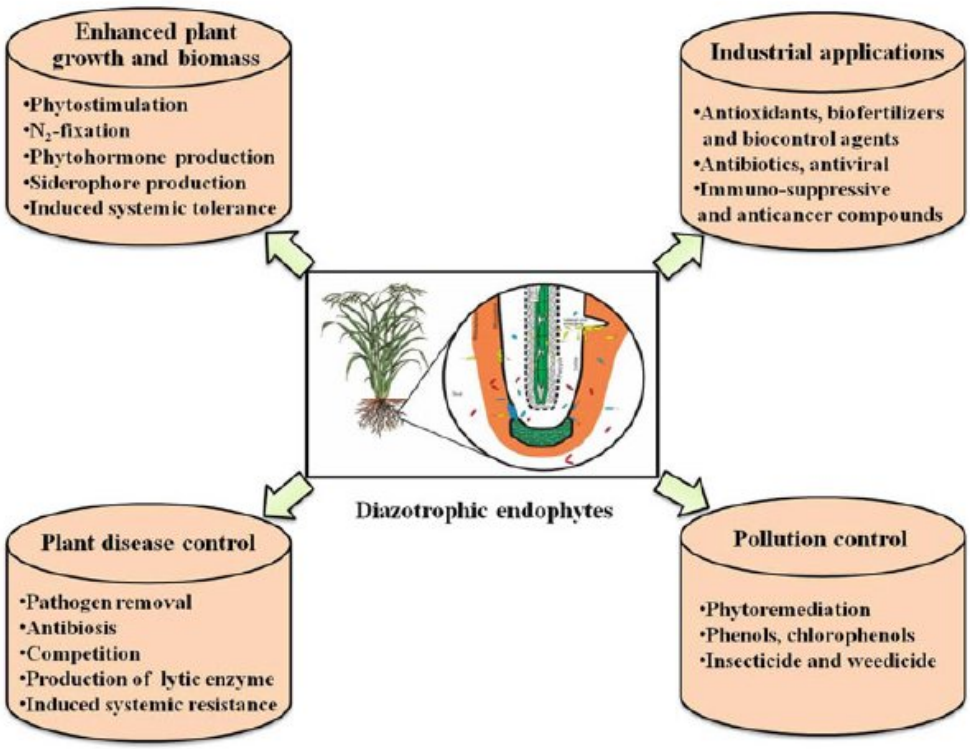


Fig. 1.1. Multiple applications of diazotrophic (N_2 fixing) endophytic bacteria in various fields including agriculture practices, industries and environment (Modified from Hardoim et al. (2008).

First, microaerophilic condition is required for optimal nitrogen fixation process and, second, it is highly expensive reaction in terms of energy requirement. Closer association of these bacteria with plants especially in roots can minimize above-mentioned problems as the host plant provides various carbon-rich exudates to the associated bacteria, and partial oxygen pressure (pO_2) present inside roots is amenable for nitrogen fixation. Abilities of endophytic bacteria in promoting plant growth have been demonstrated in various laboratory and field studies (Sturz et al. 2000, Vessey 2003). One of the well-studied plant-growth promoting endophytic bacterial strains is *Azoarcus* BH72, a Gram-negative N_2-fixing bacterium originally isolated from kallar grass. It can also colonize rice both in the laboratory and the field conditions (Hurek et al. 1994), though their association under aseptic systems is cultivar-dependent (Reinhold-Hurek et al. 2002).

Baldani et al. (1992) first reported *Herbaspirillum* as a N_2-fixing bacterium associated with the roots of rice, maize and sorghum. Until now, this bacterium has been reported in 13 members of the graminaceae including sorghum, maize, sugarcane, rice and others, particularly within roots (Olivares et al. 1996). *Herbaspirillum* sp. grow and fix N_2 under relatively high pO_2 (3%) compared with *Azospirillum* spp. (2%). *Herbaspirillum seropedicae* fix N from the atmosphere contributes 31–54% of total N in rice plants (30-d-old rice seedlings) (Baldani et al. 2000). In a greenhouse study, inoculation with *Herbaspirillum* increased rice yield significantly by 7.5 g^{-1} plant (Mirza et al. 2000). Additionally, it increased shoot and root length, 1000-grain weight and grain yield of rice (Arangarasan et al. 1998). *Herbaspirillum* can also enhance seed germination significantly in rice (Pereira et al. 1988). Further, diverse host range and effectivity of *Herbaspirillum* are evident to its ability to promote growth of another crop, maize plant, both in green house and field conditions (Canellas et al. 2012). In a very recent study, higher N (11%), P (30%) and K (17%) content was observed in maize plants treated with the combination of *Herbaspirillum* and NPK chemical fertilizer than that of plants grown with chemical NPK fertilizer only. Greater consistency and stability response of the host plant to bacterization along with chemical fertilizer indicate a promising biotechnological approach for improving growth of maize plants (Baldotto et al. 2012). Improved growth under biofertilization with endophytic bacteria in both greenhouse and field studies has been reported for several important crop plants,

including wheat (El-Mohandes 1999; Ganguly et al. 1999), cotton (Bashan 1998) and sugarcane Muthukumarasamy et al. 1999). Some of the major plant growth promoting bacteria and their contributions are listed in Table 1.2.

1.5. Ecology and diversity of endophytic bacteria

The health and development of plants depend on various biological processes that are mediated or carried out by endophytic bacterial community. Therefore, understanding of composition and community structure of endophytic bacteria are important for plant growth and yield (Andreote et al. 2009). 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 and filter out specific microbial groups growing inside or in the vicinity of plant roots (Rosenblueth and Martinez-Romero 2006). Thus, various factors such as genotype and physiological status of host-plants, bacterial colonization traits, abiotic conditions and agricultural management regimes can influence diversity of bacterial communities in plant 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, Lundberg et al. 2012).

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. and different *Rhizobium etli* strains in rice (Verma et al. 2004) and maize (Rosenblueth and Martinez-Romero 2006) respectively. Many endophytes have a broad host range, though it has not been studied in a systematic 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).

Structure and composition of microbial community in host plants are one of the major determinants of plant health and productivity. 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.

Table 1.2. Reports on yield increase on inoculation of plant growth promoting bacteria under actual farming condition.

Name of bacteria	Host plant	Grain yield/Fruit yield/% increase	Conditions	References
Klebsiella sp.	Maize	10.9-15.8 t ha^{-1}	Field experiment, Under different locations	Kennedy et al. 2004
H. seropedicae	Maize	7.55-14.86 t ha^{-1}	Field experiment, Under different locations	Kennedy et al. 2004
G. diazotrophicus	Maize	7.88-9.50 t ha^{-1}	Field experiment, Under different locations	Kennedy et al. 2004
K. pneumoniae	Maize	5.60-16.39 t ha^{-1}	Field experiment, Under different locations	Kennedy et al. 2004
Bacillus sp.	Maize	17.00 t ha^{-1}	Field experiment	Kennedy et al. 2004
Pantoea agglomerans	Maize	13.56 t ha^{-1}	Field experiment	Kennedy et al. 2004
G. diazotrophicus Pal5	Tomato	7.33-17.77 t ha^{-1}	Field experiment, Under different N levels (30-150 N Kg ha^{-1})	Luna et al. 2012
Azospirillum sp. RAM 7	Maize	2.93-6.72 Kg ha^{-1}	Field experiment, Under different N levels (30-150 N Kg ha^{-1})	Santa et al. 2004

Azospirillum sp. RAM 7	Maize	3.34-7.31 Kg ha^{-1}	Field experiment, Under different N levels (30-150 N Kg ha^{-1})	Santa et al. 2004
Azospirillum brasilense	Wheat	28.7-44.4 dt ha^{-1}	Field experiment, In different years	Swędrzyńska and Sawicka 2000
Azospirillum brasilense	Oat	44.2-43.5 dt ha^{-1}	Field experiment, In different years	Swędrzyńska and Sawicka 2000
Azospirillum brasilense	Maize	10.7%	Field experiment	Puente 2009
Burkholderia vietnamensis MGK3+other strains	Rice	9.5-23.6%	Field experiment, Under two pot and four field experiments	Govindarajan et al. 2008
Burkholderia vietnamensis MGK3	Rice	5.6-12.2%	Field experiment, Under two 2 and 4 field experiments	Govindarajan et al. 2008
Azotobacter	Wheat	30%	Field experiments	Gholami et al. 2009; Kloepper and Beauchamp 1992
Burkholderia vietnamensis	Rice	8 t ha^{-1}	Field experiments	Tran Vân et al. 2000
Herbaspirillum	Rice	7.5 g $plant^{-1}$	Field experiments	Mirza et al. 2000
Rhanella sp. in soil and seed	Maize	4553.0-5098.4 Kg ha^{-1}	Field experiments, Under different N levels (0-120 Kg ha^{-1})	Montañez and Sicardi 2013

Azospirillum sp. in soil and seed	Maize	4327.2-4994.2 Kg ha^{-1}	Field experiments, Under different N levels (0-120 Kg ha^{-1})	Montañez and Sicardi 2013
A. brasilense and urea	Rice	35.25 kg $block^{-1}$ (27.37 and 32.12 kg $block^{-1}$ individually)	Field experiments	Pedraza et al. 2009
Azorhizobium	Sugarcane	1.8 t ha^{-1} (22% increase)	Field experiments	Balandreau (2002)

The genetic principles lying behind the derivation of host-specific endophyte communities from soil communities are poorly understood. In two independent recent studies, Lundberg et al. (2012) and Bulgarelli et al. (2012) did 454 sequencing of 16S rRNA gene amplicons to compare soil, rhizosphere and endophytic communities of pot-grown *Arabidopsis* under controlled conditions in various soils. In these studies, they reported that community structure strongly depends on soil factor (less on plant genotype and age) (Lundberg et al. (2012) as well as it also depends on the plant cell wall features (Bulgarelli et al. 2012). Still it is important to conduct full metagenomic sequencing to identify the bacterial genes involved in surface and internal colonization of plant and it further requires transcriptomic and proteomics studies to pinpoint conditions required for expression of these genes. These studies will reveal the contribution of bacteria in disease resistance and nutrient cycling in plant systems. Application of these above-mentioned molecular approaches can be helpful in understanding the plant-microbe interaction which in turn will be useful in developing sustainable agriculture using endophytic bacterial biofertilizer.

The genetic diversity of plant-associated bacteria can be evaluated using one or more of the several methods, including amplified ribosomal DNA restriction analysis (ARDRA), rep-PCR (Repetitive-Polymerase chain reaction) based genomic fingerprinting and small subunit (SSU) ribosomal DNA (rDNA) sequencing (Grange and Hungria 2004). Sequence analysis of amplified *nifH* has also been used to study the diversity of diazotrophic bacteria isolated from plants (Chowdhury et al. 2007). The clusters based on *nifH* sequence of several known diazotroph families were found to be similar to that of 16S rDNA sequence analysis. Therefore, *nifH* analysis can also be used for identification of diazotrophic bacteria (Venieraki et al. 2011). Denaturing gradient gel electrophoresis (DGGE) of amplified rDNA and *nifH* of culturable or unculturable bacteria has also been used for studying molecular diversity (Burbano et al. 2010; West et al. 2010). In addition, other methods like single strand conformation polymorphism (SSCP), Terminal restriction fragment length polymorphism (T-RFLP) of rDNA are used to elucidate the prevalence of molecular diversity and study of phylogenetic relationship among bacteria. Advances in high-throughput sequencing technology and tremendous drop in cost of sequencing have revolutionized the community structure study based on 16S rDNA sequences.

Solexa 454 pyrosequencing and Illumina are two most used sequencing platform for study of microbial diversity. In a recent review, comparison of two next-generation sequencing technologies were done suggesting that latest developments of 454 and Illumina technologies offered higher resolution compared to their previous versions, and showed relative consistency with each other (Claesson et al. 2010). Community diversity in terms of desired metabolic activity or rRNA-based approach can also be studied comprehensively by stable isotope probing (SIP) method (Kiely et al. 2006). Apart from mere understanding of community structure, identification of functionally active microorganisms can be done employing few recent molecular techniques. Active endophytic bacterial community was identified by analysis of 16S rRNA sequences or other functional genes from density resolved DNA using SIP (Stable isotope probing) (Rasche et al. 2009) and RT-PCR. In recent years, metagenomic approaches are being used to define functional map and diversity of plant-associated bacterial community (Sessitch et al. 2012). Metagenomic methods can be one of the most instrumental tools in understanding community structure and functional attributes of both cultivable and uncultivable bacteria. Various approaches used in diversity studies are shown in Fig. 1.2.

1.6. 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 the root surface i.e., chemotaxis, (b) attachment and microcolony formation, (c) distribution along root and (d) growth and survival of the population.

1.6.1. 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. These plants signals favour colonization by some bacteria, while

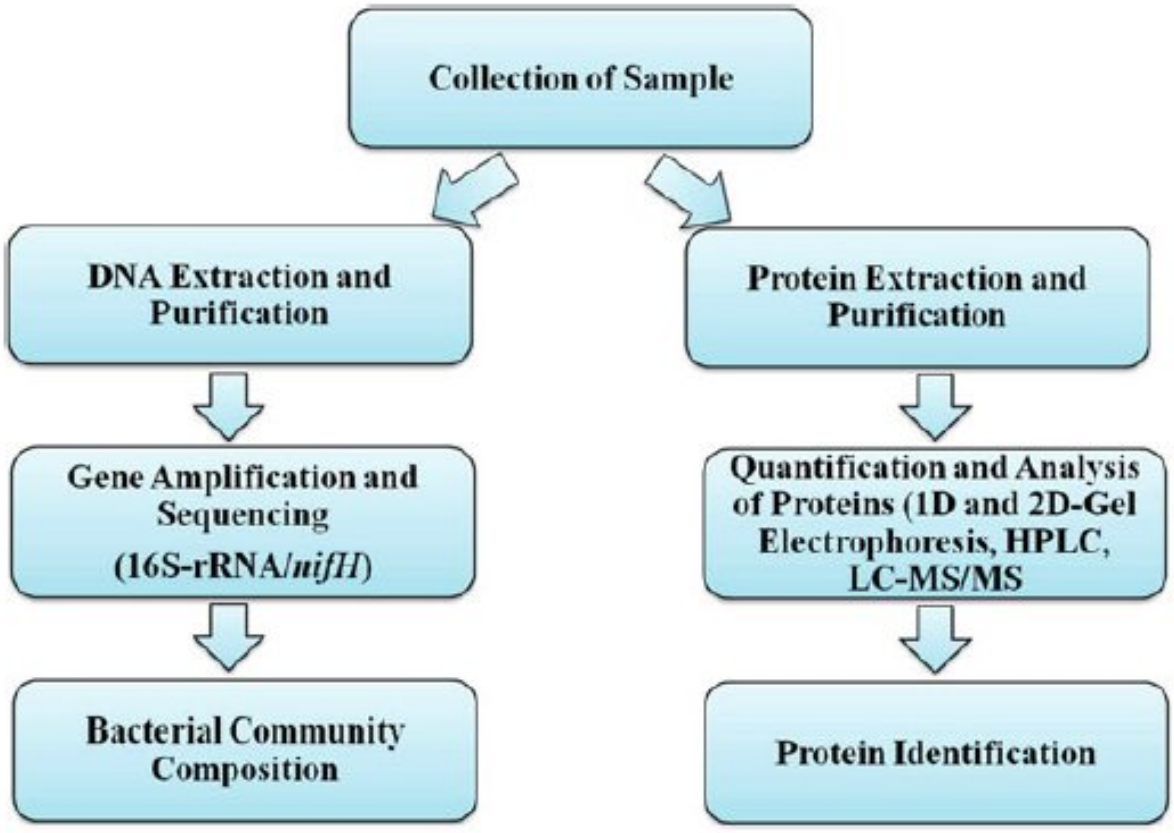


Fig. 1.2. Diagramatic representation of meta-proteogenomics sample analysis methods.

inhibit the others (Compant et al. 2010). Like plant-rhizobia interaction, plant root exudates do influence the expression of genes in associative bacteria. Stimulation of colonization of wheat and *Brassica napus* by *Azospirillum brasilense* and *Azorhizobium caulinodans* in response to flavanoid from host exudates indicates that flavanoid may be the determinant for endophytic colonization (O'Callaghan et al. 2000). In a more recent study, naringenin, a flavanoid present in exudates of plants, has been reported to modulate the expression of genes in *Herbaspirillum seropedicae* and this alteration in gene expression were observed to be 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).

1.6.2. Attachment on the root surface

Chemotaxis or electrotaxis driven migration of bacteria to roots is followed by adhesion of bacteria on the root surface to get entry into the plant tissue. Adherence of these bacterial cells depends on various cell surface molecules such as cell appendages (flagella or pili), major outer membrane proteins (MOMP) and secretion system of bacteria, which play major role for invasion (Croes et al. 1993; 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 (LPS) present on the surface of bacteria might be determinative for successful colonization in host plants (Gough and Cullimore 2011). For attachment on plant surface, biofilm formation is important which also protect bacteria from adverse conditions. Bacterial surface components, environmental and quorum-sensing signals are required for autoaggregation and biofilm development in most bacterial species (Bogino et al. 2013).

Bacterial cells are equipped with various secretion systems, which enable them to interact successfully with the host plant. Preston et al. (2001) identified SSIII (Type III secretion system) (hrp) in *Pseudomonas fluorescens* SBW25 by *in-vitro* expression technology (IVET), a promoter trapping technique used to identify genes expressed *in-vivo* during the colonization process. In a

very recent report, a 21 Kb region of *Herbaspirillum rubrisubalbicans* genome containing a cluster of 26 hrp/hrc genes encoding for Type III secretion system proteins have been identified. On Tn5-transposon based mutation of these genes, hrpE and hrcN mutant strains failed to colonize the Poaceae family plants suggesting their role in endophytic colonization (Schmidt et al. 2012). In *Azoarcus* sp. BH72, Type III and IV were not present, but the genes for Type I and II secretion system has been reported by Krause et al. (2006). Similarly, *Klebsiella pneumoniae* 342 contains all the secretion system Type I-VI except Type III which is present in most plant-associated bacteria (Fouts et al. 2008). Type IV and some part of Type II have been reported in *Gluconoacetobacter diazotrophicus* pal5 (Bertalan et al. 2009). Recently, a type III secretion system was detected in *Burkholderia* sp. KJ006 genome, former of which is known to play an important role in the endophytic colonization (Kwak et al. 2011).

1.6.3. 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* (Green fluorescent protein)-tagged rhizobia in crop plants begin with surface colonization of the rhizoplane at lateral root emergence followed by endophytic colonization within roots, and then exhibit ascending endophytic migration into the stem base, leaf sheath, and leaves where they develop high populations. Crack entry of *Azorhizobium 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 (Webster et al. 1997). Compant et al. (2008) reported the chronological detection of endophytic *Burkholderia phytofirmans* PsJN on the root surfaces, in the endorhiza and inside inflorescence stalks of *Vitis vinifera*.

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 *Azospirillum irakense* (Khammas and Kaiser 1991), *Azoarcus* sp. (Reinhold-Hurek et al. 2006) and others, has been demonstrated

as one of the efficient methods to get entry into the host plant. *Azospirillum 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 roots radial walls presumably by digesting the middle lamella and then proceeds between cells in intercellular spaces. 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), which indicates passive mode of its colonization in plants.

Azoarcus sp., an obligate endophyte of kallar grass, has been critically studied by using transposon-based mutants carrying β-glucuronidase (GUS) as a reporter gene (Hurek and Reinhold-Hurek 2003). *Azoarcus* sp. BH72 colonizes intensively in apical region of roots behind the meristem and penetrate the rhizoplane preferentially in the zone of elongation and differentiation. It colonizes 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 the xylem vessels which suggests its systemic spreading into shoots through transport vessels. However, shoot colonization of Gramineae appears to be more pronounced in *G. diazotrophicus* (James and Olivares 1998) and *H. seropedicae* (Gyaneshwar et al. 2002). Intercellular colonization of endophytic bacteria in cortex and xylem of root has been reported in recent studies as well (Prieto et al. 2011). Compant et al. (2011) noticed the colonization of endophytic bacteria in epidermis and xylem of even reproductive organ of grapevine. Based on the colonization pattern of *Pseudomonas 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. 1.3.

1.6.4. 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

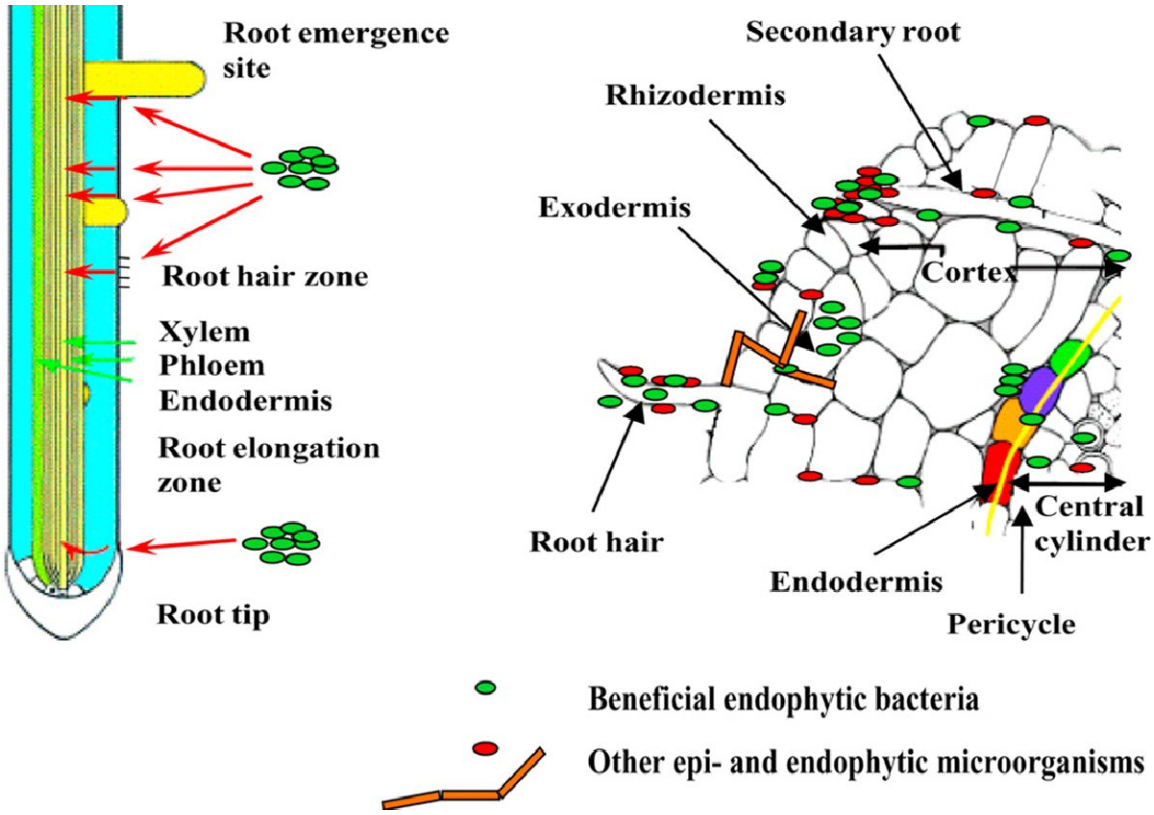


Fig. 1.3. Sites of plant colonization by endophytic bacteria. Figure adopted from Compant et al. (2010) with permission of Elsevier.

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 (SA) and ethylene restrict the endophytic colonization. Ethylene, a signal molecule of ISR in plants, decreases endophytic colonization as observed in *Arabidopsis thaliana* inoculated with *Klebsiella pneumoniae* 342 (Iniguez et al. 2005). However, proteomic approach used to study the bacterial colonization indicated that instead of ethylene and SA, it is the jasmonic acid (JA) which contributes in restricting endophytic colonization in grasses (Miché et al. 2006). Expression of JA-induced PR proteins (pathogenesis-related protein) depends upon the compatibility of plant variety and endophytic bacteria. Inoculation of *Azoarcus* sp. to more compatible rice (*Oryza sativa*) cv. IR36 led to the expression of fewer JA-induced PR proteins than that of less compatible cv. IR42. Antimicrobial peptides synthesized by some plants like rice and maize may reduce endophytic colonization (Fuentes-Ramirez et al. 1999). Understanding of molecular mechanisms and conditions limiting the colonization process need to be elucidated for exploiting the beneficial endophytic or associative interaction with plants.

1.7. Plant immune system and beneficial microbes

As described above, immune response of host plants indeed operates and limits colonization of endophytic bacteria. Therefore, modulation of plant immune response by endophytic bacteria plays an instrumental role in their successful establishment inside host environment. Except few recent studies, most of the information of plant defense response to beneficial-microbe is based on rhizobiaceae-leguminaceae relationship (Zamioudis et al. 2012). Mechanistic insights in plant defense response during plant-endophyte interaction may elucidate the differential behavioral pattern of plant in terms of defense strategies towards endophytic and pathogenic bacteria during the interaction. It can further explicate that how endophytic bacteria

gain entry and manage inside to cope up host environment. Before going into the details of plant immune system against beneficial bacteria, a brief introduction of plant immune system is described below.

1.7.1. Plant immune system

Like animals, plants do generate highly specific immune responses without any self-reactivity, which develops a long term memory for future attack, although it is less complex and lacks circulatory immune system and mobile immune cells to circumvent the pathogenic attack with few exceptions (Spoel and Dong 2012). Plants get quickly colonized by a diverse micro-flora of soil-borne bacteria and fungi that may have either beneficial or deleterious effects on the plant. However, the microbial community associated with plant roots are different from that in rhizospheric and bulk soil, suggesting that plants are able to shape their microbiome (Mendes et al. 2011).

Both beneficial and parasitic microorganisms gain entry in the plant interior, either directly by penetrating leaf or root surface or indirectly through wounds or natural openings such as stomata and cracks in lateral roots. The next step is to breach the rigid cellulosic plant cell wall, from where the plant defense system comes into play. Like mammalian immune system, plant defense system consists of two lines of defense i.e., innate immunity and adaptive immunity. Plant innate immunity is divided into two modes: Pattern triggered immunity (PTI) and effector triggered immunity (ETI). Under adaptive immunity, generation of memory in plants is related to the development of systemic acquired resistance (SAR) by pathogens and induced systemic resistance (ISR) by beneficial bacteria. SAR developed in plants against pathogens is governed by SA responsive pathways mainly and leads to PR gene induction and various downstream signaling cascades. On the other hand, ISR is the sensitization of plant immune system on contact with beneficial bacteria, and PR genes get induced only on future pathogenic attack.

1.7.2. Pattern triggered immunity (PTI)

Once invading microbes come into contact of plant cells, first line of plant defense initiates to ward them off. The host cell wall surface receptor (Pattern-recognition receptors-PRRs) recognizes microbial surface molecules known as the microorganism-associated molecular patterns (MAMPs) and triggers PTI. Some of the common MAMPs are lipopolysaccharides, peptidoglycans, and bacterial flagellin present in bacteria and, chitin and ergosterol present in the fungal cell wall (Erbs and Newman 2012). Induction of PTI initiates several other processes such as MAP kinase signaling, transcriptional induction of pathogen-responsive genes, production of reactive oxygen species (ROS) and deposition of callose to reinforce the cell wall at sites of infection, which restrict microbial growth. However, mechanistic details of triggering PTI are not thoroughly understood.

1.7.3. Effector-triggered immunity (ETI)

Many microbes have coevolved effector molecules that dodge PTI by directly delivering them inside the plants through secretion system (Type III secretion system-TTSS) and cross the first line of defense. These microbial effector molecules interfere in the plant defense system at various levels, which include: interference of host gene expression (Zhu et al. 1998), interfering the signaling cascades involved in defense responses (for eg., coronatine (COR) interfering SA signaling) (He et al. 2004), suppression of cell wall fortification/defense (DebRoy et al. 2004), interference in HR (hypersensitive responses, causing rapid death of cells in the infected region for preventing the pathogen spread in plant) responses (for instance, AvrRpt2 interferes with the HR triggered by AvrRpm1) (Ritter and Dangl 1996) and/or inhibition of ETI-associated programmed cell death (Abramovitch et al. 2003). Many other examples have been described for bacteria as well as fungal effectors in a review by Chisholm et al. 2006.

To counter deleterious effect of the effector cells, plants generate ETI. ETI is highly specific for polymorphic effectors of different pathogens, which results in either release of certain hydrolytic enzymes like chitinase, β-1,3-glucanase and programmed cell death of infected cells (Nimchuk et al. 2003). During the course of co-evolution, plants have developed

intracellular immune receptors called as 'R-proteins' (resistance proteins) encoded for recognition of effector molecules resulting in initiation of defense signaling and host resistance (Dangl and Jones 2001). These R-proteins can be divided into two classes on the basis of domain structure i.e, NB-LRR (Nucleotide binding-Leucine rich repeat) receptors and R-genes encoding extracellular LRR (e-LRR) proteins. As there are many effectors, which act on similar host targets, plants have developed indirect pathogen recognition strategy in which plants recognize the function of effectors and do not require receptors for every possible effector (Van der Biezen and Jones 1998).

1.7.4. Signal transduction in the plant immune response

After recognition of the effector molecule by plants R-protein, surviving pathogens counterattack host by generating other effector molecules and this constant fight keeps on going between plant and pathogen. This mode of action has been described as a zig-zag model of plant defence by Chisholm et al. (2006). Apart from this, downstream of PTI or ETI activation, diverse hormones play the central role in triggering the plant immune signaling network (Katagiri and Tsuda 2010). Changes in hormone concentration, sensitivity triggered during parasitic interaction, composition and timing of blend of various hormones like ethylene (ET), abscisic acid (ABA), gibberellins (GAs), auxins, cytokinins (CKs), brassinosteroids, and nitric oxide (NO) (Pieterse et al. 2012) function as modulators of the plant immune signaling and mediates a whole range of adaptive immune response.

The earliest and important detectable cellular events during plant-microbe interaction are ion fluxes across the plasma membrane and oxidative burst producing reactive oxygen intermediates (ROIs) i.e., superoxide (O^{2-}) and H_2O_2 (Spoel and Loake 2011). This interaction further lead to transfer of the signals to various signaling cascades and activation of various defense genes. A variety of secondary signaling molecules including SA, JA, ET, etc are responsible for downstream signal transduction and all these signal molecules lead to different defense pathways. Further, different subsets of defense genes get induced for providing resistance to host against specific pathogens. Salicylic acid mediated signaling pathway acts against biotrophic pathogens. Biosynthesis of SA occurs by two enzymatic pathways involving

phenylammonialyase (PAL) and isochorismate synthase (ICS/SID2) (Garcion and Métraux 2006). SA production is activated during both PTI and ETI upon recognition of MAMPs and effectors of pathogens respectively (Mishina and Zeier 2007). SA production is regulated by NPR1 monomer (Non-expressor of PR1- containing ankyrin repeat domain) onto which basic leucine zipper (bZIP) family transcription factor binds. Further this complex (NPR1+bZIP) binds to the promoters of SA-responsive genes, such as *PR-1*, resulting in their activation (Fan and Dong 2002). Thus, NPR1 plays an important role in SAR development as well as in regulation of ISR development, although some unknown mechanism is involved as in ISR NPR1 does not lead to PR gene activation (Choudhary et al. 2007). NPR1 is negatively regulated by NPR1-interacting proteins such as NIM1-INTERACTING1 (NIMIN1), 2, and 3, and SUPPRESSOR OF *npr1* INDUCIBLE1 (SNI1) (Pieterse et al. 2012).

Both JA (Jasmonic acid) and ET signaling are involved in the development of ISR (independent of SA with few exceptions) by plant growth promoting bacteria on pathogenic challenge (Pieterse et al. 1998), which further lead to induction of different subset of PR genes, including defensin (PR12) and thionin (PR13) genes (Dong 1998). MAP (Mitogen activated protein) kinases are involved in JA regulation. Two other main additional regulators are JAR1, which control JA signaling by metabolizing JA molecules (Staswick et al. 2002), and leucine-rich-repeat-containing F-box protein encoded by COI1 gene, which might function by recruiting transcriptional repressors and targeting them for removal by ubiquitination (Xie et al. 1998).

ET signaling occurs in response to pathogenic attack and EIN2 (ethylene insensitive 2) acts as a main positive regulator in the absence of ethylene (Hua and Meyerowitz 1998). Ethylene binding on ethylene receptors (membrane-localized protein), negatively regulates the activation the ethylene response. CTR1 (CONSTITUTIVE TRIPLE RESPONSE1) is another factor negatively regulating (downstream of ethylene receptors) ET signaling on the absence of ethylene signal, via a MAPK (Mitogen activated protein kinase) cascade. While, phosphorylation of EIN2 by CTR1 prevents EIN2 from ethylene signaling in the absence of ethylene. ET regulates SA induction both negatively and positively while acts synergistically with JA against necrotrophic pathogens (Pr´e et al. 2008). ET signaling regulates the JA-SA crosstalk by

modulating the NPR1 expression, although the exact mechanism is unknown (Koornneef et al. 2008).

In addition to above-described hormones, several other phytohormones are known to be involved in immune response pathways. ABA (Abscisic acid) plays an important role in biotic stress (Spoel and Loake 2011) and was shown to have a negative effect on JA or ET mediated defense gene expression. On the contrary, ET and JA exert both positive and negative effects (Lackman et al. 2011) on ABA regulated gene expression. Plants seem to balance ABA-mediated abiotic stress tolerance and SA-mediated biotic stress tolerance (Anderson et al. 2004). Another phytohormone, auxin, mediates signaling for immune response against biotrophs. Usually, SA and auxin signaling suppress each other's induction (Robert-Seilaniantz et al. 2011). Apart from this, suppressor of JA signaling is also expressed during the auxin response, making it more susceptible to necrotrophs (Grunewald et al. 2009). Further, Gibberllins, an important plant hormone, leads to degradation of DELLA protein, a growth repressing protein, which in turn shift SA-JA balance, resulting in enhanced SA signaling and resistance against biotrophs (Navarro et al. 2008). Similarly, cytokinins act on SA signaling sector against biotrophic pathogen and play its role in modulation of plant immunity (Choi et al. 2011). All these suggest that different cross-talks among these hormones occur on microbial challenge in the plant system.

1.7.5. Pathogenesis-related protein: PR-genes

PR proteins are antimicrobial proteins which are either not present or present at basal concentrations detectable in healthy tissues, but they get induced and accumulated upon pathogenic invasion. PR proteins have dual cellular localization depending on their chemical nature. Acidic and basic PR proteins are localized in vacuolar and apoplast respectively (Van Loon and Van Strien 1999). Most of the PR proteins are antifungal, although some act as antibacterial, insecticidal, nematicidal or antiviral. Generally, PR proteins inhibit microbial activity by their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability. All PR proteins and their mechanism of action have been listed in Table 1.3.

Table 1.3. Recognized families of pathogenesis related proteins in plants.

Families	Type member	Properties	Reference
PR-1	Tobacco PR1	Antifungal	Antoniw et al. 1980
PR-2	Tobacco PR2	ß-1,3-glucanase	Antoniw et al. 1980
PR-3	Tobacco P,Q	Chitinase-I,II,IV,V,VI,VII	van loon 1982
PR-4	Tobacco 'R'	Chitinase-I,II	van loon 1982
PR-5	Tobacco S	Thaumatin like	van loon 1982
PR-6	Tomato inhibitor I	Proteinase inhibitor	Green and Ryan 1972
PR-7	Tomato P69	Endoproteinase	Vera and Conejero 1988
PR-8	Cucumber chitinase	Chitinase Type III	Metraux et al. 1988
PR-9	Tobacco peroxidase	Lignin forming peroxidase	Lagrimini et al. 1987
PR-10	Parsley 'PRI'	Ribonuclease-like	Somssich et al. 1986
PR-11	Tobacco chitinase	Chitinase Type I	Melchers et al. 1994
PR-12	Raddish Rs-AFP3	Defensin	Terras et al. 1992
PR-13	Arabidopsis TH12.1	Thionin	Epple et al. 1995
PR-14	Barley LTP4	Lipid transfer protein	Garcia-Olmedo et al. 1995
PR-15	Barley OxOa	Oxalate-oxidase	Zhang et al. 1995
PR-16	Barley OxOLP	Oxalate-oxidase like	Wei et al. 1998
PR-17	Tobacco PRp27	Unknown	Okushima et al. 2000

1.7.6. Immune system against beneficial bacteria

Establishment of plant-beneficial association occurs by mutual recognition and a high degree of coordination or a constant molecular dialogue between plant and symbiotic bacteria (Zamioudis and Pieterse 2012). This molecular dialogue between the two partners helps the latter to get established inside the plant, whereas the attacking pathogen is warded-off by plant being recognized as a potential threat for plant health.

A longstanding paradigm existing about pathogen and mutualists is that, they evolved in different ways. For pathogens, 'Antagonistic arm race' model for its relationship with host has been proposed, in which both host and pathogen increase their own fitness during interaction. A traditional model for mutualists is 'Mutualistic environment' in which both microbes and hosts benefit each other by enhancing other's fitness. Although a recent theory similar to 'Antagonistic arm race' model predicts that slowly evolving mutualists, which increase their own fitness are preferred and they are selected by host plants to minimize costly contributions to host (Sachs et al. 2011).

Evidences are emerging that beneficial bacteria have developed decoy strategies to short-circuit hormone regulated defense responses, which paves the way for long term association for mutualism (Jacobs et al. 2011). Certain beneficial microbes undergo phenotypic or phase variation by reversibly switching into different morphologies in different conditions (Davidson and Surette 2008). For example, in colonizing *Arabidopsis* root tips *Pseudomonas brassicacearum* uses a flagellin-overproducing hypermotile phase variant (Achouak et al. 2004). There could be several mechanisms behind this phenomenon such as site-specific rearrangements and epigenetic modifications (Wisniewski-Dye and Vial 2008). Modulation of host immunity by PGPF (Plant growth promoting rhizobacteria/fungi) and PGPR has been depicted in Fig. 1.4. Many PGPF and PGPR suppress plant immune response to gain entry inside the plants. For instance, *Piriformospora indica* (PGPF) and *Pseudomonas flourescens* WCS417 (PGPR) suppresses JA (Jacobs et al. 2011) and ET pathways (Verhagen et al. 2004) respectively. Similarly, many free-living PGPR/PGPF (Plant growth promoting rhizobacteria/ fungi) release certain hormones such as auxins and GAs (Lugtenberg and Kamilova 2009) that potentially

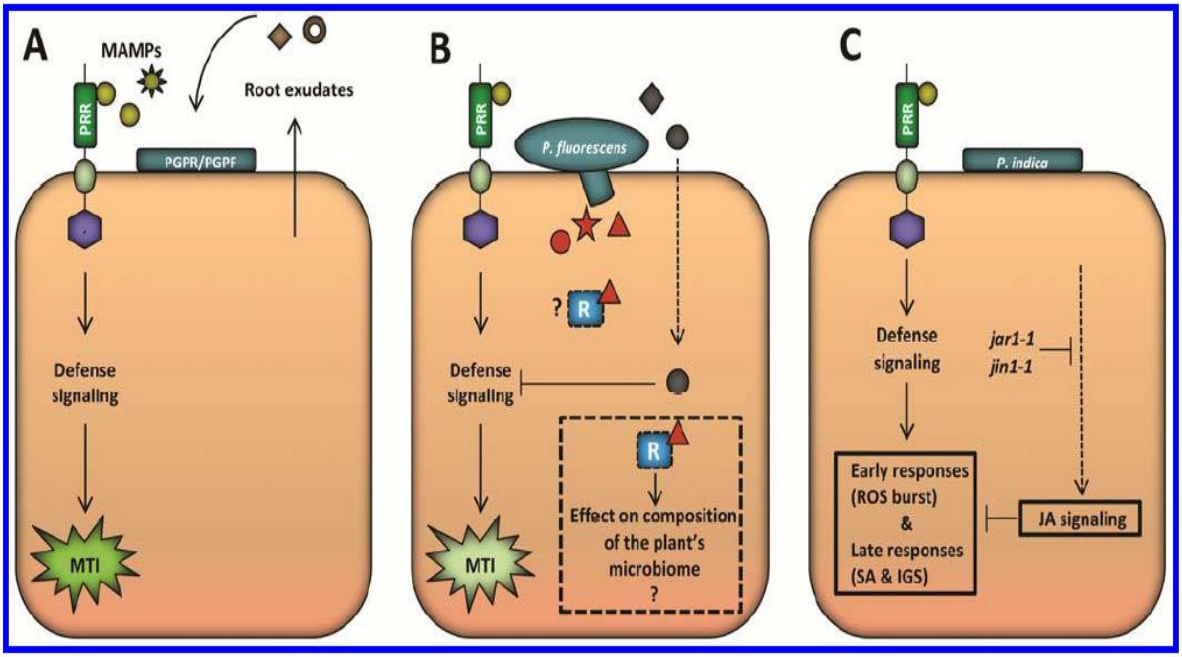


Fig. 1.4. Model for the modulation of host immunity during interactions with nonsymbiotic plant-growth-promoting rhizobacteria (PGPR) and fungi (PGPF). **A,** Root exudates recruit PGPR and PGPF and prime them for interaction. Host plants initially recognize PGPR and PGPF as potential invaders; pattern recognition receptors (PRR) in the host perceive microbe-associated molecular patterns (MAMPs, yellow-colored shapes) and a signaling cascade is initiated, resulting in MAMP-triggered immunity (MTI). **B,** The PGPR *Pseudomonas fluorescens*WCS417 suppresses the MTI response via apoplastic secretion of one or more thus-far-unidentified effector molecules (gray-colored shapes). Whether the secreted molecules act as apoplastic or cytoplasmic effectors and the mechanisms by which they interfere with the host immune system remain currently unknown. Effector molecules (brown-colored shapes) that are secreted via the type III secretion apparatus of *P. fluorescens* and other PGPR are likely to assist but seem not to be essential for MTI suppression. In analogy to root nodule symbiosis, certain type III effectors may be recognized by host resistance (R) proteins which, in turn, may impact the composition of the microbial community in the rhizosphere. **C,** The PGPF *Piriformospora indica* recruits the jasmonic acid (JA) signaling pathway to suppress both early (reactive oxygen species [ROS] production) and late (salicylic acid [SA]-mediated responses and indole glucosinolate [IGS] production) defense responses. This is mediated via the JA signaling components JAR1 (Jasmonate resistance 1) and MYC2 (JIN1) because the JA-related mutants *jar1-1* and *jin1-1* of *Arabidopsis* are unable to suppress MTI. Adopted from Zamioudis et al. (2012).

attenuate SA signaling. PGPR *Pseudomonas fluorescens* WCS417 induces ISR conferring resistance in *Arabidopsis* and it suppresses flagellin-triggered PTI responses in the roots via apoplastic secretion of one or more low-molecular-weight molecule(s) (Millet et al. 2010).

Priming and induction of hormone responses by beneficial bacteria are a kind of auto-regulatory mechanism, which controls colonization of mutualistic bacteria (Zamioudis and Pieterse 2012). Beneficial bacteria share some strategies with that of pathogens to avoid plant-immune response. These include: (i) release of certain aberrant elicitors, e.g., flagellin, which do not lead to induction of defence response, (ii) degradation of flagellin protein by bacterial alkaline protease (AprA) as observed in *Pseudomonas aeruginosa* (Bardoel et al. 2011) (iii) injecting effector molecules in the host by TTSS (Type III secretion system), (iv) role of surface polysaccharides (SPSs) in protection from plant defense responses such as, preventing entry of toxic hydrogen peroxide and oxidative burst, calcium ion chelation and global downregulation of the elicitor-induced transcriptional reprogramming (Tellstrom et al. 2007; Moreau et al. 2011).

In a recent study, two waves of transcriptional reprogramming have been observed during colonization of *Sinorhizobium meliloti* in *Medicago truncatula* (Maunoury et al. 2010). In the first wave, infection of bacteria repressed expression of defense-related genes, which was followed by the activation of a nodule-specific transcriptome. This switch in transcription module depends upon a molecular dialogue between both partners. Rhizobial Nod factors downregulate expression of *MtBGLU1* which encodes glucanase activity in host plant and thus, assists in symbiotic interaction (Mitra and Long 2004). Genomic studies have revealed that plant has flexible and overlapping response towards aggressors (pathogenic) and acquisition of benefits, and can balance the regulation of adaptive immune response between these two classes (Van wees et al. 2008). Simultaneously, attackers and beneficial bacteria have also co-evolved with host to manipulate the latter for their own benefit by altering immune response through modulation of signaling cascades in plants (Pieterse et al. 2012). The fitness cost of priming is lesser than the constitutive expression of defense genes. Therefore, generation of SAR and ISR by pathogen and beneficial bacteria respectively is cheaper as adaptive response at the required time (Saskia et al. 2008). Thus, priming functions as an ecological adaptation of the plant to respond faster to its hostile environment. With few exceptions, beneficial bacteria trigger low

levels of defense response in compatible interactions or even specialized signaling cascades. However, thorough understanding of immune response to endophytic bacteria and its exact mechanism are lacking.

1.8. Gap in existing research

Diazotrophic endophytic bacteria have been isolated from diverse crop plant species such as rice, wheat, maize, barley, corn and other plant species and have been found to enhance plant growth in laboratory studies (Lodewyckx et al. 2002). Pearl millet is of similar importance to other cash crops in several parts of India. However, very few studies have been conducted on isolation and characterization of diazotrophic bacteria from *Pennisetum glaucum* (L.) R. Br. (Hameeda et al. 2006; Pallavi et al. 2010). In earlier studies, rhizoplanic and endophytic association of diazotrophic *Azospirillum* sp. and their impact on growth of pearl millet plant has been observed (Tilak and Subba Rao, 1987). During a field study, Umali-Garcia et al. (1980) reported that pearl millet root hairs adsorb higher number of *Azospirillum* in comparison to other beneficial bacteria (*Azotobacter, Klebsiella* or *Pseudomonas*). Therefore, present work aimed to investigate community structure and biology cultivable endiphytic diazotrophic bacteria. Based on lieterature survey, following gaps was identified in present study.

- The lack of information about the diversity of bacteria specifically isolated from internal tissues leaves a gap in our understanding of an important niche in the microbial ecology of crop plants such as rice, pearl millet and wheat. A few, if any, work has been conducted for the study of diversity of endophytic bacteria from pearl millet plants, a crop of equal importance to other staple food crops in many parts of India. Moreover, dynamics of endophytic bacterial community at various growth stages of any plant growing in natural conditions is not well understood. Therefore, pattern of endophytic bacterial colonization in pearl millet plants has been studied in this work. Additionally, bacterial isolates recovered from pearl millet were also tested for different plant growth promoting and biocontrol activities to exploit them as suitable biofertilizer/biocontrol agent.
- Many research groups across the globe have reported isolation and characterization of PGP bacteria. However, performance of very few PGP bacterial isolates has been tested in actual farming conditions. Therefore, present study makes an effort to test utility of the selected biofertilizer agent at different levels, which include experiments at laboratory green-house and actual farming conditions. Test for the growth of plants in field condition will be helpful in selecting the candidate biofertilizer agent for enhancing crop productivity.

- Despite availability of large literature for endophytic bacterial colonization, clear-cut understanding of mechanisms involved in colonization of endophytes is still required. Since, most of the endophytic bacteria en-route to root interior of host plants from rhizosphere, there must be certain bacterial/host properties or strategies, which make endophytic bacteria a successful colonizer. Among such strategies, modulation of host-immune response may be one of the important ways for compatibility of endophyte-host plant. Knowing the fact that endophytic bacteria too are alien to host-plants, it is intriguing that how do endophytic bacteria evade from immune response of host plants and establish their colonization. To best of our knowledge, study of host plant immune response to endophytic bacteria has not been studied thoroughly. Therefore, present study also aims to characterize various properties critical for colonization process, and to compare immune response of host plants to endophytic and pathogenic bacteria. Understanding of colonization mechanism will be useful in optimal exploitation of endophytic bacteria as biofertilizer agent.

1.9. Objectives of the Proposed Research

Based on the gap identified in present status of endophytic bacterial research, following objectives were taken into consideration in the present study:

1. Isolation of diazotrophic endophytic bacteria from the field grown pearl millet plants at one week interval starting from sowing to harvesting of the plants.
2. To study the plant growth promoting properties of all the pearl millet isolates obtained from pearl millet.
3. Study of temporal dynamics and molecular diversity of cultivable diazotrophic endophytic bacteria isolated from pearl millet plant and identification of the most dominant diazotroph obtained in the entire study.
4. To characterize colonization properties of the efficient endophytic colonizer bacterial isolates.
5. To study immune response of host plants (wheat) to selected endophytic bacteria.

CHAPTER II

Study of community structure and diversity of diazotrophic endophytic bacteria at various growth stages of field grown *Pennisetum glaucum* (L.) R. Br.

"Science arises from the discovery of identity amid diversity."

-William Stanley Jevons

Part of this chapter has been published in the following research article:

1. **Gupta G.**, Panwar J., Jha P.N. Temporal dynamics of *Pseudomonas aeruginosa*: A dominant diazotrophic endophytic bacterium of *Pennisetum glaucum* (L.) R. Br. Applied soil ecology 2013, 64, 252-261.

2.1. Introduction

Cereals are the most consumed food worldwide and with the burgeoning population each year, world food production has to be increased to reach rising food demands around the globe for the projected population in 2025 (www.nwf.org/population). Most of the modern agriculture practices use industrially manufactured nitrogen fertilizer, latter of which is associated with economical and environmental concern (Bhattacharjee et al. 2008). In recent years, use of plant growth promoting diazotrophic (nitrogen fixing) endophytic bacteria (DEB) and their potential to replace or minimize the use of chemical fertilizer has been gaining immense attention for environmental friendly and sustainable agriculture. A diverse array of crop plant species has been screened for the isolation of diazotrophic endophytic bacteria such as rice, wheat, maize, barley, corn and other plant species (Lodewyckx et al. 2002). Impact of endophytic diazotrophic bacterial community on plant growth has been demonstrated both in laboratory and farming conditions (Yanni et al. 1997).

The endophytic bacterial community is responsible for various biological processes important for plant health and development. Thus, studies on shifts in community structure of endophytic bacteria are important for plant growth and yield (Andreote et al. 2009). Community structure of diazotrophic endophytic bacteria and their rate of nitrogen fixation, in turn, depends on various factors such as plant growth stage, nutrient content of substrate, soil composition, plant genotype, type of plant tissue (Hardoim et al. 2008), soil pollution (Oliveira et al. 2010), plant species (Duc et al. 2009), plant rhizosphere (Sato et al. 2009) and temperature (Hsu and Buckley 2009). Composition of bacterial community inhabiting plant root differs from rhizospheric and bulk soil, which suggests that plants play an instrumental role in shaping their microbiome (Lundberg et al. 2012). However, an interesting question arises whether these endophytic bacteria enter the plants through soil or are they seed borne? Depending on biotic and abiotic factors of soil, endophytic community is selected from rhizosphere and rhizoplanic community only, or endophytes can be transmitted inside the plant either horizontally (cross-infection of other plants) or vertically (through seed to next generation) (Gao and Mendgen 2006). With above context, the diversity and richness estimates of diazotrophic bacteria associated with plants are key to our understanding of the role, function and significance of the

nitrogen fixation process in farming conditions (Hong et al. 2006). As the bacterial community structure is dynamic and may subject to changes depending on plant part and age, study of the shifts in microbial community structure in different parts of plant with plant age, is of major ecological and agricultural importance (Andreote et al. 2009). Thus, the understanding of diazotrophic microbial community and its function in a given plant can be utilized to harness beneficial bacteria-plant association for enhancing growth and yield of the plant. However, very few studies have been conducted to analyze periodical changes in the diversity of endophytic bacteria at various plant growth stages (Mahaffee and Kloepper 1997; Cavaglieri et al. 2009; Andreote et al. 2010).

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a staple cereal of the hottest, driest areas of tropics and subtropics that reliably produces both grain and forage. It is the main cereal crop grown during '*Kharif*' (rainy to autumn) season (Manga and Yadav 1995) in Rajasthan, the largest state of India. Its life cycle span is 70-75 days. India is the largest producer of pearl millet, which suffice its demand as staple food, and other purposes. It is a rain-fed crop and can grow well in drought prone arid/semi-arid regions unlike other main crops such as rice, wheat, etc. With the increasing food demands in the populated developing countries, its production can substitute the requirement of main food grains. Therefore, it is important to develop a robust and sustainable agriculture to produce additional food grains in same or even lesser land area (Basavaraj et al. 2010). Despite being the crop of similar importance to other cash crops in several parts of India, very few studies have been conducted on isolation and characterization of diazotrophic endophytic bacteria from pearl millet plants (Hameeda et al. 2006; Pallavi et al. 2010). In earlier studies, rhizoplanic and endophytic association of diazotrophic *Azospirillum* sp. and their impact on growth of pearl millet plant has been observed (Tilak and Subba Rao 1987).

To the best of our knowledge, so far no work has been carried out to systematically study the natural population dynamics of cultivable endophytic bacteria in pearl millet growing in actual farming condition. Therefore, in order to elucidate community structure of endophytic diazotrophic bacteria at various growth stages of plant, the present work aimed to study changes in the microbial community with the growth of pearl millet plants present in natural growth conditions as well as to identify the dominant diazotrophic bacterial species among these. In

addition, the present study also aimed to explore efficient plant growth promoting diazotrophic bacteria, which can be used as a potential biofertilizer to attain sustainable agriculture.

2.2. Materials and methods

2.2.1. Sampling site

The study site was a pearl millet field (60.96×152.40 m^2) located in Pilani, Shekhawati region in the extreme north eastern part (28.37°N 75.6°E) of Rajasthan (India). The study area has an arid climate, uncertain and erratic rainfall (300-500 mm annually), high wind velocity (20.62 Kmph) and nutrient deficient sandy clay loam soil belonging to Typic haplocamborthid class (USDA). In summer, temperature ranges from 35-48°C, while it varies from 1-10°C in winter season and sometimes it falls below 0°C (Ghosh 1991; Shyampura et al. 2002). Healthy plant samples were collected at one week interval from the same plot. For plants collected after 56 days after sowing (DAS), the aerial portion of the plant was divided equally into lower (LS) (closer to root), middle (MS) and upper parts (US) each of 50-60 cm in length. Plant samples were transferred to the laboratory and processed immediately. Each part was processed separately for isolation and analysis of diazotrophic endophytic bacteria.

2.2.2. Preparation of reagent and medium

LGI medium (Composition: per liter, $CaCO_3$ 1.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, $FeSO_4.7H_2O$ 0.1 g, $Na_2MoO_4.2H_2O$ 0.005 g, Sucrose 5 g, pH 5.5) (Tapia-Hernández 2000) was used for isolation and maintenance of the diazotrophic bacteria. Nutrient agar (NA) (HiMedia, India) was used to assess the sterilization effectiveness**.** The 1.5 and 0.3% agar-agar was added respectively for solid and semi-solid medium, wherever required. All reagents were of analytical grade and were prepared using distilled water obtained from a Milli-Q system.

2.2.3. Sampling and soil analysis

Rhizospheric soil samples were collected along with plant samples randomly from the experimental field in triplicate. The uppermost layer of soil (1-2 cm) was removed before taking the samples so as to avoid foreign particles and litter. Soil and plant samples were placed separately in self-sealing polythene bags inside an insulated container and transported

immediately to the laboratory for analysis. Soil adhering to roots was collected carefully using fine brush and treated as rhizospheric soil. Before processing, soil samples were passed through a sieve (2 mm mess size) to remove coarse roots. One portion of each soil sample was air-dried and used for the estimation of various physico-chemical properties. Soil samples were analyzed for pH and electrical conductivity (EC) using digital pH and EC meter respectively, on a suspension of soil and water in the ratio 1:2.5. Available phosphorous (Olsen P) in soil was determined using chlorostannus-reduced molybdophosphoric blue color method by extraction with 0.5 M sodium bicarbonate for 30 min (Olsen et al. 1954). Estimation of organic carbon (OC) was done by the method of Walkley and Black (1934) using 1 N potassium dichromate and back titrated with 0.5 N ferrous ammonium sulphate solution. Total nitrogen was estimated by the method described by Jackson (1967). Available Fe, Mn, Cu and Zn were estimated as described by Lindsay and Norvell (1978). All the physico-chemical analysis mentioned above were carried out at Central Arid Zone Research Institute (CAZRI), Jodhpur.

2.2.4. Isolation of endophytic bacteria

Roots were washed thoroughly under tap water to dislodge soil or dust particles followed by vigorous shaking with sterile water and glass beads to eliminate rhizoplanic bacteria. Seeds and different plant parts (roots and different aerial portions) were processed separately and surface sterilized by immersing in 70% ethanol for 2 min washing three times with sterile water and treatment with 4% sodium hypochlorite for 12 min. Surface sterilized plant samples were thoroughly rinsed with sterilized water (3 times) to remove all the traces of sterilants. A 100 µl of the last wash was spread on Nutrient Agar (NA) media to assess the sterilization effectiveness of the treatment. Surface sterilized seeds and plant parts (root and shoot) were homogenized separately in sterile phosphate-buffered saline (PBS) solution (Composition: per liter, NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.4 g, and KH_2PO_4 0.24 g, pH 7.0) and serially diluted (decimally) to 10^{-3} in PBS. To enrich the growth of diazotrophic bacteria, 100 µl from each dilution was spread on the LGI Agar plates and incubated at 30°C for four days. After incubation, bacterial colonies were counted and grouped on the basis of their morphological characteristics. The most suitable dilution series was selected for counting number of colony-forming units (cfu). Each morphotype was maintained as pure culture and sub-cultured for several generations to ensure their

diazotrophic nature. Glycerol stock (15% w/v) for each isolates was prepared and stored at -70°C until further use.

2.2.5. Extraction of genomic DNA

DNA template for PCR was prepared by the boiling lysis method (Misra et al. 2012). Briefly, a 100 µl sample of overnight grown culture (in Nutrient broth) was harvested by centrifugation at 12,000 g for 1 min, the pellet was washed twice using 50 mM Tris-Cl (pH 8) and suspended in 100 µl Milli-Q water, and the OD_{600} (optical density) of culture was adjusted to 1.0. The sample was boiled in water bath for 15 min and immediately placed on ice. Then, the sample lysate was centrifuged at 16,000 g for 15 min. The supernatant that contained the DNA was quantified at A_{260} in a spectrophotometer (JASCO, USA). For amplification of *nifH* gene (section 2.2.10.1.2), genomic DNA of bacteria was prepared using genomic DNA extraction kit (Qiagen, USA) following the manufacturer's instructions.

2.2.6. Enterobacterial repetitive intergenic consensus sequences-PCR (ERIC-PCR)

ERIC PCR is a repetitive DNA based DNA finger-printing method, which can be used for typing bacteria upto strains level. ERIC is so named as this stretch of repetitive DNA was first described in *Esherichia coli* and Enterobacteriaceae family members (Sharples and Lloyd 1990). DNA template for ERIC-PCR was prepared by "Boiling lysis method" as described above. Fifty ng of DNA template was used as the template for ERIC-PCR, which was performed using PTC thermal cycler (MJ Research, Inc., Walthon, MA, USA) as described by Versalovic et al. (1991). Final volume of the PCR reaction mixture was 50 µl, which consisted of 1.5 units of Taq DNA polymerase, 125 µM each dNTPs (Deoxynucleotide triphosphate), 1.5 mM $MgCl_2$ and 30 pmol of each primer. The specific primers (Sigma-Aldrich, USA) used were 1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and 2R (5′-AAGTAAGTGACTGGGGTGAGCG-3′). PCR reaction included an initial denaturation at 94^{o}C for 5 min followed by 35 cycles of 1 min at 94^{o}C, 1 min at 55^{o}C and, 1 min at 72^{o}C with a final extension of 5 min at 72^{o}C. The amplified products were analyzed on 2% agarose gel using a gel documentation unit (Bio-Rad, Hercules, California, USA). The ERIC profiles were analyzed by studying the pattern of DNA amplicons

in gel of all the isolates obtained at different weeks. Isolates showing identical banding pattern were considered to be the same strain.

2.2.7. Amplification of 16S ribosomal RNA gene

The DNA template was prepared as described above. The specific primers (Sigma-Aldrich, USA) used were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1494R_C (5′-TACGGCTACCTTGTTACGAC-3′) where M=A or C (Weisburg et al. 1991). The PCR mixture included 50 ng of template, 1.5 unit of Taq DNA polymerase, 125 µM each of dNTPs, 1.5 mM $MgCl_2$ and 30 pmol of each primer. The PCR products were amplified by an initial denaturation step at 94^oC for 5 min, 35 cycles of 1 min at 94^oC, 1 min at 55^oC, and 1 min at 72^oC with a final extension of 5 min at 72^oC. Amplified PCR products were visualized on 1% agarose gel and further sequencing of PCR product was carried out to identify the particular isolate.

2.2.8. 16S ribosomal RNA gene sequencing

The 1.5 kb amplicons of 16S rRNA gene obtained by PCR were purified using QIAquick PCR purification kit (Qiagen, USA) following manufacturer's instructions. The 16S rRNA genes were sequenced by the dideoxy-chain termination method using an automated DNA sequencer (ABI Prism, USA). Bacterial taxonomic affiliations were assigned based on the closest match to sequences available at the NCBI (National center for biotechnology information) database (http://www.ncbi.nlm.nih.gov/) using the BLAST (Basic local alignment search tool) algorithm and submitted to NCBI GenBank (Altschul et al. 1997). A 98% threshold of 16S rRNA gene sequence match was used to assign the taxa of isolates. Phylogenetic tree was constructed using partial rRNA gene sequences of bacterial isolates obtained in this study using Neighbour Joining method in MEGA 4.0 (Tamura et al. 2007).

2.2.9. Diversity index and relative species abundance (RSA)

Calculation of diversity (H) index (e.g., Shannon diversity index) (Magurran 2004) and preparation of the diversity curve was done for weekly obtained isolates (using PAST software version 2.10) for all plant parts separately. It was based on the data corresponding to the total number of bacterial isolates (cfu) per identified species obtained at different growth stages of pearl millet. Identification of species was based on 16S rRNA gene sequence analysis. In

Shannon's diversity index zero corresponds to the dominance. As the value increases from zero, it depicts an increase in the diversity. The term "Dominance" means that certain species were dominating in the particular sample, while "evenness" means that none of the isolate was dominant and diversity was high.

The relative species abundance (RSA) was calculated to study the relative abundance of bacteria isolated from various plant parts at different time intervals (Atlas and Bartha 1987). Formula used for calculating RSA (Relative species abundance) was: cfu of i^{th} species/total cfu of endophytic bacterial species, where i^{th} species refers to the species of interest. The rank abundance curves (Preston curve) were also prepared (using PAST software 2.10) for identification of species ranked on the basis of abundance at particular sampling time.

2.2.10. Estimation of plant growth promoting properties

2.2.10.1. Confirmation of diazotrophy

As mentioned earlier, primary confirmation for diazotrophy was done by subculturing the different bacterial isolates in selective media (LGI) lacking any organic or inorganic source of fixed nitrogen for several generations. Nitrogen fixation ability was quantified using acetylene reduction assay. Further, amplification of *nifH*, a structural gene encoding nitrogenase reductase of nitrogenase complex, was carried out to confirm nitrogen-fixing ability of isolates on the molecular level.

2.2.10.1.1. Acetylene reduction assay: Acetylene reduction assay was used to quantify activity of nitrogenase (a key enzyme in nitrogen-fixation reaction) following method of Hardy et al. (1971). All the isolates were inoculated on N-free LGI slants incubated for 96 h at 30°C. Acetylene was injected into each tube using a hypodermic syringe to attain 10% final concentration, and the tubes were incubated at 30°C without shaking. After 24 h of incubation, 0.2 ml of gas phase was removed and the amount of ethylene produced from reduction of acetylene was analyzed using Nucon 5700 Gas Chromatograph (Nucon Engineers Ltd., New Delhi) equipped with a Porapak N column and flame ionization detector. Nitrogen was used as the carrier gas. Culture of standard *Escherichia coli* was used as negative control. Three replications for each treatment and control were subjected to analysis. Culture present on the

slant was carefully scrapped and used for estimation of soluble protein by Lowry's method (Lowry et al. 1951). ARA activity was expressed in n moles of ethylene produced mg^{-1} protein hr^{-1}.

2.2.10.1.2. Amplification of *nifH* gene: To confirm diazotrophy at the molecular level, a structural gene, *nifH* encoding dinitrogenase reductase, was amplified using the pair of *nifH* specific universal primers: Pol F (5′-TGCGAYCCSAARGCBGACTC-3′) and Pol R (5′-ATSGCCATCATYTCRCCGGA-3′) (Sigma Aldrich, USA), where Y=C/T, S=G/C, R=A/G, B=G/T/C (Poly et al. 2001). Each PCR reaction mix contained 1X Taq DNA polymerase buffer, 50 pmol of each primer, 125 µM each dNTPs, 1 U Taq DNA polymerase and 50 ng of template DNA. The cycling conditions used were: 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 30 s followed by extension at 72°C for 5 min. The amplified product was analyzed on 2% agarose gel. Genomic DNA of *E. coli* and *Azotobacter chroccum* were used as negative and positive controls respectively. The amplified product was electrophoresed on 2% agarose gel containing ethidium bromide and analyzed using the Gel documentation system.

2.2.10.2. Test for indole acetic acid production

Colorimetric assay was used for estimating IAA production as per method of Gordon and Weber (1951). Cultures were grown in Nutrient broth containing 100 µg ml^{-1} tryptophan for 72 h at 30°C with shaking at 180 rpm (revolution per min) in BOD shaker-incubator. Uninoculated media was used as control. After incubation, 1.5 ml culture was centrifuged at 12,000 g for 2 min. In tubes, 1 ml supernatant of culture was added along with 2 ml IAA reagent (as per following details) and kept for 30 min at room temperature. Development of cherry-red color was scored positive for the test. On the basis of intensity of red color, qualitatively isolates were ranked poor (+), average (++) and good (+++) for IAA production. The optical density of the samples was read at 530 nm (blank from respective medium was used). The amount of IAA was quantified using standard of pure IAA prepared separately.

IAA Reagents: A) 1 ml 0.5 M $FeCl_3$, B) 50 ml 35% $HClO_4$

Both reagents A and B were made separately and mixed on requirement, as the IAA reagent should be used freshly for estimation.

2.2.10.3. Phosphate solubilization

Screening for phosphate solubilization activity of different isolates was done using NBRIP (National botanical research institute-phosphate) medium (Composition: per liter, glucose 10 g, $Ca_3(PO_4)$ 5 g, $MgSO_4.7H_2O$ 1 g, KCl 0.2 g, NaCl 1 g, NH_4Cl 5 g and 2% general purpose agar-agar, pH 7.0) by the method of Mehta and Nautiyal (2001). Freshly grown cultures were point-inoculated on NBRIP medium and incubated at 30^oC for 4 days. Clear zone around the inoculated culture was observed and on the basis of zone size, isolates were ranked poor (+), average (++) and good (+++) for Phosphate solubilization activity.

Free phosphate released by bacterial isolates was quantified by the method of Marinetti (1962). For this, 1.5 ml of 3 days old culture was pelleted down by centrifuging at 12,000 g for 2 min. 3.5 ml of reagent C (as per following details) was added to the supernatant of culture, mixed properly and, the reaction mix was incubated at room temperature for 1 h. Standard curve was prepared using varying concentrations of K_2HPO_4 by following the same procedure described above, and OD (optical density) was taken at 660 nm against the blank.

Reagent A: 10% Ascorbic acid (stored at 4^oC)

Reagent B: 42% Ammonium molybdate in 1 N H_2SO_4

Reagent C: 1 volume of Reagent A + 6 volumes of Reagent B

2.2.10.4. Lowry's method

The reagents were prepared as given in Appendix I. Different dilutions of BSA (Bovine serum albumin) ranging from 0.1 to 1.0 mg ml^{-1} was prepared. 0.2 ml of protein samples and standards were added to 2 ml analytical reagent and incubated at room temperature for 10 min. Then, 0.2 ml of 1 N Folin-Ciocalteau reagent was added and incubated at room temperature for 30 min in dark conditions. After that, OD of the resulting reaction mix was measured at 660 nm and the concentration of protein was estimated using standard curve method.

2.2.10.5. Siderophore production

Siderophore production was determined following the method of Schwyn and Neilands (1987). For this, bacterial isolates were spot inoculated on Chrome-Azurol-S (CAS) agar medium. Composition of CAS-reagent was: 1 mM CAS, 10 ml $FeCl_3.6H_2O$ (1 mM stock) made in 10 mM HCl and 2 mM N,N-cetyl trimethyl ammonium bromide (CTAB). It was autoclaved separately and added to 300 ml of NA medium. Cultures were incubated for four days at 28°C. Appearance of yellowish or orange zone around bacterial growth was considered positive for siderophore production.

Isolates showing positive results for siderophore production in the plate assay were subjected for quantitative estimation of siderophore. Siderophore was quantified using the CAS shuttle assay (Schwyn and Neilands 1987) in which the percent siderophore units were calculated according to the formula: $[(A_r-A_s)/A_r]\times100$, where A_r and A_s represent absorbance after reaction and absorbance of shuttle solution respectively.

Siderophore produced by bacterial isolates were subject for their typing using standard protocols. They were tested for two of the main siderophore types namely catacholate and hydroxymate.

2.2.10.5.1. Catacholate type

Test for the catacholate type of siderophore was done by the method of Arnow (1937). Bacterial isolates were grown in $JNFb^-$ medium supplemented with 1 μM $FeCl_3$. 1 ml each culture was harvested at 6500 g for 5 min and culture supernatant was saved for analysis.

Reagents

a. 0.5 N Hydrochloric acid

b. Nitrite-molybdate reagent: 10 g of sodium nitrite and 10 g of sodium molybdate were dissolved in 100 ml distilled water.

c. 1 N Sodium hydroxide

1 ml each of 0.5 N hydrochloric acid and nitrite-molybdate reagent was added to 1 ml culture supernatant. Thereafter, 1 ml of 1 N sodium hydroxide was added, and volume was maintained to 5 ml with water. Appearance of bright-red colour was considered positive for the presence of catecholate type of siderophore.

2.2.10.5.2. Hydroxamate type

Detection of hydroxamate type of siderophores was done using the Csaky (1948) assay. It detects the presence of secondary hydroxamates and depends on the oxidation to nitrite and formation of a colored complex via diazonium coupling. As described in catacholate typing, the culture supernatant was obtained and hydrolyzed with 1 ml of 6 N sulfuric acid in a boiling-water bath for 6 h in an assay tube. It was then buffered by adding 3 ml sodium acetate solution. One ml of sulfanilic acid solution was added, followed by the addition of 0.5 ml iodine solution. After 5 min, excess iodine was neutralized by adding 1 ml sodium arsenite solution. Thereafter, 1 ml α-naphthylamine solution was added. Finally, 1.5 ml distilled water was added to make final volume to 10 ml and incubated for 30 min. At this point, a cherry color indicated the presence of hydroxamate type of siderophores.

2.2.10.6. Antagonistic activity

The antagonistic activity of the dominant diazotrophic bacterial species was determined by the standard agar well diffusion. The antagonistic activity assay was carried out against other plant associated bacteria [both Gram positive (*Arthrobacter woluvensis*, *Bacillus* sp., *Bacillus subtilis*, *Microbacterium* sp.) and Gram negative (*Ochrobactrum* sp., *Pseudomonas oryzihabitans, Enterobacter* sp.)] to understand the possible reason for community shift of endophytes with the growth of pearl millet plants. Briefly, 6 mm diameter wells were prepared using sterile metallic borer in fresh lawn cultures of selected endophytic bacteria growing on NA. Fresh broth culture (100 µl) of dominant endophyte was inoculated in wells. Boiled culture was used as controls. The plates were incubated at 30°C for 24 h. The experiment was performed in triplicate. Antagonistic activity was determined by measuring the zone of inhibition for which

following criteria were used: zone of inhibition <1 cm = poor (+), between 1-2 cm = average (++), >2 cm = good (+++).

2.2.10.7. Test of cellulolytic and pectinolytic activity

Production of certain hydrolytic enzymes may be required by endophytic bacteria to enter inside the plant. Therefore, all the isolates were evaluated for cellulolytic and pectinolytic enzymes.

2.2.10.7.1. Endoglucanase activity: Production of endoglucanase by endophytic bacteria was tested employing Gram's iodine method with slight modifications (Kasana et al. 2008). Bacterial cultures were point inoculated onto $JNFb^-$ agar media supplemented with 0.2% carboxymethyl cellulose (CMC) and 0.3% tryptone. After inoculation, plates were incubated for four days at 30°C. Gram's Iodine solution was poured onto bacterial growth and kept for 5 min at room temperature. Different cultures were scored as poor (+), average (++) and good (+++) for endoglucanase activity on the basis of diameter of clear zone.

2.2.10.7.2. Cellobiohydrolase activity: Exponentially growing cultures of test bacteria were spot inoculated on $JNFb^-$ solid plates containing 0.5 mM ammonium chloride and ethanol (6 ml $litre^{-1}$) as a nitrogen and carbon sources respectively. The cultures were grown for 3 days at 30°C and then incubated at 37°C with the appropriate overlay containing 8 ml of 0.05 M potassium phosphate (pH 7.0), 0.7% agarose and 0.5 µg 4-methylumbelliferyl-β-cellobioside (MUC). After 4 to 10 h, plates were exposed to 302 nm of UV light on a trans-illuminator, and the active colonies were identified by the appearance of violet fluorescence (Reinhold-Hurek et al. 1993). Scoring for activity was based on the level of fluorescence under UV light.

2.2.10.7.3. Glucosidase activity: For determination of glucosidase activity, bacteria were grown and assayed as described for the test of cellobiohdrolase activity with minor change. 4-methyllumbelliferyl-β-cellobioside (MUC) was used instead of 4-methyllumbelliferyl-β-D-glucoside (MUG) was used in overlay (Reinhold-Hurek et al. 1993).

2.2.10.7.4. Pectinase activity: Overnight grown cultures were spot-inoculated onto NA plates containing 0.5% pectin and grown for 72 h at 30°C. Bacteria growing on plates were overlaid

with 2% CTAB (N-cetyl-N,N,N trimethyl-ammonium bromide) and kept for 30 min at 30°C. This was followed by washing three times with 1 N NaOH for 10 min each. Plates were then screened for appearance of clear zones around the colonies visually.

2.2.11. Statistical analysis

Standard deviation was calculated for various replicates in different experiments using Microsoft Excel 2007 software. All population data (triplicates) were converted to log colony-forming units per gram fresh weight tissue. All populations below the detection limits were scored as 0 for calculation of means (Kloepper and Beauchamp 1992). Difference in population data of a particular sample at different time intervals was statistically analyzed by ANOVA (Analysis of variance). Least Significant Difference (LSD) test at probability level 0.05 was used to separate the means when the ANOVA F-test indicated a significant effect of the treatments (weekly isolation of bacterial population) as well as to analyze the significant changes in PGPR treated plants than control. Further, means of different samples were compared by Duncan's Multiple Range Test (DMRT), wherever applicable (Quinn and Keough 2002).

2.3. Results

2.3.1. Soil physico-chemical characteristics

Pre-sowing soil properties of selected field was as follows: pH 9.3, EC (Electrical conductivity) 0.43 dSm^{-1}, OC (Organic Carbon) 0.06%, Olsen P (Available Phosphate) 11.9 mg Kg^{-1}, total N 102.7 mg Kg^{-1}, Zn 0.56 µg g^{-1}, Mn 13.63 µg g^{-1}, Fe 24.24 µg g^{-1}, Cu 1.09 µg g^{-1}. The selected field was not supplemented with any kind of organic or inorganic fertilizers.

2.3.2. Isolation of endophytic bacteria

Surface sterilization effectiveness was achieved throughout the study. Bacterial colonies appearing on nitrogen deficient medium were considered as diazotrophic bacteria. Bacterial isolates recovered from various plant samples collected at different stages of plant growth were selected randomly on the basis of their morphology for further study. Overall, 210 different morphotypes of diazotrophic endophytes were obtained during the entire study.

In order to elucidate dynamics of diazotrophic bacterial community in pearl millet, change in population of diazotrophic bacteria was compared with total endophytic bacterial population at various growth stages of plants (Fig. 2.1). The pearl millet seeds used in this study were found to be colonized with five different diazotrophic morphotypes with a total population of 0.5×10^4 g^{-1} seed. However, out of these, only two morphotypes were observed in plant roots at 7 DAS. The overall diazotrophic population size attained during the growth period of plants ranged from 0.2×10^4 to 8.6×10^4 cfu g^{-1} fresh weight of plant material with maximum population size observed at 42 DAS in the root (Fig. 2.1. a) and at 70 DAS in shoot. In roots, the total population of diazotrophic endophytic bacteria significantly increased up to 42 DAS followed by subsequent reduction from 56 DAS (n=10, $p<0.05$). While, in shoots, the total diazotrophic population increased between 35 and 49 DAS (n=10, $p<0.05$). At 63 and 70 DAS, a significantly higher population of diazotrophic bacteria was observed in the middle portion of shoot in comparison to that of lower and upper portion (n=10, $p<0.05$). The total endophytic population was always greater than the diazotrophic endophytic population. However, population of diazotrophic bacteria increased during vegetative growth of the plants. During most part of the study period, bacterial population in root samples was higher than shoot samples except at 63 DAS where it was much higher in middle part of shoot than in the root (Fig. 2.1. b). Diazotrophic population varied in lower, middle and upper part of the plants during different stages of plant growth (Fig. 2.1. b). More diverse bacterial species, i.e., 13 and 21 morphotypes, were recovered during early (upto 28 DAS) and late (63 DAS MS to 70 DAS) growth stages of plants respectively than that of active growth stage of plants (from 35 DAS to 63 DAS). A single morphotype (*Pseudomonas aeruginosa* PM389) pre-dominated the diazotrophic endophytic community in the middle stage of plant growth from 28-56 DAS and 28-42 DAS in root and shoot respectively (Fig. 2.2). A gradual increase was observed in the cfu count of *P. aeruginosa* PM389 in root and shoot portion up to 42 DAS (8.6×10^4) and 35 DAS (1.4×10^4) respectively (n=10, $p<0.05$) (Fig. 2.2). However, a lesser colony count was observed during initial weeks (21 and 28 DAS) in shoot portion as compared to roots. A significant decrease in the cfu count of *P. aeruginosa* PM389 was observed from 42 DAS in roots and from 49 DAS in shoots (n=10, $p<0.05$). This diazotroph was not recovered in root samples from 63 DAS. The population of *P.*

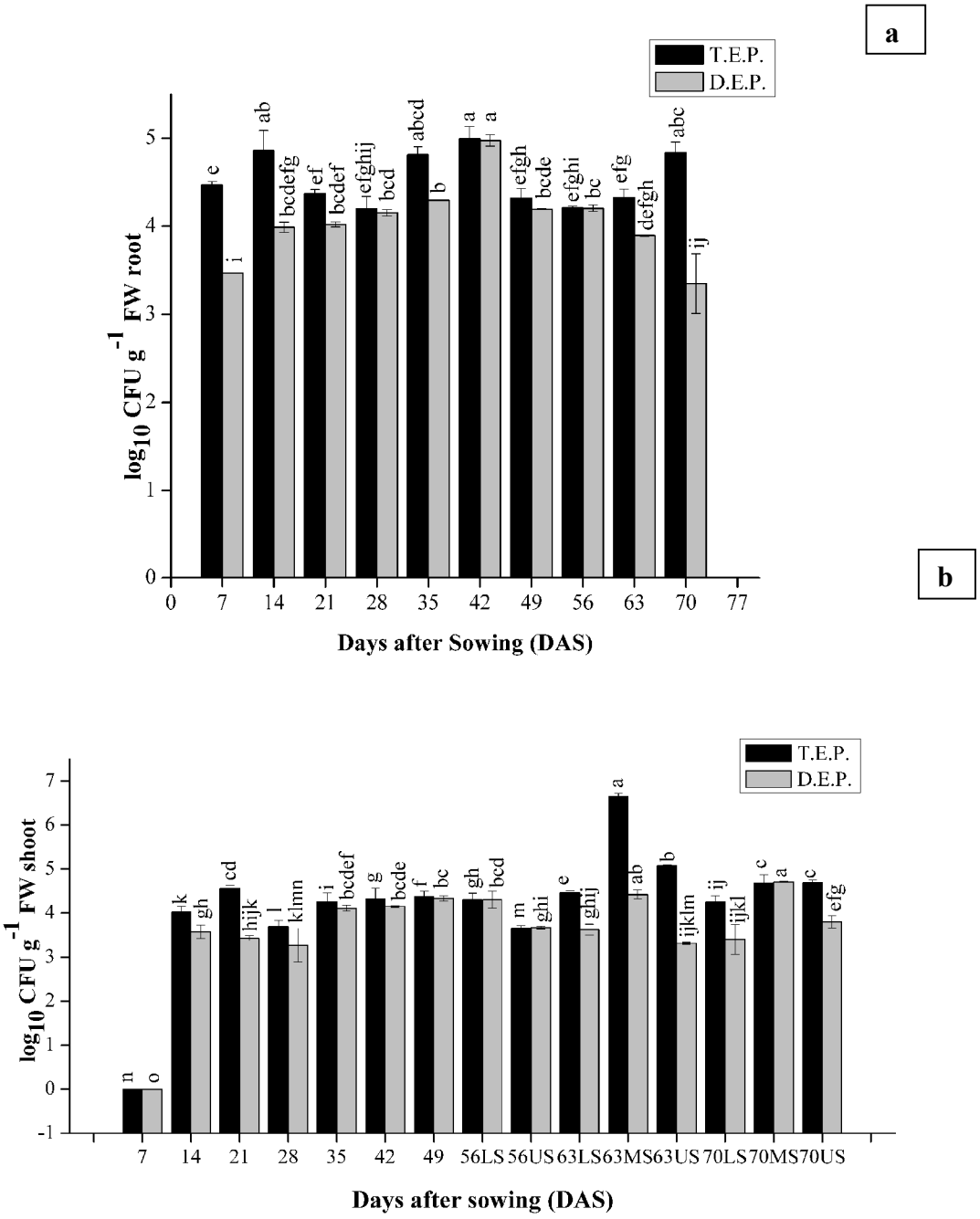


Fig. 2.1. Comparison of total endophytic (T.E.P.) and diazotrophic endophytic population (D.E.P.) with the growth of *Pennisetum glaucum* in (a) Root and (b) Shoot. Letters L, M, U denote lower, middle and upper parts of stem. Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters in different samples.

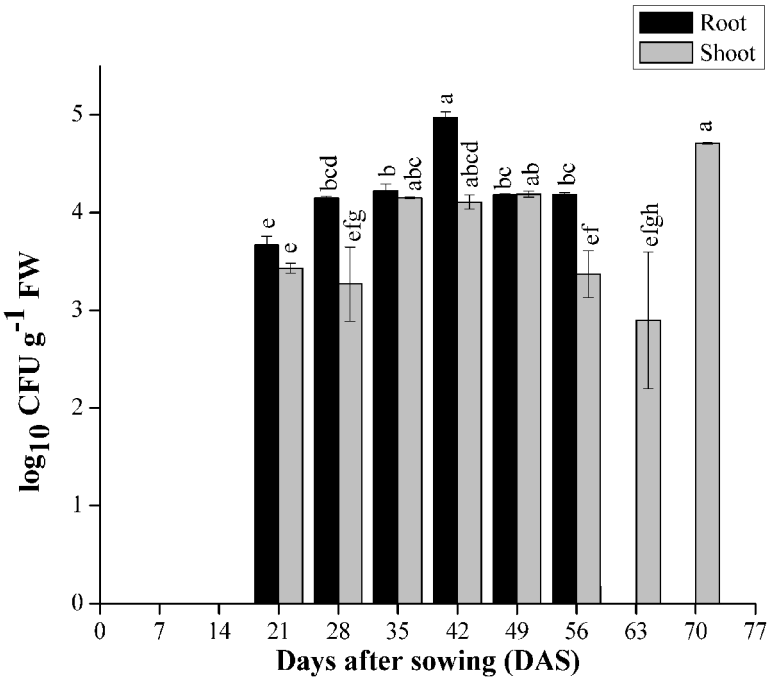


Fig. 2.2. Changes in the *Pseudomonas aeruginosa* PM389 population in root and shoot of pearl millet plants at various growth stages. At 56 and 63 DAS, data for lower shoot and at 70 DAS, data for middle shoot population are represented. Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters.

aeruginosa PM389 was found to be reduced in the lower shoot from 49 DAS with a significant increase in the middle shoot portion in the last week (70 DAS) (n=10, $p<0.05$) of plant growth. No diazotrophic bacterial population was observed in seeds formed in the cob obtained at crop maturity.

2.3.3. Study of molecular diversity using ERIC-PCR as biomarker

With a view to study molecular diversity among various isolates obtained at different growth stages of pearl millet plants, ERIC-PCR based DNA fingerprinting was carried out. ERIC profile of each isolate was also used as a marker to track the presence of corresponding endophytic diazotrophic bacteria at various growth periods of plants. Out of 210 isolates, amplification of DNA segments appeared in only 174 isolates. Therefore, the remaining 36 isolates were directly sequenced, identified, and given data was used for diversity analysis. Based on the similarity in patterns of amplicons resulting from ERIC-PCR, bacterial isolates were grouped in 40 different ERIC types (Fig. 2.3. a). Out of 40 ERIC types, recurrence of representative strains from 5 different ERIC-types was observed at different time periods of plant growth (Fig. 2.3. b). Strain belonging to ERIC type IV was found to be the most dominant and was present in most of the growth period (from 21 DAS to 70 DAS). It was tracked in both root and shoot from 21 DAS to 56 DAS. However, at 63 DAS, recovery of type IV strain was obtained only in the lower part of shoot, while it was tracked only in middle part of the shoot at 70 DAS. In addition, representative strains of a few other ERIC types could also be recovered from plant samples collected at two to three subsequent weeks (Fig. 2.3. b). ERIC profile specific to dominant bacterial strain appeared from 21 DAS. At 21 DAS, out of nine root isolates, three showed identical banding pattern corresponding to that of *P. aeruginosa* PM389. In shoot isolates, other ERIC-types having banding pattern similar to that of *P. aeruginosa* PM389 (40% banding pattern) were also present. At 28 DAS, two ERIC-types were observed in root. Further, single morphotype was prevalent from 28 to 63 DAS in shoot and from 35 to 49 DAS in the roots. ERIC results from randomly picked colonies belonged to ERIC-type of dominant isolate *P. aeruginosa* PM389. However, abundance of *P. aeruginosa* PM389 decreased in roots from 49 DAS. ERIC profile specific to *P. aeruginosa* PM389 was not obtained after 63

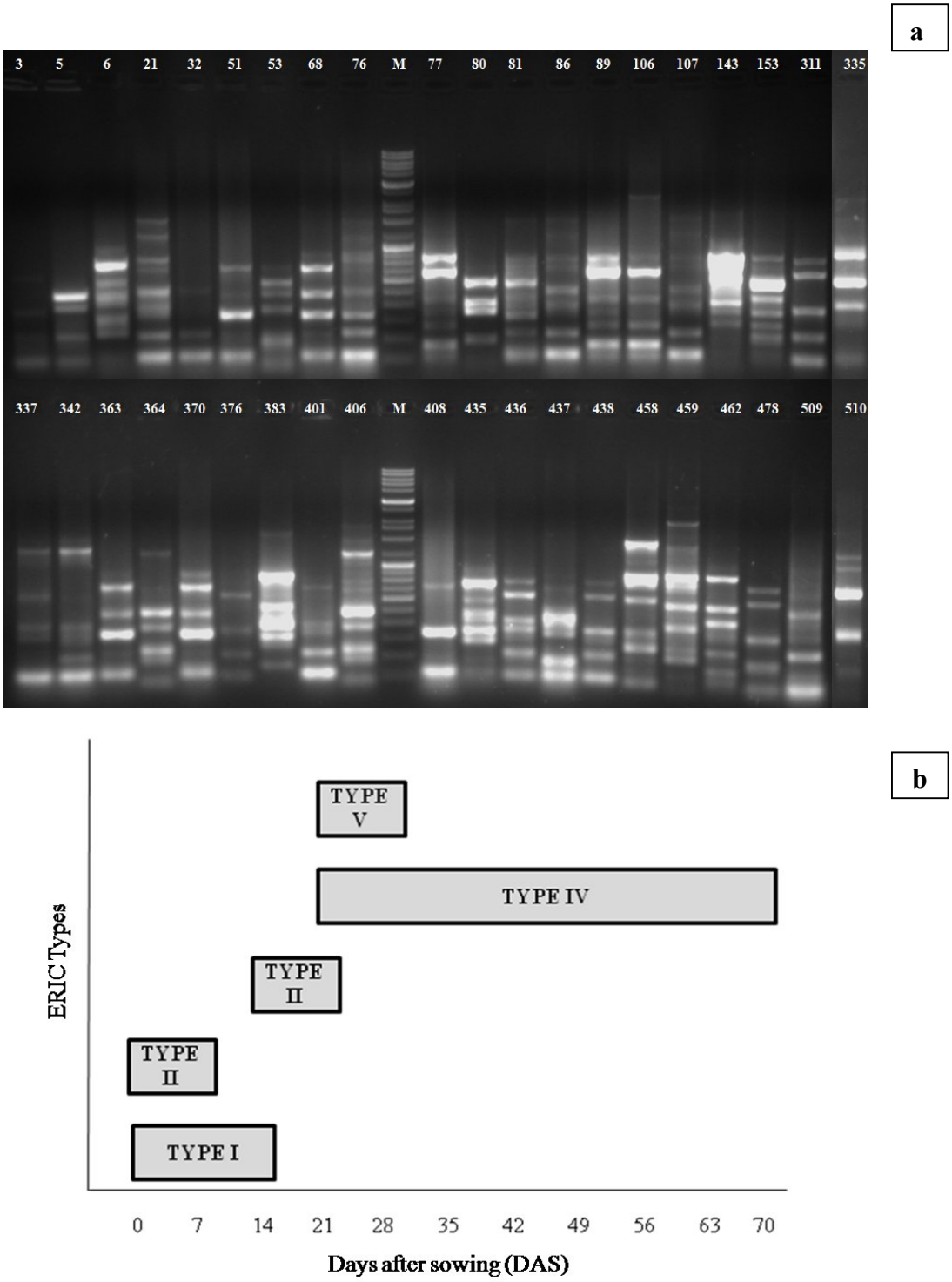


Fig. 2.3. a) Different ERIC types (Labeled by isolate last no., for eg PM389 PM-Pearl millet, Week-3, isolate last No.-89) obtained in the entire study period. b) Depiction of recurrence of ERIC-types at different growth stages of *Pennisetum glaucum* under field growth condition.

DAS in roots. On the other hand, it was present only in the lower part of shoot at 56 and 63 DAS and in the middle part of shoot at 70 DAS. In addition to *P. aeruginosa* PM389, one other strain showing similar DNA profile was also identified as *P. aeruginosa* on the basis of rRNA gene sequence. Moreover, out of 36 isolates showing no result in ERIC PCR, 3 more isolates were further identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence.

2.3.4. Phylogenetic analysis based on 16S rRNA gene sequence analysis

Overall, 41 Gram-negative and 35 Gram-positive isolates were obtained from plant samples at different growth stages. Phylogenetic analysis based on 16S rRNA gene sequence revealed affiliation of these 76 isolates to three phyla namely Proteobacteria, Firmicutes, and Actinomycetes (Fig. 2.4. a), which constituted 54, 21 and 25 percent respectively. In proteobacterial population 83% and 17% bacteria belonged to γ-Proteobacteria and α-Proteobacteria, respectively (Fig. 2.4. b). Various bacterial species obtained in the present study are listed in Table 2.1. In addition to *P. aeruginosa* PM389, few other isolates were also identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence analysis.

2.3.5. Analysis of diversity of diazotrophic bacterial species obtained in different weeks

Once the isolates belonging to each ERIC types were identified, pattern of species distribution at different period of plant growth was analyzed. As described above that ERIC results were obtained for 36 isolates, they were directly identified by 16S rDNA sequencing. Thus, some species could be similar to the member of ERIC-types classified earlier and therefore, the data for recurrence of different species during various stages of plant growth vary from the data based on ERIC-pattern.

Seed borne isolates, identified as *Microbacterium assamensis* and *Pantoea* sp. were recovered till 14 DAS. *Enterobacter cloacae* showed its presence from 14 to 28 DAS in root and at 21, 56 and 63 DAS in shoot. Another isolate belonging to *Enterobacter* sp. was also observed in roots at 28 and 56 DAS in upper shoot region. Isolate *P. aeruginosa* PM389 belonging to ERIC type IV identified as *Pseudomonas aeruginosa* was first recovered in both root and shoot at 21 DAS and regularly observed up to 70 DAS. However, its spatial distribution varied between 56 and 70 DAS.

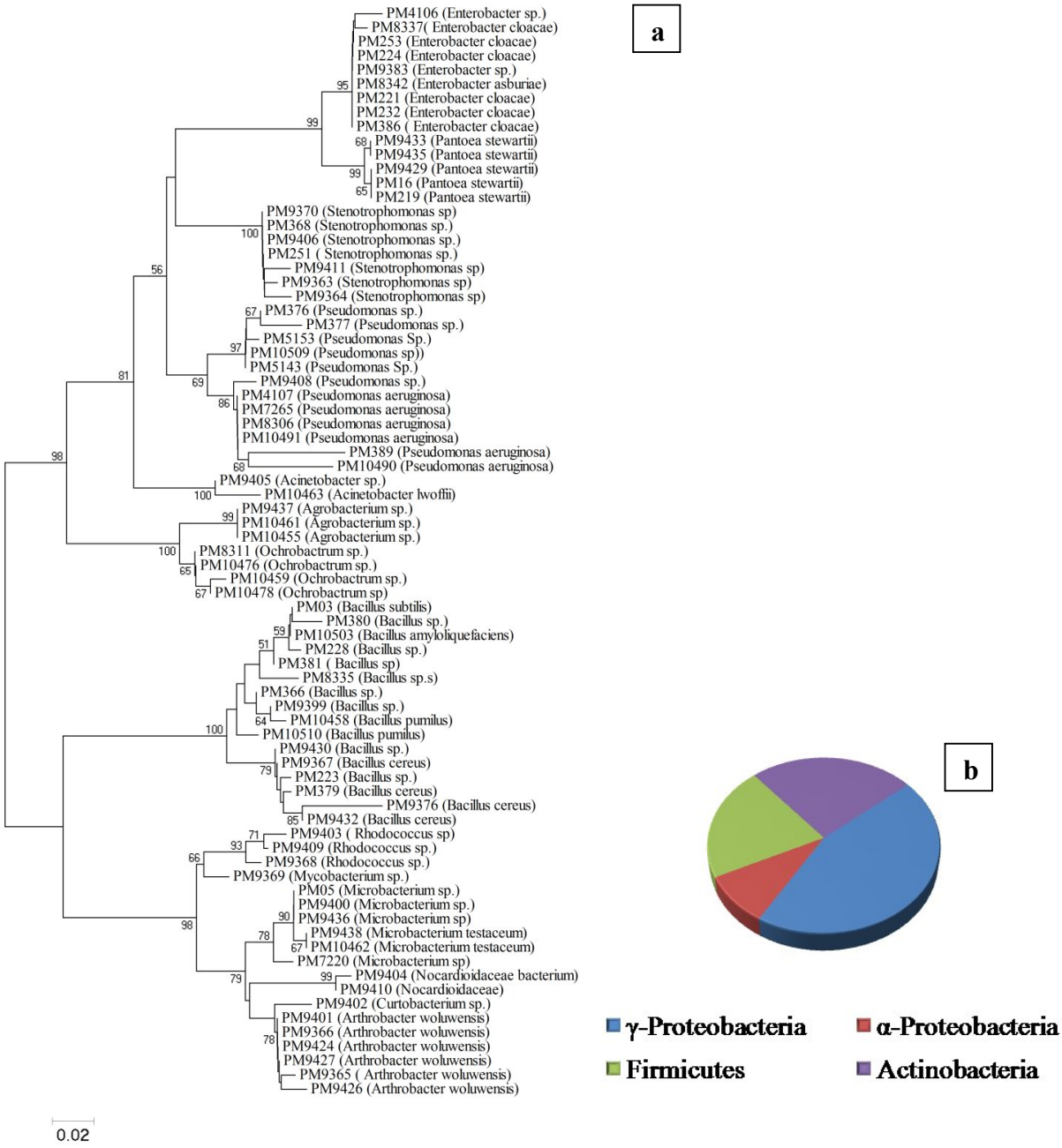


Fig. 2.4. a) Phylogenetic tree based on partial 16S rRNA gene sequence showing relationship among various bacterial isolates obtained from pearl millet during entire growth period of plant. Bootstrap values greater than 50 are indicated at the corresponding nodes. b) Pie-diagram showing different classes of bacteria isolated during the present study.

Table 2.1. Summary of the closest affiliations of the representative isolates in the GenBank according to the 16S-rRNA gene sequences.

Isolate No.	Accession Number	Sequence Coverage	Closest NCBI match/Closest type strain	% Identity
PM03	KC683731	100	*Bacillus amyloliquefaciens* strain BVC18 (JQ660601)	100
PM05	HQ878378	100	*Microbacterium assamensis* (FR832516)	100
PM16	KC683732	100	*Pantoea stew*artii AIMSTTP2S (JN835507)	93
PM219	KC683733	100	*Pantoea stewartii* strain S9-116 (JQ660286)	99
PM221	KC683734	100	*Enterobacter cloacae* strain OVC28 (JQ660744)	100
PM223	KC683735	100	*Bacillus cereus* Kt7-14 (JF460754)	99
PM224	HQ878379	100	*Enterobacter cloacae* strain MSSRFS8 (HQ701802)	100
PM228	KC683736	100	*Bacillus* sp. ZW2531-1(EF567395)	99
PM232	HQ878380	100	*Enterobacter cloacae* strain MSSRFS8 (HQ701802)	100
PM251	KC683737	100	*Stenotrophomonas maltophilia* strain YPA 1-1 (JQ308611)	100
PM253	KC683738	100	*Enterobacter cloacae* strain MSSRFS8 (HQ701802)	100
PM366	KC683739	100	*Bacillus nealsonii* strain KTNB19 (FJ864726)	99
PM368	KC683740	100	*Stenotrophomonas maltophilia* strain EU84(JF681290)	100
PM376	KC683741	100	*Pseudomonas putida* strain DS8 (JQ791183)	100
PM377	KC683742	100	*Pseudomonas* sp. AB_13 (JQ033386)	97
PM379	KC683743	99	*Bacillus cereus* DF2C3 (DQ298085)	98
PM380	KC683744	100	*Bacillus subtilis* strain 1A337 (JQ746567)	99
PM381	KC683745	100	*Bacillus amyloliquefaciens* MH18B1 (JN558839)	99
PM386	KC683746	100	*Enterobacter cloacae* strain MSSRFS8 (HQ701802)	100
PM389	JF899310	100	*Pseudomonas aeruginosa* ASFP-38 (HQ018741)	97
PM4106	KC683747	99	*Enterobacter* sp. J54C13 (GQ901869)	96
PM4107	JF899311	100	*Pseudomonas aeruginosa* KZ3 (FJ695213)	99
PM5143	KC683748	100	*Pseudomonas fluorescens* A506 (CP003041)	100
PM5153	KC683749	100	*Pseudomonas fluorescens* A506 (CP003041)	99
PM7220	KC683750	100	*Microbacterium arborescens* strain S1-81 (JQ660028)	100
PM7265	JF899312	100	*Pseudomonas aeruginosa* strain BP C2 (JQ866912)	100
PM8311	KC683751	100	*Ochrobactrum anthropi* strain BDUEBTVRMK33 (GU596495)	99
PM8335	KC683752	100	*Bacillus flexus* strain NIO24 (JQ818411)	100
PM8337	KC683753	95	*Enterobacter* sp. Wy2-D9 (JN986806)	98
PM8342	KC683754	100	*Enterobacter asburiae* strain M-T-MRS_23 (JQ795793)	100

PM9363	KC683755	99	*Stenotrophomonas maltophilia* strain YPA 1-1 (JQ308611)	99
PM9364	KC683756	100	*Stenotrophomonas maltophilia* (HE664167)	99
PM9365	KC683757	100	*Arthrobacter woluwensis* ED (HM536961)	99
PM9366	KC683758	99	*Arthrobacter woluwensis* ED (HM536961)	99
PM9367	KC683759	100	*Bacillus cereus* Kt7-14 (JF460754)	99
PM9368	KC683760	99	*Rhodococcus kroppenstedtii* strain AHJ_3 (JN873342)	99
PM9369	KC683761	100	*Mycobacterium neoaurum* strain OS29 (JQ348111)	99
PM9370	KC683762	100	*Stenotrophomonas maltophilia* strain YPA 1-1(JQ308611)	99
PM9376	KC683763	96	*Bacillus cereus* AIMST Musa3 (HQ694153)	99
PM9383	KC683764	100	*Enterobacter cloacae* strain RCT8 (HM805113)	100
PM9399	KC683765	97	*Bacillus stratosphericus* strain 290 (JQ860101)	99
PM9400	KC683766	100	*Microbacterium testaceum* StLB037 (AP012052)	99
PM9401	KC683767	100	*Arthrobacter woluwensis* ED (HM536961)	99
PM9402	KC683768	100	*Curtobacterium oceanosedimentum* strain S9-644 (JQ660303)	99
PM9403	KC683769	100	*Rhodococcus* sp. Eu-32 (DQ386111)	98
PM9404	KC683770	100	*Nocardioides* sp. DWM128 (HM854514)	99
PM9405	KC683771	99	*Acinetobacter calcoaceticus* (AY800383)	99
PM9406	KC683772	100	*Stenotrophomonas maltophilia* (HE664167)	100
PM9408	KC683773	100	*Pseudomonas oryzihabitans* (JQ388741)	100
PM9409	KC683774	100	*Rhodococcus qingshengii* strain HWG-A33 JQ684256)	99
PM9410	KC683775	100	*Nocardioides* sp. S2-186 (JQ660079)	99
PM9411	KC683776	100	*Stenotrophomonas maltophilia* strain C-J-R2A1	99
PM9424	KC683777	100	*Arthrobacter woluwensis* ED (HM536961)	100
PM9426	KC683778	100	*Arthrobacter woluwensis* MS4_481 (HM032831)	99
PM9427	KC683779	100	*Arthrobacter woluwensis* ED (HM536961)	99
PM9429	KC683780	97	*Pantoea stewartii* strain S9-116 (JQ660286)	99
PM9430	KC683781	100	*Bacillus cereus* Kt7-14 (JF460754)	100
PM9432	KC683782	92	*Bacillus cereus* strain H9B-3 (HQ238861)	99
PM9433	KC683783	98	*Pantoea stewartii* AIMST Nmie4 (JF819695)	99
PM9435	KC683784	99	*Pantoea stewartii* isolate 40G RT2A (EF189919	100
PM9436	KC683785	100	*Microbacterium testaceum* StLB037 (AP012052)	100
PM9437	KC683786	100	*Agrobacterium larrymoorei* strain S3-89 (JQ660107)	99
PM9438	KC683787	100	*Microbacterium testaceum* StLB037 (AP012052)	98
PM10455	KC683788	100	*Rhizobium* sp. HPCKc-Ca(i) (HE802763)	100
PM10458	KC683789	100	*Bacillus pumilus* FS55 (AF260751)	99
PM10459	KC683790	99	*Ochrobactrum* sp. MVSV1(JN089705)	97
PM10461	KC683791	100	*Rhizobium* sp. HPCKc-Ca(i) (HE802763)	100
PM10462	KC683792	100	*Microbacterium testaceum* StLB037 (AP012052)	99

PM10463	KC683793	100	*Acinetobacter* sp. XJ104-YF-7(JQ726509)	98
PM10476	KC683794	100	*Ochrobactrum intermedium* strain TND41(JQ660549)	100
PM10478	KC683795	100	*Ochrobactrum intermedium* strain TND41(JQ660549)	99
PM10490	JF899314	99	*Pseudomonas aeruginosa* NQ8 (EU939706)	97
PM10491	JF899315	100	*Pseudomonas aeruginosa* PB28 (JN408334)	100
PM10503	KC683796	92	*Bacillus amyloliquefaciens* Kk3-3 (JF460724)	99
PM10509	KC683797	100	*Pseudomonas fluorescens* strain hswx163 (JQ236807)	100
PM10510	KC683798	100	*Bacillus pumilus* strain Y24 (JQ798393)	100

After 63 DAS, in both root and all the parts of shoot, presence of diverse bacterial species was observed except in case of middle part of shoot where dominance of *P. aeruginosa* was observed at 70 DAS. Only *P. aeruginosa* showed a regular presence in the later weeks of study based on isolation studies. In addition to *P. aeruginosa,* endophytic bacterial species which showed its recurrence at various stages of plant growth were *Arthrobacter woluvensis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Enterobacter* sp., *Microbacterium testaceum, Pseudomonas* sp. (PM377) and *Stenotrophomonas maltophilia.* On the other hand, species such as *Acinetobacter* sp., *Agrobacterium larrymoorei*, *Bacillus flexus*, *Bacillus nealsonii*, *Bacillus pumilus*, *Bacillus subtilis*, *Enterobacter asburiae*, *Microbacterium arborescens*, *Ochrobactrum anthropi*, *Ochrobactrum intermedium*, *Ochrobactrum* sp., *Pseudomonas fluorescens, Pseudomonas putida*, *Pseudomonas* sp. (PM5143), *Rhodococcus qingshengii* and *Rhizobium* sp., were recovered only once during the entire study period.

Shannon's diversity index is important to compare the differences between two communities. Analysis of diversity based on shannon's diversity index indicated higher diversity of endophytic diazotrophic bacterial species in root and shoot till 21 DAS than in the rest of the study period where increase in abundance of *P. aeruginosa* PM389 was observed from 28 DAS to 56 DAS (Table 2.2). Evenness (lack of dominance and more diversity) was higher at time intervals where *P. aeruginosa* was not observed to be dominant. The highest diversity was observed at 21 DAS in the root followed by 63 DAS in middle part of shoot during the entire study period (Fig. 2.5). In shoots, population as well as diversity of diazotrophic endophytic bacteria was lower than the roots (Fig. 2.1 and 2.5). Similar to the results of roots, dominance of *P. aeruginosa* was also observed in shoot from 28 to 49 DAS. At 56 DAS, diversity of diazotrophic bacteria was higher in upper part of shoots than the lower one. Shoot was also dominated by *P. aeruginosa* PM389 from 28 DAS to 49 DAS. Dominance continued in lower part of shoot, but the diversity was observed in the upper part of shoot at 56 DAS. At 63 DAS, *P. aeruginosa* was approximately 50% of the bacterial population in the lower part of shoot. In the last week, the middle part of shoot was completely dominated by *P. aeruginosa,* which affected the diversity observed at 63 DAS. At 70 DAS, the diversity was observed in the lower and upper

Table 2.2. Changes in diversity of total diazotrophic endophytic population with the growth of pearl millet under field condition. R and S represent root and shoot respectively.

Sample (DAS)	Shannon's Index (H)	Evenness ($e^{H/S}$)	Dominance (D)
0 (Seeds)	1.0890	0.9903	0.3400
7	0.0000	1.0000	1.0000
14R	1.1270	0.5146	0.4683
14S	0.3046	0.6781	0.8347
21R	1.7990	0.6714	0.2256
21S	0.4506	0.7846	0.7222
28R	0.1446	0.3852	0.9463
28S	0.0000	1.0000	1.0000
35R	0.1888	0.6039	0.9109
35S	0.0000	1.0000	1.0000
42R	0.0000	1.0000	1.0000
42S	0.0000	1.0000	1.0000
49R	0.0938	0.5492	0.9629
49S	0.5890	0.9011	0.6005
56R	0.1976	0.6092	0.9056
56(L)S	0.3514	0.7105	0.8006
56(U)S	0.8439	0.7751	0.4588
63R	1.3910	0.8036	0.2764
63(L)S	0.7724	0.7217	0.5678

63(M)S	1.9810	0.7254	0.1667
63(U)S	1.2680	0.7106	0.3504
70R	1.1600	0.6377	0.4380
70(L)S	0.0000	1.0000	1.0000
70(M)S	0.0000	1.0000	1.0000
70(U)S	0.9652	0.8751	0.4289

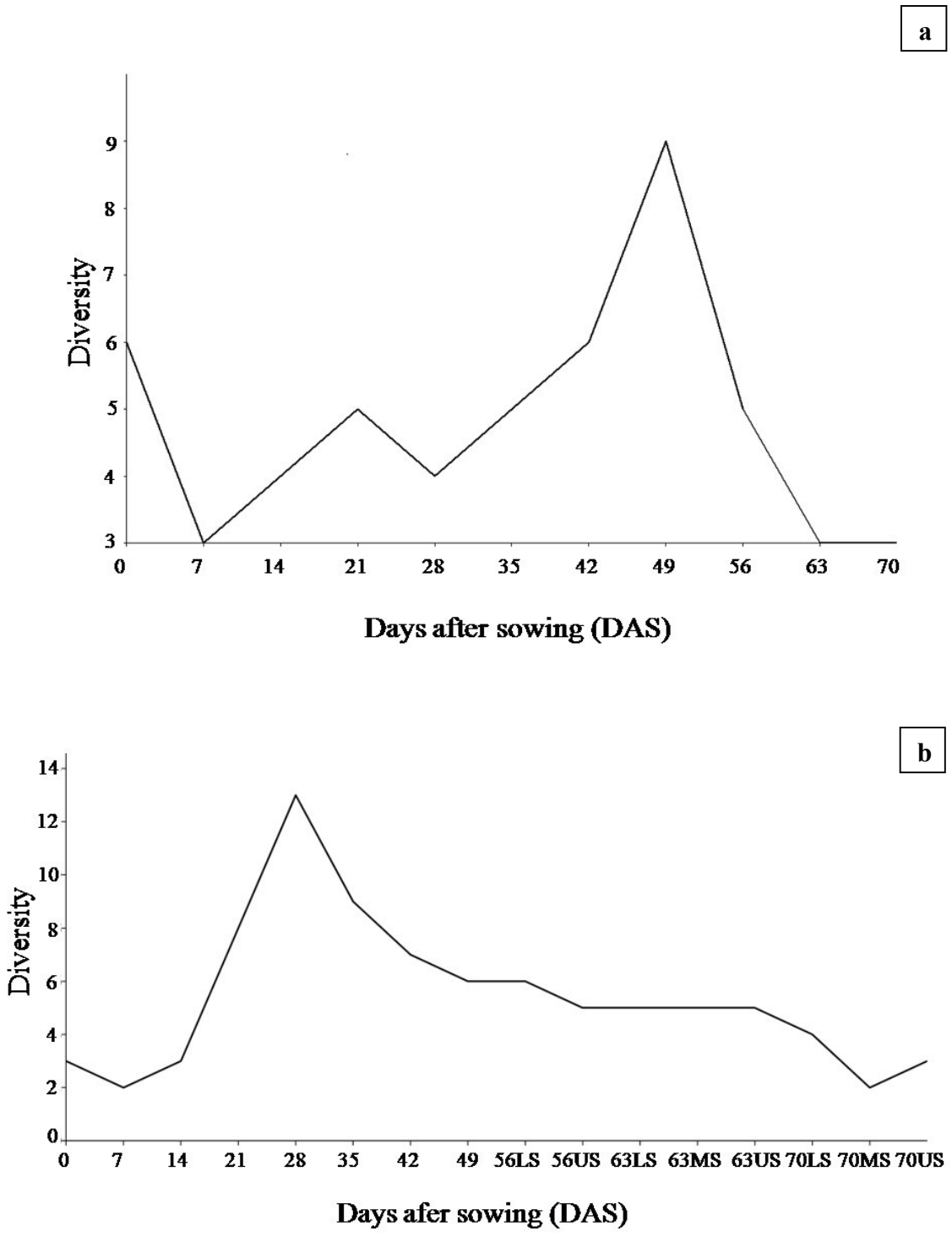


Fig. 2.5. Change in diversity of diazotrophic endophytic bacteria with growth of pearl millet plants grown under field condition. X-axis represents the plant sample (a. root, b. shoot) at subsequent days after sowing.

part of the shoot and *P. aeruginosa* was not recovered at all from either part. However, it was recovered from middle part.

2.3.6. Relative species abundance

Pseudomonas aeruginosa PM389 was found to be the most dominant bacteria (11-100%) throughout the growth period of pearl millet plant. The relative abundance of *P. aeruginosa* ranged from 0.48 to 1 (Fig. 2.6) with its maximum at 42 DAS. It was unrecoverable after 56 DAS in roots. In shoot samples the relative abundance of *P. aeruginosa* remained maintained at the maximum level from 28 to 42 DAS followed by its subsequent decrease. It was clearly evident from the results that with the maturity of plant, the relative abundance of *P. aeruginosa* decreased to 0 in the lower stem and increased to 1 in the middle portion of stem. This result suggests an upward movement of bacterial colonization in pearl millet plants with time. The next most dominant genera was *Enterobacter cloacae* and it's abundance ranged between 1 and 99%. As described above, other than these two, few other species repeatedly showing their occurrence were also found to be abundant at different growth stages. These include *Arthrobacter woluvensis* (6-22%), *Bacillus amyloliquefaciens* (10-58%), *Microbacterium testaceum* (6-44%), *Pseudomonas* sp. (PM5143) (5-27%) and *Stenotrophomonas maltophilia* (1-42%), which were abundant at different growth stages as shown in Fig. 2.7. Various species showed their occurrence at different time point, but some of them showed great abundance like *Pantoea* sp. (100%), *Ochrobactrum anthropi* (89%), *Bacillus flexus* (91%), *Ochrobactrum intermedium* (100%) at 7 DAS in root, 56 DAS in lower shoot part, 56 DAS in upper shoot part and 70 DAS in lower shoot part respectively.

Rank abundance curve (Preston's plot) was obtained for various species recovered at different time intervals (Fig. 2.8). On the basis of % abundance of species, they were ranked in decreasing order in different weeks. In certain samples such as a, d, g, j and k, one species dominated the entire population, and the other species present were almost equally abundant. In some samples many species were equally abundant (b, c, e, h) at a particular time point and thus, clustered at a particular level of abundance. In other samples, most of the species had differences in their abundance level and showed scattered pattern in Preston's plot (f and i).

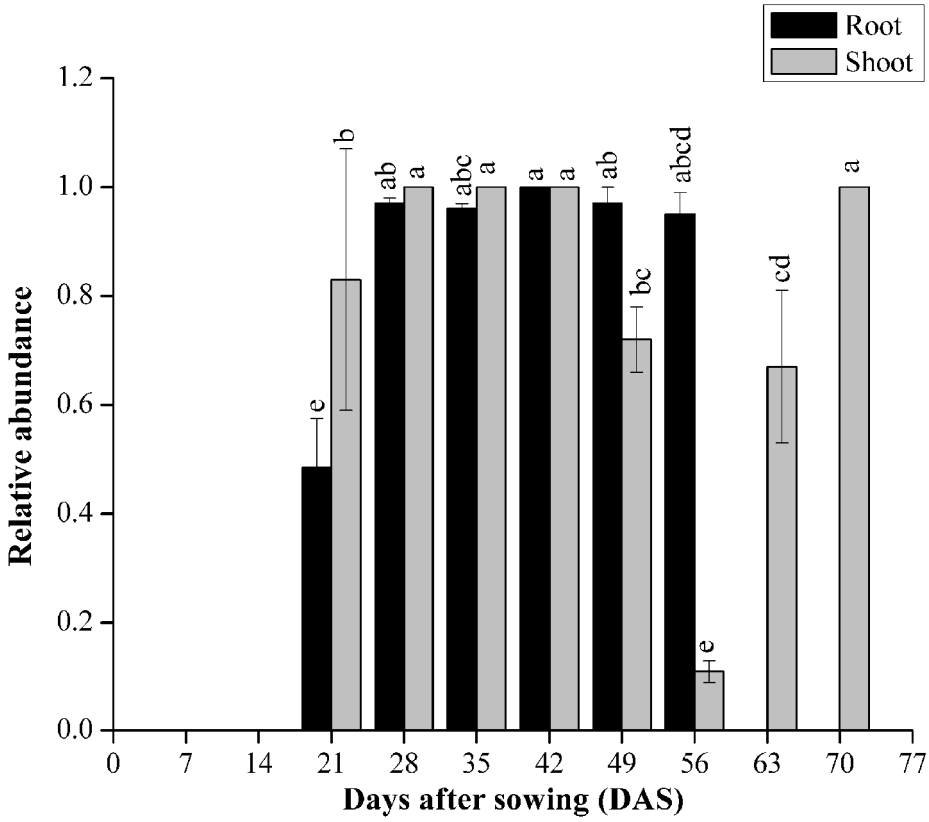


Fig. 2.6. Changes in the relative abundance of *Pseudomonas aeruginosa* PM389 in root and shoot of pearl millet plants at various growth stages. At 56 and 63 DAS, data for lower shoot and at 70 DAS, data for middle shoot population are represented (L-Lower, M-Middle, U-Upper shoot).Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters.

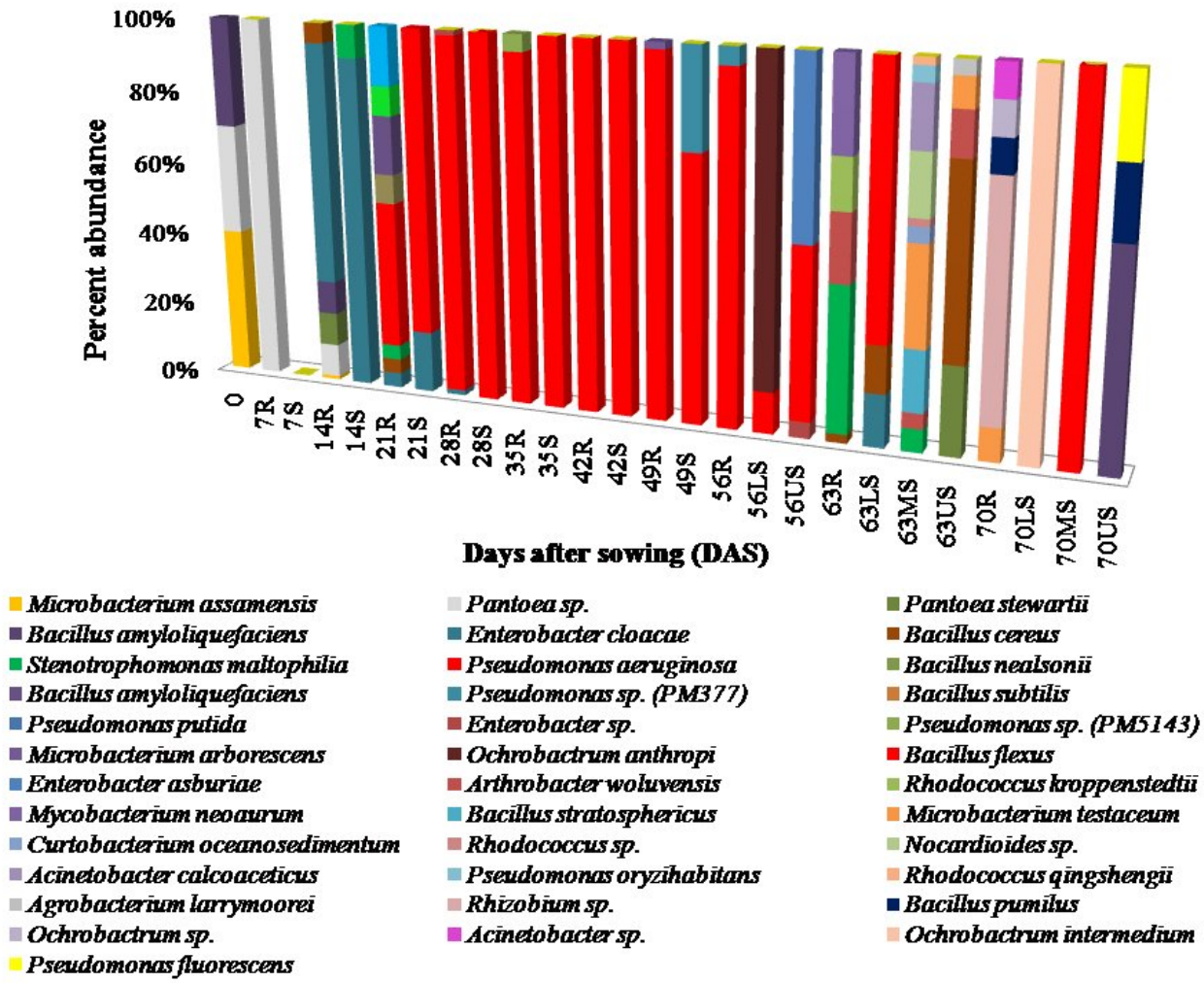


Fig. 2.7. Representation of percent abundance of variety of bacterial species obtained in different weeks of plant growth under field conditions (R-root, S-shoot, LS-lower shoot, MS-middle shoot, US-upper shoot).

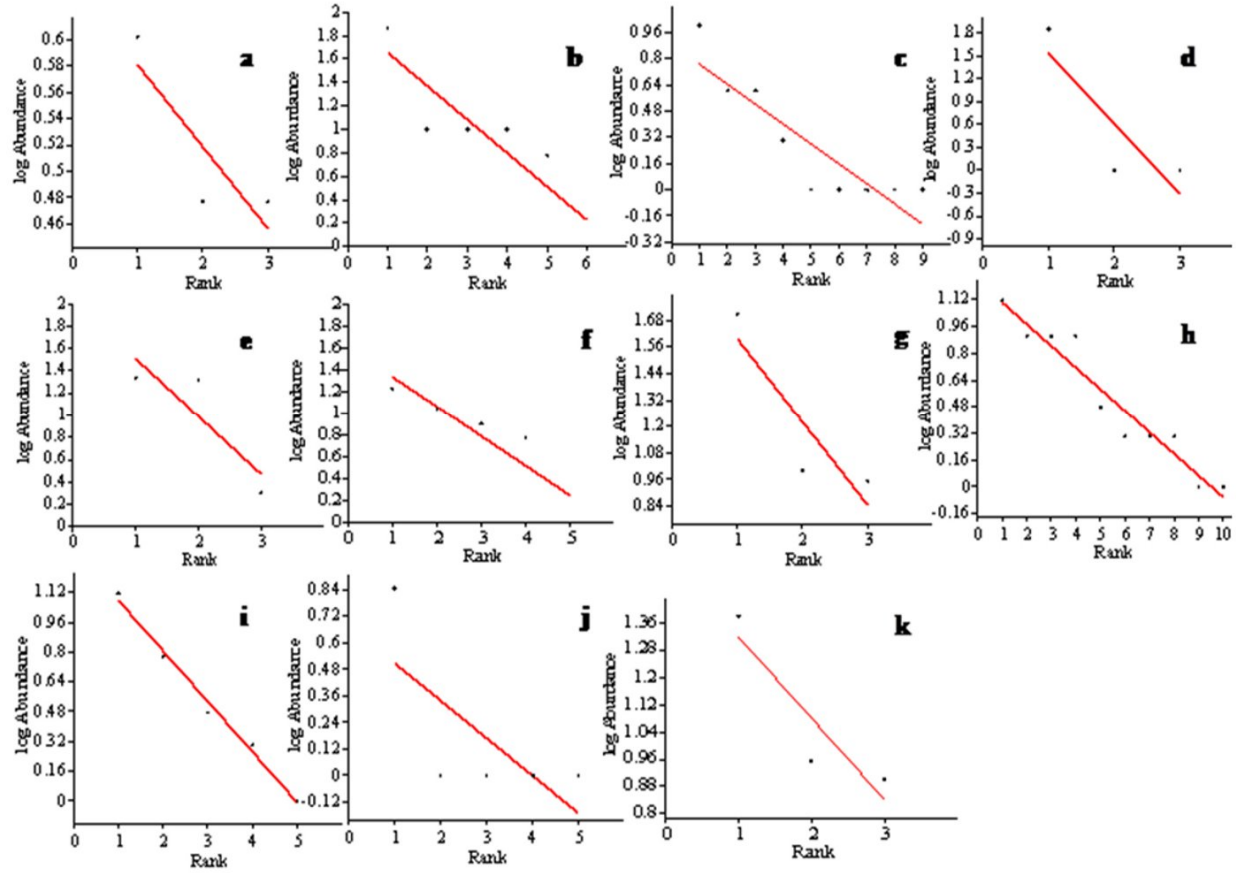


Fig. 2.8. Preston's Rank abundance curve for different growth stages of pearl millet a) 0 DAS b) 14 DAS (Root) c) 21 DAS (Root) d) 28 DAS (Root) e) 56 DAS (Above Shoot) f) 63 DAS (Root) g) 63 DAS (Below shoot) h) 63 DAS (Middle shoot) i) 63 DAS (Above shoot) j) 70 DAS (Root) k) 70 DAS (Above shoot).

2.3.7. Test of plant growth promotion activities

Qualitative analysis for plant growth promoting activities was done for all 210 isolates (Appendix II). Based on the results, one representative strain showing positive for PGP properties each from 76 classified groups were used for quantitative estimation of different PGP activities. Result of quantitative analysis is shown in Table 2.3.

2.3.7.1. Confirmation of diazotrophy

All isolates showed positive results for nitrogenase activity in ARA. Nitrogenase activity in different isolates ranged from 1.83 to 128.36 nmol ethylene mg protein^{-1} h^{-1} (Table 2.3), which indicated varied nitrogen fixation efficiency of different isolates. As depicted in Table 2.3, highest nitrogenase activity was exhibited by isolate PM10455 (128.36 nmol ethylene mg protein^{-1} h^{-1}) followed by PM10461. Appreciably good nitrogenase activity was observed in PM10461, PM9437, PM9405, which showed, 103.93, 100.89, 95.56 nmol ethylene mg protein^{-1} hr^{-1} respectively. Amplification of *nifH* gene was performed using the pair of *nifH* specific universal primers to confirm diazotrophy of bacterial isolates on the molecular level. However, only 13 isolates showed amplification for *nifH* gene (Table 2.3). The desired amplicon of 342 bp corresponding to *nifH* gene was obtained in all 13 isolates. There are various reasons of it, which have been explained in discussion section 2.4.

2.3.7.2. Phosphate solubilization, IAA and siderophore production

All 210 isolates were subjected to the tests for mineral phosphate solubilization activity, production of IAA and iron chelator siderophore. Out of 210 isolates, 96, 83 and 119 were found positive for phosphate solubilization, IAA and siderophore production respectively (Appendix II). Representative strains belonging to each ERIC-type showing best activity in qualitative tests were subjected to quantification of above mentioned properties. In quantitative assays, highest P solubilization activity was shown by PM03 (9.4 mM ml^{-1}) followed by PM10462 (9.1 mM ml^{-1}) and PM386 (8.9 mM ml^{-1}) (Table 2.3). Highest production of IAA was observed in PM253 (81.3 µg ml^{-1}) followed by PM224 (80.25 µg ml^{-1}). Twenty representative isolates belonging to various ERIC types showing positive test for production of siderophore were subjected to sidero-

Table 2.3. Plant growth promoting traits of various bacterial isolates obtained in this study.

Isolate No.	IAA (µg/ml)	Phosphate-solubilization (mM/ml)	% Siderophore (Typing)	ARA (nmol ethylene mg protein^{-1} hr^{1})	*nifH*
PM03	-	9.4	-	40.24	-
PM05	10.74	-	12.8 (H)	7.70	-
PM16	-	2.5	2.5 (H)	21.09	+
PM219	11.80	-	-	30.36	+
PM221	73.70	6.4	0.3 (C)	24.84	-
PM223	-	-	-	35.53	-
PM224	80.25	7.5	-	26.22	-
PM228	-	-	-	32.40	+
PM232	19.76	6.8	14.2 (C)	26.28	-
PM251	-	-	-	3.45	-
PM253	81.30	6.0	6.77 (C)	28.84	-
PM366	8.58	-	3 (H)	30.23	-
PM368	-	-	0.5 (C)	4.55	-
PM376	9.40	-	63.5 (C) (H)	10.09	-
PM377	-	-	78.5 (C) (H)	9.78	-
PM379	-	-	-	45.83	-
PM380	-	-	-	20.84	+
PM381	-	-	-	23.20	-
PM386	67.30	8.9	-	18.98	-
PM389	-	V.L.	0.6 (C) (H)	28.91	+
PM4106	50.35	-	-	20.34	-
PM4107	-	V.L.	13 (H)	25.68	-
PM5143	14.09	-	-	9.87	+
PM5153	4.47	-	0.3 (H)	8.87	+
PM7220	-	-	-	5.67	-
PM7265	-	V.L.	13.43 (H)	26.00	-
PM8311	3.40	-	-	6.72	+
PM8335	-	-	-	31.79	-
PM8337	15.47	5.9	11.7	22.98	+
PM8342	31.81	2.9	13.05	29.98	-
PM9363	-	-	-	2.98	+
PM9364	-	-	-	1.83	+
PM9365	-	-	-	34.63	-
PM9366	-	2.5	5.3 (H)	28.89	-
PM9367	-	-	-	20.79	-

PM9368	-	-	-	10.96	-
PM9369	-	-	-	18.94	-
PM9370	17.78	-	-	4.32	-
PM9376	-	-	-	15.67	-
PM9383	-	-	-	7.58	-
PM9399	-	-	-	28.89	-
PM9400	6.91	-	-	6.86	-
PM9401	15.43	-	-	26.78	-
PM9402	-	-	-	24.32	-
PM9403	-	-	-	13.58	-
PM9404	-	-	0.5 (H)	56.03	-
PM9405	10.77	-	-	95.56	-
PM9406	-	-	-	4.58	-
PM9408	6.50	-	8.8 (H)	16.43	-
PM9409	-	-	-	13.08	-
PM9410	-	-	-	5.90	-
PM9411	7.89	-	-	5.25	-
PM9424	-	-	-	30.90	-
PM9426	8.99	-	-	32.96	-
PM9427	10.23	-	-	30.34	-
PM9429	-	-	-	18.65	-
PM9430	-	-	-	16.90	-
PM9432	-	-	-	8.92	-
PM9433	-	-	-	7.89	-
PM9435	41.49	-	-	14.56	-
PM9436	17.62	-	-	5.67	-
PM9437	15.85	-	-	100.89	-
PM9438	14.86	-	-	31.86	-
PM10455	12.08	-	-	128.36	+
PM10458	-	-	-	25.43	-
PM10459	-	-	-	15.98	-
PM10461	19.48	-	-	103.93	-
PM10462	6.34	9.1	-	5.68	-

PM10463	-	-	-	15.02	-
PM10476	-	-	-	8.98	-
PM10478	-	-	-	15.67	-
PM10490	-	V.L.	3.5 (H)	23.34	-
PM10491	-	V.L.	8.8 (H)	25.67	-
PM10503	-	-	-	47.11	+
PM10509	-	-	-	18.96	-
PM10510	-	-	-	57.73	-

*- - no activity, +- activity present, H-hydroxamate, C-catacholate, V.L.-very low

phore typing. Out of these 20 isolates, eleven isolates produced hydroxamate and four isolates produced catacholate type siderophore (Table 2.3). Three isolates produced both hydroxamate and catacholate types, while two of them showed production of some other kind of siderophores (not tested).

2.3.7.3. Antagonistic activity

P. aeruginosa PM389 showed considerably good antagonistic activity against the majority of bacterial species tested in the present study (Table 2.4). It showed strong antagonistic activity against *Arthrobacter woluwensis* (Gram positive) and *Enterobacter* sp. (Gram negative). While, it showed least activity against *Bacillus* sp. (8 mm) and no activity against *Pseudomonas oryzihabitans* and *Microbacterium* sp.

Table 2.4. Antagonistic activity of *Pseudomonas aeruginosa* PM389 against plant associated bacterial species.

Organisms tested (Gram's reaction)	**Zone of Inhibition (mm)**	**Results**
Arthrobacter woluwensis (+)	39.0±0.49	+++
Bacillus sp. (+)	8.0±0.11	+
Bacillus subtilis (+)	33.0±0.14	+++
Microbacterium sp.(+)	NA	-
Ochrobactrum sp. (-)	28.0±0.07	++
Pseudomonas oryzihabitans (-)	NA	-
Enterobacter sp. (-)	35.0±0.74	Good

-= absent, + = poor, ++ = average, +++=Good, NA-No Activity. ± represents standard deviation.

2.3.7.4. Tests for cellulolytic and pectinolytic activities

Presence of cellulolytic (endoglucanase, cellobiohydrolase, β-glucosidase) and pectinolytic activities (pectinase) are among the few important traits required for endophytic colonization of bacteria. Out of 76 strains, 26 strains produced only one type of above mentioned four hydrolytic enzymes tested, while 20 and 3 (PM228, PM379, PM9401) isolates were found positive for the production of two and three types of these enzymes, respectively. None of the isolate was observed to produce all four kinds of enzymes tested. PM9411 and PM10462 showed highest production of β-glucosidase, but none of the isolates showed good activity for cellobiohydrolase. Highest endoglucanase activity was shown by PM228 and PM380. Isolates PM5143 and PM7220 showed highest pectinase activity as mentioned in Table 2.5. Surprisingly, the most dominant isolate (*P. aeruginosa* PM389) showed no enzymatic activity for all the enzymes tested.

Table 2.5. Test for the cellulolytic and pectinolytic activities in various endophytic bacteria obtained in present study.

Isolate No.	β-glucosidase	Cellobiohydrolase	Cellulase	Pectinase
PM03	+	-	-	-
PM05	-	-	-	-
PM16	+	-	++	-
PM219	-	-	+++	-
PM221	-	+	-	-
PM223	++	-	++	+
PM224	++	+	-	-
PM228	++	+	++++	-
PM232	++	+	-	-
PM251	-	-	-	-
PM253	+	+	-	-
PM366	++	-	-	-
PM368	++	-	-	-
PM376	-	+	+	-
PM377	-	-	-	-
PM379	+	+	+	-
PM380	-	+	++++	-
PM381	-	+	++	-
PM386	-	+	-	-
PM389	-	-	-	-
PM4106	-	+	-	-
PM4107	+	-	-	-
PM5143	-	-	-	+++
PM5153	-	-	-	++
PM7220	-	-	-	+++
PM7265	-	-	-	-
PM8311	-	-	-	-
PM8335	-	-	-	-
PM8337	++	+	-	-
PM8342	++	+	-	-
PM9363	-	-	-	-
PM9364	-	-	-	-
PM9365	++	+	-	-
PM9366	+	+	-	-
PM9367	+	-	-	-

PM9368	+	-	-	-
PM9369	+	+	-	-
PM9370	+	+	-	-
PM9376	-	-	-	-
PM9383	-	+	-	-
PM9399	+	-	-	-
PM9400	+	+	-	-
PM9401	+	+	+++	-
PM9402	-	+	+	-
PM9403	-	-	-	-
PM9404	-	-	-	-
PM9405	+	-	-	-
PM9406	+	-	-	-
PM9408	-	-	-	-
PM9409	+	+	-	-
PM9410	-	-	-	-
PM9411	+++	-	-	-
PM9424	-	-	-	-
PM9426	-	-	++	-
PM9427	-	-	-	-
PM9429	-	-	+	-
PM9430	-	-	++	-
PM9432	-	-	-	-
PM9433	-	-	-	-
PM9435	-	-	-	-
PM9436	-	-	-	-
PM9437	-	-	-	-
PM9438	++	-	-	-
PM10455	+	-	-	-
PM10458	-	-	-	-
PM10459	-	-	-	-
PM10461	-	+	-	-
PM10462	+++	-	-	-
PM10463	++	+	-	-

PM10476	++	+	-	-
PM10478	++	+	-	-
PM10490	-	-	-	-
PM10491	-	-	-	-
PM10503	-	-	+++	-
PM10509	-	-	-	-
PM10510	++	+	-	-

-= absent, + = poor, ++ = average, +++=good

2.4. **Discussion**

Present study delivers the first insights into the population dynamics and diversity of diazotrophic endophytic bacteria in the pearl millet plant during its vegetative growth period in actual farming conditions. A systematic study to examine the population dynamics of diazotrophic endophytic bacterial species at different growth stages of the host plant can provide a sustainable way to use the dominant one as possible biofertilizer. Understanding of the population dynamics and bacterial diversity during various stages of plant growth can unveil several important aspects, including effect of plant age on diazotrophic endophytic community (Duineveld et al. 1998), influence of the community shift on nitrogen fixation (Hsu and Buckley 2009), migration of endophyte in the plant parts under field conditions (Andreote et al. 2009**)** and effect of diversity on soil factors (Garbeva et al. 2006). To the best of our knowledge, this is the first report on temporal changes in population and diversity of diazotrophic bacterial endophytes at different plant growth stages. Present study demonstrated the exclusive dominance of *P. aeruginosa* at various growth stages of pearl millet and dynamic changes in bacterial community at various growth stages of plant. The results of this study were based exclusively on growth of cultivable bacteria. Overall, 210 cultivable diazotrophic endophytes were recovered and characterized in this study by analyzing their phenotypic properties, taxonomic position, and features likely to contribute for promoting plant growth.

In the early stage of pearl millet plant growth, population of diazotrophic endophytic bacteria was less, which gradually increased in subsequent weeks upto middle growth period of the plant (28-56 DAS). In accordance with our results, trends in population change was observed in the field grown cucumber (Mahaffee and Kloepper 1997). Enhanced population of endophytic bacteria during vegetative growth of plant may have resulted due to release of root exudate rich in nutrition. Contents of the root exudate act as probable chemoattractant and lead to successful colonization of compatible bacteria. In the latter stage of plant growth, bacterial population increased in the middle part of stem. This observation supports the notion that the migration of bacteria to aerial portion was in search of more nutrition, and space resulted due to shift of the sink (source-sink relationship) from roots to aerial parts of the plant (Gupta et al. 2013). However, population of diazotrophic bacteria was lesser in the upper part of stem than other

parts. This indicated that the decreased bacterial population in uppermost aerial portion probably arose due to slow systemic spreading problem in xylem connectivity or occluded pits in tracheary elements. Thus, restriction of the passage between xylem elements lengthens time required to reach the uppermost part of the stem (Compant et al. 2008). Greater populations of endophytic bacteria in the root than shoot during the majority of growth stages of plant correlate with the fact that the vast surface area provided by roots is an ambient habitat for harbouring a variety of microorganisms (Welbaum et al. 2004). Thus, root growth leads to substrate production in the root zone, which in turn promotes rhizobacterial proliferation providing a carbon-rich environment as well as the primary site of entry for endophytes (Lodewyckx et al. 2002).

One of the purposes of using ERIC-PCR approach was to track the fate of seed-borne isolates during plant growth in natural conditions. ERIC and other similar fingerprinting techniques have been used to type, and track plant associated bacteria (Yanni and Dazzo 2010). Recovery of isolates from the root at 14 and 21 DAS showing ERIC-profile identical to that of seed isolates indicated their migration from seed to root. This observation was in agreement with the findings of Ferreira et al. (2008) in which transfer of various endophytes from seeds to seedling was reported. In our recent publication, we have reported that bacterial isolate *P. aeruginosa* PM389 belonging to *P. aeruginosa* species was recovered from plant parts during the majority of the growth period of the plant (Gupta et al. 2013). Absence of *P. aeruginosa* PM389 in the pearl millet seeds used for sowing suggested its non-vertical transfer in plants. However, seed borne isolates could not be recovered during each stage of plant growth, which suggests replacement of the former with soil borne bacteria. This observation was supported by previous study of Lilley et al. (1996) where similar finding were described. It indicated that seed-borne endophytes were replaced by soil-borne *P. aeruginosa* and other bacteria during plant growth in natural soil conditions. Predominance of *P. aeruginosa* PM389 and elimination of other bacteria during the active growth period of plants could have occurred due to the production of siderophores or antagonistic compounds by *P. aeruginosa* PM389 (Fgaier and Eberl 2011). Antagonistic activity of *P. aeruginosa* PM389 against various bacterial species (Table 2.4) indicates its ability to compete for the given niche over other bacterial species (Ikeda et al. 2010).

Contribution of antagonistic activity in modulation of community structure has also been demonstrated in other studies (Long and Azam 2001; Grossart et al. 2004). Similar to our results predominance of one species over others was also reported in earlier study where *Pantoea* sp. was found to severely inhibit the colonization of *Ochrobactrum* sp. co-inoculated in rice plants (Verma et al. 2004). This observation posits that *P. aeruginosa* PM389 is a successful colonizer of pearl millet plant. As observed in our study, Pseudomonads have been found to be diverse, numerous, dominating, and aggressive colonizer in several studies (Germida et al. 1998; Santoyo et al. 2012). The possible reasons for predominance of *P. aeruginosa* PM389 can be a result of higher population leading to its efficient colonization, and the fact that this genera is nutritionally or environmentally favored by the host plant (Mahaffee and Kloepper 1997).

Next most abundant species recovered during the study period was *Enterobacter.* Similar findings were observed in Farina et al. (2012), in which *Pseudomanas* and *Enterobacter* genera were the abundant genera associated with canola plant (*Brassica napus*). Although unlike our findings none of the genera was most dominant in their study. In the initial stages of plant growth, some other species like *Enterobacter* sp. *and Bacillus* sp., were also recovered in addition to *P. aeruginosa* from different parts of plants. Later on, *P. aeruginosa* predominated in the middle stage of plant growth, although above mentioned species reappeared in the last stage of plant growth during the decline of *P. aeruginosa* population. It can be inferred that these endophytic bacteria were present in the middle stage of pearl millet growth, but the population was very less due to the antagonistic nature of *P. aeruginosa* and could not be recovered (Gupta et al. 2013). In a recent review, it has been explained that many bacteria become dormant metabolically in unfavourable conditions like stress or presence of growth inhibiting compounds, and revive on return of favourable conditions (Nemergut et al. 2013). A significant increase in population of *P. aeruginosa* PM389 in the middle portion of the shoot at 70 DAS suggests the upward migration of endophyte possibly through xylem vessels of stem under the influence of the transpiration stream, passively (Thorne et al. 2006). The recovery of these endophytic bacteria from plant samples collected in late stage of plant growth indicates suboptimal condition for the growth of *P. aeruginosa*. Decline in the population of *P. aeruginosa* might also have resulted due to intraspecies competition for nutrition and space (Bacon and Hinton 2006).

Qualitative changes in plant metabolism and various plant associated bacterial communities can vary during various plant growth phases (Andreote et al. 2010). Several factors described above may contribute towards altered endophytic bacterial population at later stages of plant growth. Moreover, the absence of *P. aeruginosa* PM389 in the upper part of stem may be due to certain obstruction in transportation vessels described above and lengthening of time taken by it to reach the uppermost part of stem (Compant et al. 2008).

The diversity of diazotrophic bacteria was observed higher in the roots as compared to the shoots in the initial weeks because of the difference in niches w.r.t. nutrient supply, atmospheric conditions and competitiveness with other components of these communities (Rao et al. 2006). In roots, the carbon exudates are released which act as nutrient source as well as chemo-attractant for bacteria, which leads to the colonization of endophytes inside the plant. Roots are the primary entry site for endophytic bacteria. Endophytic bacteria require the penetration mechanism to enter and establish a colonization niche inside the plant (Lodewyckx et al. 2002). The colonization behavior of bacterial population inside the plant depends on various environmental factors like biofilm formation and avoiding migration to other plant parts through sieve transportation (Andreote et al. 2009). These factors are important for the establishment and selection of niche inside the plant by endophytes, which are responsible for the changes in composition and magnitude of community structure.

Diversity of endophytic bacteria in shoots was lower than roots during early stage of plant growth, while it showed a stable community structure in the middle stage of plant growth. On the other hand, endophytic bacterial diversity was high in both roots and shoots during late stages of plant growth in comparison to initial weeks of plant growth in which diversity was higher only in roots. Given results are similar to the results obtained by Mahaffee and Kloepper (1997), in which the diversity and genera richness of all the habitats studied tended to increase over the growing season, with the highest diversity and genera richness values at 70 days after planting. Moreover, diversity was higher in the upper part of shoot as compared to the middle part of the shoot which might be due to aerial colonization in the plants (Verma et al. 2004). The present study also demonstrated higher diversity of endophytic bacteria but with low total population count. Similarly, relative increase in diversity of bacteria with the decline in

population has also been observed in some genera in a previous report (Mahaffee and Kloepper 1997).

From 210 isolates, 76 different isolates obtained were further screened for various plant growth promoting activities. These 76 isolates were different strains identified on the basis of ERIC-PCR analysis. There was no amplification of *nifH* obtained for 63 isolates, although all isolates showed ARA activity. It has been previously reported in many studies that due to high variability of this gene in nature, amplification could not be obtained (Zehr et al. 2003, Chowdhury et al. 2007). The set of conserved sites of these genes suitable for designing primers is rather limited. Apart from this, high degree of degeneracy of the *nifH* gene nucleotide sequences virtually rules out the possibility of the existence of sufficiently extended invariant sequence sites. Thus, no direct correlation exists between the presence of *nifH* and the ARA activity of the bacterial isolates (Dean and Jacobson 1992).

CHAPTER III

Screening of potential PGPR candidates as future biofertilizers-A strategic approach from lab to field.

"Look first to Nature for the best design before invention"

-Leonardo da Vinci

3.1. Introduction

Tremendous increase in World human population, changes in lifestyle, urbanization and industrialization have shrunken the total arable land for agriculture. This whole scenario has thus affected demand vs. supply ratio of food. If this issue is not resolved in time it will lead to world-wide famine in the future. Aforesaid problem is of major concern for several developing countries, including India where majorities of population depend on Agriculture. Contribution of agriculture in India has fallen down to 13.7% of the GDP (Gross domestic product) in FY (Financial year) 2012-13 (estimated by Central Statistics Office (CSO)). On the other hand, it was estimated by Population Reference Bureau (PRB) in 2007 that India may emerge as the most populated country by 2037 if it is not controlled and to feed this population, India will need about 50 Tg (1 Tg or Tera gram = 10^{12} g = 1 million tonne) more food grains than produced in 2010-2011 (National council of applied economic research, 2012). Several crops namely rice, wheat, maize and pearl millet are staple food in India. Despite the fact of being second largest producer of wheat (80.6 million T in 2008-2009), it will require 109 million T for feeding 1.25 billion population by year 2020 (Singh 2010). Similarly pearl millet, for which India is the largest producer, needs to be produced in the higher amount to suffice its demand as staple food, and other purposes. Therefore, it is high time to develop a robust and sustainable agriculture to produce additional food grains in same or even lesser land area (Basavaraj et al. 2010).

To attain the aim of increasing food yield in limited agriculture land, improved agriculture practices must be followed. Use of inorganic fertilizer for increasing crop productivity has been a great success, and its demand is still on an increase (Tilman et al. 2001). However, environmental and economic concerns associated with industrially manufactured inorganic fertilizers has motivated scientists to adopt an alternative for enhanced and sustainable agriculture practices (Keneddy et al. 2004). Use of organic fertilizers, including animal wastes and compost, has gained its market value in recent days and is being adopted by concerned farmers. It promises to be cost-efficient, equivalent in performance to inorganic fertilizer and lead to sustainable agriculture. Use of compost in unique agriculture strategy called as systematic rice intensification (SRI) program enhanced the crop yield three times more than N fertilizer supplemented soil. In this strategy, one rice seedling was planted per hill instead of three per hill in soil supplemented with compost (Uphoff et al. 2012). However, wide practice of this

technique is still lacking. More importantly, environmental issue and slow utilization of organic fertilizer still discourage its adoption at a larger scale.

Exploitation and manipulation of beneficial bacteria-plant association have been acknowledged as one of the most effective and eco-friendly approaches to enhance plant growth and productivity. Potential of plant growth promoting bacteria (PGPB) has been demonstrated in several studies (Babalola 2010; Baset Mia et al. 2013). Effect of PGPR on various cereal crops like sorghum (Raju et al. 1999), maize (Nezarat 2009), pearl millet (Niranjan et al. 2003), wheat (Shaukat et al. 2006a), rice (Govindarajan et al. 2007) and cash crops like sunflower (Shaukat et al. 2006b) has been studied earlier. Among PGPRs, endophytes have several advantageous factors over rhizospheric and rhizoplanic bacteria as endophytes can provide direct benefits to host, face less competition for nutrient and space, are less vulnerable to biotic and abiotic stress and have wide host range than legume-associated bacteria (Gupta et al. 2012; Jha et al. 2013). Beneficial effects of bacteria have already been discussed in section 1.3. Several endophytic bacteria such as *Azoarcus* sp., *Burkholderia* sp., *Glucanoacetobacter diazotrophicus* and *Herbaspirillium* sp. have been reported as promising endophytic bacteria to enhance crop yield in various crops (Vessey 2003). On several crops, cross-infection and consortia studies have been conducted using PGPR and endophytic isolates (Sturz et al. 2000). It includes a recent study which demonstrated that PGPR and endophytic bacteria recovered from willow and poplar plants enhance various growth parameters in maize as well as its original host plants (Knoth et al. 2013). Bacterization of plant with endophytic/associative bacteria can replace or minimize the use of chemical fertilizer at a greater extent (Dazzo and Yanni 2006). In a large-scale field study, inoculation with single strains or multi-strain consortia of endophytic *Rhizobium leguminosarum* bv *trifolli* significantly increased grain yield of rice in 19 of the 24 trials. By combining superior rhizobial inoculants with agricultural extension training, grain yield increased up to 47% in farmer's fields, with an average increase of 19.5%. The same study also reported that application of rhizobial treatment in combination of minimal dose of chemical fertilizer influenced the maximal growth (Yanni and Dazzo 2010). In contrast, there are several conflicts on their usage of combination of these two (Krey et al. 2013). These contributions have advanced scientific knowledge on beneficial plant–microbe association. Inevitably, it will be helpful to low-income farmers who produce crops on marginally fertile soils deficient in N and many other nutrients. However, one of the major limitations of successful plant-bacteria interaction is genetic

compatibility with one another, which may vary for different plants with particular bacterial isolates. Apart from genetic compatibility, success of bacterial inoculants greatly depends on their competence with other natural bacterial communities and performance in the field. It necessitates broader investigation of bacterial inoculation to the different varieties of plants in actual farming conditions. Incompatible association has been reported to have neutral or deleterious effect on plant growth (Nguyen et al. 2002).

Another major issue which is of economic as well as practical importance is broad host-range of bacteria. For using as a successful biofertilizer, it must be applicable to different crop varieties. Field studies with inoculation of endophytic bacteria *Rhizobium leguminosarum* bv *trifolli* E11 has been done on several non-host plants such as corn and wheat where it was successful in increasing productivity corresponding crop plants (Personal communication with Prof. Frank B Dazzo, Michigan State University). Successful cross-infection of endophytic bacteria has been highlighted in the couple of reports (Jha and Kumar 2007, 2009). These results are encouraging, but isolates showing appreciably good response in laboratory and pot level did not work well on field level and showed inconsistencies in performance in several studies (Çakmakçi et al. 2006; Martínez-Viveros et al. 2010)

According to the reports for the year of 2011 of IPNI (International Plant Nutrition Institute, USA), certain major countries using biofertilizer are North America, Brazil, Southern Cone, Africa, Russia, South Asia, China, Southeast Asia (http://www.ipni.net/ipniweb). In India, concept of biofertilizer has been adopted in last 20 years only and the production of biofertilizer has increased six times from 1993-2003 according to FAO (Food and Agriculture Organization of United States). Indian government is promoting usage of biofertilizer by National Project on Development and Use of Biofertilizers (NPDB). However, lack of awareness amongst farmers and, of efficient biofertilizer agents and anticipated risks, its usage on the farm level is highly dissatisfactory (http://www.ipni.net/ipniweb/portal.nsf/). To our knowledge, very few studies have been done on several levels as mentioned above (Frohlich et al. 2012; Hahm et al. 2012). A systematic approach from lab to natural conditions can provide better and stable bioinoculants as a potential biofertilizer in actual farming conditions. Therefore, present work aimed to examine the efficiency of selected bioinoculants at several levels namely laboratory, pot level with sterilized and non-sterilized soil, microcosm and field trial. To broaden the application of selected bacterial inoculant, cross infection studies were performed. Moreover, the cross-

infection capability and endophytic nature of potent biofertilizer isolate was proved using 16S rDNA species-specific primers (Matsuki et al. 2004).

3.2. Material and methods

3.2.1. Bacterial isolates and growth medium

Based on different plant growth promoting activities, 14 out of 76 representative isolates from each ERIC type, were selected to test their effect on growth of pearl millet plant and their ability to cross-infect wheat plant. The isolates used in this study are listed in Table 3.1. For preparation of inoculum, bacterial cultures were grown in LB (Luria Bertani) broth for 24 h at 37^{o}C and then harvested by centrifuging at 6,500 g for 10 min. The bacterial cell pellet was washed with 1X phosphate-buffered saline (PBS), resuspended in PBS and cultures were adjusted to 10^{7}-10^{8} cells ml^{-1} for further use.

3.2.2. Plant varieties used for experimental studies

HHB 67 variety of pearl millet was used for plant growth experiment. HHB 67, an old hybrid released in 1990 by CCS Haryana Agricultural University (HAU), India, is a single-cross pearl millet hybrid. It is highly popular because of its extra-early maturity (it needs less than 65 days from sowing to grain maturity). Another variety HHB 67-improved has downy mildew (DM) disease resistance and is the first product of marker-assisted breeding to reach cereal producers in India. For cross-infection studies, wheat plant (*Triticum aestivum*) variety GW322 was used. GW322 is a wheat variety suitable for timely sown and irrigated conditions, and gives an average yield of 41-45 qtls/ha (Quintals/Hectare). Plants of this variety attains a height of 90-100 cms. The variety is resistant to stem rust (black rust) and leaf rust (brown rust).

3.2.3. Soil parameters

Soil used in pot (sand:clay:vermiculite-2:7:1) and field studies were analyzed in triplicate before experiment as described in section 2.2.3. Physico-chemical properties of soil are given in Table 3.2.

3.2.4. Test of cross-infection ability of endophytic bacteria

In order to evaluate host range and to test effect of endophytic bacteria on non-host plants, 14 selected bacterial isolates were tested for their ability to cross-infect wheat plant, a crop of similar economic importance to pearl millet. Experiment was performed in four replicates for each treatment under *in vitro* axenic conditions. Seeds of wheat were surface sterilized with 70% ethanol for 2 min followed by 0.2% $HgCl_2$ solution for 3 min and washed by

Table 3.1. List of isolates used in this study and their taxonomic affiliation based on 16S rRNA gene sequence.

Isolate	Bacterial Species	NCBI Accession number
PM9438	*Microbacterium testaceum*	KC683787
PM10455	*Rhizobium* sp.	KC683788
PM380	*Bacillus subtilis*	KC683744
PM389	*Pseudomonas aeruginosa*	JF899310
PM9426	*Arthrobacter woluwensis*	KC683778
PM9402	*Curtobacterium oceanosedimentum*	KC683768
PM9363	*Stenotrophomonas maltophilia*	KC683755
PM9405	*Acinetobacter calcoaceticus*	KC683771
PM10461	*Rhizobium* sp.	KC683791
PM9404	*Nocardioides* sp.	KC683770
PM9408	*Pseudomonas oryzihabitans*	KC683773
PM9368	*Rhodococcus kroppenstedtii*	KC683760
PM9364	*Stenotrophomonas maltophilia*	KC683756
PM9311	*Ochrobactrum anthropi*	KC683751

Table 3.2. Physicochemical properties of soil used in pot and field studies.

Soil	Pot studies	Field studies
pH (1:2.5)	8.20±0.02	8.72±0.03
EC (1:2.5)	0.01±0.00	0.23±0.00
Olsen's P (μg g^{-1})	27.65±0.54	13.14±0.10
O.C. (%)	0.48±0.03	0.21±0.20
Available nitrogen (μg g^{-1})	37.80±0.66	31.96±0.80
Ca^{+2} (mg g^{-1})	0.04±0.00	0.64±0.08
Mg^{+2} (mg g^{-1})	0.03±0.00	0.19±0.02
Cu (μg g^{-1})	0.47±0.06	0.19±0.07
Mn (μg g^{-1})	11.44±1.75	0.93±0.05
Zn (μg g^{-1})	2.23±2.30	0.25±0.10
Fe (μg g^{-1})	3.78±1.66	1.64±0.20
K (μg g^{-1})	90.88±0.11	55.00±0.23
N (μg g^{-1})	5.20±0.00	-

sterile distilled water thoroughly (Gupta et al. 2013). Six seeds were kept in each petri-dish having moist sterile Whatman filter paper No.1 in four replicates. The overnight grown cells of endophytic (10^8 cells ml^{-1} in PBS) bacteria were applied to each petri-dish containing surface sterilized seeds. Effectivity of surface sterilization of seeds was checked as described earlier in section 2.2.4. Uninoculated seeds served as control. All the petri-dishes were incubated in dark for initial 4 days and subsequently maintained on 16:8 photoperiod up to 10 days at 28±2°C. Two ml of water was added to the petri-dishes at every alternate day. After 10 days of growth, plants were examined for root length, shoot length, fresh weight and vigor index. Vigour index was calculated by the formula: (Shoot length + Root length) × Percent Germination.

3.2.5. Effect of endophytic inoculation on plant growth under green house conditions

To test and formulate effective bio-inoculum for the growth of plants, pearl millet seeds were treated with single and various combinations of endophytic bacterial consortia in different experimental set-ups. Bacterial isolates which induced plant growth in cross-infection studies and *in-vitro* growth study of wheat were selected for further test of plant growth of pearl millet in potted soil in green house conditions.

3.2.5.1. Pot level (Sterilized condition)

Twelve best isolates, which promoted plant growth in above-mentioned growth conditions, were selected for further pot studies conducted on pearl millet (HHB-67 Improved). These isolates were PM380, PM9426, PM9402, PM9408, PM9363, PM389, PM10461, PM9404, PM9405, PM10455, PM9368 and PM10438. The experiment was performed in five replicates for each treatment. For bacterization of seeds, two g surface sterilized pearl millet seeds were soaked in 25 ml PBS containing 1% carboxymethyl cellulose (CMC) and 10^8 cells ml^{-1} of endophytic bacteria, in a 250 ml conical flask. Bacterization was carried out under shaking condition (180 rpm) for 6 h at 30°C. Unbacterized seeds served as a control treatment and manure (3 soil:1 manure) treated plants were taken as positive control. Inoculated seeds were air dried for two hours under aseptic conditions. Ten seeds from each treatment were sown in sterilized soil filled in a plastic pot each with 8 cm diameter and 6 cm length. Soil was sterilized by autoclaving in a discontinuous manner for three days alternatively at 121°C for 15 min. Seedlings were grown at 25-30°C with 50-80% relative humidity in green house. Sterile water was added to the pots as and when required to maintain the moisture. After the growth period of 15 days, the plantlets were measured for shoot length using a measuring scale having 1 mm of

spatial resolution. Root lengths were measured using grid line intersect method (Tennant 1975). Other parameters monitored for plant growth promotion effects were fresh weight per plant, dry weight per plant, R:S (Root:Shoot) ratio, germination rate, total height and vigor index. The vigor index was calculated as described above.

3.2.5.2. Pot studies under unsterilized condition

Based on the results obtained in pot studies conducted in sterilized soil, nine (PM9426, PM9402, PM9408, PM389, PM10461, PM9405, PM10455, PM380, PM9404) best isolates were selected to assess their effect on plant growth of pearl millet in unsterilized soil. Experimental set-up for bacterization, plant growth and measurement criteria for pearl millet plant growth were similar as described above. Tap water was used as and when required to maintain the moisture.

3.2.5.3. Treatment of pearl millet plants with bacterial consortia, a microcosm study

Finally, 8 best isolates (PM9426, PM9402, PM9408, PM389, PM10461, PM9405, PM10455, PM9404), which promoted plant growth both under sterilized and unsterilized soil conditions, were selected for microcosm studies. To test performance of bacterial isolates in natural condition, microcosm study was conducted using unsterilized soil in pots. Different combinations of above eight selected isolates were prepared, and plant growth promotion studies were carried out in the similar manner as described above. Comparative analysis of performance of eight bioinoculants at petri-dish and pot studies (both sterilized condition and unsterilized soil) was done. Different formulations were made according to the ranking of selected isolate in equal proportion of different isolates (final concentration of 10^8 cells ml^{-1}). Altogether, 28 combinations (10^8 cells ml^{-1}) were evaluated on the growth of pearl millet in pot studies (unsterilized soil) under green house conditions. All the combinations were compared with control and manure-treated samples.

3.2.6. Effect of endophytic inoculation on plant growth: Field experiment

Seeds were bacterized as explained above. Field trial was designed and done in triplicates in actual farming conditions in a farm at Pilani, Rajasthan (India) during *kharif* season (August to October). PGPR-treated seeds (pearl millet) were hand sown with 100-150 seed in five rows (each separated by 15 cm) in each block (90×110 $inch^2$) with replicate. After emergence of seedlings, each plant was maintained at a distance of 5 cm and extra seedlings were removed. Treatments were arranged in randomized complete block design and inoculants used were PM9408, PM10461, PM9405, PM9426, PM389, and PM9402. The field was maintained

according to the pearl millet growing conditions and irrigated once at every 15 days. The field was devoid of any kind of organic and inorganic fertilizer. At the time of harvest (75 DAS), plant samples were collected for studying various growth parameters such as height of plant, number of cobs, length of cob, girth of cob, 1000 seeds wt and yield (Kg ha^{-1}).

3.2.7. Amplification and sequencing of 16S ribosomal RNA gene

Amplification and sequencing of 1.5 kb amplicons of 16S rRNA gene of six isolates (used in field studies) was done by using similar protocol as mentioned in section 2.2.7 and, full length of 16S rRNA gene was sequenced by primer walking method (section 2.2.8). Bacterial taxonomic affiliations were assigned based on the closest match to sequences available at the NCBI database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm and submitted to NCBI GenBank (Altschul et al. 1997).

3.2.8. Detection of endophytic bacterial inocula in plants

With a view to confirm endophytic nature of selected bacterial isolates, wheat plants were treated with candidate bacterial isolates and endophytic colonization of each bacterial inoculant was confirmed by PCR of 16S rDNA using the species-specific pair of primers from surface sterilized plants under bacterial treatment.

3.2.8.1. Inoculation of wheat plant with test bacterial isolates (six isolates)

3.2.8.1.1. Surface sterilization and plant growth: The surface sterilized seeds were placed on moistened sterile Whatman filter paper No.1 in petri-dishes for germination and watered every alternate day for four days under dark conditions as described in section 3.2.4. Healthy seedlings were transferred on 4^{th} day in tubes containing Hoagland media (0.3% Agar) (Appendix III).

3.2.8.1.2. Inoculation of plants: Bacterial inoculum was prepared as described in section 3.2.1. On 10^{th} day of plant growth, plants were challenged with different putative endophytic bacteria with a population density of 10^7-10^8 cells ml^{-1} (in 1X PBS) separately. Plants inoculated with 1X PBS served as control. All the treatments were carried out in triplicates. Plant samples were collected after 7 days post-inoculation of bacterial isolates, surface sterilized and sterilization efficiency of the protocol was assessed in a similar manner as described in section 3.2.4.

3.2.9. Designing of species-specific primers

Based on consistent plant growth promoting abilities in petri-dish, pot and field experiments, six bacterial isolates were selected for tracking their colonization by PCR. Full

Table 3.3. List of species-specific primers used in PCR analysis.

Strains	Primers	Sequence (5´-3´)	Size of Amplicons (bp)
PM10461	PM10461F	5'GGAGACGATGTCCTTCAGTTAGGC3'	145
PM10461	PM10461R	5'CCCTTAGAGTGCCCAACTAAATGC3'	
PM9405	PM9405F	5'GCGCTAATAGATGAGCCTAAGTCGG3'	265
PM9405	PM9405R	5'CCACTATCCAGTAGTATTAATACTAGTAGC3'	
PM9426	PM9426F	5'GAGCTTGCTCTCTGGGTGGCG3'	125
PM9426	PM9426 R	5'CCCCTGCTTTCACCCGTAGG3'	
PM389	PM389F	5'GAGCTTGCTCCTGGATTCAGC3'	138
PM389	PM389R	5'CGTGAGGTCCGAAGATCCCCC3'	
PM9402	PM9402F	5`AGCTTGCTGGGTGGATTAGTGGC3`	133
PM9402	PM9402R	5`TCTTTCCACCACCAGACCATGC3`	
PM9404	PM9404F	5'GCTGCAGAGATGTGGCCTCC3'	130
PM9404	PM9404R	5`AAAGGCGTGCTGGCAACATAGG3`	

length 16S rDNA sequences of selected (6) bacterial species used in present study and similar species available in public DNA database of NCBI were used for primer designing. DNA segments of respective bacterial species were aligned using multiple sequence alignment tool of clustal W (Thompson et al. 1994) to identify the variable region present in particular species. Species-specific primers were designed based on the variable region of each isolate. Specificity of each primer was cross-checked by BLAST analysis. Primers for different isolates have been described in Table 3.3. Oligonucleotides were synthesized by Eurofins scientific, India (Bangalore).

3.2.10. DNA isolation and PCR

Culture independent method was used for amplification of species-specific 16S rRNA genes. Collected plant tissues were crushed in liquid nitrogen and total DNA was isolated using ultraclean soil DNA isolation kit (MO BIO, USA) as per the manufacturer's instruction. Isolated DNA was confirmed by running on 0.8% agarose gel and stored at -20°C for further use. The 100 ng of DNA template was used as a template for PCR, which was performed using PTC thermal cycler (MJ Research, Inc., Walthon, MA, USA). Final volume of PCR reaction mixture was 50 µl, which consisted of 1.5 unit of Taq DNA polymerase, 125 µM each dNTPs, 1.5 mM $MgCl_2$ and 30 pmol of each primer. The specific primers used for different cultures are mentioned in Table 3.3. Thermal profile of PCR reaction included an initial denaturation at 94^{o}C for 5 min followed by 35 cycles of 1 min at 94^{o}C, 58^{o}C for 30 s and, 30 s at 72^{o}C with a final extension of 5 min at 72^{o}C. The amplified products were analyzed on 2% agarose gel using a gel documentation unit (Bio-Rad, USA).

3.2.11. Statistical analysis

Microsoft Excel 2007 was used for statistical processing of the data (standard deviation, and student's *t*-test).

3.3. Results

3.3.1. *In vitro* studies of plant growth

Based on the presence of appreciable plant growth promoting properties, 14 isolates were selected and used to inoculate wheat seeds for: (i) evaluation of their effect on seed germination, (ii) plant growth stimulation in plate assay, and (iii) their ability to cross-infect. Inoculation of all

Table 3.4. Effect of selected bacterial inoculants on growth of wheat plants under *in vitro* condition.

Isolates	Root length (cm)	Shoot length (cm)	Fresh Wt. (g)	Vigour index
Control	2.9±1.0	6.7±0.9	0.08±0.02	960
PM9438	3.4±1.9 (17%)*	7.6±1.9 (13%)	0.10±0.05 (35%)	1100 (15%)*
PM10455	3.65±1.3 (26%)*	8.2±1.7 (22%)*	0.15±0.09 (88%)*	1185 (23%)*
PM380	3.1±1.6 (7%)	8.45±1.5 (25%)*	0.13±0.03 (63%)*	1155 (20%)*
PM389	3.5±0.1 (21%)*	7.9±1.3 (18%)*	0.1±0.02 (25%)*	1140 (19%)*
PM9426	3.8±1.5 (31%)*	9.6±2.2 (43%)*	0.10±0.03 (25%)*	1340 (40%)*
PM8311	3.3±1.3 (14%)*	6.9±1.7 (3%)*	0.08±0.03 (0%)	1020 (6%)
PM9364	3.32±1.5 (14%)*	6.9±2.4 (3%)*	0.08±0.02 (0%)	1022 (6%)
PM9402	4±1.8 (38%)*	7.8±1.0 (16%)*	0.09±0.01 (13%)*	1180 (23%)*
PM9363	3.7±0.9 (28%)*	8.2±1.0 (22%)*	0.11±0.02 (38%)*	1190 (24%)*
PM9368	3.7±1.3 (28%)*	7.1±1.3 (5%)	0.09±0.02 (13%)	1080 (23%)*
PM9405	3.8±1.2 (31%)*	7.8±1.2 (16%)*	0.11±0.02 (38%)*	1160 (21%)*
PM10461	3.4±1.1 (17%)	7.6±1.5 (13%)*	0.09±0.03 (13%)*	1100 (15%)*
PM9404	3.7±1.0 (28%)*	7.6±1.0 (13%)*	0.10±0.02 (25%)*	1130 (18%)*
PM9408	3.7±1.4 (28%)*	7.5±1.2 (12%)*	0.10±0.02 (25%)*	1120 (17%)*

*$p<0.05$, n=37 (Student's *t*-test). ± represents standard deviation of mean. Values in parenthesis indicate percent increase in comparison to un-inoculated control plants.

selected isolates led to increase in root/shoot length and fresh weight in comparison to un-inoculated control (Table 3.4). Out of 14 isolates, isolate PM10455 was noted as the most efficient isolate, which enhanced plant growth by 23 and 88% w.r.t. total height and biomass respectively. Except few, all the isolates showed significant difference in various parameters in comparison to control ($p<0.05$, n=37). For instance, inoculation of PM380 and PM10461 resulted into insignificant changes in root length of treated plants, while PM9438 and PM9368 showed no significant changes in shoot length. In case of fresh weight, no significant changes were observed in PM9438, PM8311, PM9364 and PM9368. Similarly, PM8311 and PM9364 showed no significant changes in vigor index.

3.3.2. Effect of endophytic inoculation on plant growth under green house conditions: Pot level (Sterilized condition)

Out of 14 isolates subjected to *in vitro* cross-infection studies of wheat described above, 12 best isolates were selected for pot studies (pearl millet) under sterilized conditions in green house (Table 3.5). In this study, most of the PGPR formulations except PM380 significantly enhanced the root length higher than manure treated and untreated control. Isolate PM9408 showed highest increment in root length (87% than control) amongst all PGPRs, followed by PM10461 and PM9405. Very few inoculants such as PM9404 and PM9408 enhanced the shoot length of pearl millet in this experiment, which were 9 and 2% higher respectively than untreated control. R:S index (Root:Shoot ratio) was significantly highest in PM9408 followed by PM10461. Isolates PM380, PM9363, PM389, PM10455 showed no increase in fresh weight, and none of the isolates showed increment in fresh weight in comparison to the manure treated sample. PM9408 was the only isolate showing significant induction in fresh weight (37%) than untreated control as shown in Table 3.5. Highest enhancement in dry weight was recorded by PM9363 and PM10461. PM9405 showed significantly high germination rate and vigor index, while PM9408 enhanced the total height over control. Two isolates, PM10438 and PM9368, showed no significant changes in any of the parameters of plant growth promotion. Based on the results of all above-mentioned parameters cumulatively, PM9405 showed best results followed by PM10461 and PM9402 in the pot studies (sterilized conditions) under green house conditions.

Table 3.5. Effect of selected bacterial inoculants on growth of pearl millet plant in pot (sterilized soil) studies under green house condition.

Treatment	**Root length (cm)**	**Shoot length (cm)**	**Root:Shoot Index**	**Fresh Wt. (g)**	**Dry Wt. (g)**	**Germination rate (%)**	**Total height (cm)**	**Vigor index**
Control	17.13 ±2.38	22.68 ±2.28	0.80 ±0.40	0.30 ±0.07	0.03 ±0.01	42 ±29	41 ±10.09	1788 ±854
Manure	21.33 ±6.18	27.18 ±2.00	0.78 ±0.21	0.63 ±0.12	0.03 ±0.03	25 ±10	49 ±7.25	1394 ±573
PM380	18.32 ±6.50 (7*, -)	18.46 ±2.56 (-, -)	0.87 ±0.42 (9, 12)	0.29 ±0.06 (-, -)	0.02 ±0.01 (-, -)	45 ±17 (7, 80)	36.93 ±8.23 (-, -)	1700 ±572 (-, 22)
PM9426	25.15 ±3.94 (47*, 18)	20.18 ±1.78 (-, -)	1.24 ±0.14 (55*, 43*)	0.35 ±0.08 (16, -)	0.03 ±0.04 (-, -)	43 ±17 (2, 72)	45.33 ±5.37 (11, -)	1714 ±724 (-, 23)
PM9402	27.99 ±4.04 (63*, 31*)	21.48 ±2.51 (-, -)	1.31 ±0.15 (64*, 68*)	0.35 ±0.13 (17, -)	0.01 ±0.01 (-, -)	43 ±15 (2, 72)	49.47 ±5.95 (21, 1)	2096 ±697 (17, 50)
PM9408	32.31 ±5.80 (87*, 51*)	23.17 ±2.28 (2, -)	1.40 ±0.24 (75*, 79*)	0.41 ±0.08 (37*, -)	0.02 ±0.04 (-, -)	28 ±8 (-, 12*)	55.48 ±7.09 (35*, 13)	1520 ±327 (-, 9*)

PM9363	25.45 ±5.13 (52*, 22)	21.97 ±3.13 (-, -)	1.17 ±0.26 (46*, 50*)	0.26 ±0.06 (-, -)	0.04 ±0.04 (33, 33)	34 ±18 (-, 36)	47.42 ±6.58 (16, -)	1598 ±742 (-, 15)
PM389	26.03 ±4.74 (34*, 21)	17.97 ±2.44 (-, -)	1.48 ±0.43 (85*, 90*)	0.27 ±0.01 (-, -)	0.03 ±0.02 (-, -)	38 ±17 (-, 52)	44.00 ±3.66 (7, -)	1640 ±707 (-, 18)
PM10461	28.58 ±5.77 (67*,34*)	21.73 ±3.35 (-, -)	1.32 ±0.22 (65*, 69*)	0.38 ±0.14 (27, -)	0.04 ±0.01 (33*, 33)	37 ±14 (-, 48)	50.32 ±8.20 (23*, 3)	1622 ±616 (-, 16)
PM9404	24.95 ±4.26 (44*, 17)	24.77 ±1.58 (9, -)	1.01 ±0.17 (26*, 29*)	0.34 ±0.09 (13, -)	0.03 ±0.02 (-, -)	44 ±15 (4, 76*)	49.72 ±4.94 (21, -)	2206 ±790 (23, 58*)
PM9405	28.41 ±2.71 (66*, 33*)	22.67 ±4.08 (-, -)	1.29 ±0.25 (61*, 65*)	0.34 ±0.10 (13, -)	0.02 ±0.01 (-, -)	48 ±17 (14, 92*)	51.09 ±5.03 (25*, 4)	2407 ±900 (35, 73*)
PM10455	26.52 ±2.32 (55*, 24*)	21.53 ±2.57 (-, -)	0.90 ±0.63 (13, 15)	0.27 ±0.07 (-, -)	0.02 ±0.01 (-, -)	41 ±20.88 (-, 64)	40.47 ±12.84 (-, -)	1796 ±1023 (-, 29)
PM9438	15.23 ±1.08 (-, -)	20.01 ±1.22 (-, -)	0.76 ±0.40 (-, -)	0.20 ±0.00 (-, -)	0.01 ±0.01 (-, -)	24 ±4 (-, -)	35.24 ±9.09 (-, -)	845 ±60 (-, -)

PM9368								
	11.33 ±2.05 (-, -)	18.01 ±3.24 (-, -)	0.63 ±0.22 (-, -)	0.15 ±0.05 (-, -)	0.02 ±0.00 (-, -)	22 ±7 (-, -)	29.34 ±8.99 (-, -)	645 ±100 (-, -)

*p<0.05 (Student's t-test). ± represents standard deviation of mean. Values in parenthesis indicate percent increase in comparison to un-inoculated control and manure treated plants respectively.

3.3.3. Effect of endophytic inoculation on plant growth under green house conditions: Pot level (Unsterilized condition)

Pearl millet plants were treated with endophytic bacteria to test their effect on plant growth in natural soil under green house condition. Nine best isolates were selected on the basis of results obtained in pot studies under sterilized conditions. Similar to the results of pot studies under sterilized conditions, most of the bioinoculants showed an increment in root length. However, the changes noticed in root length increment were not significant. *P. aeruginosa* PM389 significantly enhanced the root length and R:S index over control and manure treated sample, while none of the PGPR treatments showed an increment in shoot length, fresh weight, dry weight and total height (Table 3.6). Inoculation of most of the isolates resulted into significant changes in R:S index than control and manure treated sample. Isolates PM9408, PM10461 and PM9426 equally (approx.) and significantly increased the germination rate.

No significant increment was observed in vigour index in bacterized plants. Positive results could not be obtained in PM380 and PM9404 in any of the parameters assessed. On overall assessment of all the parameters computed in Table 3.6, PM389 followed by PM10461 and PM9426 showed best outcomes as bioinoculants in this experiment.

3.3.4. Effect of endophytic inoculation on plant growth under green house conditions: Microcosm studies (Unsterilized condition)

On the basis of performance shown by various isolates in the results described above for different studies, a comparative analysis was done as described in Table 3.7. Comparative analysis suggested that all the isolates showed difference in plant growth promotion with the varying conditions viz., *in vitro* studies, pot studies under sterilized and unsterilized conditions. From above results, isolates were ranked on the basis of stable and best results. Finally, 8 best bacterial isolates were selected to be inoculated in various combinations (Table 3.8) of bacterial consortia for microcosm studies. With few exceptions, most of the PGPR formulations enhanced root (T-3, T-4, T-16, T-19, T-25) and shoot length (T-1, T-3, T-8, T-12, T-14, T-20, T-25) as well as R:S index (T-16) except few. PM9402 was the only isolate, which showed maximum activity along with PM9408 than consortium of PM9402 with many other isolates. In microcosm studies, treatments T-6 and T-17 showed the maximum increase in root length. PM10455 was the common isolate in both of the above treatments (Table 3.9). T-7 was the only combination which enhanced the shoot length significantly by 18%.

Table 3.6. Effect of selected bacterial inoculants on growth of pearl millet plants in pot (Unsterilized soil) studies under green house condition.

Treatment	Root length (cm)	Shoot length (cm)	Root:Shoot Index	Fresh Wt. (g)	Dry Wt. (g)	Germination rate (%)	Total height (cm)	Vigor index
Control	11.23 ±1.14	21.16 ±2.27	0.44 ±0.25	0.18 ±0.03	0.02 ±0.00	76 ±9	32.78 ±3.34	2629 ±271
Manure	11.30 ±1.19	24.00 ±3.25	0.48 ±0.08	0.23 ±0.04	0.02 ±0.00	89 ±13	35.30 ±3.59	2930 ±272
PM9426	12.34 ±0.97 (10, 9)	17.20 ±1.75 (-, -)	0.72 ±0.09 (64*, 50*)	0.15 ±0.02 (-, -)	0.02 ±0.00 (-, -)	90 ±5 (18*, 1)	29.54 ±1.96 (-, -)	2654 ±163 (1, -)
PM9402	11.50 ±1.95 (2, 2)	17.05 ±1.51 (-, -)	0.67 ±0.11 (52*, 40*)	0.16 ±0.01 (-, -)	0.02 ±0.00 (-, -)	80 ±12 (5, -)	28.55 ±3.00 (-, -)	2293 ±474 (-, -)
PM9408	10.82 ±0.51 (-, -)	16.95 ±0.78 (-, -)	0.64 ±0.05 (45*, 33*)	0.15 ±0.03 (-, -)	0.02 ±0.00 (-, -)	93 ±6 (22*, 4)	27.77 ±0.81 (-, -)	2565 ±125 (-, -)
PM389	13.60 ±1.30 (21*, 20*)	14.86 ±2.35 (-, -)	0.92 ±0.08 (100*, 92*)	0.14 ±0.02 (-, -)	0.02 ±0.00 (-, -)	79 ±12 (4, -)	28.46 ±3.50 (-, -)	2263 ±511 (-, -)

PM10461	12.80 ±1.44 (14, 13)	17.92 ±0.65 (-, -)	0.71 ±0.07 (61*, 48*)	0.16 ±0.02 (-, -)	0.02 ±0.01 (-, -)	92 ±12 (21*, 3)	30.72 ±1.86 (-, -)	2822 ±367 (7, -)
PM9405	12.60 ±1.74 (12, 12)	17.85 ±1.74 (-, -)	0.71 ±0.07 (61, 48)	0.18 ±0.02 (-, -)	0.02 ±0.00 (-, -)	86 ±13 (13, -)	30.45 ±3.19 (-, -)	2652 ±602 (1, -)
PM10455	12.93 ±1.08 (15, 14)	18.68 ±0.46 (-, -)	0.69 ±0.05 (57*, 44*)	0.19 ±0.02 (-, -)	0.02 ±0.00 (-, -)	85 ±9 (12, -)	31.60 ±1.40 (-, -)	2682 ±269 (2, -)
PM380	10.53 ±2.20 (-, -)	19.04 ±3.00 (-, -)	0.55 ±0.22 (-, -)	0.16 ±0.03 (-, -)	0.01 ±0.00 (-, -)	72 ±4 (-, -)	29.57 ±1.22 (-, -)	2129 ±190 (-, -)
PM9404	9.26 ±1.09 (-, -)	20.09 ±2.78 (-, -)	0.46 ±0.15 (-, -)	0.17 ±0.03 (-, -)	0.01 ±0.00 (-, -)	74 ±8 (-, -)	29.35 ±1.05 (-, -)	2172 ±303 (-, -)

*$p<0.05$ (Student's *t*-test). ± represents standard deviation of mean. Values in parenthesis indicate percent increase in comparison to un-inoculated control and manure treated plants respectively.

Table 3.7. Comparative analysis of results obtained in different conditions of plant growth promotion studies.

Strains	***In vitro* test**	**Pot studies (Sterilized condition)**	**Pot studies (Unsterilized condition)**	**Final Ranking**
PM380	VI	X	IX	-
PM9426	III	VII	III	V
PM9402	IV	III	VII	VI
PM9408	VI	IV	VI	I
PM9363	II	V	-	-
PM389	VII	VIII	I	VIII
PM10461	VIII	II	II	III
PM9404	V	VI	VIII	II
PM9405	III	I	V	IV
PM10455	I	IX	IV	VII

#Roman letters suggests the ranking of each strain on the basis of overall parameters studied.

Table 3.8. Different combinations of strains on the basis of comparative analysis of plant growth tests used in microcosm studies.

Treatments	Combinations
T-1	PM9408+PM9404
T-2	PM9408+PM10461
T-3	PM9408+PM9405
T-4	PM9408+PM9426
T-5	PM9408+PM9402
T-6	PM9408+PM10455
T-7	PM9408+PM389
T-8	PM9408+PM9404+PM10461
T-9	PM9408+PM9404+PM9405
T-10	PM9408+PM9404+PM9426
T-11	PM9408+PM9404+PM9402
T-12	PM9408+PM9404+PM10455
T-13	PM9408+PM9404+PM389
T-14	PM9408+PM9404+PM10461+PM9405
T-15	PM9408+PM9404+PM10461+PM9426
T-16	PM9408+PM9404+PM10461+PM9402
T-17	PM9408+PM9404+PM10461+PM10455
T-18	PM9408+PM9404+PM10461+PM389
T-19	PM9408+PM9404+PM10461+PM9405+PM9426
T-20	PM9408+PM9404+PM10461+PM9405+PM9402
T-21	PM9408+PM9404+PM10461+PM9405+PM10455
T-22	PM9408+PM9404+PM10461+PM9405+PM389
T-23	PM9408+PM9404+PM10461+PM9405+PM9426+PM9402
T-24	PM9408+PM9404+PM10461+PM9405+PM9426+PM10455
T-25	PM9408+PM9404+PM10461+PM9405+PM9426+PM389
T-26	PM9408+PM9404+PM10461+PM9405+PM9426+PM9402+PM10455
T-27	PM9408+PM9404+PM10461+PM9405+PM9426+PM9402+PM389
T-28	PM9408+PM9404+PM10461+PM9405+PM9426+PM9402+PM10455+PM389

Table 3.9. Effect of mixture of selected inoculants on the growth of pearl millet plants in pot (Unsterilized soil) studies under green house condition.

Treatment	Root length (cm)	Shoot length (cm)	Root:Shoot Index	Fresh Wt. (g)	Dry Wt. (g)	Germination rate (%)	Total height (cm)	Vigor index
Control	11.23	21.16	0.44	0.18	0.02	76.25	32.78	2630
	±1.14	±2.27	±0.25	±0.03	±0.00	±8.54	±3.34	±270.57
Manure	11.30	24.00	0.48	0.23	0.02	89.00	35.30	2930
	±1.19	±3.25	±0.08	±0.04	±0.00	±12.94	±3.59	±271.57
T-1	11.55	20.80	0.56	0.17	0.02	82.50	32.35	2671
	±1.48	±1.70	±0.12	±0.01	±0.00	±17.68	±0.21	±589.73
	(3, 3)	(-, -)	(27*, 17)	(-, -)	(-, -)	(8, -)	(-, -)	(2, -)
T-2	12.65	22.70	0.56	0.16	0.02	90.00	35.35	3189
	±0.75	±1.82	±0.06	±0.03	±0.00	±14.14	±1.92	±585.19
	(13, 12)	(7, -)	(27, 17)	(-, -)	(-, -)	(18, 1)	(8, -)	(21, 9)
T-3	11.00	20.55	0.54	0.18	0.02	97.50	31.55	3075.13
	±0.67	±0.62	±0.04	±0.01	±0.00	±2.89	±0.56	±55.43
	(-, -)	(-, -)	(19, 11)	(-, -)	(-, -)	(22*, 9)	(-, -)	(14*, 5)
T-4	10.70	22.00	0.49	0.17	0.02	92.50	32.70	3019.88
	±1.65	±1.44	±0.09	±0.03	±0.01	±6.45	±1.39	±146.50
	(-, -)	(4, -)	(11, 2)	(-, -)	(-, -)	(21*, 4)	(-, -)	(15*, 3)
T-5	12.63	23.15	0.55	0.17	0.02	91.25	35.78	3267.25
	±0.79	±1.17	±0.06	±0.05	±0.00	±7.50	±0.50	±313.99
	(12, 12)	(9,-)	(20, 13)	(-, -)	(-, -)	(20*, 3)	(9, 1)	(24*, 12)
T-6	13.57	21.43	0.63	0.21	0.02	88.33	35.00	3079.83
	±2.12	±0.81	±0.09	±0.05	±0.00	±7.64	±2.63	±125.58
	(21, 20)	(1, -)	(43, 31)	(17, -)	(-, -)	(16, -)	(7, -)	(17*, 5)

T-7	12.33 ±0.48 (10, 9)	24.93 ±1.21 (18*, 4)	0.50 ±0.02 (14, 4)	0.19 ±0.01 (5, -)	0.02 ±0.00 (-, -)	77.50 ±9.57 (2, -)	37.25 ±1.49 (14*, 6)	2886.75 ±367.83 (10, -)
T-8	11.20 ±0.71 (-, -)	19.65 ±1.48 (-, -)	0.57 ±0.08 (23, 16)	0.16 ±0.02 (-, -)	0.01 ±0.00 (-, -)	92.50 ±3.54 (18*, 4)	30.85 ±0.78 (-, -)	2855.00 ±181.02 (-, -)
T-9	12.37 ±0.25 (10, 9)	23.30 ±1.57 (10, -)	0.53 ±0.03 (20, 10)	0.18 ±0.02 (-, -)	0.03 ±0.00 (50*, 50*)	83.33 ±5.77 (9, -)	35.67 ±1.81 (9, 1)	2965.33 ±56.19 (13, 1)
T-10	11.85 ±1.14 (6, 5)	22.73 ±2.73 (7, -)	0.52 ±0.04 (18, 8)	0.18 ±0.04 (-, -)	0.02 ±0.00 (-, -)	80.00 ±14.72 (5, -)	34.58 ±3.65 (5,-)	2771.50 ±616.88 (5, -)
T-11	11.63 ±1.28 (4, 3)	21.75 ±2.01 (3, -)	0.53 ±0.03 (20, 10)	0.17 ±0.03 (-, -)	0.01 ±0.00 (-, -)	76.25 ±11.09 (-, -)	33.38 ±3.16 (2, -)	2567.25 ±586.18 (-, -)
T-12	13.10 ±1.97 (16, 15)	24.00 ±1.36 (-, -)	0.55 ±0.08 (25, 15)	0.23 ±0.03 (28, -)	0.02 ±0.00 (-, -)	92.50 ±9.57 (21*, 4)	37.10 ±2.68 (13, 5)	3432.00 ±442.51 (30*, 17)
T-13	11.89 ±1.90 (5, 5)	21.88 ±1.69 (3, -)	0.54 ±0.09 (23, 13)	0.19 ±0.02 (5, -)	0.01 ±0.00 (-, -)	78.75 ±9.46 (4,-)	33.77 ±2.99 (3, -)	2649.65 ±322.12 (1, -)
T-14	12.15 ±1.72 (8, 8)	19.55 ±4.88 (-, -)	0.66 ±0.23 (50, 38)	0.19 ±0.01 (5, -)	0.01 ±0.01 (-, -)	81.25 ±11.09 (7,-)	31.70 ±5.39 (-, -)	2531.13 ±100.97 (-, -)
T-15	11.40 ±0.80 (2, 1)	22.90 ±2.69 (8, -)	0.50 ±0.06 (14, 4)	0.21 ±0.02 (17, -)	0.02 ±0.01 (-, -)	86.25 ±7.50 (13, -)	34.30 ±3.11 (5, -)	2967.63 ±464.68 (13, 1)

T-16	10.83 ±2.45 (-, -)	21.93 ±1.90 (4, -)	0.49 ±0.07 (11, 2)	0.22 ±0.04 (17, -)	0.02 ±0.01 (-, -)	85.00 ±8.66 (11, -)	32.77 ±4.35 (-, -)	2784.33 ±440.94 (6, -)
T-17	13.58 ±0.49 (21*, 20*)	22.08 ±0.49 (4, -)	0.62 ±0.03 (41*, 29*)	0.23 ±0.03 (28*, -)	0.02 ±0.00 (-, -)	65.00 ±8.16 (-, -)	35.65 ±0.45 (9, 1)	2318.75 ±310.13 (-, -)
T-18	12.26 ±1.05 (9, 8)	23.12 ±1.92 (9, -)	0.53 ±0.05 (20, 10)	0.18 ±0.04 (-, -)	0.02 ±0.01 (-, -)	76.00 ±8.22 (-, -)	35.38 ±2.56 (8, -)	2682.10 ±268.08 (2, -)
T-19	11.30 ±1.06 (-, -)	22.10 ±0.78 (4, -)	0.51 ±0.03 (16, 6)	0.24 ±0.02 (33*, 4)	0.03 ±0.01 (50, 50)	86.67 ±5.77 (14, -)	33.40 ±1.84 (2, -)	2887.67 ±47.01 (10, -)
T-20	12.00 ±0.73 (7, 6)	21.08 ±1.71 (-, -)	0.57 ±0.07 (30, 19)	0.22 ±0.02 (22*, -)	0.02 ±0.00 (-, -)	88.00 ±7.58 (15, -)	33.08 ±1.64 (1, -)	2919.20 ±367.57 (11, -)
T-21	- (-, -)	- (-, -)	- (-, -)	- (-, -)	- (-, -)	- (-, -)	- (-, -)	- (-, -)
T-22	11.89 ±0.64 (6, 5)	21.82 ±2.68 (3, -)	0.55 ±0.06 (25, 15)	0.21 ±0.03 (17, -)	0.02 ±0.01 (-, -)	79.00 ±15.17 (4, -)	33.71 ±3.14 (3, -)	2660.02 ±561.64 (1, -)
T-23	12.06 ±1.35 (7, 7)	21.84 ±1.86 (3, -)	0.56 ±0.11 (27, 17)	0.20 ±0.04 (11, -)	0.02 ±0.01 (-, -)	78.00 ±14.83 (3, -)	33.90 ±1.55 (3, -)	2652.80 ±567.80 (-, -)
T-24	12.23 ±0.35 (9, 8)	23.23 ±1.99 (10, -)	0.53 ±0.03 (20, 10)	0.20 ±0.03 (11, -)	0.03 ±0.01 (50*, 50*)	86.25 ±2.50 (-, -)	35.45 ±2.28 (-, -)	3058.63 ±233.30 (-, -)
T-25	11.90 ±0.85 (-, -)	22.70 ±2.11 (-, -)	0.52 ±0.01 (18, 8)	0.17 ±0.04 (-, -)	0.02 ±0.00 (-, -)	70.00 ±13.23 (-, -)	34.60 ±2.96 (5, -)	2440.17 ±600.02 (-, -)
T-26	11.73 ±1.31 (4, 4)	23.15 ±0.59 (9, -)	0.51 ±0.05 (16, 6)	0.19 ±0.03 (5, -)	0.02 ±0.01 (-, -)	86.25 ±6.29 (13, -)	34.88 ±1.61 (6, -)	3004.75 ±201.81 (14*, 2)

T-27	12.38 ±0.37 (10, 10)	23.83 ±1.35 (13, -)	0.52 ±0.02 (18, 8)	0.21 ±0.04 (17, -)	0.02 ±0.00 (-, -)	78.75 ±4.79 (3, -)	36.20 ±1.70 (10, 2)	2844.88 ±54.11 (8, -)
T-28	12.35 ±0.41 (10, 9)	23.53 ±0.96 (11, -)	0.53 ±0.02 (20, 10)	0.20 ±0.02 (11, -)	0.02 ±0.00 (-, -)	83.75 ±13.15 (10, -)	35.88 ±1.25 (9, 2)	2994.75 ±395.44 (14, 2)

*$p<0.05$ (Student's *t*-test). ± represents standard deviation of mean. Values in parenthesis indicate percent increase in comparison to un-inoculated control and manure treated plants respectively.

Most of the combinations of PGPRs significantly increased the R:S index (Table 3.9) where, it was highest in T-14 treatment (PM9408+PM9404+PM10461+PM9405). For fresh weight, very few (T-17, T-19, T-20) combinations were observed to be effective. However, T-19 (PM9408+PM9404+PM10461+PM9405+PM9426) showed significant increment in fresh weight. T-9, T-19 and T-24 were the only combinations which equivalently enhanced the dry weight of the plants by 50%. In all the three combinations, PM9408, PM9404 and PM9405 were the common isolates. Most of the mixture inoculants increased germination rate, total height as well as the vigor index with certain exceptions. T-3, T-7 and T-12 enhanced germination rate, total height and vigour index by 28, 14 and 30% respectively. On overall estimation based on all the parameters, T-12 (PM9408+PM9404+PM10455) showed maximum activity followed by T-9 (PM9408+PM9404+PM9405) and T-6 (PM9408+PM10455). It revealed that PM10455 influenced plant growth higher in combination with PM9404 than when it was used alone. Next best isolate was PM9405 which again showed more activity in the presence of many other isolates in comparison to when used alone (T-3>T-19>T-9). Other than these two isolates, both PM10461 and PM9426 showed good activity in the presence of other isolates. Combination of all the selected isolates (8) in this study showed good activity in terms of plant growth promotion. However, combination of *P. aeruginosa* PM389 showed the least activity among all the isolates, it showed better activity in combination with PM9408 than in the microcosm including PM9408. Unexpectedly and exceptionally, inoculation of T-21 showed no seed germination in any of the replicates. It was contrasting to observe that T-21 was a combination of PM10461, PM9405, and PM10455, in which all the isolates showed appreciably good effect on plant growth on individual inoculation.

3.3.5. Effect of endophytic inoculation on plant growth: Field experiment

Once, the effect of endophytic bacteria was tested in laboratory and green house, the study was extended to actual farming condition. Six isolates namely PM9408, PM10461, PM9405, PM9426, PM389, PM9402 were selected for conducting field studies. Only PM10461, PM9405, PM9426 showed positive results under field condition, while for other three isolates positive results could not be obtained (data not shown). PM10461 showed the best results and significant enhancement in all the parameters observed similar to that obtained in pot studies (2^{nd} best isolate in both sterilized and unsterilized conditions) followed by PM9405 and PM9426 (Table 3.10). PM9405 enhanced all the parameters significantly except girth of earhead, while

Table 3.10. Effect of selected bacterial inoculants on growth of pearl millet plant under field condition.

Treatment	Height of plant (cm)	No. of earhead/plant	Length of earhead (cm)	Girth of earhead (cm)	1000 seeds Wt. (g)	Yield (Kg/Ha)
Control	80.7±21.8	2±0.5	9.1±1.4	3.9±2.0	5±1.1	125.2±90.7
PM10461	136.2±5.5 (68%)*	3±0.6 (50%)*	17.4±1.8 (91%)*	8.1±1.9 (100%)*	7.2±0.6 (44%)	219.0±36.3 (74%)
PM9405	131.7±13.8 (63%)*	3±0.5 (50%)*	16.2±1.2 (78%)*	6.4±0.7 (64%)	7.6±0.8 (52%)*	200.6±47.9 (60%)
PM9426	107.7±18.2 (33%)	2±0.6 (0%)	14.3±2.8 (57%)*	5.7±1.5 (46%)	7.2±0.4 (44%)	167.0±39.8 (33%)

*$p<0.05$ (Student's *t*-test). ± represents standard deviation of mean. Values in parenthesis indicate percent increase in comparison to un-inoculated control plants.

PM9426 enhanced only the length of earhead significantly. PM9426 produced higher increments in all the parameters observed than that of untreated control.

3.3.6. Sterilization efficiency and species-specific PCR

Absence of bacterial growth from last wash sample confirmed effectivity of surface sterilization. Surface sterilization was done to eliminate rhizoplanic bacteria. Specificity of each pair of primers was ensured by amplification of 16S rDNA segment from isolated pure culture as well as from mixed population. Six isolates were subjected to species-specific PCR, and for all isolates positive amplification was obtained as shown in Fig. 3.1. Exceptionally, in PM9426 non-specific amplification was obtained in PM9426 infected plant samples, although during optimization single band was obtained from PM9426 F and PM9426 R primers.

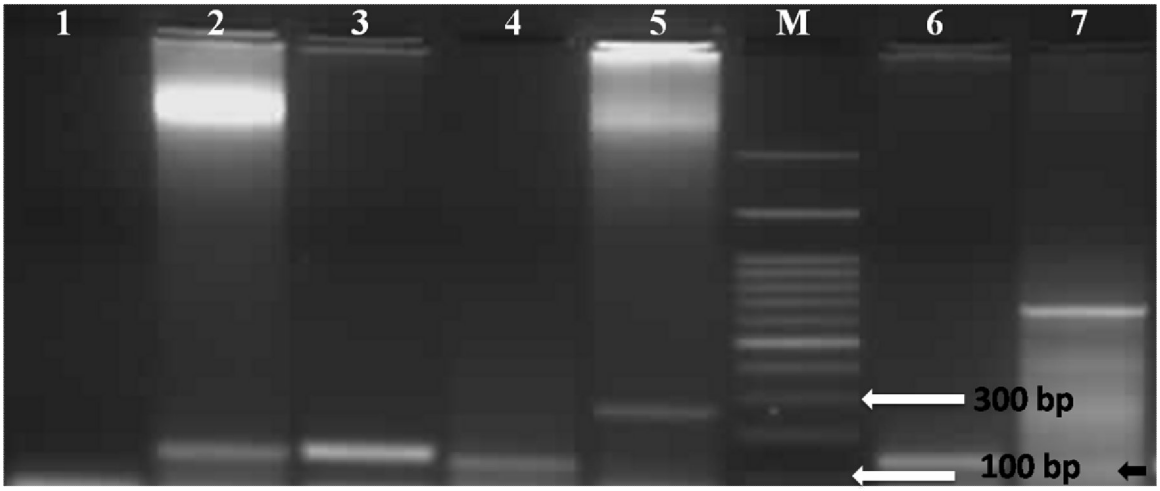


Fig. 3.1. Species-specific PCR of different isolates. Each lane (treatment) represents desired amplicon for the respective isolate such as: Lane1-control (plant without infection), Lane 2- PM9402, Lane 3- PM10461, Lane 4- PM9404, Lane 5- PM9405, M; molecular weight marker (100-1000 bp ladder; MBI Fermentas, USA), Lane 6- PM389, Lane 7- PM9426. An arrow in lane 7 (PM9426) is showing the specific desired band among the non-specific bands.

3.4. Discussion

Application and potential of plant growth promoting endophytic bacteria has largely been acknowledged and authenticated by several researchers across the globe in last few decades. Stimulation of plant growth and yield by endophytic bacteria has been reported on laboratory, greenhouse (Figueiredo et al. 2011) and field level (Vessey 2003) in several studies (Hayat et al. 2010). However, application of endophytic bacteria on large-scale field studies is still lacking. In particular, use of endophytic and other PGPR to increase plant growth and yield is still in nascent stage mostly in developing countries, where demand of food crop is very high (Adesmoye and Egamberdieva 2013). Therefore, present work made an effort to study the efficiency of endophytic diazotrophic bacteria obtained from pearl millet at various levels from laboratory conditions to field level. Bacterial isolates, which showed significant increment in growth parameters as well as stability at different conditions, may serve as potential bioinoculants for future use.

Similar to *in vitro* experiment in wheat, increment in rooting was observed in pearl millet plants by most of the isolates in pot studies under green house conditions, which confirmed that preliminary results are important in the strategic approach for identifying the potential biofertilizer agent. Under sterilized condition in pot studies, most of the isolates improved the growth of pearl millet plants in terms of growth parameters tested when compared to untreated control. Moreover, some of the isolates showed significant increments in growth which were higher than the manure treated plants as well. However, the efficiency varied significantly with different isolates. Similar results were reported by Holguin and Glick (2001). Extensive proliferation of the root system has an important role in growth promotion and resistance development in plants under adverse conditions. Generation of rooting may be attributed to bacterial production of auxin which proliferates roots. In turn, healthier root system led to increase in nutrients acquisition due to increase in root surface area and contribute to overall plant growth and the crop yield (Lalande et al. 1989). This observation and its relation to increased plant growth have been reported in several studies (Baset Mia et al. 2010). Earlier greenhouse and pot level studies with the endophytic rhizobial inoculum indicated the significant increase in N, P, K and Fe uptake in rice plants and led to increased biomass and yield of rice plants (Biswas et al. 2000). R:S index was also significantly higher in almost all PGPR treated plants than untreated control, which suggest that these inoculants are efficient in making the

plant system healthier. R:S index is an important parameter of plant growth and plays an important role in ameliorating stress in plants. Increment in the R:S index indicates that plant is growing under favorable conditions and long rooting systems can increase the availability of nutrients and water to plants (Vavrina 1996). However, some of the endophytic isolates in this study induced a little increase in the shoot length, which may be due to suboptimal condition for the given isolates in natural conditions. Increase in total height, germination rate and vigor index by almost all the PGPR inoculants generalized that they have an important role in enhancing the vegetative and early stage growth of plants. Increase in biomass of treated plants is also due to early stage development in growth of root and shoot (Saharan and Nehra 2011). Our study observed increase in rate of seed germination, which might be resulted from the release of bacterial gibberellins leading to the synthesis of α-amylase responsible for early growth promotion and increase in starch assimilation. However, better seedling vigor index could be due to increase in synthesis of auxins (Bharathi et al. 2004), which is also responsible for root initiation, breaking seed dormancy, increase in cell division and delaying leaf senescence (Faure et al. 1999). Other possible reasons for increasing plant growth are one or more of several properties, including synthesis of hormones and vitamins, increase in availability of nutrients, inhibition of plant ethylene synthesis due to bacterial ACC (1-aminocyclopropane-1-carboxylate) deaminase, solubilization of inorganic P and nitrogen fixation (Burd et al. 2000; Dobbelaere et al. 2003; Lucy et al. 2004).

In case of pot studies under unsterilized conditions, most of the isolates produced either insignificant or lesser changes in most of the growth parameters studied in this experiment. However, changes in response to some of the bacterial inoculants were noticed mainly in root length, R:S index and germination rate. Similar results were obtained in previous studies, in which PGPR effects of *Azotobacter* sp. get reduced in the unsterilized soil due to changes in soil factors (Kobabe et al. 2004) as well as the decrease in the number of *Azotobacter* sp. in unsterilized soil than sterilized soil (similar number of bacteria present under *in vitro* condition) (Toledo et al. 1988). Under unsterilized conditions, these inoculants could not perform well possibly due to increase in rhizospheric competence and increase in performance pressure. Some of the inoculants increased certain growth parameters in unsterilized soil than sterilized soil, which infers that these inoculants exerted beneficial effects in early growth stage of plants. Moreover, autoclaving of soil might have affected the nutrient status suitable for desired

bacterial isolates. This observation suggests that the bacterial endophytes have competitive nature and can work well under stress conditions and in the presence of indigenous microflora (Roesti et al. 2006; Khalid et al. 2004).

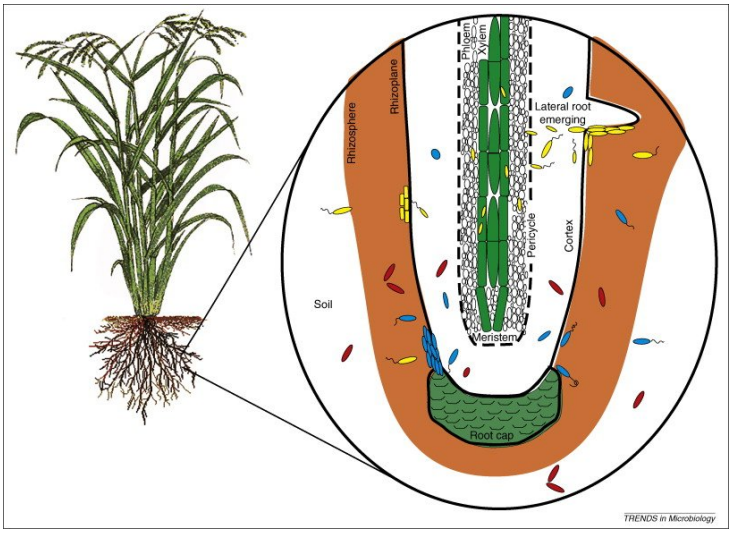
Comparative analysis of performance of different isolates suggested that most of the PGPR inoculants are showing fluctuations in their level of performance in different conditions. It is similar to the previous studies in which changes in performance has been reported from lab to field conditions (Çakmakçi et al. 2006). Therefore, based on the stability in performance under various conditions these isolates were ranked. Several formulations were designed as described and were further applied to plants in the microcosm studies. In microcosm studies, it was observed that many isolates showed enhancement in growth parameters in the presence of other PGPR isolates. In accordance with our results, synergistic effects of mixed inoculants (*Rhizobium*+PGPR) on growth promotion of *Vigna radiata* L. has been observed, which infers that the consortium of PGPR can serve as better performers in terms of supporting plant growth than single inoculants (Ahmad et al. 2012). In contrast, single inoculants exerted more beneficial effects than the consortia in few studies. Reduced plant growth in presence of mixed population can result from the incompatibility among inoculated bacteria or antagonistic effects of certain bacterial species on other isolates. Our result for *P. aeruginosa* PM389 is in agreement with the observation where, reduction or no effect under unsterilized conditions (in microcosm) has been recorded (Toledo et al. 1988). In all the studies done at pot level, best results were shown by PM10455, PM9405, PM10461, PM9426, PM9402 and PM389, although some of the isolates showed fluctuations in the performance in different conditions as discussed earlier. Out of these six best isolates, PM10461, PM9426 and PM9405 showed consistent growth promotion in all the studies carried out. These three isolates showed significantly higher increase in all the parameters considered in the field trial. These results suggested that these isolates can serve as better, consistent and promising biofertilizer agents in the future.

Further, selected potent biofertilizer isolates were subjected to identify their endophytic colonization property using non-culture based approach. 16S rDNA based species-specific primers were designed to identify the endophytic bacteria colonized inside the plant. As discussed earlier, endophytic bacteria have several advantages over their rhizospheric and rhizoplanic counterparts for lending beneficial effects to their host plant. Therefore, identifying the potent endophytic colonizers can be more beneficial in the agriculture sector for decreasing

the agriculture demands around the globe. In this study selected isolates were subjected to identify the colonization potential of endophytic bacteria. As 100% surface sterilization efficiency was obtained it ensured the elimination of rhizospheric and rhizoplanic bacteria. Amplification of desired DNA segment in samples from bacteria treated plant samples suggested that all these bacteria are capable of endophytic colonization inside the plant, and they can cross-infect wheat plant. Changes in intensity of desired fragment indicates colonization efficiency of each bacterial inoculants. Similar approach was used to track colonization of *Xylella fastidiosa* causing infection in citrus trees (Oliviera et al. 2002). This non-culture based approach for identification of endophytic colonization can further be important and easier to identify the cross-infection capability of these PGPR isolates in various other important crops.

CHAPTER IV

Evaluation and characterization of *Pseudomonas aeruginosa* PM389.



"Colonialism. The enforced spread of the rule of reason. But who is going to spread it among the colonizers?"
- Anthony Burgess

4.1. Introduction

Plant rhizosphere houses a plethora of microorganisms some of which establish intimate relationship with plants and influence their growth and physiology. Success of associative and endophytic bacteria depends on how effectively they compete with competitor microbes present in rhizosphere and colonize the plant roots effectively. Molecular mechanisms involved in colonization of endophytic bacteria are still not understood in greater detail. However, in general, colonization of bacteria in the interior of plant is a complex process, which involves interplay between several bacterial traits and genes, and plant responses. Endophytes show chemotaxis towards a variety of plant exudates, which are rich in nutrient sources. Plants generate signals in response to the bacterial invasion in rhizosphere, former of which select certain bacteria while inhibit others to enter inside the plant (Compant et al. 2010). Colonization by microorganisms creates "biased" rhizosphere with exudation of specific metabolic products, which in turn induce flagellar motility that directs their colonization on plant roots. It is the most important aspect for the successful colonization events (Lugtenberg et al. 2001). Followed by chemotaxis, these bacteria attach and spread on plant surface and the interior on plant tissues.

Many plant-associated bacteria are known to form biofilm on the plant surface which helps bacteria to establish and survive on host plant tissue (Irie 2012). Adherence on the plant root surface has been found to be mediated by exopolysaccharide produced from partnering bacteria with host plants (Bogino et al. 2013). 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 (LPS) present on the surface of bacteria might be determinative for successful colonization in host plants (Gough and Cullimore 2011). Detail of the general colonization mechanisms by endophytic bacteria is reviewed in chapter I.

In our study of temporal dynamics of diazotrophic endophytic bacteria in pearl millet plants, *Pseudomonas aeruginosa* PM389 emerged as the dominant endophytic bacteria during the vegetative growth period of plants. The dominance of this bacteria in pearl millet may be attributed to antagonistic activities to several other co-inhabitants, compatible host environment and efficient colonization process. Prominent colonization of *P. aeruginosa* PM389 in natural conditions makes it an ideal candidate for plant microbe-interaction studies. Thus, a study of

colonization strategies of *P. aeruginosa* PM389 and their compatibility with their host-plant will be instrumental in understanding biology of endophytic bacteria-host plant interaction, which in turn help selects suitable biofertilizer/biocontrol agent for attaining optimal yield in sustainable agriculture. In addition to be efficient colonizer, presence of plant growth promoting features, their positive effect on plant growth and ability to cross-infect non-host plant make *P. aeruginosa* PM389, a potential biofertilizer isolate. Therefore, *P. aeruginosa* PM389 was selected for further characterization in detail. Present chapter deals with characterization of this isolate for their growth properties, basic biochemical characterization and colonization mechanisms.

4.2. Material and methods

4.2.1. Bacterial isolate, media and growth conditions:

Pure culture of *Pseudomonas aeruginosa* (PM389) was used for study. For routine tests and maintenance, bacterial culture was grown on Luria Bertani (LB) media at 37°C for 24 h. Culture in LB broth was grown with shaking at 180 rpm in a BOD incubator. Isolate was stored at -70°C in glycerol (15%, v/v) until experimental use.

4.2.2. Biochemical characterization

Basic microbiological and biochemical tests namely Gram staining, amylase, caseinase, urease, oxidase, IMViC (Indole, Methyl Red, Voges Proskauer, Citrate utilization) and lipase tests were performed following standard protocols (Harley and Prescott 2002). Lipase test was done using Tributyrin agar plates. The isolate was also tested for its ability to utilize various carbon sources like lactose (MA (MacConkey agar) media), mannitol (Mannitol salt agar), glycerol (King's B agar), fructose, sucrose and galactose (at the final concentration of 1%). Production of fluorescent pigment was tested on MA media (McConkey's agar) (HiMedia, India), which is a rapid detection method for *P. aeruginosa* (Brodsky and Nixon 1973).

4.2.3. Estimation of generation time

Growth of *P. aeruginosa* PM389 was determined turbidometrically at 600 nm. Absorbance at 600 nm was measured at desired time intervals in a UV-VIS spectrophotometer. The bacterial isolate was grown in LB broth, and OD_{600} was taken at each hour to plot a standard

curve of OD_{600} against time. The generation time was calculated according to the standard formula (Kratz and Myers 1955).

Generation time (g) was calculated using the formula of

$$g = \frac{t_2 - t_1}{2.303 (\log N_2 - \log N_1)}$$

Where

N_1 = Initial growth (OD_1)

N_2 = Final growth (OD_2)

$(t_2 - t_1)$ = The time elapsed between N_2 and N_1

4.2.4. Growth of *P. aeruginosa* PM389 at various pH and salt concentration

In order to evaluate optimal conditions for growth, the bacterial isolate was grown in LB broth medium at various salt concentrations (2-12% NaCl) and different pH (2-12). pH of the medium was adjusted using 1 M NaOH and 1 N HCl. Bacterial culture was grown at 37°C for 12 h and growth of bacteria was measured at 600 nm in a spectrophotometer.

4.2.5. Motility assays

Motility of bacteria is one of the most important prerequisites for successful colonization in hosts and chemotactic responses. Therefore, *P. aeruginosa* PM389 was assayed for test of various types of motility namely swimming, swarming and twitching, following standard protocol described by Deziel et al. (2001). *Enterobacter cloacae* was used as a positive control.

4.2.5.1. Swimming

For the test of swimming motility, bacterial isolate was grown overnight on LB agar plates at 37°C and individual colonies were then stab-inoculated onto swimming plates containing 1% tryptone, 0.5% NaCl and 0.3% agar. Culture was incubated at 32°C for 16 h and observed for the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

4.2.5.2. Swarming

Bacterial culture was point-inoculated on petri-plates containing 0.5% Bacto-Agar, 0.8% nutrient broth powder (both from Difco, USA), and 0.5% dextrose and grown at 30°C for 24 h. Media was dried overnight at room temperature after pouring.

4.2.5.3. Twitching

Freshly grown individual bacterial colonies of given isolate were stab-inoculated through the agar to the bottom of 1% LB agar plates. The plate was incubated at 32°C for 24 h. Bacterial growth at the interface between the plastic surface, and the agar was indicative of twitching. Agar media was removed from the plate and bacteria attached onto the polystyrene surface was washed under the tap water, and stained with 1% crystal violet to visualize.

4.2.6. Estimation of exopolysaccharide production

Since, exopolysaccharide play an important role in attachment to plant surface and in the endophytic colonization, *P. aeruginosa* PM389 was tested for production of exopolysaccharide. For extraction of exopolysccharide, bacterial culture grown in NB (Nutrient broth) for 24 h was heated at 100°C for 15 min to denature exopolysaccharide (EPS) degrading enzyme and, centrifuged at 10,000 g for 30 min at 4°C. Three volumes of ice-cold ethanol were added to 1 volume of supernatant, and kept at 4°C for overnight precipitation of EPS. The precipitate was washed with 70–100% ethanol–water mixtures. After washing with ethanol, the EPS was dried in a desiccator and stored at room temperature until needed. Residual protein was removed using TCA (Trichloroacetic acid) precipitation (60%) method (Jiang 2004). To remove extra salts, the EPS was re-dissolved in distilled water and dialyzed at 4°C for 24 h (molecular weight cut-off of 13 kDa; Sigma-Aldrich Chemie GmbH, Seelze, Germany) against distilled water. Excess water was removed under vacuum using lyophilizer (Heto PowerDry®LL6000 Freeze Dryer, Thermoscientific USA) at 3000 psi at 4°C. The lyophilized EPS was stored at room temperature until further use (Bramhachari and Dubey 2006). Quantification of EPS was done using phenol sulphuric test (DuBois 1956). In this method, the carbohydrate content of the EPS sample was determined using glucose as standard. One mg of dried EPS dissolved in 1 ml water was mixed with 50 µl of 80% (w/v) phenol/water and 5 ml of H_2SO_4. The mixture was heated at 100°C for 5 min and then cooled to 20°C. An aliquot of the sample was diluted 20 times in concentrated H_2SO_4 and absorbance was noted at 490 nm for pentoses. To detect functional groups present in the EPS, FTIR (Fourier transform infrared) spectroscopy was done by micro-KBr pellet

technique (DuBois 1956). Lyophilized EPS (10 µl of EPS (stock-1mg ml^{-1}) with 200 mg of dry potassium bromide powder) was ground and fine pellet of EPS was made. A spectrum was obtained using FTIR spectrophotometer (Shimadju, Japan) in the frequency range of 400-4000 cm. Potassium bromide was used as *P. aeruginosa* background reference.

4.2.7. Test for biofilm formation

Biofilm forming ability of *P. aeruginosa* PM389 was tested following method of O'Toole and Kolter (1998). 5 µl fresh culture of *P. aeruginosa* PM389 grown in LB was inoculated in polystyrene microtiter plate containing 0.2 ml of BDT (Bushnell Haas mineral salts) medium (HiMedia, India) supplemented with 0.2% dextrose and 0.5% tryptone in each well. The test was done in triplicate. At regular intervals, plate was rinsed thoroughly with water, and attached cells were stained for 10-15 min with 1% solution of crystal violet at room temperature. The plate was rinsed with water and biomass of attached cells (biofilm) was quantified by solubilization of dye with two ml 95% ethanol. Ethanol (95%) was used as a blank sample. The absorbance was measured at 600 nm with a spectrophotometer. *Escherichia coli* was used as a negative control for the test.

4.2.8. Formation of biofilm on the plant surface

Wheat seeds were surface sterilized, and plants were grown in Hoagland media as described in section 3.2.4. On 4^{th} day, the healthy seedlings were transferred into tubes containing Hoagland media and treated with *P. aeruginosa* PM389 with a population of 10^7-10^8 cells ml^{-1} suspended in 1X PBS. After 15 days of inoculation, segments of roots were individually dipped into a solution of acridine orange (0.1%, pH 7.0) for two min. Bacterial cells on various parts of plants were visualized at 100X using flourescens Olympus-CKX41 microscope (Olympus, Japan) (Morris 1997).

4.2.9. Test of cellulolytic and pectinolytic activity

P. aeruginosa PM389 was screened for cellulolytic tests namely endoglucanase, β-glucosidase and cellobiohydrolase. Presence of endoglucanase activity was estimated by the method described by Kasana et al. (2008) using Gram's iodine method. Appearance of a clear zone around bacterial growth after overlaying culture plate with Gram's iodine was considered positive for endoglucanase activity. The β-glucosidase and cellobiohydrolase activities were determined by the method of Reinhold-Hurek et al. (1993) (Section 2.2.10.7). Briefly, for testing

the presence of cellobiohydrolase and glucosidase, test plates were exposed to 302 nm of ultraviolet (UV) light on a transilluminator, and the active colonies were identified by the appearance of violet fluorescence. The positive colonies for β-glucosidase and cellobiohydrolase enzymatic activities were identified by the appearance of violet fluorescence on illumination with ultraviolet (UV) light of 302 nm. Similarly, pectinase activity was tested by spot-inoculating exponentially grown culture on NA plates containing 0.5% pectin and incubated for 72 h at 30°C. Colonies appearing on plates were overlaid with 2% CTAB (N-cetyl-N,N,N trimethyl-ammonium bromide) and kept for 30 min at 30°C. This was followed by washing three times with 1 N NaOH for 10 min each. Plates were then screened for appearance of clear zones around the colonies.

4.2.10. Reinfection/reisolation of *P. aeruginosa* PM389 from plant

Wheat plants were grown and inoculated as described for test of biofilm formation on the plant surface. Healthy plants were uprooted and washed thoroughly with sterilized tap water to remove media. *P. aeruginosa* PM389 was isolated following protocol described earlier in section 2.2.4 for isolation of endophytic bacteria. Bacterial colonies were observed for the presence of *P. aeruginosa* PM389 and thus, ensuring it as an endophyte.

4.3. Result

4.3.1. Biochemical characterization of dominant diazotrophic endophytic bacteria

Isolate *P. aeruginosa* PM389 was found to be a Gram-negative bacillus. It showed positive reactions for catalase, citrate agar, oxidase, urease and negative reaction for indole, methyl red, voges-proskauer, caseinase and amylase. On the basis of biochemical tests, it was identified as *Pseudomonas aeruginosa*. The isolate showed fluorescence on MacConkey agar media, which indicated production of fluorescein by bacteria. This rapid biochemical test further confirmed it as *Pseudomonas aeruginosa.* It was also identified at the molecular level by 16S rRNA sequence (JF899310) analysis as described earlier in section 2.2.8. It can utilize various carbohydrates like mannitol, glycerol, galactose, and fructose. However, it showed negative test for sucrose, lactose and dextrose.

4.3.2. Optimization of growth characteristics of *P. aeruginosa* PM389

Growth kinetics of *P. aeruginosa* PM389 is shown in Fig 4.1. Based on the given formula, the generation time of the *P. aeruginosa* PM389 was 1.69 h. Considering its potential

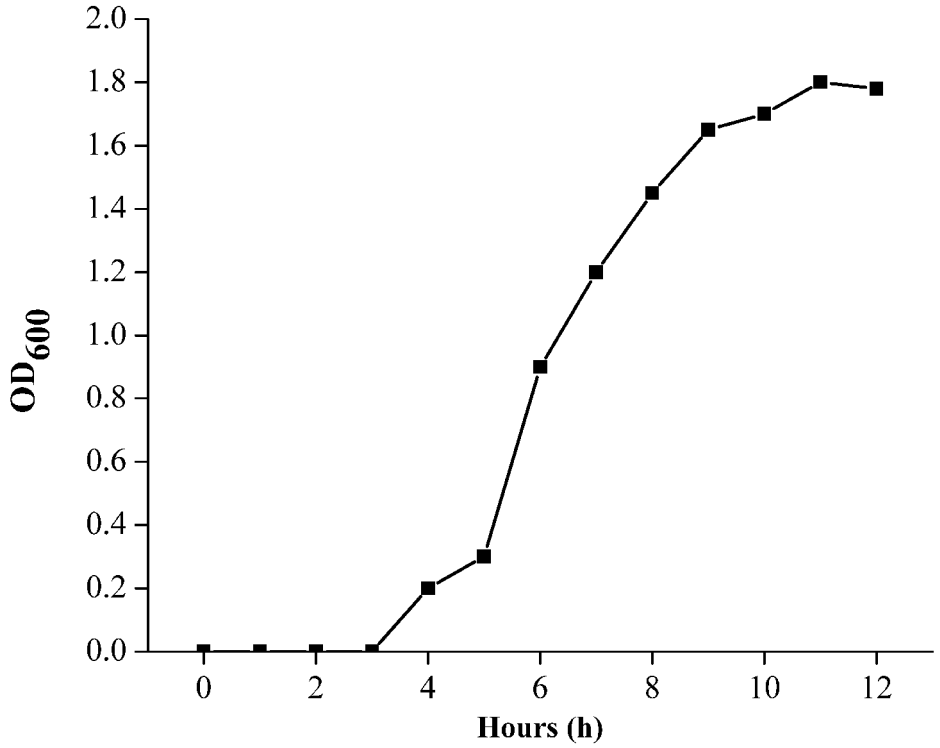


Fig. 4.1. Growth curve of *P. aeruginosa* PM389.

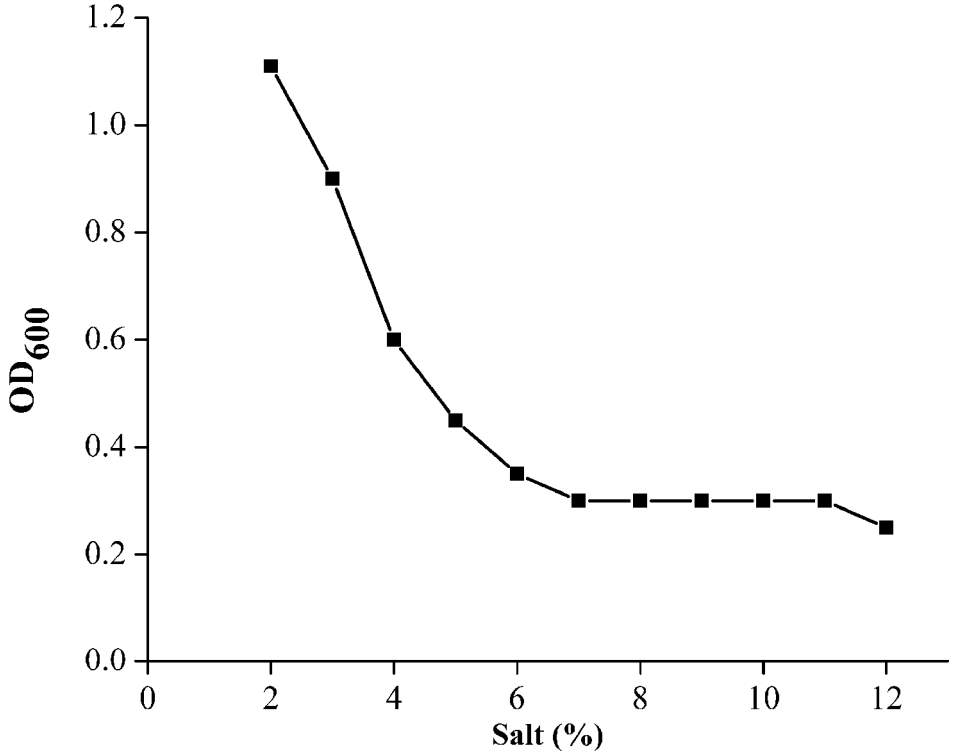


Fig. 4.2. Effect of salt stress on the growth of *P. aeruginosa* PM389.

as successful PGPR, *P. aeruginosa* PM389 was grown at different pH and salt concentrations to test its tolerance in stress conditions. In salt stress, maximum growth was observed at 3% NaCl. Inhibition of growth was noted in more than 3% NaCl concentration, although growth of *P. aeruginosa* PM389 was observed upto 12% of NaCl (Fig. 4.2). On the other hand, the optimum growth of *P. aeruginosa* PM389 was obtained between 6.8 and 7 pH (Fig. 4.3). *P. aeruginosa* PM389 can tolerate pH variation upto 4 (acidic condition) and 9 (basic condition).

4.3.3. Motility assays

P. aeruginosa PM389 showed all three different types of motility like positive control (*Enterobacter cloacae*) as shown in Fig. 4.4. (A-D). In swimming test, circular turbid zone was formed; in swarming bacteria spreaded on the media plate and; in twitching test, bacterial cells were found to be attached on the plastic plate surface.

4.3.4. Estimation of exopolysaccharide production

Exopolysaccharide produced (8.5 μg ml^{-1}) by *P. aeruginosa* PM389 was screened for the partial structure elucidation by FTIR. Presence of various chemical bonds in the EPS of *P. aeruginosa* PM389 were evident in Table 4.1 and Fig. 4.5. It showed three aromatic C-H bonds ranging between 681-838 cm^{-1}, C-O-C (1141.25 cm^{-1}) and aromatic C=C (1693.36 cm^{-1}) bonds. It also showed the presence of two carboxyl group (2549.79, 2914.53 cm^{-1}) and alkyl C-H (2922.78 cm^{-1}).

4.3.5. Test for biofilm formation

Understanding the role of biofilm formation in successful plant-microbe interaction, *P. aeruginosa* PM389 was assessed for its ability to form the biofilm. Biofilm formation was measured in terms of OD_{600} of cells solubilized in ethanol after growth of cells in microplate at each hour. *P. aeruginosa* PM389 showed optimum biofilm production between 4 and 6 hour in comparison to control (*E. coli*) as shown in Fig. 4.6. After confirming the biofilm formation in microplate, *P. aeruginosa* PM389 was investigated for the same *in-vivo*. Plants were inoculated, grown for 15 days and was observed for biofilm formation on the plant root surface. On staining with acridine orange, in the *P. aeruginosa* PM389 treated sample bacterial cells were observed in both root and shoot under the microscope confirming successful colonization of *P. aeruginosa* PM389 in plants. It is evident from Fig. 4.7, that *P. aeruginosa* PM389 formed microcolony formation, one of the characteristic of plant-microbe interactions and biofilm formation.

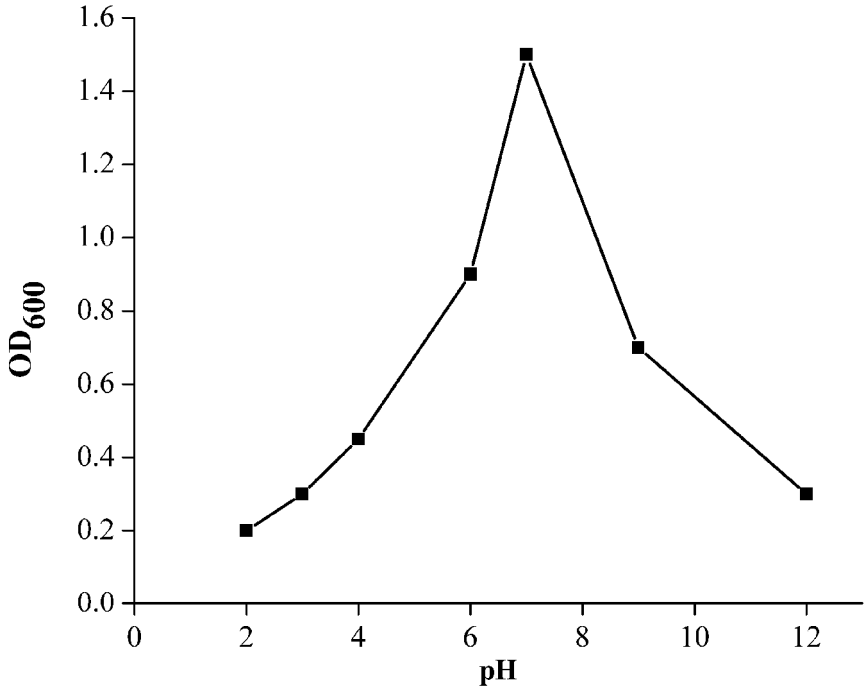


Fig. 4.3. Effect of pH on the growth of *P. aeruginosa* PM389.

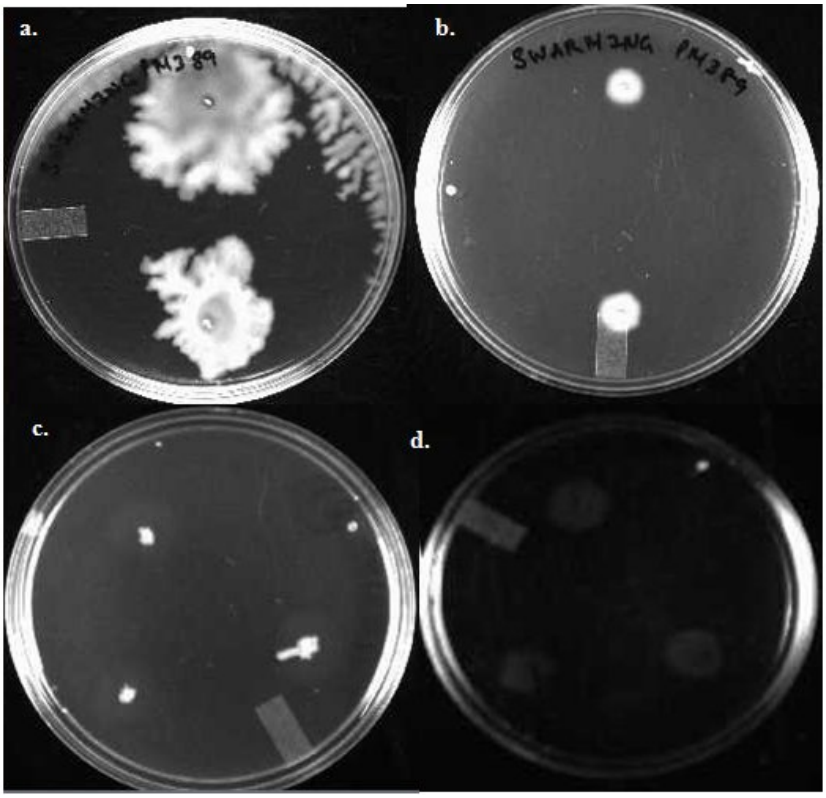


Fig. 4.4. Agar plate-based method for the test of different types of motility in *P. aeruginosa* PM389. (a) Swimming, (b). Swarming, (c). Twitching motility and, (d) Detection of twitching motility by staining with 1% Crystal violet. All these tests were done in triplicate.

Table 4.1. Functional groups present in exopolysaccharide of *P. aeruginosa* PM389.

Wavenumber	Groups
681.59	Aromatic C-H Bending
750.67	Aromatic C-H Bending
837.16	Aromatic C-H Bending
1141.25	Aryl alkyl C-O-C
1693.36	Aromatic C=C Bending
2549.79	Carboxylic Acid O-H Stretch
2914.53	Carboxylic Acid O-H Stretch
2922.78	Alkyl C-H Stretch

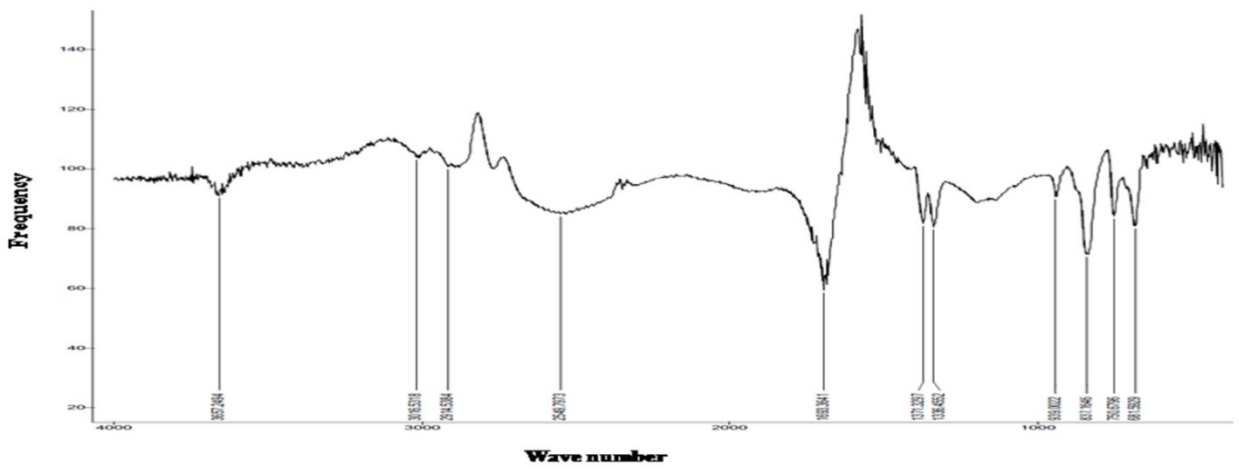


Fig. 4.5. FTIR spectrum of exopolysaccharide extracted from *P. aeruginosa* PM389.

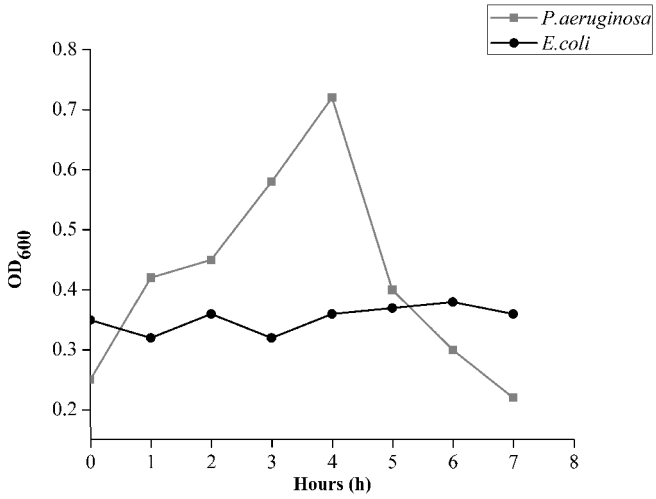


Fig. 4.6. Biofilm formation test of *P. aeruginosa* PM389.

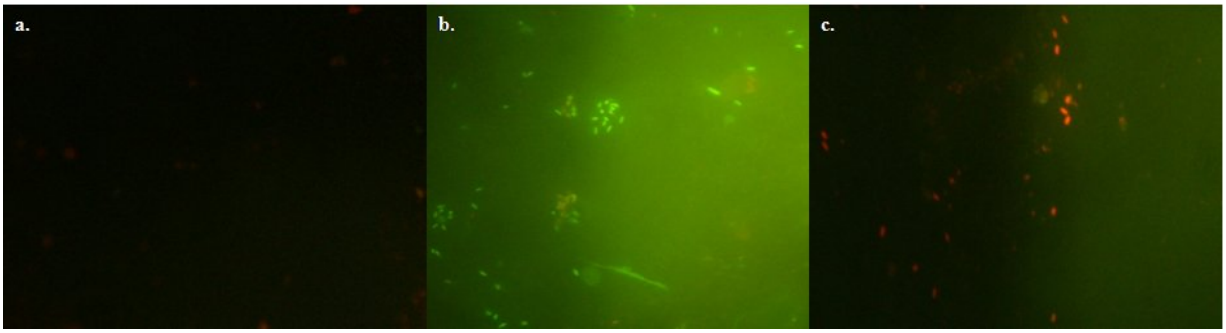


Fig. 4.7. Staining of *P. aeruginosa* PM389 treated plant sample with acridine orange and visualization under epiflourescence microscope, a. Control, b. Root sample (*P. aeruginosa* PM389 Treated), c. Shoot sample (*P. aeruginosa* PM389 Treated).

4.3.6. Test of cellulolytic and pectinolytic activity

With a view to confirm the role of hydrolytic enzyme in endophytic colonization, detection of cellulolytic and pectinolytic enzymes were made in the isolate employed in the present study. It showed negative reaction for all the three cellulolytic activities namely endoglucanase, β-glucosidase and cellobiohydrolase as well as for pectinase activity.

4.4. Discussion

Pseudomonas aeruginosa, a common soil inhabitant, is popularly known as an opportunistic pathogen. However, its existence as abundant endophytic bacteria in pearl millet associated microbial community extends its ecological and functional role in environment (Gupta et al. 2013). Present study found *P. aerugionsa,* a dominant colonizer during plant growth in natural conditions. In addition, it also possesses plant growth promoting and antagonistic properties, which can be exploited in sustainable agriculture. Since, ability to colonize and establish inside the plant tissue are important features for successful plant-microbe interaction, aim of the present study was to characterize plant growth promoting *P. aeruginosa* PM389 for important traits required for endophytic colonization and successful interaction with host plant. To our knowledge, few studies have demonstrated colonization behaviour of plant growth promoting *P. aeruginosa* (Pandey et al. 2012). Most of the studies related to colonization traits of *P. aeruginosa* have been conducted for human pathogenic strains PAO1 and PA14 on animal as well as in plants.

P. aeruginosa PM389 could utilize most of the carbohydrates tested in the present work. It suggests that it has an ability to use a diverse array of nutritional sources for their survival in natural conditions (Mahaffee and Kloepper 1997). We assume that the ability of *P. aeruginosa* PM389 to utilize the different carbon sources may be one of the possible reasons for its presence as a dominant endophyte in our study (Chapter II). Various biochemical and molecular tests confirmed it as *Pseudomonas aeruginosa*, which is well known from Pseudomonads genus being diverse, numerous, dominating, and aggressive colonizer in earlier studies (Germida et al. 1998).

The generation time of *P. aeruginosa* PM389 was observed to be 1.69 h, which is much lesser than its pathogenic counterpart PAO1 having generation time of 36 min (Ohman et al. 1980). With a view to evaluate survival of *P. aeruginosa* PM389 in stress condition, it was tested for its viability in different concentration of NaCl (salt stress) and pH. The results revealed that *P. aeruginosa* PM389 can tolerate the salt stress to a certain limit. It was also found to be capable of growing in a broad pH range that was between 4 and 9. The ability of *P. aeruginosa* PM389 to tolerate stress indicates its usefulness as biofertilizer agent that can support growth of plants growing in stress conditions often observed in field environment. There are mechanisms of salt tolerance in *P. aeruginosa*, which might be involved in increasing tolerance against salt stress (D'Souza-Ault 1993). Mechanism involved for osmotic tolerance in human pathogenic strain

PAO1 of *P. aeruginosa* has been studied suggesting the role of N-acetylglutaminylglutamine and glycine betaine (D'souza –Ault et al. 1993). Recently, salt tolerance has been reported in endophyte *P. aeruginosa* strain PW09 obtained from wheat (Pandey 2012).

As described earlier in section 1.6, successful colonization of endophytic bacteria involves multiple steps. Root colonization is the first and foremost step for plant-microbe association, in which microorganisms move towards rhizosphere in response to root exudates. Thus, motility and chemotaxis play a key role in the root colonization. First step of the colonization process is mobility of bacteria in the plant root in response to root exudates. *P. aeruginosa* PM389 exhibited all the three types of motilities (swimming, swarming and twitching) tested. These three motilities are driven for different purposes. The role of twitching motility in endophytic colonization has been demonstrated in several reports. Twitching motility is mediated by type IV pili, suggesting the presence of type IV pili important in colonization inside the plants (Burrows 2012). Moreover, flagellar and twitching motility of *P. aeruginosa* PA14 are also known to play major role in biofilm development (O'Toole and Kolter 1998).

After chemotaxis, next step in rhizoplanic and endophytic colonization is adhesion of bacteria to plant root surface. EPS plays an important role in bacterial attachment and is also instrumental in biofilm formation at the plant surface (Bogino et al. 2013). FTIR analysis suggested the presence of phenyl substitution ring (aromatic C-H), and aromatic ring (aromatic C-H) in the exopolysaccharide of *P. aeruginosa* PM389. It also showed the presence of certain aryl-alkyl ethers (C-O-C) and carboxyl group as well, it suggests that EPS of *P. aeruginosa* PM389 might be containing some kind of carbohydrates which are responsible for binding divalent cations. It also showed alkyl C-H group, which indicates the presence of certain alkane in EPS. Thus, FTIR results suggested that EPS is highly complex compound containing various functional groups in addition to previously reported groups in polysaccharide structure. Presence of certain aromatic functional group such as aromatic ring has already been reported in the EPS of *Lactobacillus fermentum* CFR 2195 using FTIR (Yadav et al. 2011). EPS production has been reported in a plant growth promoting phosphate solubilizing *P. aeruginosa* strain PS1 (Ahemad and Khan 2009) and many human pathogenic strains (Mian et al. 1978).

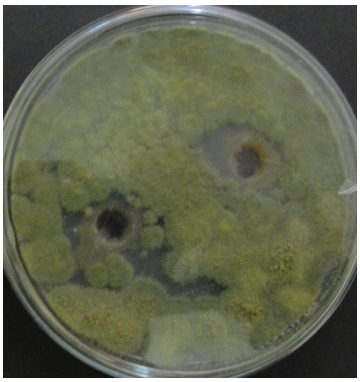
Knowing the ability of *P. aeruginosa* to form biofilm and the importance of biofilm formation in plant-microbe interaction, the ability of *P. aeruginosa* PM389 to form the biofilm in microplate as well as *in-planta* was tested. Biofilm formation by PGPR on the plant surface is an

important trait of endophytes required for colonization and further, plant growth promotion in the plant (Ramey et al. 2004). In order to confirm colonization and biofilm formation, plants were inoculated with *P. aeruginosa* PM389 and, roots and shoots were stained with acridine orange for visualization of *P. aeruginosa* PM389 in plants. Plants were grown in axenic condition. Absence of fluorescent bacterial cells in control indicated that fluorescent bacterial cells in treated plant roots and shoots were of *P. aeruginosa* PM389. Visualization of plant-associated bacteria using acridine orange has been performed in several studies (Morris et al. 1997). It was evident from the result that *P. aeruginosa* PM389 was aggregated to form microcolonies and biofilm on the plant surface, which is one of the characteristics of successful microbe-plant association. Biofilm formation by *Pseudomonas syringae* on the leaf surface of bean (*Phaseolus vulgaris*) was reported earlier by Monier and Lindow (2004). More accurate information about the structure of biofilm could be obtained using laser scanning confocal microscopy (LSCM), which eliminates stray and out-of focus light that interferes with the formation of the object's images (Dazzo et al. 2007). Extensive biofilm formation by pathogenic *P. aeruginosa* strain PAO1 at the plant surface has been demonstrated in earlier studies (Walker et al. 2004). Recently, biofilm formation by endophytic *P. aeruginosa* strain PW09 isolated from wheat was studied and compared with *P. aeruginosa* rhizospheric strain such as PHU094 and PJHU15 (Pandey et al. 2012).

It has been demonstrated in several studies that many endophytic bacteria produce the moderate amount of cellulolytic and pectinolytic enzymes to get entry and spread in the interior plant roots and other parts of plant. Therefore, *P. aeruginosa* PM389 was tested for these activities to ascertain whether it is an active or passive colonizer of plants. Lack of all these hydrolytic enzymes indicated towards the passive mechanism (i.e., through conductive tissues like xylem and phloem) of colonization adopted by *P. aeruginosa* PM389 (Compant et al. 2010). Similar to these results, another well characterized endophytic bacteria *Herbaspirillum. seropedicae* strain SmR1 does not possess cellulolytic activities, which were evident from the lack of genes encoding plant cell wall degrading enzymes in given strain (Pedrosa et al. 2011). Reisolation of *P. aeruginosa* PM389 from surface sterilized plants inoculated with the same and amplification of 16S rDNA using species specific-approach (in chapter III) confirmed the colonization ability of *P. aeruginosa* PM389 inside the plant.

CHAPTER V

Mechanistic insights of antagonistic activity of *P. aeruginosa* PM389 and its potential as biocontrol agent.



"No wise combatant underestimates their antagonist."

- Johann Wolfgang von Goethe

5.1. Introduction

In present study, *Pseudomonas aeruginosa* PM389 was found to be an important plant growth promoting endophyte due to its natural abundance and ability to colonize plants efficiently. It has several plant growth promoting traits such as N_2-fixation, phosphate solubilization and siderophore production (Chapter I), and various endophytic traits (Chapter III) described earlier. Apart from plant growth promoting activities, *P. aeruginosa* PM389 showed strong antagonistic activity against other endophytic bacteria, which indicated possible reason for their abundance during the vegetative period of plant growth. It suggests that *P. aeruginosa* PM389 possess certain kind of antagonistic mechanism, and thus, can be exploited as a biocontrol agent. Therefore, a thorough investigation for its antagonistic properties and its potential use as biocontrol agent against certain phytopathogens, are required.

Owing to drastic changes in the climatic conditions, soaring of temperature encourages propagation of pathogens in new regions and renders some plants more susceptible to diseases. According to FAO (Food and Agriculture Organization of United States) estimates, 20-40% global crop yields get affected every year due to various pests and infectious diseases. In last few decades, to evade pests and pathogens, use of pesticides is gaining importance among farmers. Pesticides have important credits like increase in crop yield and disease management. However, use of chemical pesticides is indeed associated with several adverse effects which include various kinds of pollutions, hazardous for human health, impact on non-target beneficial microorganisms and drastic effect on soil fertility (Akhtar 2009). Therefore, scientific communities are looking for other environmental friendly and sustainable alternatives. Application of biocontrol is one of the most important approaches, which has the ability to prevent microbial infection in ecofriendly manner. Although these agents have been observed to be lesser effective in reducing diseases than chemical pesticides, a proper exploitation of their biocontrol properties may improve plant health without compromising with environmental concern (Walters et al. 2013).

Certain biocontrol agents can be used to overcome the infestation of microbial diseases. These biocontrol agents prevent plant pathogens through one or more antagonistic mechanisms,

such as competition with other soil micro-organisms for nutrients, production of antibiotics or lytic enzymes, siderophore production and induced systemic resistance (ISR) (Gupta et al. 2012). In recent past, studies on induced systemic resistance have gained immense importance due to its several applications as described by Kuć (2001). Induced systemic resistance is an enhanced defense capacity of plant before or after pathogenic challenge to generate various defense-related compounds throughout the plant (Gupta et al. 2012). Development of ISR can result in generation of reactive oxygen species (ROS; e.g., increase of peroxidase (PO)), ion exchange, deposition of callose, lignin and phenolics (Duijff et al. 1997); increase in lytic enzymes (chitinase and glucanses), polyphenol oxidases (PPO), phenylalanine ammonia lyase (PAL) activity (Magnin-Robert et al. 2007) and phytoalexins production as well as the induced expression of other defense and stress-related genes in host plants (Verhagen et al. 2004). In ISR, usually exposure of appropriate bacteria sensitize plants for the future pathogenic attack called as 'priming' and a rapid or enhanced induction of defense response occurs on the subsequent pathogenic challenge. Priming is a cost-effective process than that of constitutive expression of defense and stress-related genes (Van Wees et al. 2008). Thus, those organisms which are capable of inducing systemic resistance can be utilized as an inexpensive and eco-friendly method for disease management. For instance, *Pseudomonas aeruginosa* 7NSK2 has been used for inducing systemic resistance against *Blumeria graminis* in wheat (Muyanga et al. 2005).

Wheat (*Triticum aestivum* L.) is the major crop of *Rabi* season in India. *Fusarium* head blight (FHB) of wheat is one of the devastating diseases occurring across the broad range of environmental condition. It is caused by a group of different *Fusarium* species mainly by *F. graminearum* and *F. monaliforme,* and various other species such as *F. subglutinans, F. avenaceum*, *F. culmorum*, *F. poae* (Marasas 1984). *Fusarium oxysporum* is also responsible for causing root rot of wheat (Al-Abdalall 2010). Other than this, there are reports of secondary infection of *Erwinia carotovora* enhancing the disease in wheat crop after infection of *Fusarium* species during crop rotation (Newton et al. 2003). Practice of such crop rotation is very common in India where wheat (November to February) is cultivated after potato crop (July to October). *Erwinia carotovora* is the major pathogen causing bacterial soft rot of potato (*Solanum tuberosum*) (Czajkowski et al. 2011). There are various reports which indicate that both of these

pathogens can infect some of the major crops of India such as sorghum, pearl millet, maize, potato, carrot, onion and tobacco (Hingorani et al. 1960).

Acknowledging the importance of biocontrol agent in environmental friendly and sustainable agriculture, present work makes an attempt to investigate different mechanisms of antagonistic activities of *P. aeruginosa* PM389, which are inhibitory to fungal and bacterial pathogens. In addition, this work also deals with the ability of *P. aeruginosa* PM389 to induce ISR in wheat plants against fungal pathogen *Fusarium monaliforme* and bacterial pathogen *Erwinia carotovora* for its potential application.

5.2. Materials and methods

5.2.1. Cultures used

Pseudomonas aeruginosa PM389 was used to test its ability to develop induced systemic resistance against pathogens in host-plant and, for antagonistic properties as mentioned above in section 5.1. Antagonistic activity of *P. aeruginosa* PM389 was assessed against various fungal phytopathogens namely *Fusarium monaliforme*, *F. graminearum*, *F. pallidoroseum*, *F. oxysporum*, *Aspergillus* sp., and *Verticillium* sp. Pathogens used for ISR studies were *Erwinia carotovora* and *Fusarium monaliforme*, procured from IARI, Pusa, India. All the fungal isolates were stored as described in section 2.2.4, until use.

5.2.2. Antagonistic activity

The antagonistic activity of *P. aeruginosa* PM389 against important plant pathogenic fungal species (*Fusarium oxysporum, Fusarium monaliforme*, *F. graminearum*, *F. pallidoroseum*, *F. oxysporum*, *Aspergillus* sp., and *Verticillium* sp.) was determined by the standard agar well diffusion assay. Briefly, 100 µl of suspension containing approx. 4000 spores ml^{-1} fungal spores was spread uniformly on potato dextrose agar (PDA) plates, and 6 mm diameter wells were made using sterile metallic borer. Fresh broth culture (100 µl) of *P. aeruginosa* PM389 was inoculated in each well. Boiled culture of *P. aeruginosa* PM389 was used as control. The plates were incubated at 28°C for 96 h. Antagonistic activity was determined by measuring the zone of inhibition for which following criteria were used: zone of

inhibition <1 cm = Poor (+), between 1-2 cm = Average (++), >2 cm = Good (+++). The experiment was performed in triplicate.

5.2.3. Mechanistic studies behind antagonism

P. aeruginosa PM389 showed antagonistic activity against several endophytic bacteria and fungal pathogens as reported in our recent publication (Gupta et al. 2013). To confirm the mechanism for antagonistic activity, *P. aeruginosa* PM389 was tested for the production of allelochemicals as well as other antagonistic mechanisms as described below.

5.2.3.1. Pyoverdin and pyocyanin production

P. aeruginosa PM389 was grown on King's B Agar media (HiMedia, India) at 37°C for 48 h. Flourescin (pyoverdin) production of isolate was determined by illuminating culture plate on UV-illuminator fitted with UV-lamp of 360 nm to observe florescence around bacterial growth. For determining production of pyocyanin, isolate was grown for 24 h at 37°C in *Pseudomonas* broth medium (PB) containing (per liter): Bacto Peptone (Difco Laboratories), 20 g; $MgCl_2$, 1.4 g and; K_2SO_4, 10 g (Mavrodi 2001). Cultures were harvested by centrifugation at 6500 g for 25 min and chloroform was added to the culture supernatant in the ratio of 1:1, which in turn resulted to the formation of blue color. Further, organic layer was removed and extracted with 1.5 ml of 0.2 N HCl, which converted pyocyanin to acidic (red) form. Resulting mixture was used for quantification by measuring absorbance at 520 nm in UV-Vis spectrophotometer.

5.2.3.2. Production of hydrogen cyanide (HCN)

Isolate was streaked on Nutrient agar plates supplemented with 4% glycine. A piece of filter paper soaked in 0.8% picric acid were placed onto agar-media, and 8% Na_2CO_3 was spread in the lid of petri-dish. Culture plates were then incubated at 37°C for 48 h in the inverted position and observed for a change from yellow to orange, red, brown, or reddish brown as an indication of weak, moderate, or strongly cyanogenic potential, respectively. It is based on the principle that cyanogenesis from glycine lead to production of HCN (volatile) which in turn develop color on reaction with picric acid in the presence of Na_2CO_3.

5.2.3.3. Test of chitinolytic and chitosanolytic activity

Colloidal chitin was prepared by the method of Roberts and Selitrennikoff (1988) from crab shell chitin (HiMedia, India). Briefly, one g colloidal chitin was slowly dissolved in 40 ml of concentrated HCl kept with constant stirring at 4ºC. Thereafter, it was incubated at 37ºC until viscosity was reduced. Further, 400 ml of sterile distilled water was added and kept overnight at 4ºC. Thus prepared colloidal chitin was collected on filter paper and washed extensively with distilled water to attain neutral pH. Finally, the chitin was dissolved in 25 ml sterile distilled water and added in the media for chitinolytic test. For the test of chitinolytic and chitosanolytic activities, 1% (w/v) colloidal chitin and chitosan were used, respectively, as sole carbon source in a minimal salt medium comprising (composition: per liter) of Na_2HPO_4 6 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 0.5 g, yeast extract 0.05 g, agar 15 g. Test isolate was streaked onto above medium and incubated at 30ºC. Colonies showing zones of clearance against the creamy background were considered positive for chitinase/chitosanase production.

5.2.3.4. Siderophore production

Siderophore production for *P. aeruginosa* PM389 was determined as described earlier in section 2.2.10.4.

5.2.4. Effect of *P. aeruginosa* PM389 on plant growth on the pathogenic challenge

On the basis of various plant growth promoting properties and antifungal property shown by *P. aeruginosa* PM389, its effects on plant growth as well as its ability to prevent fungal pathogen (*Fusarium oxysporum* causing root rot of wheat) were tested in wheat (*Triticum aestivum* L.) plants under axenic conditions. Surface sterilization of wheat seeds, bacterization of seeds and growth of seedlings in petriplate were done in the similar manner as described in section 3.2.4. After 4 days of plant growth, plants pretreated with *P. aeruginosa* PM389 and control plants were challenged with *Fusarium oxysporum* (4000 spores ml^{-1}). Sterile water was added to the petri-dishes as and when required to maintain the moisture. After 10 days of plant growth, seedlings were measured for the root length, shoot length and vigor index as described earlier in section 3.2.4.

5.2.5. Test of fungal growth inhibition by extracellular product of *P. aeruginosa* PM389

Extracellular fraction of *P. aeruginosa* PM389 was used to test if the fungal growth inhibition is mediated by secreted compound. Estimation of fungal growth was based on dry weight of fungal culture. For preparation of test samples, *P. aeruginosa* PM389 was cultured in 100 ml LB broth at 37°C for 48 h with shaking at 150 rpm. Bacterial culture was centrifuged at 8000 g for 20 min at 4°C and the supernatant (B_{sup}:broth supernatant) was collected. Separately, one fraction of B_{sup} was used for precipitation of protein. Precipitated protein (P_o) and protein supernatant (P_{sup}) were also used for growth inhibition study (methods for protein precipitation, purification and quantification is described in section 5.2.6). To ensure antifungal activity mediated by protein fraction, extracted protein sample was treated with proteinase K. Loss of fungal growth by proteinase K treated protein sample indicates antifungal activity of secreted bacterial protein fraction. A 20 mg ml^{-1} stock of proteinase K was prepared and stored at -20°C until use. To the crude protein sample, working concentration of 100 μg ml^{-1} of proteinase K was added and incubated in a water-bath at 55°C for 2 h.

The B_{sup} (broth supernatant), P_{sup} (Protein supernatant) and P_o (protein) samples were filtered through a 0.2 μm membrane filter (Axiva, India) and stored at -20°C until use. Inhibition of fungal growth by these three fractions was tested separately against *F. oxysporum*. In a similar experimental set-up, proteinase K-treated protein fraction was also used in the culture medium inoculated with fungal spores. For the antifungal assay, 1 ml each of B_{sup}, $P_{sup,}$ and proteinase K-treated sample and, 10 μg ml^{-1} of P_o were added in 20 ml of Czapek Dox broth (HiMedia, India) in 150 ml flasks. These culture media were inoculated with fungal spores (4,000 fungal spores ml^{-1}) and grown at 28°C for 3 days. Sterile LB broth was used as control for B_{sup} and sterile distilled water was used as control for other samples. After 7 days growth, fungal mycelia from each treatment were harvested on Whatman filter papers, kept for drying at 50°C for 2 h and measured for their dry weight of the fungal biomass. Antifungal activity of protein was also tested against *Fusarium monaliforme*, *F. graminearum*, *F. pallidoroseum*.

5.2.6. Isolation and partial characterization of antifungal protein

Based on the results of growth inhibition by culture supernatant and its protein fraction of *P. aeruginosa* PM389, role of secreted protein in fungal growth inhibition was assumed as one of the main reasons. Method for preparation of extracellular proteins and their partial characterization are described below.

5.2.6.1. Ammonium sulphate precipitation

Broth supernatant (B_{sup}) (described in section 5.2.5) was collected for precipitation of proteins. It was done to assess the role of extracellular protein in fungal growth inhibition. Protein precipitation was done by ammonium sulphate precipitation method (Saturation-50%, 80%). Ammonium sulphate was added slowly to the bacterial culture supernatant (ice-cold B_{sup}) and kept on a magnetic stirrer to dissolve salt as well as to avoid frothing. Resulting solution was incubated overnight at 4°C for precipitation of protein. Precipitated proteins were collected by centrifugation at 19000 g for 20 min at 4°C and supernatant was separated (P_{sup}:Protein supernatant). To remove $(NH_4)_2SO_4$, pellet of protein (P_o) was dissolved in 50 mM Phosphate buffer (pH 7.2) and dialyzed extensively against the same buffer as described below.

5.2.6.2. Dialysis

Appropriate size of dialysis bag (>12,000 Da, Sigma-Aldrich, USA) was washed under running tap water for 3 h. Dialysis bag was treated with 0.3% sodium nitrite at 80°C for 1 min and then washed with hot water (at 60°C) for 2 min. To remove residual sodium nitrite, dialysis membrane was treated with sulphuric acid solution (0.2% in water) for 15-20 sec followed by washing three times with distilled water and incubation in distilled water at 4°C for later use. The precipitated protein was added into the pre-treated dialysis bags and dialyzed against phosphate buffer (50 mM, pH 7.2) with stirring. The first two buffer changes were done at every one and a half-hour and then the set up was kept for overnight. All the steps were carried out at 4°C. Concentration of protein was estimated by Lowry's method as described in section 2.2.10.4.

5.2.6.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide resolving (12%) and stacking gels (5%) were prepared in Mini-Protean® 3 system (Bio-Rad Laboratories, Alfred, Hercules, CA, USA) (Appendix IV). The

crude protein sample P_o was subjected to SDS PAGE for 2 h at 100 V. A 25 µl of sample (P_o) was mixed with 25 µl of sample loading buffer (2X) and boiled at 100°C for 5-7 min. After boiling, samples were immediately transferred to ice. The 20 µl (0.6 mg ml^{-1}) of the sample was loaded into the wells. The protein marker (10 µl) (NEB, USA; 10-250 kDa) was used as a standard for molecular weight determination. Coomassie brilliant blue G-250 staining was done overnight according to the modified protocol by Candiano et al. (2004) and de-staining was done in a mixture containing water, methanol and acetic acid in the ratio 45:45:10 for 2 h. Gel was observed under bright light to analyze the protein bands.

5.2.7. Test for development of ISR by *P. aeruginosa* PM389 in wheat plants

ISR is an important biocontrol mechanism having several advantages over other methods as it has less chances of resistance development, ecofriendly, has broad-spectrum, long-term effect and does not introduce any resistance gene like plant breeding method leading to release of genes in the environment (Kuc 2001). Therefore, *P. aeruginosa* PM389 was studied for the development of ISR.

5.2.7.1. Preparation of test inoculums

Bacterial inoculum (*P. aeruginosa* and *E. carotovora*) was prepared by growing bacteria in LB broth for 24 h at 37°C. *Fusarium monaliforme* was chosen as a fungal pathogen for ISR studies. *F. monaliforme* causes Fusarium head blight of wheat. For preparation of fungal culture, *F. monaliforme* was grown in PDB (Potato dextrose broth) (HiMedia, India) at 28°C for 72 h. Cultures were harvested by centrifuging at 8,000 g for 10 min. Pellet was washed with 1X PBS solution, resuspended in PBS and cultures were adjusted to 10^7-10^8 cells ml^{-1} for bacteria and 4000 spores ml^{-1} for fungus.

5.2.11.2. Inoculation of plants

Wheat seeds were surface sterilized and grown in Hoagland media as described earlier in section 3.2.4. At 4^{th} day of plant growth, plants were treated with endophytic bacteria (*P. aeruginosa* PM389) with a population of 10^7-10^8 cells ml^{-1} (in 1X PBS). On 10^{th} day plants were

challenged with *Erwinia carotovora* (10^7-10^8 cells ml^{-1}) and *Fusarium monaliforme* (4000 spores ml^{-1}) in endophyte pretreated and untreated plants separately. Plants inoculated with 1X PBS was used as control (untreated). There were six treatments in this study. Plants treated with (a) only endophyte (b) only *Erwinia carotovora* (c) only *Fusarium monaliforme* (d) endophyte + *Erwinia carotovora* (e) endophyte + *Fusarium monaliforme*, and (f) uninoculated control, were used for comparative analysis of defense responses. Plant samples were harvested at a regular interval of 24 h up to six days after inoculation (DAI). All the treatments were in four replicates, and each sample was further assayed in triplicates (4 biological and 3 technical replicate).

5.2.12. Estimation of plant defense enzyme

Collected plant samples (entire plant) were crushed in liquid nitrogen and stored at -70°C for further analysis of defense enzymes namely peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and β-glucanase using standard methods described below. All the samples were homogenized in double volume of respective buffer for the particular enzyme assay and centrifuged at 8000 g for 10 min at 4°C. The resulting supernatant was used for enzyme assays. Protein was estimated by the method as described by Lowry (1951) as described in section 2.2.10.4.

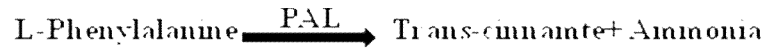
5.2.12.1. Test for β-glucanase activity

To test presence of β-glucanase, plant samples were extracted in 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C. A 10 µl crude enzyme extract was added to 10 µl of 4% laminarin and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent (0.8% NaOH, 0.25% dinitrosalicylic, 0.2% phenol, 0.05% Na_2SO_3) and heating for 10 min on a boiling water bath followed by addition of 20 µl of 40% sodium potassium tartarate. The resultant coloured solution was diluted three fold with Milli Q water, vortexed and read its absorbance at 575 nm in UV-Vis spectrophotometer (JASCO, USA). Various concentrations of glucose (0-1 mM) were used to obtain the calibration curve. One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmol of glucose equivalent per 10 min under the above conditions (Kurt et al.

1991). This method tests for the presence of free carbonyl group (c=o), called as reducing sugar. This involves the oxidation of the functional group present; for example; in aldehyde in glucose and the ketone in fructose. Simultaneously, 3,5 dinitrosalicylic acid (DNS) is reduced to 3-amino, 5 nitrosalicylic acid under alkaline conditions, which absorbs light strongly near 540 nm.

5.2.12.2. Estimation of phenylalanine ammonia lyase (PAL) activity

Plant sample was crushed in 50 mM Tris, pH 8.8. To 40 µl of total enzyme extract obtained, 24 µl of 10 mM phenylalanine and 176 µl Tris (pH 8.8) were added in successive order. Mixture was incubated for 60 min at 30°C, and the reaction was stopped by adding 200 µl of 2 N HCl and 300 µl toluene. The reaction mix was spun at 750 g for 5 min and upper layer was collected to take absorbance at 290 nm. Cinnamic acid (1-5 µg ml^{-1}) was used to prepare standard curve (Ramamoorthy et al. 2002). This method is based on the principle that L-phenylalanine acts as a substrate for PAL, which convert it to cinnamate showing maximum absorbance at 290 nm. One enzyme unit was defined as the amount of protein catalyzing the appearance of 1 µmol of cinnamate per min at 30°C.



5.2.12.3. Estimation of peroxidase activity

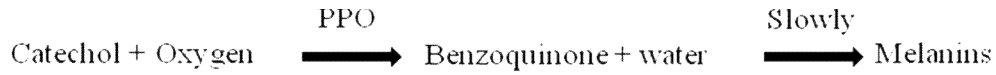
Plant tissue was extracted in 10 mM phosphate buffer (pH 6.0) and 6 µl of enzyme extract was added to reaction mix containing 24 µl 0.25% guaiacol dissolved in 0.01 M potassium phosphate (pH 6.0) and 0.1 M H_2O_2. Enzyme activity was measured by taking OD every 30 sec for 3 min at 470 nm and expressed as changes in the absorbance min^{-1} mg^{-1} protein (Hammerschmidt et al. 1982). It is based on the principle that rate of decomposition of hydrogen peroxide by peroxidase in presence of guaiacol as a H-donor, is determined by measuring the rate of color development spectrophotometrically.

$$4 \text{ guaiacol} + 4\ H_2O_2 \rightarrow \text{tetraguaiacol} + 8\ H_2O$$

formation of tetraguaiacol (increase of absorbance at 470 nm)

5.2.12.4. Estimation of polyphenoloxidase (PPO) activity

PPO catalyzes conversion of colorless polyphenols to colored compound in presence of molecular oxygen. This reaction results in formation of black-brown colored complex called as melanins.



To estimate PPO activity, plant tissues were homogenized in 0.1 M sodium phosphate buffer (pH 6.5). The reaction mixture consisted of 20 µl of the enzyme extract and 320 µl of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 40 µl of 0.01 M catechol was added to reaction mix, and the activity was expressed as changes in absorbance (measured at every 30 sec for 1 min) at 495 nm min^{-1} mg^{-1} protein (Mayer et al. 1966).

5.2.13. Estimation of pathogenic microbial infection in endophyte-treated plants

In a separate experiment with similar experimental set-up as described above, changes in population of pathogenic bacteria and fungi was monitored in the endophyte-pretreated and untreated plants at the end of the study. Experiment was carried out in duplicates. Wheat plants were uprooted and washed thoroughly with sterilized water to remove media. Plants of different treatments were homogenized separately in sterile phosphate-buffered saline (PBS) solution. 100 µl homogenized sample was spread on the NA and PDA (Potato dextrose agar) plates to grow pathogenic bacteria (*E. carotovora*) and fungi (*F. monaliforme*) respectively. After growth, colonies growing on media plates were counted for each treatment using standard protocols (Harley and Prescott 2002). Before microbial isolation from plants, seedlings were measured for the root length, shoot length, R:S index, fresh weight, dry weight and total height. Root and shoot length were measured by using the scale of 1 division, which equals to 1 mm.

5.2.14. Effect of *P. aeruginosa* PM389 on plant growth after pathogenic challenge: Pot studies

Based on various plant growth promoting properties and antifungal properties shown by *P. aeruginosa* PM389 in previous studies (Gupta et al. 2013), *P. aeruginosa* PM389 was checked

for its ability to prevent or minimize infection of fungal (*Fusarium monaliforme*) and bacterial pathogen (*E. carotovora*) in wheat (*Triticum aestivum* L.) plants in pot studies kept in green house. Surface sterilization of wheat seeds and experimental setup was similar as described above for *in vitro* studies.

5.2.15. Statistical analysis

One-way ANOVA, standard deviation and Student's t-test were calculated wherever applicable using Microsoft excel. Least Significant Difference (LSD) and the Duncan's Multiple Range Test were used to compare the means wherever applicable (Quinn and Keough 2002).

5.3. Results

5.3.1. Antagonistic activity

The antifungal activity of *P. aeruginosa* PM389 was tested against various fungal pathogens. It showed good antifungal activity against different fungal plant pathogens as shown in Table 5.1. It showed highest activity against *Fusarium graminearum* and appreciable antifungal activity against all tested fungus except *Colletotrichum acutatum* and *Aspergillus niger* in which no activity was observed.

5.3.2. Identification of antifungal mechanism

In order to address possible mechanisms involved in antagonism, it was screened for various antagonistic tests to identify the cause of antifungal activity of *P. aeruginosa* PM389. The bacterial isolate was found to be positive for pyoverdin (fluorescin). Siderophore production is one of the biocontrol mechanisms in Pseudomonads, it is also used as biochemical marker for identification of Pseudomonads (Fig. 5.1).

P. aeruginosa PM389 showed a positive result also for the production of pyocyanin, though the amount was meager. On assaying antifungal activity of pyocyanin, no activity was detected against fungal pathogens (*F. graminearum* and *F. pallidoroseum*) as shown in Fig. 5.2. The isolate *P. aeruginosa* PM389 showed a zone of inhibition (control), but the isolated pyocyanin didn't, thus it can be said that inhibitory activity of pyocyanin was almost negligible.

Table 5.1. Antagonistic activity of *Pseudomonas aeruginosa* PM389 against plant pathogenic fungal species.

Fungal pathogen	**Zone of Inhibition (cm)**	**Results**	**Inference**
F. pallidoroseum	3.13±0.11	+++	Good
F. oxysporum	3.30±0.10	+++	Good
F. graminearum	3.37±0.32	+++	Good
F. monaliforme	3.27±0.12	+++	Good
Verticillium sp.	2.80±0.19	+++	Good
Aspergillus flavus	2.50±0.00	+++	Good
Aspergillus niger	NA	-	NA
Colletotrichum caspasci	2.06±2.50	+++	Good
Colletotrichum acutatum	0.53±2.50	+	Poor

± represents standard deviation.

<1 cm = Poor (+), between 1-2 cm = Average (++), >2 cm = Good (+++), NA= No Activity.

Other than above mentioned tests, partial browning of filter (moderate activity) paper from the corners did confirm positive test for HCN production as shown in Fig. 5.3. For the test of hydrolytic enzymes, *P. aeruginosa* PM389 was found to be negative for chitosanolytic and chitinolytic activities while positive for the production of lipase activity (Fig. 5.4) and siderophore production (section 2.3.7.2).

5.3.3. Effect of *P. aeruginosa* PM389 on plant growth after pathogenic challenge: Pot studies

The plant growth promoting as well as biocontrol properties were further tested by conducting *in vitro* test in which *P. aeruginosa* PM389 showed 37% (n=12, $p<0.05$) increase in shoot length in pathogen (*F. oxysporum*) challenged plants than the control plants not treated with endophytic bacteria (Fig. 5.5). However, there was not a significant change in germination rate, vigour index (Control-1506, *P. aeruginosa* PM389+F-1502) and total height (Control-15.06 cm, *P. aeruginosa* PM389+F-15.02 cm) in *P. aeruginosa* PM389 pretreated plants than control. Inoculation of fungal pathogen affected plant growth in terms of all the growth parameters tested when compared to uninoculated control plants. However, growth of plants pretreated with endophytic bacteria had equivalent growth in terms of plant vigor index, total height and shoot length. However, no effect was observed on root growth on endophytic treatment in pathogen challenged plants.

5.3.4. Antifungal assay of protein

In the antifungal assay, all the three components (B_{sup}, P_{sup}, P_o) (Broth supernatant, Protein supernatant, Precipitated protein) inhibited growth of *Fusarium oxysporum*. Further, isolated protein was tested against different plant fungal pathogens and percent inhibition in dry weight was calculated. Dry weight analysis revealed that the antifungal protein inhibited growth of *F. oxysporum*, *F. monaliforme*, *F. pallidoroseum* and *F. graminearum* by 69%, 55%, 51% and 15% respectively.

These protein components were further separated by gel electrophoresis (SDS-PAGE) which revealed seven discrete bands of different sizes (Fig. 5.6).

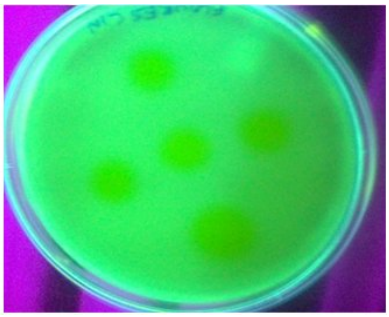
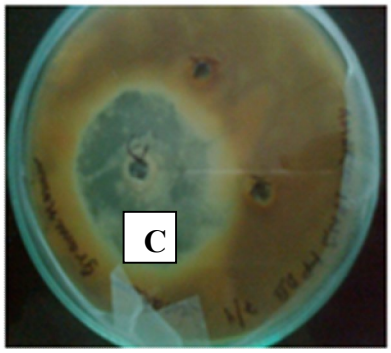
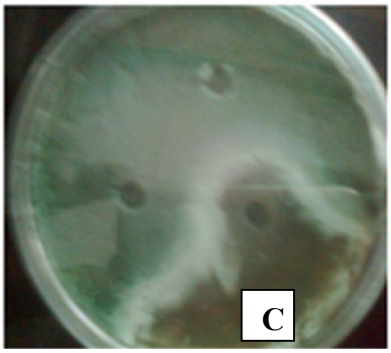


Fig. 5.1. Production of florescein (Pyoverdin) by *P. aeruginosa* PM389.



Fusarium graminearum



Fusarium pallidoroseum

Fig. 5.2. Antagonistic test of isolated pyocyanin against fungal pathogens.

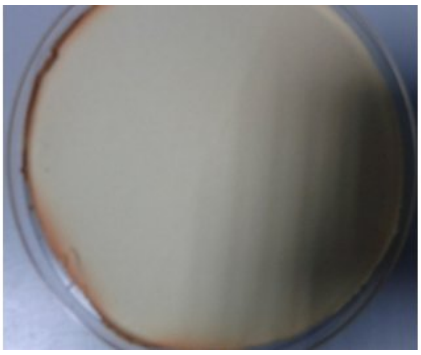


Fig. 5.3. HCN production by *P. aeruginosa* PM389.

Fig. 5.4. Lipase activity of PM389.

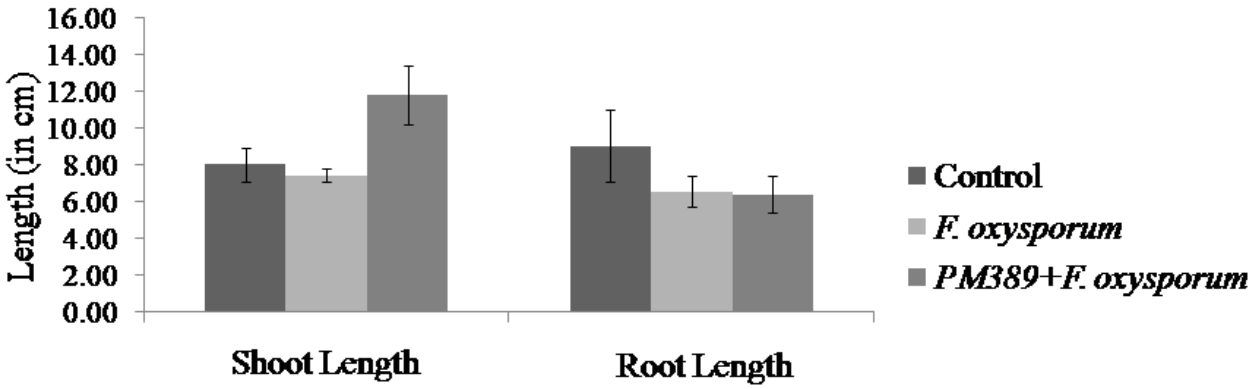


Fig. 5.5. Effect of pretreatment of *P. aeruginosa* PM389 on wheat plant challenged with *Fusarium oxysporum*: *In vitro* studies.

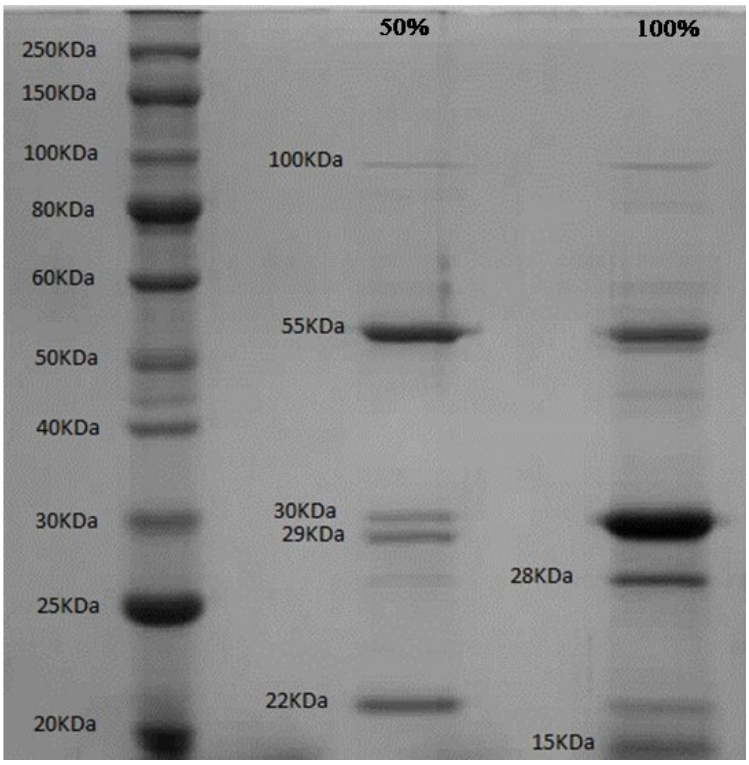


Fig. 5.6. SDS-PAGE of extracellular protein of endophyte *P. aeruginosa* PM389.

Antifungal activity of protein was confirmed from the results of treatment in which protein component was treated with Proteinase k (loss of action). Proteinase treatment resulted in reduction of inhibitory activity of extracellular components to 147 ± 9%.

5.3.5. Induced systemic resistance by *P. aeruginosa* PM389

As described in chapter I, ISR inducing bacteria prime plants for protection from future attack of pathogen by highly and rapidly activated immune responses. It suggests that the immune responses should be higher in case of endophyte pretreated plant than untreated one on pathogenic attack for the onset of ISR.

5.3.5.1. β-glucanase activity

Higher glucanase activity was observed in bacterial pathogen-treated plants at 1st DAI, which followed drastic reduction in the later sampling period. Plants responded differently to fungal pathogen. In response to fungal pathogen only, glucanase activity was higher initially and remained almost constant in the entire study period (Table 5.2. a). Unlike pathogen-treated and control plants, no induction in activity of β-glucanase was observed in only endophyte pretreated plants on inoculation of bacterial pathogen. Higher induction in glucanse activity was noticed at 1st, 5th and 6th DAI (n=12, p<0.05) in endophyte pretreated fungal pathogen challenged plant than untreated fungal challenged plant and control.

5.3.5.2. Phenylalanineammonia lyase (PAL) activity

Increase in PAL activity was noted on the bacterial pathogenic challenge at different time interval except at 2nd and 6th DAI. In case of fungal pathogen-challenged plants, PAL activity increased suddenly at 2nd DAI. Similarly, significantly (n=12, p<0.05) higher PAL activity was observed in endophyte pretreated plant challenged with the bacterial pathogen in comparison to only pathogen challenged and the control plants at all time intervals except at 4th and 5th DAI. In the entire study, endophyte-pretreated plants showed the significant (n=12, p<0.05) increase in the enzyme activity on the pathogenic (fungal) challenge to plants except at 2nd DAI (Table 5.2. b).

5.3.5.3. Peroxidase activity

Plants challenged with the only bacterial pathogen showed significant induction of PO activity at 2nd DAI. In another set-up endophyte-pretreated plants challenged with the bacterial pathogen showed significantly (n=12, $p<0.05$) higher levels of PO activity from 4th and 6th DAI than the only bacterial pathogen challenged plants and control. Interestingly, enhanced PO activity in plants treated with the only fungal pathogen was observed at 3rd and 5th DAI (Table 5.2. c), while significantly (n=12, $p<0.05$) high PO activity was noted in plants, which were pretreated with endophytic bacteria before fungal challenge.

5.3.5.4. Polyphenoloxidase activity

Significantly higher (n=12, $p<0.05$) PPO activity was detected at 2nd DAI in only bacterial pathogen-challenged plant, while no change in PPO activity was observed in only fungal pathogen-challenged plant as shown in Table 5.2. d. From 3rd-5th DAI, a slightly increased level of PPO activity was noticed in endophyte-pretreated plants challenged with the bacterial pathogen than control and only bacterial pathogen-challenged plants. PPO activity was significantly higher (n=12, $p<0.05$) from 2nd-4th and 6th DAI in endophyte pretreated plants challenged with fungal pathogen than control and only fungal challenged plants.

5.3.6. Disease incidence and plant growth promotion studies after pathogen challenge under *in vitro* conditions

Based on the results of antagonistic activity and ISR, the effect of endophytic bacteria *P. aeruginosa* PM389 was tested to validate their ability to protect plants from pathogenic attack and in turn promote plant growth under biotic stress condition. As shown in Fig. 5.7, pathogenic (bacterial) challenged plant pretreated with endophytic bacteria showed lesser prevalence of pathogenic bacteria (in terms of cfu count) than in plants treated with pathogen only. Interestingly, no fungal colony was recovered from plants, which were pretreated with endophytic bacteria before fungal challenge inoculation. It was observed in plant growth promotion studies that endophyte pretreatment was more effective in inhibiting fungal pathogen than the bacterial one (Fig. 5.8). It is evident from the Table 5.3 that there was less adverse effect

Table 5.2. Changes in activity of different defense enzymes in *Triticum aestivum* (var. GW322) in response to various microbial treatments (a) β-1,3-glucanase, (b) PAL, (c) PO, and (d) PPO activity. In Control treatment, seeds were treated with PBS, endophyte (*P. aeruginosa* PM389) (inoculated on 4th day), bacterial pathogen (*Erwinia carotovora*) (challenged on 10th day), fungal pathogen (*Fusarium monaliforme*) (challenged on 10th day), *P. aeruginosa* PM389+ *Erwinia carotovora* (inoculated on 4th day with *P. aeruginosa* PM389 and challenged with *Erwinia carotovora* on 10th day), *P. aeruginosa* PM389+ *Fusarium monaliforme* (similar to *E. carotovora*).

a)

Treatments	**Days after infection (DAI)**					
	1	2	3	4	5	6
Control	0.24 ± 0.1^{c}	0.22 ± 0.0^{abcde}	0.14 ± 0.0^{bcde}	0.15 ± 0.0^{bcd}	0.15 ± 0.0^{bc}	0.10 ± 0.0^{bcde}
P. aeruginosa PM389	0.23 ± 0.1^{cd}	0.24 ± 0.0^{ab}	0.16 ± 0.0^{abc}	0.16 ± 0.0^{b}	0.08 ± 0.0^{de}	0.11 ± 0.0^{bcd}
E. carotovora	0.36 ± 0.1^{a}	0.23 ± 0.0^{abc}	0.16 ± 0.0^{abcd}	0.15 ± 0.0^{bc}	0.09 ± 0.0^{d}	0.12 ± 0.0^{bc}
F. monaliforme	0.27 ± 0.0^{f}	0.24 ± 0.0^{a}	0.18 ± 0.0^{ab}	0.18 ± 0.0^{a}	0.18 ± 0.0^{b}	0.13 ± 0.1^{b}
P. aeruginosa PM389+*E. carotovora*	0.15 ± 0.0^{e}	0.12 ± 0.0^{f}	0.08 ± 0.0^{f}	0.10 ± 0.0^{f}	0.07 ± 0.0^{def}	0.03 ± 0.0^{f}
P. aeruginosa PM389+*F. monaliforme*	0.30 ± 0.0^{b}	0.22 ± 0.1^{abcd}	0.19 ± 0.1^{a}	0.14 ± 0.0^{bcde}	0.24 ± 0.1^{a}	0.21 ± 0.1^{a}

b)

Treatments	**Days after infection (DAI)**					
	1	2	3	4	5	6
Control	7 ± 2^{cdef}	12 ± 2^{d}	17 ± 2^{cde}	13 ± 3^{de}	18 ± 2^{b}	10 ± 1^{cdef}
P. aeruginosa PM389	16 ± 4^{cd}	8 ± 2^{de}	18 ± 4^{cd}	14 ± 3^{d}	18 ± 1^{bc}	4 ± 1^{cd}
E. carotovora	21 ± 3^{cde}	8 ± 3^{def}	$20\pm^{c}$	20 ± 1^{bc}	18 ± 2^{bcd}	4 ± 1^{cde}
F. monaliforme	9 ± 6^{c}	57 ± 2^{ab}	8 ± 2^{f}	4 ± 2^{f}	5 ± 4^{ef}	6 ± 4^{c}
P. aeruginosa PM389+*E. carotovora*	34 ± 6^{b}	32 ± 6^{bc}	34 ± 9^{a}	20 ± 2^{b}	9 ± 2^{e}	27 ± 2^{b}
P. aeruginosa PM389+*F. monaliforme*	64 ± 2^{a}	62 ± 13^{a}	31 ± 3^{ab}	69 ± 4^{a}	78 ± 12^{a}	62 ± 18^{a}

c)

Treatments	**Days after infection (DAI)**					
	1	2	3	4	5	6
Control	36 ± 7^{bc}	35 ± 7^{b}	17 ± 2^{def}	21 ± 5^{cd}	20 ± 5^{cde}	16 ± 2^{cd}
P. aeruginosa PM389	30 ± 4^{d}	30 ± 6^{cde}	21 ± 5^{bcd}	21 ± 4^{c}	21 ± 3^{cd}	13 ± 2^{cde}
E. carotovora	40 ± 8^{b}	41 ± 6^{a}	19 ± 2^{cde}	19 ± 4^{cde}	18 ± 2^{cdef}	14 ± 2^{cdef}
F. monaliforme	9 ± 2^{f}	13 ± 8^{f}	31 ± 13^{a}	15 ± 5^{cdef}	47 ± 18^{b}	18 ± 4^{c}
P. aeruginosa PM389+*E. carotovora*	30 ± 7^{de}	31 ± 7^{cd}	26 ± 6^{abc}	31 ± 9^{b}	25 ± 10^{c}	28 ± 9^{b}
P. aeruginosa PM389+*F. monaliforme*	61 ± 2^{a}	34 ± 5^{c}	28 ± 6^{ab}	68 ± 14^{a}	75 ± 5^{a}	45 ± 3^{a}

d)

Treatments	**Days after infection (DAI)**					
	1	2	3	4	5	6
Control	1.44 ± 0.2^{a}	1.14 ± 0.2^{bcd}	1.30 ± 0.1^{abc}	1.35 ± 0.1^{abcd}	1.04 ± 0.2^{abcd}	0.87 ± 0.2^{abc}
P. aeruginosa PM389	1.27 ± 0.2^{bc}	1.29 ± 0.2^{ab}	1.21 ± 0.0^{abcd}	1.43 ± 0.0^{abc}	1.13 ± 0.1^{ab}	1.06 ± 0.2^{a}
E. carotovora	1.34 ± 0.1^{b}	1.75 ± 0.2^{a}	1.33 ± 0.1^{ab}	1.44 ± 0.1^{ab}	1.05 ± 0.2^{abc}	0.99 ± 0.2^{ab}
F. monaliforme	0.02 ± 0^{def}	0.03 ± 0.02^{ef}	0.03 ± 0.02^{f}	0.02 ± 0^{f}	0.01 ± 0^{ef}	0.01 ± 0^{f}
P. aeruginosa PM389+*E. carotovora*	0.09 ± 0.05^{de}	0.28 ± 0.12^{e}	1.50 ± 1^{a}	1.50 ± 0.8^{a}	1.40 ± 1.2^{a}	0.55 ± 0.23^{d}
P. aeruginosa PM389+*F. monaliforme*	0.10 ± 0.07^{d}	1.20 ± 1^{bc}	0.38 ± 0.19^{e}	1.10 ± 0.24^{abcde}	0.03 ± 0.1^{e}	0.03 ± 0^{e}

± represents standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters.

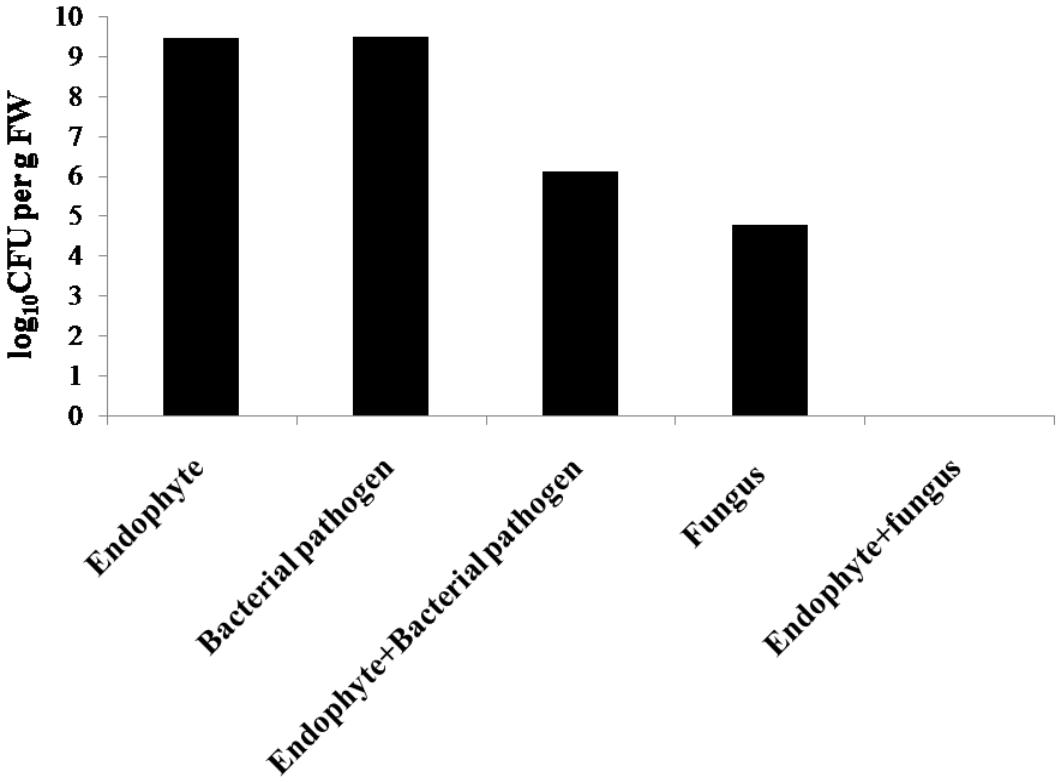


Fig. 5.7. Effect of *P. aeruginosa* PM389 treatment on growth of pathogen challenged wheat plants under *in vitro* studies.

of fungal pathogen on growth of plants, which were pretreated with *P. aeruginosa* PM389. *P. aeruginosa* PM389 increased various growth parameters slightly in fungal pathogen challenged plant. However, presence of *P. aeruginosa* PM389 exacerbated the effect of bacterial pathogen (Table 5.3).

5.3.7. Effect of *P. aeruginosa* PM389 on plant growth after pathogenic challenge: Pot studies

Based on the positive results obtained in plant growth studies in presence of pathogenic invasion in endophytic bacteria treated plants, the study was extended to pot studies to examine the ability of *P. aeruginosa* PM389 to protect plants from pathogenic infection. In pot studies, it was observed that endophyte-pretreated plant challenged with the fungal and bacterial pathogen showed significantly higher growth than other treatments (Fig. 5.9). Similar to the results of *in vitro* experiments, *P. aeruginosa* PM389 increased plant growth more efficiently in fungal pathogen-challenged plant than that of bacterial pathogen-challenged plant in pot studies as well. Pretreatment with *P. aeruginosa* PM389 significantly increased various growth parameters such as root length, shoot length, fresh weight, dry weight, total height and vigor index on the fungal and bacterial challenge. It promoted 14-59% and 10-53% higher plant growth in plants pretreated with endophyte on challenging with fungal and bacterial pathogen respectively (Table 5.4).

Fig. 5.8. Effect of *P. aeruginosa* PM389 treatment on growth of pathogen challenged wheat plants under *in vitro* studies.

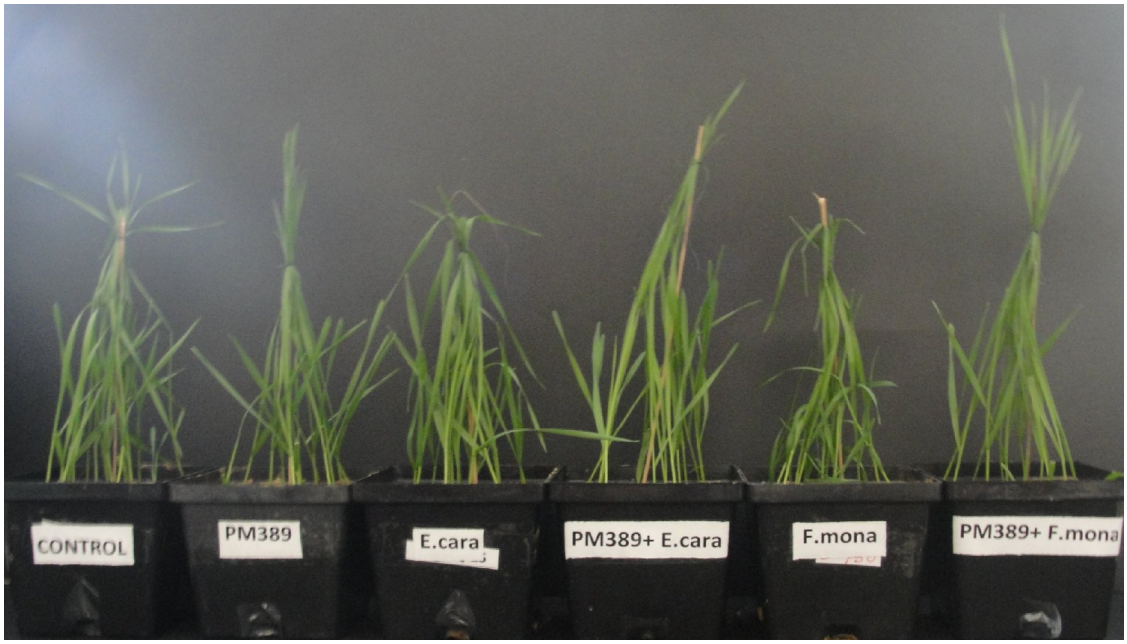


Fig. 5.9. Effect of *P. aeruginosa* PM389 treatment on growth of pathogen challenged wheat plants under pot studies in green house.

Table 5.3. Effect of *P. aeruginosa* PM389 on growth of pathogen challenged wheat plants under *in vitro* studies.

Treatment	Root length (cm)	Shoot length (cm)	Root:Shoot Index	Fresh Wt (g)	Dry Wt (g)	Germin-ation rate (%)	Total height (cm)	Vigor index
Control	9.17±0.12	21.1±0.80	0.43±0.02	0.21±0.02	0.023±0.01	100±0	30.27±0.70	3026.67±70
F.monaliforme	9.05±1.70	20.90±1.10	0.43±0.08	0.21±0.03	0.024±0.01	100±0	29.95±2.13	3002±204
E. carotovora	8.93±0.71	21.80±0.36	0.41±0.04	0.21±0.02	0.022±0.01	100±0	30.73±0.62	3073±62
P. aeruginosa PM389	11.30±0.50	22.70±0.95	0.50±0.00	0.26±0.03	0.034±0.0	100±0	34.00±1.45	3400±145
	(23, 25, 27)*	(8, 9, 4)*	(16,16, 22)*	(24,24,24)*	(48,48,54)*	(-, -, -)	(12,14,11)*	(12,13,11)*
P. aeruginosa PM389+ *F.monaliforme*	9.10±0.42	22.30±1.70	0.41±0.08	0.22±0.03	0.025±0.01	100±0	31.40±1.27	3140±127
	(-, 2)	(6, 7)	(-, -)	(4, 4)	(9, 4)	(-, -)	(4, 5)	(4, 5)
P. aeruginosa PM389+ *E. carotovora*	6.70±2.54	19.55±1.25	0.34±0.04	0.20±0.02	0.020±0.01	100±0	26.25±3.62	2625±362
	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)

*$p<0.05$, n=4 (Student's *t*-test). ± represents standard deviation of mean. Values (I, II, III) in parenthesis are percent increase than control, fungal pathogen, bacterial pathogen or control, fungal pathogen/bacterial pathogen respectively. Plant growth parameters for *P. aeruginosa* PM389 was compared with control, fungal pathogen, bacterial pathogen, *P. aeruginosa* PM389+ *F.monaliforme* with control and *F.monaliforme* (I, II), *P. aeruginosa* PM389+ *E. carotovora* with control and *E. carotovora* (I, II).

Table 5.4. Effect of *P. aeruginosa* PM389 on growth of pathogen challenged wheat plants under pot studies in green house.

Treatment	Root length (cm)	Shoot length (cm)	Fresh Wt (g)	Dry Wt (g)	Root: Shoot Index	Germination rate (%)	Total height (cm)	Vigor index
Control	17.88±2.06	19.14±0.54	0.17±0.02	0.027±0.004	0.93±0.17	100±0	37.02±2.02	3702±202
F.monaliforme	15.08±0.60	18.71±2.56	0.19±0.03	0.029±0.00	0.85±0.13	100±0	33.62±3.02	3362±302
E. carotovora	15.31±1.99	18.44±1.30	0.22±0.02	0.030±0.00	0.83±0.09	100±0	33.75±2.87	3375±287
P. aeruginosa	16.22±0.90	21.37±2.14	0.21±0.03	0.028±0.00	0.77±0.10	100±0	37.59±1.95	3759±195
PM389	(-, 8, 6)	(12, 14, 16)	(24, 2,-)*--	(-, -, -)	(-, -, -)	(-, -, -)	(2,12,11)	(2,12,10)
P. aeruginosa PM389+ *F.monaliforme*	21.14±1.17 (18, 40)**	25.86±1.15 (35, 38)**	0.27±0.02 (59, 42)**	0.033±0.00 (22,14)**	0.82±0.08 (-, -)	100±0 (-, -)	47.01±0.09 (27, 40)**	4701±9 (27, 40)**
P. aeruginosa PM389+ *E. carotovora*	16.88±1.52 (-, 10)-*	24.41±1.33 (28, 33)**	0.26±0.01 (53,18)*-	0.032±0.00 (19, 7)	0.69±0.09 (-, -)	100±0 (-, -)	41.29±1.60 (12,18)**	4129±160 (12, 18)**

*p<0.05, n=4 (Student's *t*-test). ± represents standard deviation of mean. Values (I, II, III) in parenthesis are percent increase than control, fungal pathogen, bacterial pathogen or control, fungal pathogen/bacterial pathogen respectively. Plant growth parameters for *P. aeruginosa* PM389 was compared with control, fungal pathogen, bacterial pathogen, *P. aeruginosa* PM389+ *F.monaliforme* with control and *F.monaliforme* (I, II), *P. aeruginosa* PM389+ *E. carotovora* with control and *E. carotovora* (I, II).

5.4. Discussion

Based on the presence of antagonistic activity against several bacterial and fungal isolates especially pathogenic ones, we tested *P. aeruginosa* PM389 for its potential to be used as an effective biocontrol agent. Understanding importance of biocontrol agent in sustainable and environmental friendly agriculture system, *P. aeruginosa* PM389 was investigated for various possible mechanisms, which can be exploited to prevent or minimize pathogenic microbial infestation. Out of various mechanisms, ISR is one of the most important as it develops memory for the future attack and can contribute to acquire disease resistance for long term usage. Therefore, present chapter deals with thorough investigation of biocontrol mechanisms of *P. aeruginosa* PM389 against the fungal and bacterial pathogen. Further *P. aeruginosa* PM389 was also assessed as a biocontrol agent by studying their effect on suppression of pathogenic colonization in plants under *in vitro* growth conditions and at pot level studies.

P. aeruginosa PM389 showed appreciable antifungal activity against most of the pathogens tested, which suggests it is a potential antifungal agent. There are various mechanisms involved in the antifungal activity such as production of pyocyanin and pyoverdin, HCN production, lipase production, siderophore production, production of antifungal protein and development of induced systemic resistance. Pyocyanin is known to have antifungal activity and may be involved in development of ISR in plants. Involvement of pyocyanin and siderophore (pyochelin) in ISR has been confirmed in *P. aeruginosa* 7NKS2 against *Botrytis cinerea* in tomato (Audenaert 2002). However, very weak activity of pyocyanin in test isolate *P. aeruginosa* PM389 rules out the possibility of pyocyanin to be involved in fungal growth inhibition. In addition to pyocyanin, *P. aeruginosa* PM389 was also found to be negative for the production of chitosanolytic and chitinolytic activities. These results suggested involvement of some other mechanisms responsible for the antifungal activity.

However, it did exhibit other antifungal activities such as presence of lipase activity and HCN production, which can contribute disease suppression in host plants through antifungal activity. It has been reported in a recent finding that null mutation in the cluster of HCN gene of *Pseudomonas flourescens* In5, abolished its HCN production as well as the antifungal activity against *Rhizoctonia solani* and *Pythium aphanidermatum.* Same study also reported that HCN

production is highly dependent on the medium used (Michelsen and Stougaard 2012). Alternative mechanism which can inhibit fungal growth is through production of siderophore, which chelates iron and deprive pathogens from it (Fgaier and Eberl 2011). Thus, lipase, siderophore and HCN at some extent might be the reason of *P. aeruginosa* PM389 being antifungal.

P. aeruginosa PM389 acted as good antifungal agent having several mechanisms as mentioned above. Further, the supernatant was also checked for the antifungal activity to ensure production of inhibitory compound in the extracellular components. On showing the positive test by supernatant, extracellular protein was tested as it could be a possible factor responsible for the antifungal activity. Extracellular protein showed antifungal activity and reduction in activity of antifungal protein on proteinase K treatment suggested that the extracellular proteins of *P. aeruginosa* PM389 are also responsible for its antifungal nature. Similar to this study, antifungal protein has been isolated from *Bacillus subtilis* EDR4 (Liu et al. 2010).

Another possible mechanism for antifungal nature of *P. aeruginosa* PM389 was the development of ISR in plants on the pathogenic challenge, in which induction in the level of various defense enzymes playing the key role in the plant defense system was observed. β-glucanase (PR2) has an important role as an elicitor, and it is known as an antimicrobial peptide which acts on the cell wall of fungal plant pathogens (Datta et al. 1999). After the initial increase of β-glucanase in bacterial pathogen challenged plant, activity turned down as bacterial cell wall has different composition than fungal pathogen having oligo-glucan composed cell wall. Therefore, β-glucanase activity was not observed against the bacterial pathogen afterwards. Since, the intensification in activity of β-glucanase requires the release of glucan oligomers, it was not exhibited in presence of bacterial pathogen, which lacks glucan in their cell wall (Frindlender et al. 1993). Similar result was obtained in plants which were pretreated with *P. aeruginosa* PM389, which is also a bacterium. As expected, β- glucanase activity was higher in fungal pathogen challenged plant due to release of glucan oligomers. Glucanase activity in only fungal pathogen treated plants remained higher than the control plants throughout the study period. This observation favours the notion that plant shows constant activity against the fungal pathogen by releasing the glucanase enzyme to ward-off the pathogen and released glucan

oligomers are intensifying the reaction (Frindlender et al. 1993). This finding is unlike to that of Rammoorthy et al. (2002), in which they observed induction in β-glucanase activity initially, which get further declined in only fungal pathogen *Fusarium oxysporum* challenged plant. Enhanced activity of β-glucanase was observed in endophyte pretreated plants challenged with the fungal pathogen after one day of challenge. Glucanase activity in endophyte pretreated plants challenged with the fungal pathogen was observed higher than control and only fungal pathogen treated plants in most of the samples collected at different time intervals. However, level of expression varied in different samples. These results infer that *P. aeruginosa* PM389 is involved in the development of ISR.

PAL is the first key enzyme of phenylpropanoid pathway and flavanoid pathway, which have the role in generating precursors for lignin biosynthesis, phytoalexins, and other phenolic compounds that have direct effects on pathogens. Cinnamic acid is the product of PAL, which is directly linked to cell lignification process (Daayf et al. 1997). In case of bacterial pathogen challenged plant, PAL activity was higher at 1st DAI. In case of fungal pathogen (*F. monaliforme)*, sudden induction in PAL activity was observed only at 2nd DAI followed by its decline, which suggests that *F. monaliforme* has invaded the host roots and thus, resulted in decreased PAL activity (Rammoorthy et al. 2002). While, in bacterial pathogen frequent changes in PAL activity suggested that a constant fight is occurring between pathogen and host plant similar to zig-zag model suggested by Chisholm et al. (2006). In endophyte pretreated plant challenged with pathogens, drastically induced activity was observed, which concluded role of *P. aeruginosa* PM389 in suppressing disease incidence and preventing invasion of pathogen (Liang et al. 2011).

PO is a key enzyme in the biosynthesis of lignin and other oxidized phenol, catalyzing the oxidation of hydroxyl-cinnamyl alcohol into free radical intermediates, which subsequently are coupled into lignin polymers (Bruce and West 1989). Other than oxidized phenol, oxidation mediated by PO and PPO are also highly toxic for pathogens (Sequeira 1983). PO and PPO catalyze the oxidation of phenolic compounds through a PPO-PO-H_2O_2 system (Velazhahan 1994). In various previous findings, induction of PO and PPO activity has been correlated with

increased acquired resistance and inhibition of plant pathogens (Sequeira 1983). Induction in PO activity was observed at 1st DAI in bacterial pathogen challenged plant, as ROS (H_2O_2) production is the initial defense response of sensitized plant against pathogens (Lamb and Dixon 1997). In case of endophyte-pretreated plant challenged with *Erwinia carotovora*, activity was induced more than that of untreated *E. carotovora* challenged plant as observed in previous studies of *Bacillus megaterium* against pathogen *Pythium aphanidermatum* (Liang et al. 2011). Similarly, in case of fungal pathogen-challenged plant pretreated with *P. aeruginosa* PM389, enhanced activity of PO was observed throughout the study period suggested that induced resistance lead to plant protection. Similar to the results of PO, enhanced activity of PPO was observed in endophyte pretreated plant challenged with pathogen than that of plants without pretreatment with endophyte. This confirmed the hypothesis that the increased level of defense responses in presence of *P. aeruginosa* PM389 against pathogen resulted in development of induced systemic resistance in plants.

Altogether, above results suggest that *P. aeruginosa* PM389 has several antagonistic mechanisms such as HCN, lipase, siderophore, antifungal protein and ISR for inhibition of pathogenic organisms. ISR induced by *P. aeruginosa* PM389 against pathogen was further tested under *in vitro* and pot condition for further application as a biocontrol agent. The disease incidence study revealed that *P. aeruginosa* PM389 was highly effective against fungal pathogen *F. monaliforme* than that of *E. carotovora*. Also in case of endophyte pretreated plants challenged with bacterial pathogen, lesser induction in defense responses than the fungal pathogen was observed. In some previous reports, it has been observed that ISR can be generated against fungal pathogens more efficiently than bacterial pathogens (Kuc 2001), which goes well with our result where more effectiveness of *P. aeruginosa* PM389 against fungal pathogen *F. monaliforme* was observed. The pretreatment of endophytic bacteria *P. aeruginosa* PM389 to plants prior to inoculation of pathogenic microorganisms protected plants from the negative effects of pathogenic organism, as in case of *F. oxysporum* also. *P. aeruginosa* PM389 further showed plant growth promotion in wheat plants in pot and under *in vitro* condition on challenging with pathogen in its presence, which showed the application of *P. aeruginosa* PM389 as a biofertilizer as well as biocontrol agent in future use.

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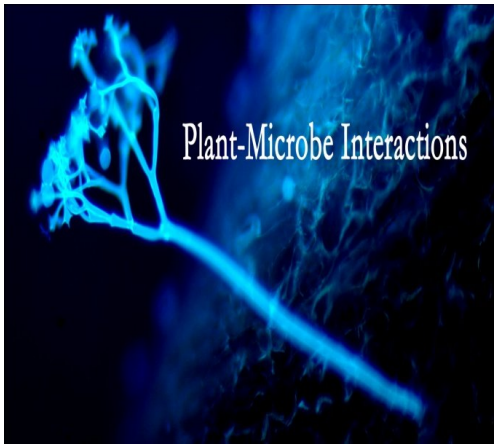
CHAPTER VI

Attenuated immune response to endophytic bacteria favours successful interaction with host plant.



"For plants, vulnerable as they are to changes in their immediate environment, the services provided by microbes are critical. In their natural, unmanaged environments, all plants are supported by a vast, invisible world of bacteria, viruses, and fungi that live in and around their roots, stems, leaves, seeds, pollen, fruits, and flowers."

- American Academy of Microbiology

6.1. Introduction

Plants are invaded and colonized by a diverse microflora of soil-borne bacteria and fungi that may have either beneficial or deleterious effects to the plant. Invasion of pathogenic microorganisms can have adverse effect on plant health. Therefore, plants have developed a strong immune response which plays the important role in protection from attack of invading microorganisms. Similar to animals, plants do have first line of defense in terms of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) and, second line of defense mediated by hormone signaling and acquired resistance. These defense mechanisms help plants respond to the intruder's invasion without any self-reactivity and develop a long-term memory (SAR in pathogen and ISR in beneficial microbes) for future attack (Spoel and Dong, 2012). Pathogenesis related (PR) proteins produced during generation of immune response are eminent in combating biotic stress conditions. PR proteins are usually absent or produced at basal concentrations in healthy tissues, but they are induced and accumulated upon microbial invasion (Van Loon *et al.*, 1999). Most of the PRs are antifungal, although some acts as antibacterial, insecticidal, nematicidal and/or antiviral (section 1.7.5).

Plants are quickly colonized by a diverse microflora of soil-borne bacteria and fungi that may have either beneficial or deleterious effects to the plant. Most of the endophytic bacteria are also *en-route* from soil and enter the plant interior to establish mutual relationship. However, the endophytic microbial communities associated with plant roots are different from that in rhizospheric and bulk soil. Thus, selective successful colonization of certain microbes suggests an instrumental role of host plants in shaping up their microbiome (Mendes et al. 2011). Both endophytic (beneficial) and pathogenic (deleterious) bacteria have the capacity to colonize an interior of the host, but their successful establishment inside the plant depends on genotypic compatibility, physiological status and extent of plant immune response. Since, both are alien to the host plant during the colonization process, they trigger host's immune response through similar or different pathways. Despite the generation of plant immune response towards beneficial microbe, host-plants do not ward them off. It suggests a well coordinated and a continuous molecular dialogue between the plant and the beneficial organism (van Wees et al.

2008). A few studies have been conducted to assess locally induced changes in plant gene expression or metabolism during plant–beneficial microbe interactions (Bordiec et al. 2011). These studies report elicitation of only weak, transient, or strictly localized defense-associated responses towards beneficial microbes. It differs greatly from the massive induction of defense responses triggered during plant–pathogen interactions (Liu et al. 2007). There are certain studies, which suggest the overlap and convergence of plant genes responsive to pathogenic and beneficial bacteria involved in signaling cascades during plant-beneficial/pathogenic bacteria interactions (S Van der Ent, PhD thesis, Utrecht University, 2008).

To establish a mutualistic interaction with the plant, endophytes need to cope with host immune responses that are triggered locally in the roots upon MAMP (microbe-associated molecular pattern) perception. Evidence is emerging that beneficial bacteria have developed decoy strategies to short-circuit hormone regulated defense responses, which pave the way for long term association for mutualism (Jacobs et al. 2011). Various microbial molecules such as flagella, EPS/LPS, peribacteroid membrane of Rhizobiaceae family have been reviewed of suppressing host immune response for symbiotic relationship (Zamioudis and Pieterse 2012). There are very few studies for PGPF and PGPR that suppress plant immune response to gain entry inside the plants (Bordiec et al. 2011). For instance, PGPF *Piriformospora indica* was reported to recruit suppressed JA pathway (Jacobs et al. 2011), while PGPR *Pseudomonas fluorescens* WCS417 suppressed flagellin-triggered PTI responses (Millet et al. 2010).

Thus, it is important to understand the strategies deployed by endophytic bacteria to subsist the host immune responses as well as the role of plants in helping endophytic bacteria to establish inside the plants. To our knowledge, a few studies have been conducted on plant immune responses during endophytic colonization (Bordiec et al. 2011). Therefore, in order to address possible differences in intensity of immune response to endophytic and pathogenic bacteria, present study aimed to estimate the level of immune response by measuring activity of defense enzymes produced after bacterial challenges and to correlate them with success of colonization of given bacteria *in-planta*. Activity of four defense enzymes namely polyphenol oxidase (PPO), peroxidase (PO), phenylalanine ammonia-lyase (PAL) and β-1,3-glucanases was

estimated. Present study has been carried out using endophytic bacteria *Pseudomonas aeruginosa* PM389 and a pathogen *Erwinia carotovara. E. carotovora* is a wide range pathogen, and it causes secondary infection in wheat for enhancing the activity of major wheat pathogens like *Fusarium* spp. (Newton et al. 2004). We chose the wheat plant as host for immune response studies as it is a major cereal crop world-wide. Such studies on wheat can be helpful in increasing the yield in the future. While, model plants such as *Arabidopsis thaliana* (weed), tomato and tobacco are not so much agriculturally important and thus, cannot play a role in reaching the increased agriculture demands.

Success of endophytic bacteria to colonize plants raises several important questions. It includes: how do endophytic bacteria evade or suppress host immune response?, or; is it plant which differentiates between pathogen and endophytic bacteria dwelling inside the plant? Present work has made an attempt to address above questions where immune response of wheat plants raised against beneficial and pathogenic bacteria has been compared. Measurement of immune response has been conducted in terms of stimulation of defense enzyme activities. It was further estimated by quantitative analysis of pathogenesis-related gene expression of endophytic bacteria. This study would be useful for understanding the different strategies of endophytes to fight against the host immune response.

6.2. Materials and methods

6.2.1. Bacterial isolates used

Plant growth promoting endophytic bacteria used in the present study was *Pseudomonas aeruginosa* PM389 isolated from pearl millet. *Erwinia carotovora,* a wide host range plant pathogen was purchased from Indian Agriculture Research Institute, Pusa, India. Pure cultures were grown and maintained as described earlier in section 2.2.4.

6.2.2. Surface sterilization of seed and plant growth

Induction of immune response towards endophytic bacteria and pathogenic bacteria was studied in wheat plants grown in axenic conditions. Seeds of wheat plants were surface sterilized

following standard protocol described in section 3.2.4. Surface sterilized seeds were germinated and grown in Hoagland medium under standard conditions (section 3.2.4).

6.2.3. Inoculation of wheat plant with bacterial isolates

Bacterial cultures were grown and prepared for inoculation to plants as described in section 5.2.11. On 10^{th} day of plant growth, axenically grown wheat plants were inoculated with endophytic and pathogenic bacteria with a population density of 10^7-10^8 cells ml^{-1} (in 1X PBS) in a separate set of experiments. Plant samples were collected at 0, 6, 12 hours of post-inoculation of bacterial isolates and at a regular interval of 24 h up to six days after inoculation (DAI). In control treatment, plants were treated with 1X PBS. All the treatments were carried out in four replicates (biological replicates), and each individual treatment was further assayed in triplicates (technical replicates). For qPCR (Quantitative PCR) studies, plant samples were collected at 0, 6, 12 and 24 h from endophyte and control treatments in the similar manner described above.

6.2.4. Estimation of plant defense enzymes

In order to monitor changes in defense responses in host plants, defense enzyme activities were measured in plants after treatment with endophytic and pathogenic bacteria separately. Plant samples collected at different time periods after inoculation of test bacteria were macerated and used for various enzymatic assays following methods explained in section 5.2.12.

6.2.5. Quantitative analysis of defense-related gene expression

With a view to estimate induced or suppressed expression of defense-related genes in response to beneficial bacteria, total plant RNA was extracted and level of cDNA (complementary DNA) was measured using qPCR.

6.2.5.1. RNA isolation and quantification

Total RNA was extracted from plant samples treated with endophytic bacteria as described in section 6.2.3. All experiments were conducted in duplicates. Plant samples were washed thoroughly with Milli Q water for the removal of adhered media. Collected plant tissues

were crushed in liquid N_2 and further RNA isolation was done using Qiagen Plant mini RNA isolation kit (Qiagen, USA) as per the manufacture's instruction. Isolated RNA samples were stored at -70°C until use. Experion RNA StdSens analysis kit (BIO-RAD, USA) was used to measure purity and quantity of RNA following manufacture's instruction (Appendix V).

6.2.5.2. cDNA preparation

RNA sample was treated with DNAse (Deoxyribonuclease) I (Thermoscientific, USA) to remove genomic DNA contamination as per the manufacturer's instruction. Briefly, 1 µg RNA was treated with 1 µl (1 U) DNAse I (in 1 µl of 10X reaction buffer with $MgCl_2$) and kept at 37°C for 30 min after making up total reaction volume to 10 µl. The reaction was stopped by adding 1 µl 50 mM EDTA (Ethylene diamine tetraacetic acid) and incubated at 65°C for 10 min. One µg of RNA sample was used for cDNA preparation. The genomic DNA-free cDNA (1 µg) was synthesized using oligo dT (0.5 µg) primer as per the protocol of "First strand cDNA synthesis" provided by the manufacturer (Thermoscientific, USA). The mixture of RNA and oligo dT was heated at 65°C for 5 min to remove secondary structures, chilled on ice and centrifuged briefly. Thereafter, 4 µl 5X reaction buffer, 20 U RNAse (Ribonuclease) inhibitor, 2 µl 10 mM dNTP mix (final concentration-1 mM) and 40 U M-MuLV (Moloney murine leukemia virus) reverse transcriptase (Thermoscientific, USA) were added to the above sample and mixed by flicking. Reaction mix was centrifuged briefly to collect the reaction components. The mixture was incubated at 37°C for 60 min and reaction was terminated by heating at 70°C for 10 min.

6.2.7. Quantitative-PCR of defense-related genes

RT-qPCR was performed in an optical clear 8 well-strips using SYBR green I based detection system in a BIO-RAD iQ5 Real-Time PCR detection system. 15 µl of each reaction mix for PCR contained 7.5 µl of 2X iQ^{TM} SYBR green supermix (contains dNTPs, 50 U ml^{-1} antibody-mediated hot-start iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, enhancers, stabilizers, 20 nM flourescein) (BIO-RAD, USA), 10 pmol of both forward and reverse gene specific primers and 25 ng cDNA. Sequence of gene specific primers and the size of expected

Table 6.1. Primer sequences used for PR gene expression studies used in real time PCR.

Primers	Sequence (5´-3´)	Amplicon (bp)	References
PR2 F	CTCGACATCGGTAACGACCAG	118	Ray et al. 2003
PR2 R	GCGGCGATGTACTTGATGTTC		
PR3 F	AGAGATAAGCAAGGCCACGTC	115	Desmond et al. 2006
PR3 R	GGTTGCTCACCAGGTCCTTC		
PR4 F	CGAGGATCGTGGACCAGTG	127	Bertini et al. 2003
PR4 R	GTCGACGAACTGGTAGTTGACG		
PR5 F	ACAGCTACGCCAAGGACGAC	90	Kuwabara et al. 2002
PR5 R	CGCGTCCTAATCTAAGGGCAG		
PR9 F	GAGATTCCACAGATGCAAACGAG	101	Pritsch et al. 2000
PR9 R	GGAGGCCCTTGTTTCTGAATG		
PR10 F	TTAAACCAGCACGAGAAACATCAG	157	Muthukrishnan et al. 2001
PR10 R	ATCCTCCCTCGATTATTCTCACG		
PR12 F	GGCGAATTCCCGGACGTGCCTGTCGC	177	Unknown
PR12 R	CGCGTCGACTGGTGTTGCTTAGCA		
PR16 F	AACAAAGGTGATGTGTTCGTCTTC	212	Schweizer et al. 1999
PR16 R	GAGCCGGTCTATTGTATTCTTTTCC		
PDI F	TTATGACTTTGGCCACACCG	100	Ray et al. 2003
PDI R	CGAGCTCATCAAATGGCTTG		
ß-Tubulin	5'-GCCATGTTCAGGAGGAAGG-3'	71	Desmond et al.2006
ß-Tubulin	5'-CTCGGTGAACTCCATCTCGT-3'		

amplicon are listed in Table 6.1. Sequence of different primers was adopted from literature as described in Table 6.1. The thermal profile used for all qPCR reactions were: 95°C for 10 min, 40 cycles of 95°C for 15 s and 58°C for 25 s. No template control, negative control (bacterial genomic DNA) and positive control (plant genomic DNA) were used in every plate reaction to ensure purity of reagents and minimal contamination carryover, primer specificity, and primer binding capability respectively. No reverse transcription control (no RT) was also included in the PCR runs to ensure the negligible possibility of genomic DNA contamination. Data was analyzed using iQ^{TM}5 optical system software, version 2.1 (BIO-RAD, USA) and Microsoft Excel. Normalization of Ct values for all genes was done using wheat ß-tubulin gene to eliminate the possibilities of variation between PCR runs or different cDNA samples. For relative quantification of PR gene expression, double-delta Ct method was used. Control samples were always run on the same plate as their respective treated samples.

6.2.8. Estimation of bacterial colonization in plants

In a separate set of experiments with similar experimental set-up used for enzyme assays described above (section 6.2.3), colonization of endophytic and pathogenic bacteria, and their establishment in plants were evaluated by re-isolation of inoculated bacteria at various time intervals. Colonization of test bacteria vis-à-vis defense response of plants was estimated by re-isolation of inoculated bacterial isolates, confirmation of its identity by ERIC-PCR and quantitative analysis of their colonization using qPCR.

6.2.8.1. Isolation of inoculated bacteria

Isolation of colonized bacteria was carried out from surface sterilized plant samples collected at above-mentioned time interval employing method described in earlier section 2.2.4. Efficiency of colonization was measured in terms of cfu (colony-forming units) count. Identity of bacterial inoculants was confirmed by ERIC PCR (section 2.2.6) using pure culture as standard.

6.2.8.2. Quantitative estimation of bacterial colonization by qPCR

An objective for performing qPCR of rRNA gene of *P. aeruginosa* PM389 was to understand the relative changes in endophytic population during initial plant-endophyte

interaction. Total RNA sample from inoculated plant samples was used for cDNA synthesis and amplification of rRNA gene specific to bacterial inoculants. Protocol for synthesis of cDNA was identical as mentioned above with minor variation. Total cDNA was synthesized using random hexamer (0.2 µg) as per the protocol of first strand cDNA synthesis provided by the manufacturer (Thermoscientific, USA). Reaction PCR mix used for qPCR was similar to that described for qPCR of defense-gene amplification. Designing of primers and their specificity has been described in section 3.2.9. The rRNA gene was amplified using thermal profile as follows: 95°C for 5 min, 40 cycles of 95°C for 1 min and 58°C for 1 min.

6.2.9. Statistical analysis

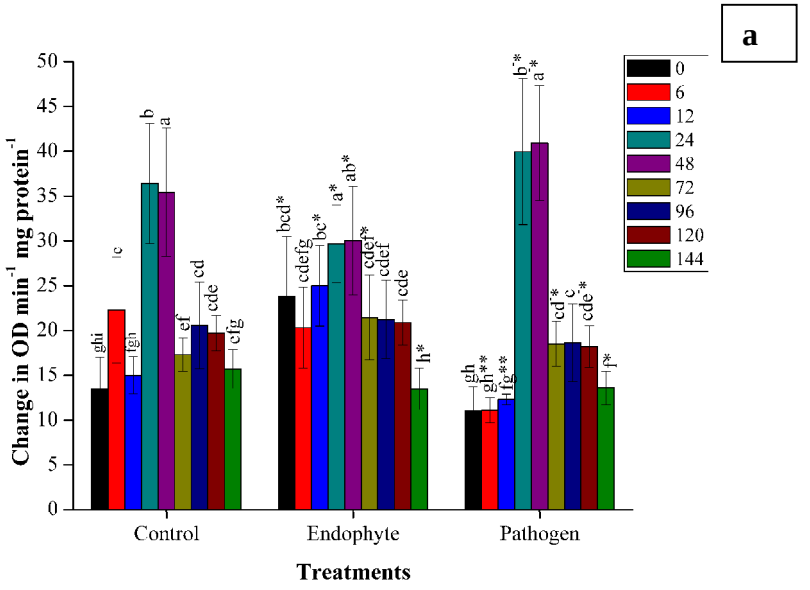
Computation of One-way ANOVA and standard deviation was done using Microsoft excel, wherever applicable. Least Significant Difference (LSD) (Quinn and Keough 2002) was done for identifying the significant difference in plant defense response towards endophytic and pathogenic bacteria. Duncan's Multiple Range Test (DMRT) was used to compare mean at different time intervals of the study period in various experiments.

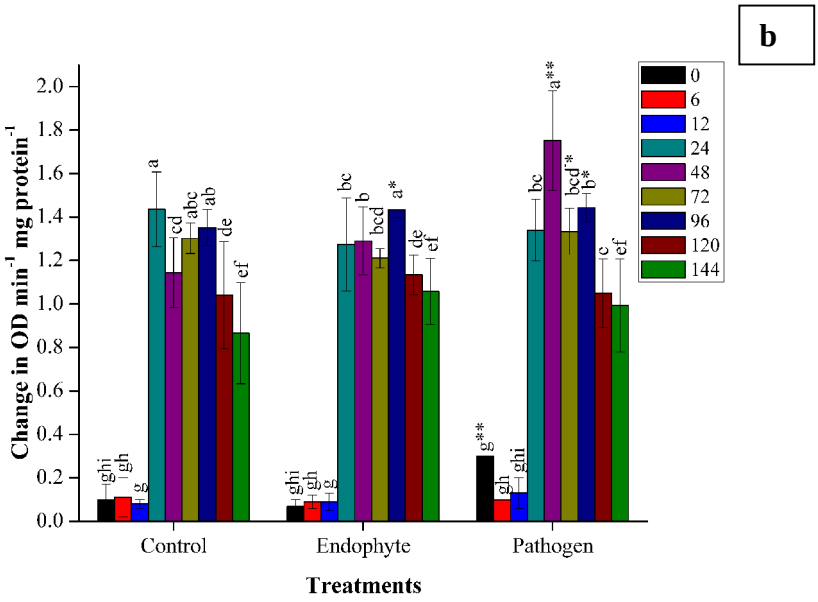
6.3. Results

6.3.1. Comparative analysis of plant defense enzymes in response to endophytic and phytopathogenic bacteria

In order to understand possible mechanism for successful establishment of endophytic bacteria in crop plants, we compared activities of defense enzymes in plants inoculated separately with endophytic bacteria *P. aeruginosa* PM389 and pathogenic bacteria *E. carotovora*. Inoculation of both types of bacteria led to significant elevation in activity of all defense enzymes at 24 HAI (Hours after inoculation) with few exceptions (Fig. 6.1. a-d).

Peroxidase activity (PO): In peroxidase assay, plants treated with *P. aeruginosa* PM389 showed significantly higher peroxidase activity (n=12, $p<0.05$) even at 0 HAI than pathogen-treated and control plants. However, high activity of PO (n=12, $p<0.05$) was noted between 24 and 48 h followed by reduction in enzyme activity in all plant samples, including control plants. In pathogen-treated plants, detectable change in enzyme activity was appeared at 12 HAI. Pathogen


a
Change in OD min-1 mg protein-1
Control
Endophyte
Pathogen
Treatments
0
6
12
24
48
72
96
120
144


b
Change in OD min-1 mg protein-1
Control
Endophyte
Pathogen
Treatments
0
6
12
24
48
72
96
120
144

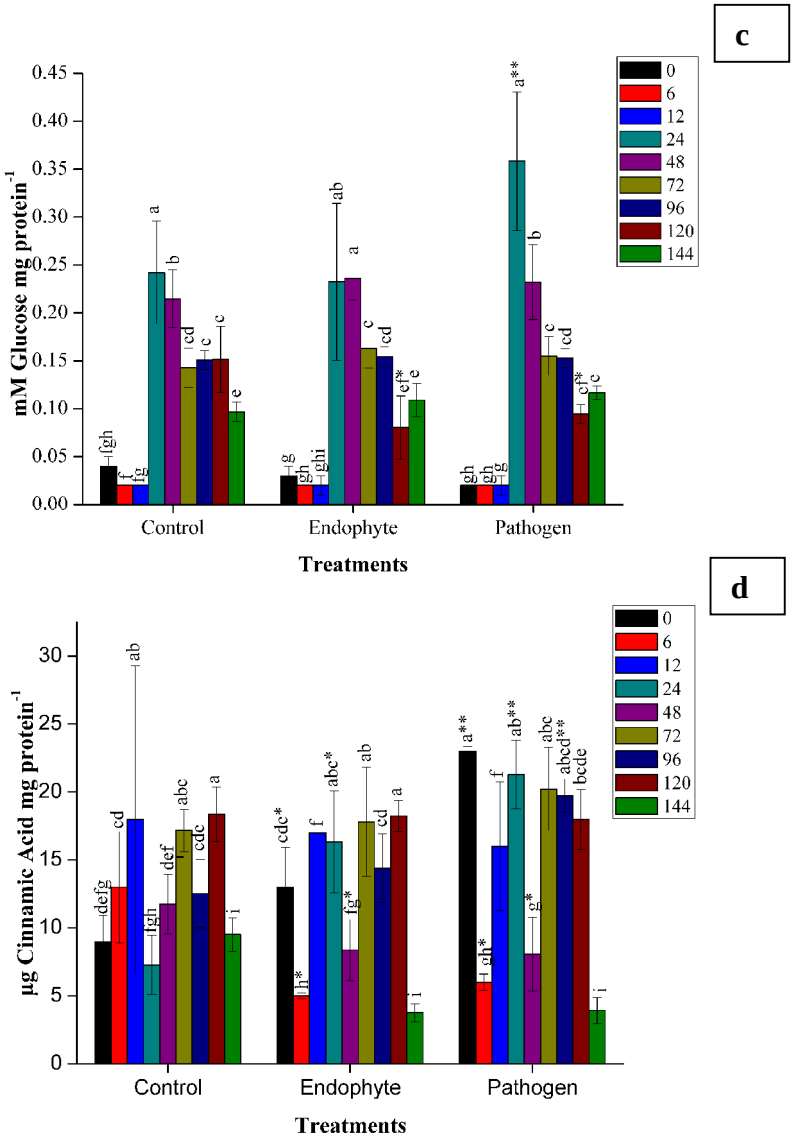


Fig. 6.1. Changes in activity of different defense enzyme in wheat (*Triticum aestivum* var. GW322) plants at various time interval in response to endophytic bacteria *P. aeruginosa* PM389 and phytopathogenic bacteria *Erwinia carotovora* (**a**) Plant peroxidase, (**b**) Polyphenol oxidase (**c**) β-1,3-glucanase, and (**d**) Phenyl-ammonia lyase. Plants treated with phosphate buffer saline was used as control (PBS) treatment. Each value represents mean of four biological and three technical replicates (12 replicates). Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters in each column of treatment.* represents significant difference in the respective column from column. ** represents significant difference from both control and endophyte in pathogen. *-/-* represents significant difference in pathogen from control/endophyte only.

treatment resulted in the highest change in activity of PO enzyme, while repressed enzyme activity was recorded in endophytic bacteria (*P. aeruginosa* PM389) treated plant in comparison to control treatment (Fig. 6.1. a).

Polyphenol oxidase (PPO): PPO activity was highly influenced in pathogenic bacteria treated plants. Maximum activity of PPO was observed at 48 HAI. There was significant (n=12, $p<0.05$) increase with 26% higher PPO activity in pathogenic bacteria-treated plants than endophytic bacteria-treated and un-inoculated control plants (Fig. 6.1. b). However, it followed a sharp decline in activity of PPO in all the treatments. On the contrary, no significant deviation in PPO activity in control plants was observed from the plants which were treated with endophytic bacteria. At 120 HAI, PPO activity was lower in bacteria-treated plants than control plants (Fig. 6.1. b).

β-glucanase: Starting from 24 HAI, activity of β-glucanase in all plants remained higher in samples collected at various time intervals. Similar to results of PPO activity, plants treated with pathogenic bacteria showed enhanced activity of β-glucanase enzyme, while endophytic bacteria *P. aeruginosa* PM389 did not elicit much higher glucanase activity than the control (Fig. 6.1. c).

Phenyl ammonia lyase (PAL): Unlike other assays, no finite trend was observed in PAL assay. In all treatments including control plants, there were alternate increase and decrease in enzyme activity at different time points. At 48 HAI, PAL activity decreased drastically for all the treatments and further, activity level enhanced suddenly at 72 HAI. Similar to the results of other enzyme assays, pathogenic bacterial treatment led to the highest change in enzyme activity in host plants. In pathogen-treated plants, significantly higher activity (n=12, $p<0.05$) was recorded at 0 HAI. At 6 HAI, activity was drastically reduced in both endophyte and pathogenic treatment and followed an increase in activity until 24 HAI. At 24 HAI, significant difference has been observed in pathogenic and endophytic treatment as shown in Fig. 6.1. d. On the other hand, *P. aeruginosa* PM389 stimulated insignificantly lesser and constant immune response than *E. carotovora* from 12-24 HAI (Fig. 6.1. d).

6.3.2. Tracking of endophytic and pathogenic colonization in wheat plants

In order to estimate and compare colonization efficiency of endophyte (*P. aeruginosa* PM389) and pathogen (*E. carotovora*), re-isolation of inoculated bacteria was done at various time intervals as mentioned in previous experiment. Efficiency was assessed on the basis of cfu count of each bacteria inoculated separately. Identity of isolated bacteria was confirmed on the basis of ERIC-PCR typing. In ERIC-PCR profiles, selected re-isolated bacterial colonies of endophyte (Fig. 6.2. a) and pathogen (Fig. 6.2. b) showed similar band patterns to that of original cultures used in this study. To elucidate the colonization pattern of test isolates, population dynamics study was conducted. In re-isolation analysis, recovery of endophytic bacterial colonies started from 24 HAI and remained constant in the further growth period. Population of endophytic bacteria was observed to be stabilized with slight variations. Maximum population was achieved at 144 HAI as depicted in Fig. 6.3. a.

Establishment of pathogenic bacteria in plants was not found to be consistent during the study period. Unlike endophytic bacteria, recovery of pathogenic bacteria was obtained as early as 6 HAI with a sudden incline in population. It followed a steep decline in population at 12 HAI. At 24 HAI, *Erwinia* get drastically increased. After 48 HAI, pathogenic population started gradually increasing and further steep decline in population was noticed at 96 HAI. In subsequent sampling time point, pathogenic population started increasing slowly (Fig. 6.3. b). Thus, population of pathogenic bacteria showed increase and decrease alternatively during colonization.

6.3.3. Analysis of PR gene expression on the endophytic challenge

Changes in PR gene expression profile on the endophytic challenge (*P. aeruginosa* PM389) were studied to understand and quantify the plant responses towards endophytic bacteria in the initial stages of colonization inside the plant. Specificity of the pair of primers was confirmed with the appearance of single amplicon of expected size on agarose gel. Change in pattern of gene expression in response to endophytic challenge appeared as early as 0 HAI.

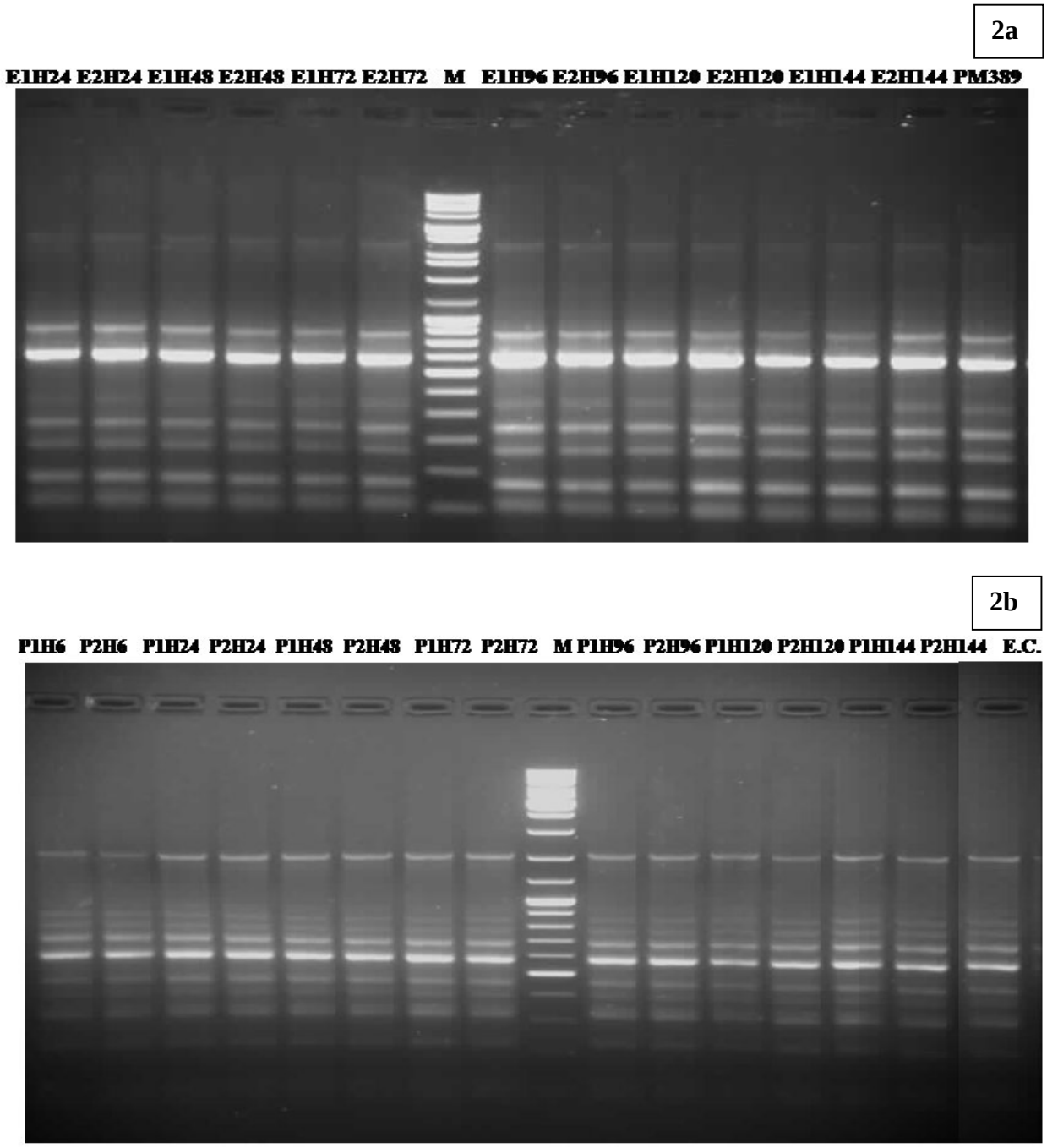


Fig. 6.2. ERIC-PCR profile of (**a**) endophyte (*P. aeruginosa* PM389) and (**b**) pathogen (*Erwinia carotovora*-E.C.) isolated from *Triticum aestivum* (var. GW322) at selected time points (0-144 HAI) on challenge inoculation. Letter of lane name refers to: E/P-Endophyte/Pathogen;. 1 and 2 refer to replicates number 1 and 2; H-Hour; Number of hours after infection by Endophyte/Pathogen. Each lane represents ERIC profile of isolates showing identical banding pattern at given sampling time. Lanes: M- Molecular weight marker, 100-bp ladder from MBI Fermentas (USA).

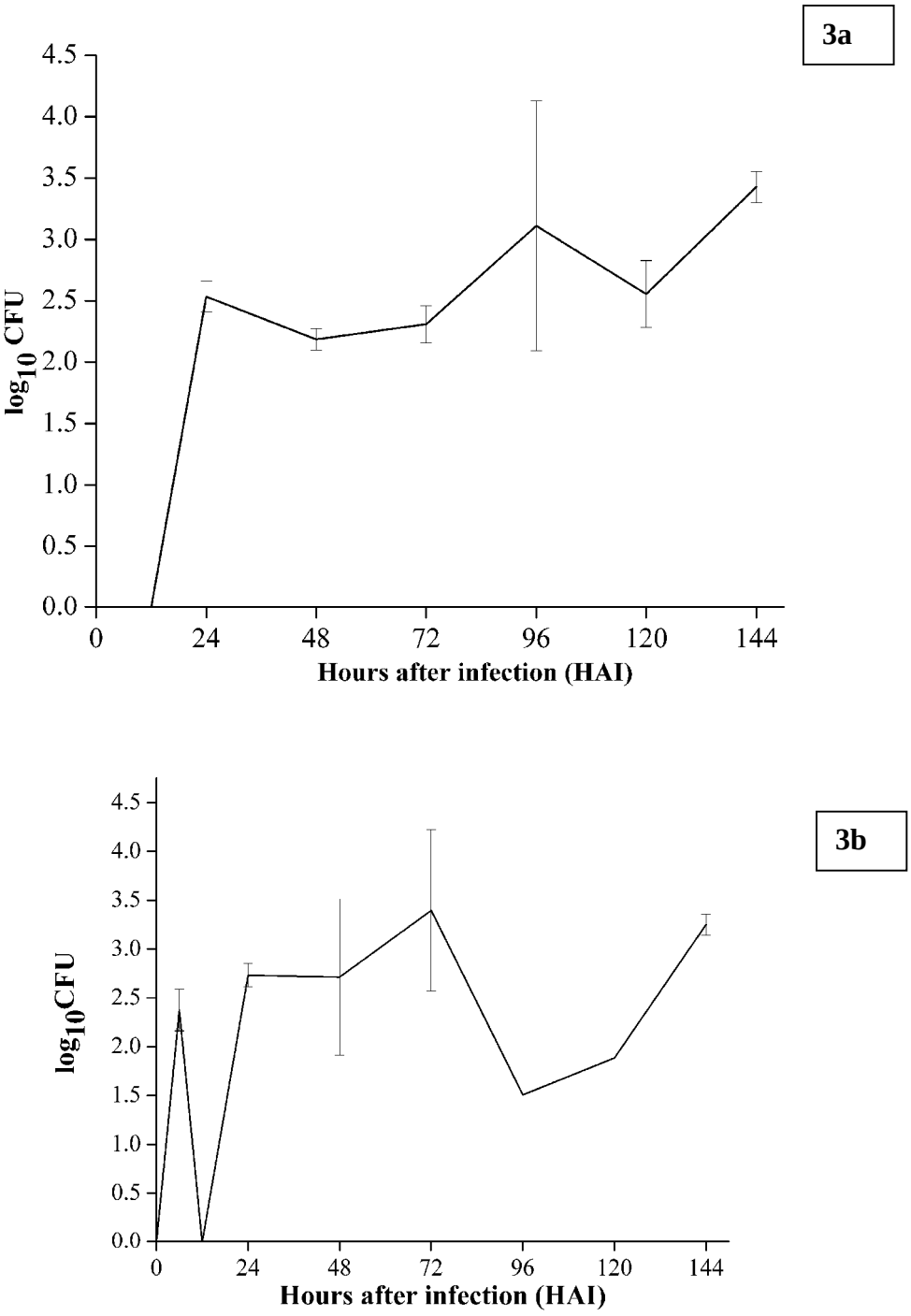


Fig. 6.3. Population dynamics of (a) endophyte (*P. aeruginosa* PM389) and (b) pathogen (*Erwinia carotovora*) on challenge inoculation in *Triticum aestivum* (var. GW322) at selected time points 0-144 HAI. Each value represents mean of duplicates and experiment was repeated two times (4 replicates). Vertical bars represent standard deviation.

Majorities of PR genes namely PR2, PR3, PR4, PR5, PR9 and PDI (Protein disulfide isomerase) showed higher level of expression than the control treatment, though the level of induction of gene varied with genes with the lowest for PR3. Induction of PR3 was noted to be marginal (Fig. 6.4). Expression of PDI was the highest with 66 fold up-regulation. On the other hand, other genes such as PR10, PR12 and PR16 were downregulated at 0 HAI in endophyte-treated plants.

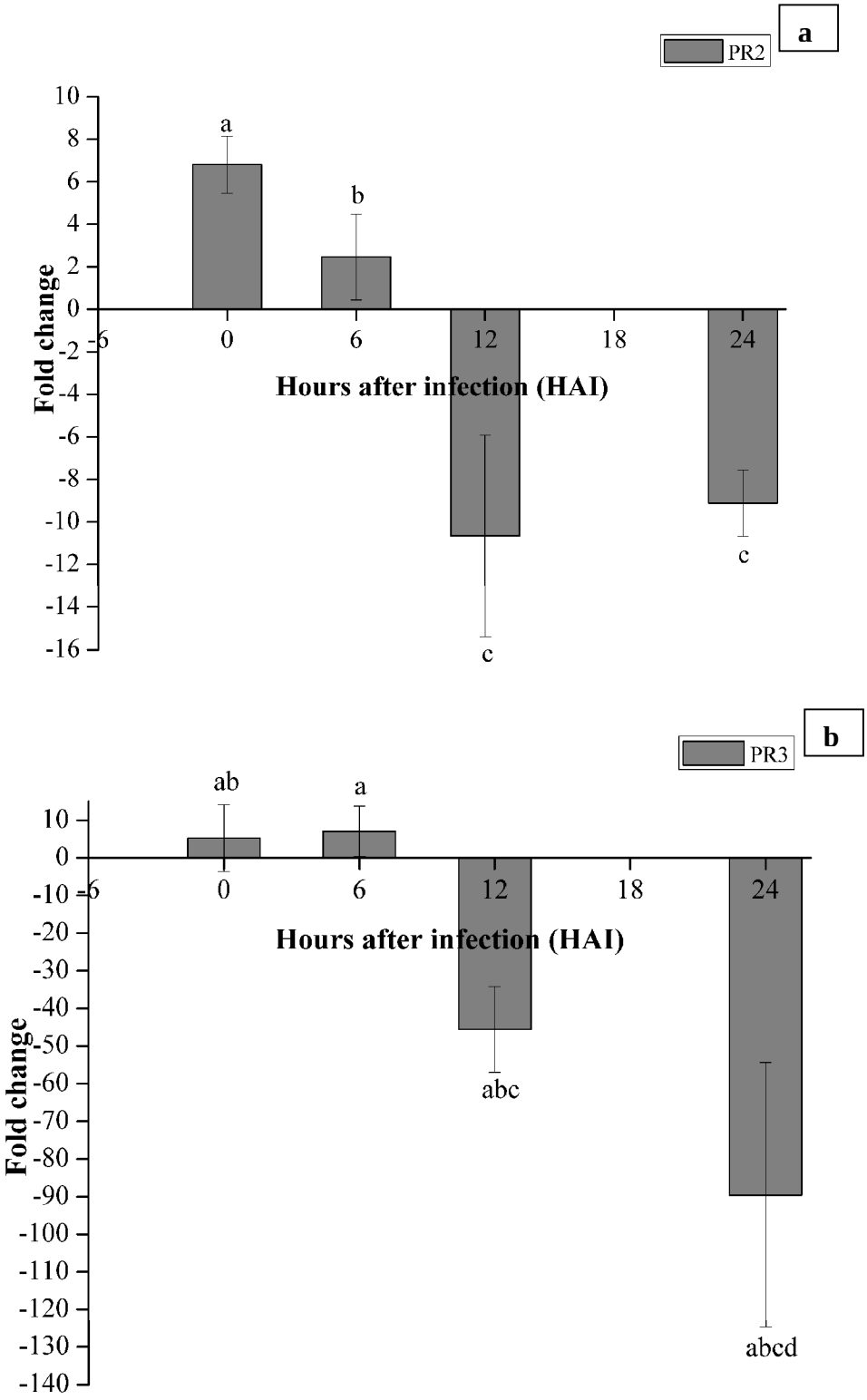
PR10 was observed to be the most down-regulated gene whose expression was 53 fold lower than the control plants at 0 HAI. However, gradual reduction of level of expression for most genes, including PR2, PR3, PR4, PR5 and PDI was observed in plant samples collected at following time intervals (from 6 HAI). Expression of PR9 and PR16 was observed to be upregulated and downregulated alternatively at the subsequent time intervals of sampling. Level of PR16 transcript, which was downregulated (~4 fold) at 0 HAI, increased 2.67 fold relative to control at 6 HAI. Recovery of expression level of PR10, though with marginal induction in comparison to that of control treatment at 6 and 12 HAI, was noted. At 12 HAI, most of the PR genes except PR9 and PR10, were downregulated by several folds. Interestingly, all PR genes except PR16 were found to be downregulated at 24 HAI, while the level of PR16 was 5.28 fold higher than the control.

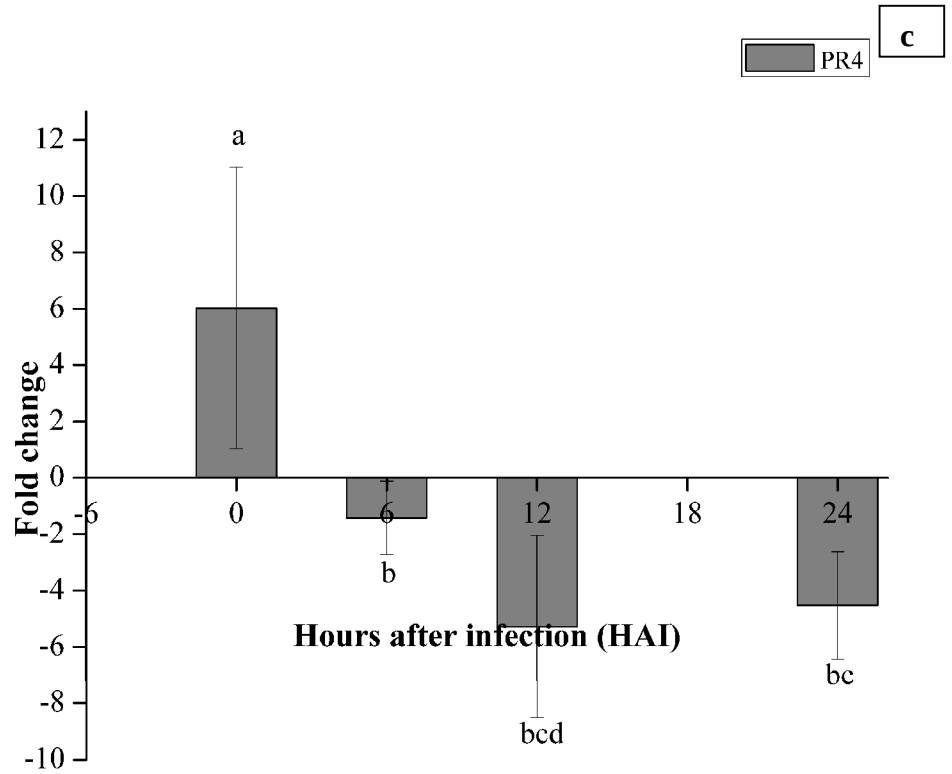
At 0 HAI, 7-fold induction (n=12, $p<0.05$) was observed in PR2 (ß-glucanase) gene, which reduced to 3-fold (n=12, $p<0.05$) in subsequent time point and finally followed approximately 10-fold (n=12, $p<0.05$) down-regulation at 12-24 HAI. Expression of PR3 gene was induced slightly at 6 HAI, though get highly downregulated (45-fold) at 12 HAI and at 24 HAI (90-fold). Similarly, after initial induction (five-fold), at 0 HAI, expression of PR4 gene decreased significantly (1-5 fold; n=12, $p<0.05$) at subsequent time points. Another gene PDI, which showed as high as 66-fold up-regulation at 0 HAI, was also down-regulated (approx.2-fold) at 24 HAI. Similar pattern was observed for PR5 which was upregulated (32 fold; n=12, $p<0.05$) initially and followed sharp downregulation (18 fold; n=12, $p<0.05$). PR9 and PR16 activity kept on fluctuating at different time points as shown in Fig. 6.4. For PR10 and PR12, 53

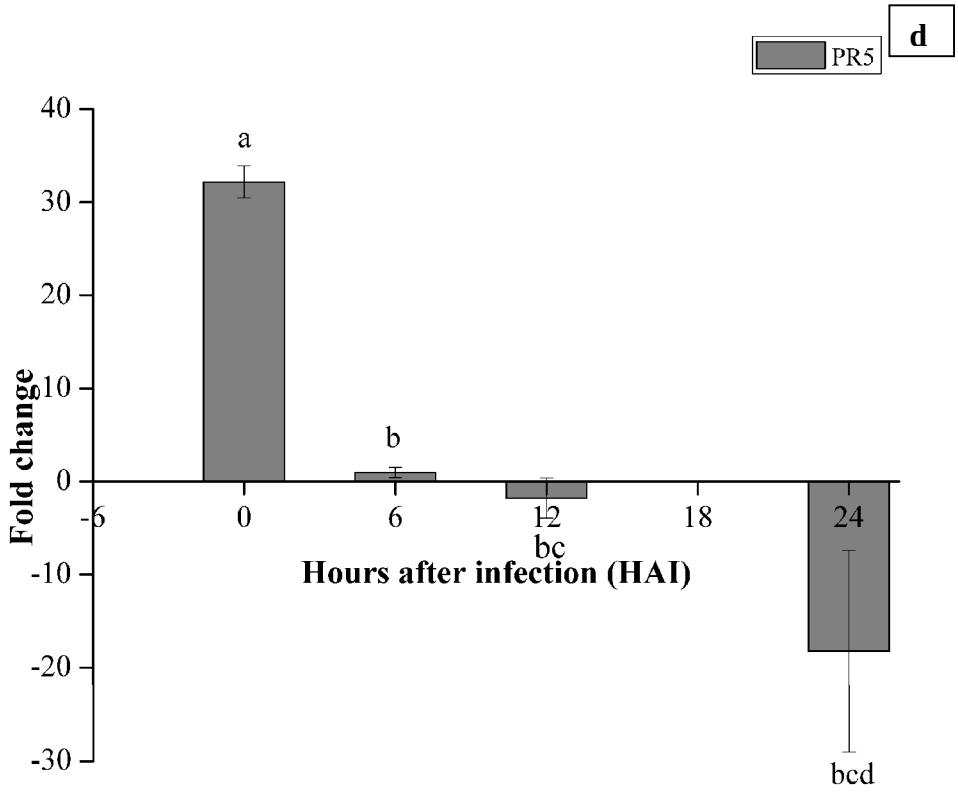
and 25 fold downregulation was observed at 0 and 24 HAI respectively, otherwise slight changes were observed at other time points.

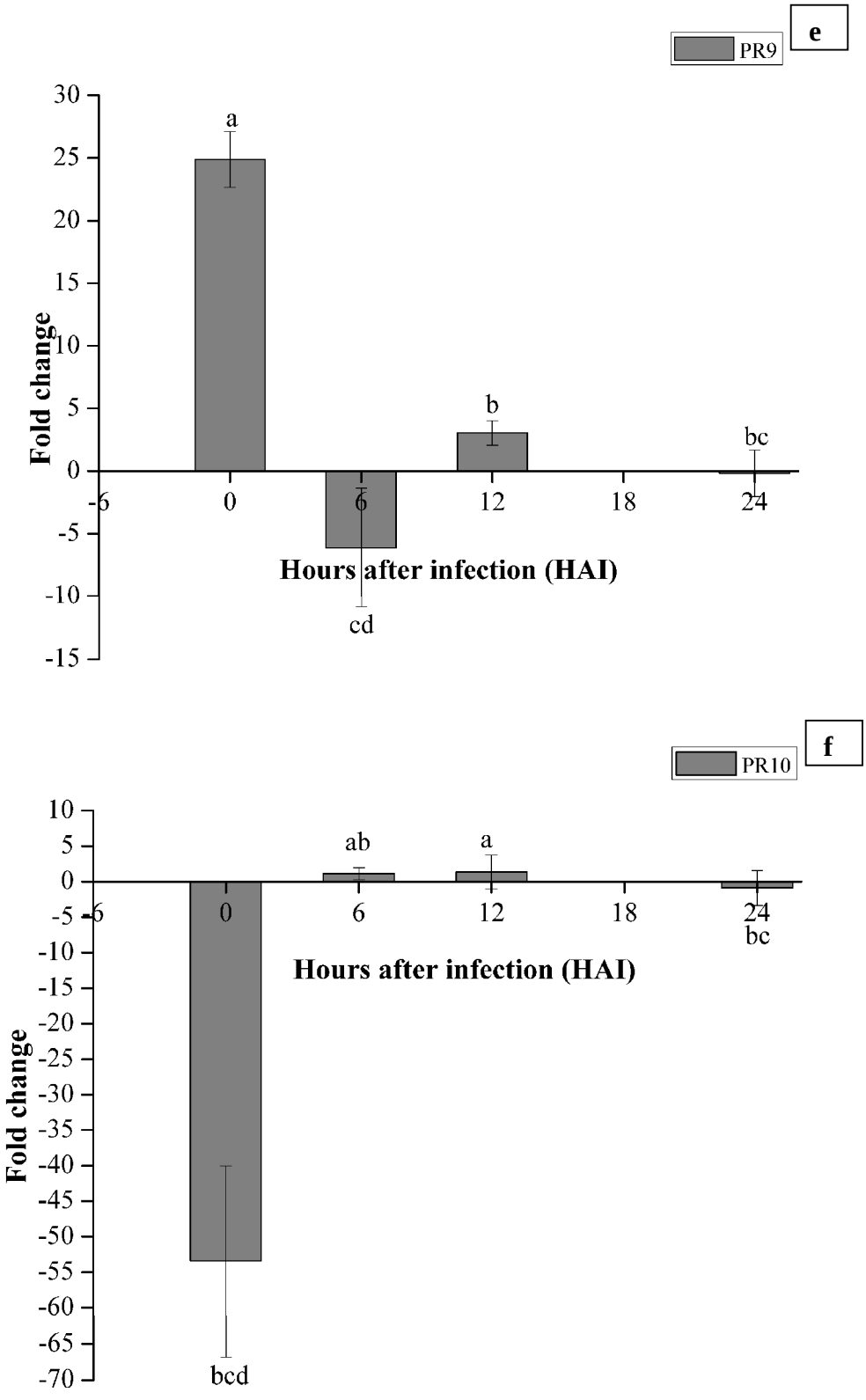
6.3.4. Colonization studies

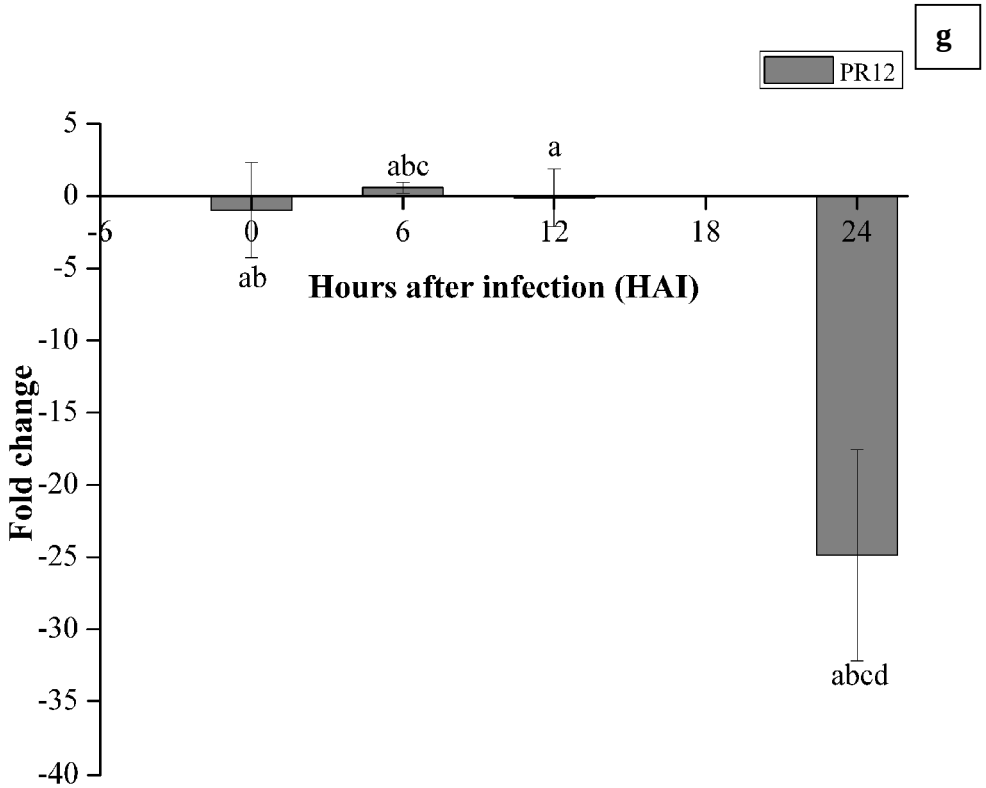
The qPCR analysis was done to quantify the extent of bacterial colonization in wheat plant. It also demonstrated functionality of colonizing bacteria. The cDNA was synthesized and amplified using the pair of primers of 16S rDNA specific to *P. aeruginosa* PM389. Based on the level of 16S rRNA gene transcript, no certain trend of colonization was observed. At 6 HAI, significant increase (n=12, $p<0.05$) of the transcript level of *P. aeruginosa* PM389 with 32 fold increase from 0 HAI treatment (Fig. 6.5). However, it observed 12 and 14 fold increase in the level of bacterial transcript from plant samples collected at 12 and 24 h. In brief, level of bacterial colonization was initially higher and then reduced to attain a stabilized population.

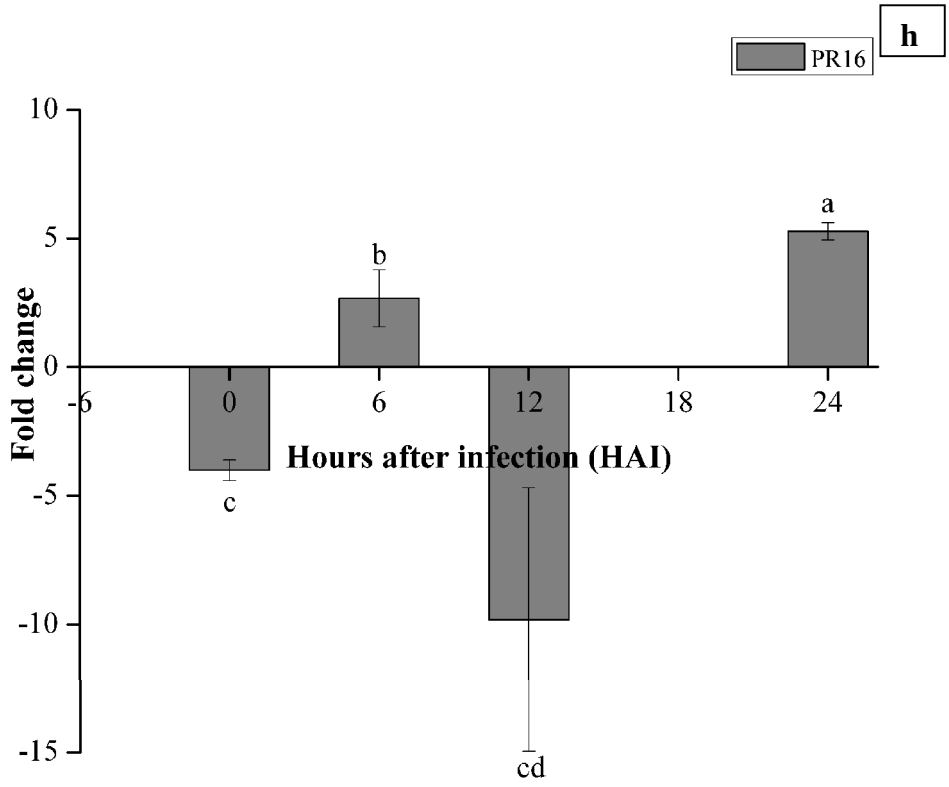

a
PR2
Fold change
Hours after infection (HAI)
a
b
c
c
b
PR3
Fold change
Hours after infection (HAI)
ab
a
abc
abcd


c
PR4
Fold change
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-6
0
6
12
18
24
a
b
bcd
bc
Hours after infection (HAI)


d
PR5
Fold change
40
30
20
10
0
-10
-20
-30
-6
0
6
12
18
24
a
b
bc
bcd
Hours after infection (HAI)


e
PR9
Fold change
Hours after infection (HAI)
a
b
bc
cd
f
PR10
ab
a
bc
bcd


g
PR12
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-6
0
6
12
18
24
abc
a
ab
abcd
Hours after infection (HAI)
Fold change


h
PR16
10
5
0
-5
-10
-15
-6
0
6
12
18
24
b
a
c
cd
Hours after infection (HAI)
Fold change

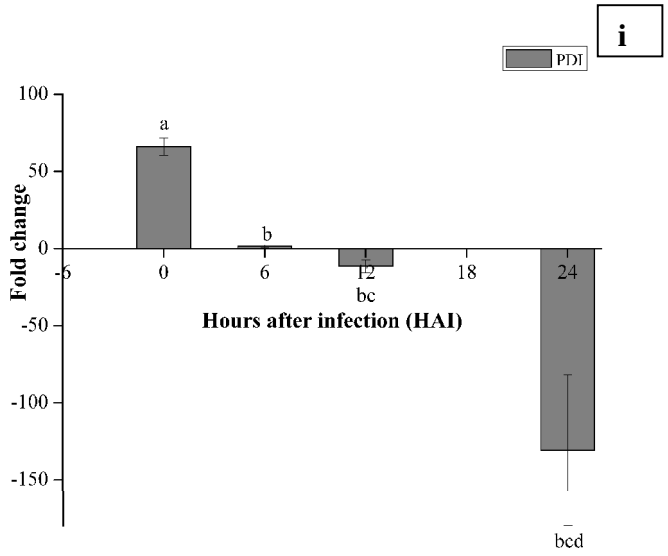


Fig. 6.4. Temporal changes in PR gene (**a.** PR2, **b.** PR3, **c.** PR4, **d.** PR5, **e.** PR9, **f.** PR10, **g.** PR12, **h.** PR16, **i.** PDI) expression of *Triticum aestivum* (var. GW322) on endophytic (PM389) challenge from 0-24 HAI. Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters in each bar of treatment.

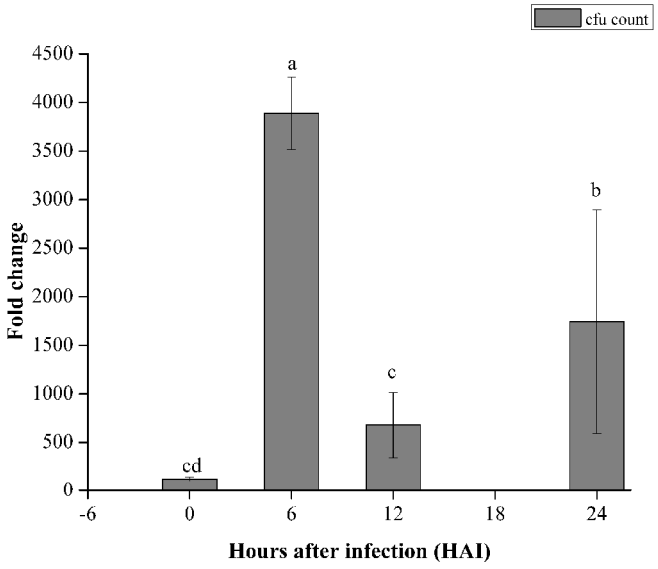


Fig. 6.5. Population dynamics of endophyte (*P. aeruginosa* PM389) on challenge inoculation in *Triticum aestivum* (var. GW322) at transcript level. Each value represents mean of duplicates and vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p<0.05$. Values that are significantly different from each other are headed by different letters in each bar of treatment.

6.4. Discussion

In order to elucidate possible mechanism of successful establishment of endophytic bacteria, we compared differences in plant immune response towards endophytic and pathogenic bacteria during the interaction. In the present study, above objective was addressed using endophytic bacteria *P. aeruginosa* PM389 (Gupta et al. 2012) and a pathogen *E. carotovora* in wheat plant. Present work highlights direct evidence for the success of endophytic colonization. Immune response was measured in terms of activity of four defense enzymes namely peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), β-1,3-glucanases as well as PR gene expression. To our knowledge, few work has demonstrated differential behavior of plants to beneficial and pathogenic bacteria (Bordiec etal. 2011). However, in a similar study, comparative studies of defense responses induced by endophytic bacteria *Burkholderia phytofirmans* strain PsJN and non-host bacterium *Pseudomonas syringae* in grapevine cell suspensions has recently been reported (Bordiec et al. 2011). Several PR proteins and their homologues including superoxide dismutase (SOD), peroxidase (PO), catalase (CAT) (oxidative enzymes) and polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), β-1,3-glucanases (PR-2 family) and chitinases (PR-3 family) (Beaudoin-Eagan and Thorpe 1985) are synthesized constitutively in plants, but their level increases in response to microbial invasion (van Loon et al. 1999).

Extracellularly released peroxidases are responsible for catalyzing the generation of reactive oxygen species (ROS) and other physiological processes such as lignifications, suberization, auxin catabolism, wound healing and defense responses against pathogenic attack (Kawano 2003). Upsurge in activity of peroxidase in plants following microbial challenge may lead to oxidative burst resulting from sudden changes in ion fluxes across the membrane (Chinchilla et al. 2007). Appearance of peroxidase activity in response to endophytic inoculation at 0 HAI suggests rapid recognition of endophytic bacteria and may have led to ROS generation further. Similar to our observation, increased ROS production (catalyzed by peroxidase) in response to microbial infection was noted as early as two min in earlier studies (Chinchilla et al. 2007). However, peroxidase activity remained decreased later in response to *P. aeruginosa*

PM389 throughout the sampling period, which indicates that endophytic bacteria attenuate the levels of peroxidase activity. Reduced activity of peroxidase on inoculation of endophytic bacteria in host plants was also noticed by Naffaa et al. (1999). It suggested that the reduction in peroxidase level may be a possible mechanism to facilitate endophytic colonization of bacteria. On the other hand, significant stimulation of peroxidase activity was noticed from 24-48 HAI in pathogen treated plant, suggesting that host plant was showing cell fortification/lignification for inhibiting the pathogen entry from 24 HAI. This activity get further reduced after certain time as ROS (reactive oxygen species) induction and cell wall strengthening activities comes under first line of plant defense (GÓMez-VÁSquez et al. 2004).

Unlike results of peroxidase activity, induction of glucanase was not observed to be rapid in either treatment. *Erwinia* showed significant induction of β-glucanase activity at 24 HAI, while no significant change was observed for above enzyme in plants inoculated with *P. aeruginosa* PM389. On the contrary, slightly repressed activity was observed in response to *P. aeruginosa* PM389 in comparison to control treatment. After 24 HAI, β-glucanase expression turned down for all the treatments. Decrease in activity in the further sampling period may rely upon the fact that the β- glucanase gets intensified further only in the presence of glucan oligomers released during the lysis of the fungal cell wall. Since in the present study, both treatments were bacterial, inoculated plants did not show further induction of β-glucanase activity due to the absence of elicitors (Fridlender et al. 1993).

Similar to the results of β- glucanase activity, significant induction of PPO was observed in only pathogen treated plants at 48 HAI. It suggests that the plants are also responsible for differentiating endophyte and pathogen during infection and help to the establishment of endophytic bacteria inside the plants. Recently, researchers reported two waves of transcriptional reprogramming in *M. truncatula* involving repression of defense-related genes followed by the activation of a nodule-specific transcriptome (Maunoury et al. 2010). Several collections of plant and bacterial mutants were used in the study, which established the fact that the transcriptome switch is dependent upon a molecular dialogue between both partners.

Fourth enzyme whose activity was assayed during plant-microbe interaction was phenyl ammonia lyase (PAL). PAL is a key enzyme of phenylpropanoid pathway and flavanoid pathway, playing the role in lignin biosynthesis, phytoalexins, other phenolic compounds that have direct effects on pathogens (Zdor and Anderson 1992). PAL activity was higher for pathogen at 0 HAI suggesting the quick recognition by plant's phenylpropanoid pathway. In the entire study period, a decrement was observed after a sudden increase in PAL activity in all treatments at the subsequent time point except 72-120 HAI. There could be two possibilities for it; one suggests that level of PAL is inhibited by pathogen, and another that it is regulated at the transcriptional or post-translational level by anaerobic condition and metabolites (trans-cinnamate) of phenylpropanoid pathways respectively (Shirsat and Nair 1986). Further, in endophyte treated plants, reduced PAL activity was recorded, again supporting the notion that plants generate weak immune response against endophytes or beneficial bacteria (Liu et al. 2007).

Overall we observed suppressed activity of defense enzymes in endophytic bacteria treated plants. In earlier studies, it has been reported that various MAMPs such as EPS, and nod-factors of beneficial bacteria are capable of suppressing immune response of plant to gain entry inside the plant (Zamioudis and Pieterse 2011). There are certain examples of beneficial bacteria, which show that roots of *Lotus japonicus*, although well able to respond to flg22, do not respond to purified flagellin from *Mesorhizobium loti*, suggesting that LjFLS2 is unable to sense flagellin molecules from the symbiotic partner (Lopez-Gomez et al. 2012). Similar to these tactics described above of suppressing immune response of host plant and gaining entry inside the plant, *P. aeruginosa* PM389 might have certain strategies to downregulate the immune response as observed in *B. phytofirmans* (Bordiec et al. 2011). It needs to be investigated in detail in future studies.

In order to verify results of biochemical assays, quantitative gene expression analysis was conducted by measuring gene expression of nine PR genes, which play the important role in plant immune response. Regulation of these genes might have influential roles in establishment of bacterial colonization of endophytic bacteria. Very few studies have been done on plant

immune responses during initial plant-endophytic bacterial interaction at the molecular level (Bordiec et al. 2011). In present study, expression of the majority of PR genes was up-regulated initially (6 HAI), although they were downregulated further from 12 HAI. Similar finding was reported in the recent study conducted on expression of defense proteins during interaction between *Medicago truncatula* and *Sinorhizobium meliloti* (De-La-Pena et al. 2008). It reported release of various defense proteins such as glucanses, chitinases, PR5, Peroxidase, PR10 in abundance until 6 HAI, which started decreasing from 12 HAI, with few exceptions. In our study, similar observation was noted for glucanases (PR2), chitinases (PR3, PR4), PR5, Peroxidase (PR9) and PR10 (ribonuclease) at the transcript level, which showed downregulation or reduction from 12 HAI except for PR10. Expression of defense genes has been studied in few other host-beneficial microbe interactions. Similar to our observation, microarray study of *Arabidopsis-Trichoderma harzianum* T34 (biocontrol agent) revealed that most of the genes mediated by SA and JA (SAR marker genes) were downregulated at 24 hours after infection, although some JA responsive genes such as *PDF1.2* (Protein disulphide isomerase 1.2)*, ETR1* (Ethylene resistant)*, LOX1* (Lipoxygenase) was not affected (Moran-Diez et al. 2012). Consistent with above-mentioned examples, suppression of SA-mediated gene expression has also been reported in *Rhizobium*-legume (Stacey et al. 2006) and mycorrhiza-plant (Pozo and Azcon-Aguilar 2007) interactions. In another study, *Bradyrhizobium* also showed downregulation of defense responses for successful nitrogen-fixation symbiosis (Brechenmachar et al. 1985). This is further supported by proteomic studies in which suppression of Prx (Peroxidase), Mn-superoxide dismutase, PR10 and stress-induced protein were significantly downregulated during nodule emergence from the root during legume-rhizobium association (Lim et al. 2010).

Trend for gene expression of peroxidase gene (PR9) was similar to glucanase (PR2) both at transcript and protein level in response to endophytic bacterial inoculation. It infers important role of modulation of level of peroxidase in interaction between plant and endophyte *P. aeruginosa* PM389. Suppression of peroxidase during colonization of *P. aeruginosa* PM389 is supported by reports, which state that decline in peroxidase (PR9) activity is important to gain entry inside the plants (Naffa et al. 1999; Den Herder et al. 2007). On the otherhand, inoculation of endophytic bacteria did induce expression of PR2 (ß-glucanase) though its level fallen down

in subsequent time period. This finding supports the result of an enzyme assay for β-glucanase explained in section 6.3.1. However, it differed from results of enzyme assays where no changes were noticed until 24 HAI. This result is in agreement with the studies conducted on *Sinorhizobium meliloti* which showed downregulation of β-glucanase (*MtBGLU1)* gene in the roots of *Medicago truncatula* after 24 h of inoculation (Mitra and Long 2004). It indicates involvement of the host or bacteria mediated factor, latter of which modulates expression of PR2 genes. Other genes that were downregulated include PR3 and PR4. PR3 and PR4 belong to several classes of chitinases and thus protect from fungal pathogens and insects. However, these proteins are protective against bacterial invasion as well. PR3 also possesses lysozyme activity and is indirectly antimicrobial, as some of the PR2 and PR3 for instance, release elicitor-active oligosaccharides and peroxidases by catalyzing cross-linking of macromolecules in the cell wall (Stintizi et al. 1993). Our observation differ from previous studies where invading *Rhizobium meliloti* was protected from chitinases of *Medicago* and *Vicia* through structural modification in nod factors. In our studies, beneficial bacteria (*P. aeruginosa* PM389) suppressed expression of chitinases (Staehelin et al. 1994). However, we have not identified mechanism of suppression by these bacteria.

In one of the previous studies, proteomic study has been carried out to elucidate immune response for compatible interaction between *Medicago truncatula* and *Sinorhizobium meliloti* during initial interaction (De-La-Pena et al. 2008). In this study, it was observed that various defense proteins such as glucanases, chitinases, PR5, Peroxidase, PR10 were released in abundance till 6 HAI in compatible interaction, which started further reducing from 12 HAI with very few exceptions. In an incompatible interaction (*Medicago truncatula-Pseudomonas syringae*), lesser type of proteins were released during the interaction. Based on the data of protein profiling obtained in response to plant-bacteria interaction, it suggested that higher number of proteins are required for initial interaction in compatible interaction (between *Medicago truncatula* and *Sinorhizobium meliloti*) than that of incompatible interaction. Especially for peroxidase it was suggested that it acts as symbiotic interaction protein, whose decline signals for the synthesis of nod factors by symbiotic bacteria (De-La-Pena et al. 2008). In our study, similar observation was noticed for glucanases (PR2), chitinases (PR3, PR4), PR5,

Peroxidase (PR9), PR10 (ribonuclease) at the transcript level, which showed downregulation or reduction from 12 HAI except for PR10.

Defensins (PR12/PDF1.2) are regulated by JA-ET pathway, and they play an important role in plant defense as the antimicrobial agent (Koornneef et al. 2008). Jacobs et al. (2011) reported that PGPR and PGPF are involved in interfering JA signaling for gaining entry inside the plant. Thus, downregulation of PR12 on colonization of *P. aeruginosa* PM389 do go along with above observations. Apart from this, Camehl and Olemuller (2010) has reported the role of ET signaling in balancing beneficial and non-beneficial traits of symbiosis in *Arabidopsis thaliana* and *Piriformospora indica*. It suggests that plants are also responsible for establishing symbiotic relationship by suppressing plant immune responses. In an another report, PDF1.2 (PR12) and PR 1 to 5 were not upregulated in *Arabidopsis* (wild type for Ethylene signaling)-*Piriformospora indica* interaction (upegulation in ethylene mutants), and ERFs (Ethylene response factors) 9 and 14 were found to be responsible for suppression of PR genes (PR1 and 2) (Camehl et al. 2010). Similarly, our results also supported above observations and showed downregulation of most of the PR genes during plant-endophytic bacteria interaction. This further suggests that it might be due to two reasons: one may be; *P. aeruginosa* PM389 is involved in interfering the JA pathway or other defense signaling cascades and; the host plant is involved in suppressing its own immune response for the establishment of *P. aeruginosa* PM389 inside the plant. Proteins strongly induced in *Oryzae sativa* roots in varieties IR36 and IR42 by JA were identified as Bowman-Birk trypsin inhibitors, a GLP (Germin and Germin-like proteins) (Accession 5852087), a putative endo-1,3-beta-D-glucosidase, a glutathione-S-transferase, a 1-propane-1-carboxylate oxidase synthase, peroxidase precursor, PR10-a, and a RAN protein (Miché et al. 2006)**.**

PR16 (oxalate-like oxidase) is involved in releasing microcidal concentration of peroxidases and eliciting hypersensitive cell death (Lane 2002). We observed downregulation of PR16 at 0 HAI on inoculation of *P. aeruginosa* PM389 and the transcript level kept on fluctuating throughout the experiment. While studying JA-mediated gene expression during *Azoarcus*-Rice interaction, Miché et al. (2006) suggested that the expression of JA-induced genes

restrict entry of endophytic bacteria and thus not apparently important in compatible interaction. Thus, downregulation of most of the JA responsive genes in our study suggest the possibility of compatible interaction between *P. aeruginosa* PM389 and wheat plant. On the contrary, on inoculation of a PGPR *Pseudomonas flourescens* to *Arabidopsis thaliana,* a wide range of defense response was generated during interaction. It demonstrated that JA plays major role during less compatible interaction (Wang et al. 2005).

Unlike other defense proteins, few proteins including PDI play the indirect role in immune response of plants. PDI acts as a chaperone and assist in forming intramolecular disulfide bridges in PR proteins for maintaining their conformation in harsh environments (Kitajima and Sato 1999). Additionally, it is also known to act in the endoplasmic reticulum (ER) quality control system for correct protein folding in *Piriformospora indica* (Anelli and Sitia 2008). Interruption in ER (endoplasmic reticulum)-quality control system leads to disturbance in MAMP-triggered immunity (MTI) responses and increase in disease susceptibility, thus enhanced colonization of invading microorganism (Saijo et al. 2009). Therefore, initial induction of the PDI expression in present study suggests that PDI help in correct PR protein generation which in turn prevents initial colonization. However, absence of PDI may lead to increase in the invasion of bacteria by altering MTI, due to interruption in ER (endoplasmic reticulum)-quality control system. In present study, PDI was also found to be down-regulated at 24 HAI after initial induction on the endophytic challenge. Absence of PDI interrupts ER (endoplasmic reticulum)-quality control system, which leads to disturbance in MAMP-triggered immunity (MTI) responses. Consequently, it enhances endophytic colonization (Saijo et al. 2009). Thus, altered expression of PDI might be one of the mechanisms of suppression of PR genes by endophytic bacteria to gain entry inside the plant.

In order to corroborate immune response to success of colonization in plants, re-isolation of *P. aeruginosa* PM389 and *E. carotovora* was done and their extent of colonization was measured in terms of cfu count. Based on the results of reisolation study, it appears that colonization of *P. aeruginosa* PM389 started late in comparison to *Erwinia,* where latter was recovered as early as 6 HAI. However, extent of colonization of *P. aeruginosa* PM389 was

found to be consistent throughout the study period with a slight intermittent decrease in cfu count. It again accounts for the similar phenomenon of *P. aeruginosa* PM389 to *Rhizobium*, in which stress and defense-related genes get upregulated on exposure in plants initially and follows their downregulation (Moreau et al. 2011). This phenomenon strengthens the fact that beneficial bacteria have evolved to establish in host plant through immuno-suppression of the host system. On the contrary, *Erwinia* was found to colonize earlier but a fluctuation in their population was observed at succeeding intervals. Consecutive increase and decrease of pathogenic population at the different time intervals clearly evince the constant fight of host plant to ward off pathogen and the latter further tries to pioneer in the plant by certain tactics. It shows a similar pattern to that of zig-zag model of immune response of plant against pathogenic establishment inside the plant (Jones and Dangl 2006).

In both pathogen and endophyte, decrement in population was recorded on an increase in either of the defense enzyme and vice-versa, with few exceptions. After diminished enzymatic activities, both endophytic and pathogenic population started increasing, suggesting the establishment of bacteria inside the host plant. The present study suggests that the immune response generated against the endophytes is similar to that of pathogen, though the intensity varies between the two. Other than that, endophytes display several mechanisms to combat plant immune response. Moreover, plant is also involved in helping endophytes to get established inside the plants. Although most of the supporting examples are for rhizobiaceae-leguminaceae (Maunoury et al. 2010) and plant-fungal endophyte (Camehl and Olemuller 2010), but being similar to that in PGPR effects, hosts of endophytic bacteria may also be utilizing similar mechanisms for endophytic establishment inside the plants. Thus, endophyte as well as host plants are responsible for successful establishment inside the plant by various molecular dialogues between plants and the microbe.

Colonization trend of endophytic bacteria was further studied by quantifying active endophytic *P. aeruginosa* PM389 in plants employing qPCR approach by measuring 16S rRNA transcript. Colonization studies of *P. aeruginosa* PM389 at the transcript level revealed that during initial interaction with host plant *P. aeruginosa* PM389 showed constant fluctuation in population count as observed at colony count level on plate. It suggested that during initial

interaction host plant detects endophyte as non-self and generates significantly higher immune response and after downregulation of immune response at 12 HAI, *P. aeruginosa* PM389 transcript level started increasing. In two recent reports about PGPR's, it was noticed that *Pseudomonas flourescens* WCS417r (Millet et al. 2010) and *Bacillus subtilis* FB17 are involved in suppressing early MAMP-triggered immune responses, which may further lead to successful establishment inside the host plant (Lakshmanan et al. 2012). As FB17 does not produce any COR (Coronatine-interferring JA signaling), nod factors (Rhizobiaceae family) or other factors to suppress immune response, authors further suggested the role of certain small diffusible protein molecules (in some cases amyloid fibres are reported) in immune suppression (Lakshmanan et al. 2012).

In the present study, endophytes were compared with the plant pathogen for induction of defense enzyme vis-à-vis establishment inside the host plant. It was observed that endophyte showed suppression of immune system of the host, which could be a possible mechanism for colonization inside the plant. Another possibility for suppressed immune response against endophyte might be due to weak immune response generated by host plants against endophytic bacteria, thus can differentiate between pathogen and endophytic bacteria. Therefore, a constant and positive molecular dialogue between host and endophytic bacteria might be involved for endophytic establishment, unlike pathogen establishment, which shows constant fight occurring between pathogen and host plant. Immune suppression was further observed on challenging plant with endophyte at the molecular level as well. Thus, this study is helpful to understand the strategy and mechanistic insight of endophytic bacteria during colonization inside the plant as well as the plant behavior during endophytic colonization.

SUMMARY & CONCLUSIONS

Endophytic bacteria are beneficial and gain entry into the plant roots from rhizosphere mainly through wounds, cracks and the points of lateral root emergence. Studies on changes in community structure of endophytic bacteria are crucial from agriculture point of view as endophytic communities are involved in various processes of plant growth enhancement and health increment. Thus, the better understanding of diazotrophic microbial community and its function in a given plant can be utilized to harness beneficial bacteria-plant association for enhancing growth and yield of the plant. Still there are very few PGPR isolates, which can perform in actual farming conditions. Therefore, a systematic approach is required for the screening of potential bioinoculants, so that PGPR isolate can respond well on scaling up from the lab to field level. Some of the endophytes can also serve as better biocontrol agent by competing with pathogens and due to other antagonistic properties. So, the screening of potential biocontrol agent and understanding the mechanism of antagonism can help in the development of future biocontrol agent. As endophytes have more potential than their rhizospheric competitors, better utilization of endophytes necessitates the proper understanding of various properties required for endophytic colonization. Albeit beneficial, endophytic bacteria do induce immune response in host plants. Thus, it is intriguing to know that how plants differentiate endophyte from their pathogenic counterpart and endophyte successfully gains entry inside the plant. Therefore, aims of the present study were to understand the community structure of diazotrophic endophytic bacteria (DEB) in pearl millet under field conditions, screening of potential biofertilizer and biocontrol agents, to elucidate the mechanisms of endophytic property, antagonistic property and successful association of the dominant diazotrophic endophytic bacteria with host plant. The major findings of the present research study are as follows:

1. Overall, 210 morphotypes were obtained in this study, out of which 76 different ERIC types were identified. Based on the data of representative isolates from each ERIC types, abundance and species richness of diazotrophic bacteria recovered at various growth stages were calculated. Diversity of DEB was found to be higher during early (upto 28 DAS) and late (63 DAS onwards) stages than the vegetative growth stages (28-56 DAS) of pearl millet plant. *Pseudomonas aeruginosa* was observed to be the most dominant endophyte during the majority of the growth period. Other than *P. aeruginosa*, abundance

of bacterial isolates belonging to other genera (*Pantoea* sp., *Ochrobactrum anthropi*, *Bacillus flexus*, *Ochrobactrum intermedium*) was also recorded at various growth stages of the plant. Plant growth promoting features such as phosphate solubilization, nitrogen fixation and siderophore production were present in most of the isolates. All endophytic bacteria tested for various PGPR properties were further tested for their ability to enhance plant growth. Certain endophytes capable of inducing plant growth can be selected for serving as potential biofertilizer and biocontrol agent in the future.

2. Various PGPR isolates were screened for consistent and better performance from the lab to field level. Also, the best six isolates were screened for the endophytic nature and cross-infection capability in wheat. Most of the isolates worked better in sterilized soil conditions than unsterilized soil. In microcosm studies conducted in unsterilized soil, some of the isolates showed synergistic and better effect on plant growth promotion as mixed inoculum than single inoculants. *Rhizobium* sp. PM10461 showed the best results in the field conditions for most of the growth parameters, followed by *Nocardioides* sp. PM9404 and *Arthrobacter woluwensis* PM9426. *Rhizobium* sp. PM10461 consistently performed well at all the levels of plant growth studies. Therefore, it can be concluded that *Rhizobium* sp. PM10461 and other two isolate (*Nocardioides* sp. PM9405 and *Arthrobacter woluwensis* PM9426) can serve as the potential biofertilizer candidate in future. All selected endophytic bacteria enhanced millet plant growth and were capable to cross-infect wheat plants as well. Present study suggests that various formulations in microcosm studies showing better plant growth promotion can be tested in future studies at the large scale in field condition, and efficient inoculants can be converted into commercial biofertilizer for wide usage and industrial production. These formulations can also be tested for their plant growth promoting abilities in other crops. Ability of selected bacterial isolates to cross-infect wheat plant was confirmed by PCR using species-specific primers for amplification of 16S rDNA. Present study can be useful in identifying the promising endophytic colonizer and manipulating plant-bacterial interaction for increasing crop yield of a wide array of crops. The systematic approach used in this study have provided conclusive and valuable results of the effect of PGPR treatments under variable circumstances, which can be further manipulated for efficient utilization of these consistent performers as commercial biofertilizer in the future.

3. *Pseudomonas aeruginosa* PM389 was observed to be the most dominant colonizer of pearl millet. In order to understand the mechanism of colonization, *Pseudomonas aeruginosa* PM389 was tested for presence of hydrolytic enzymes which may be required for active colonization process. Negative test for cellulolytic (endoglucanase, Cellobiohydrolase and ß-glucosidase**)** and pectinolytic enzymes suggest that *Pseudomonas aeruginosa* PM389 is a passive colonizer of plant. Several traits required for endophytic colonization like different kinds of motility, biofilm formation and EPS production were present in *Pseudomonas aeruginosa* PM389. For confirming endophytic colonization, sections of plant roots treated with *Pseudomonas aeruginosa* PM389 were stained with acridine orange, and bacterial cells were visualized using the fluorescent microscope. Thus, it suggested that endophyte *Pseudomonas aeruginosa* PM389 has several properties of endophytic colonization, which make it a potential colonizer.
4. One of the possible reasons for being *Pseudomonas aeruginosa* PM389 a dominant colonizer, was assumed to be antagonistic activity. *Pseudomonas aeruginosa* PM389 showed positive results for multiple mechanisms of antagonism like siderophore, lipase production, antifungal protein production, HCN production and showed strong antagonistic activity against important fungal pathogens (*Fusarium oxysporum, F. graminearum, F. pallidoroseum, and F. monaliforme*). In the present study, *Pseudomonas aeruginosa* PM389 was further checked for inducing systemic resistance in wheat against the fungal and bacterial pathogen. Highly induced defense response by endophyte pretreatment in plant on pathogenic challenge and reduction in pathogenic population count in its presence suggested the involvement of induced systemic resistance in plants against pathogens. Moreover, treatment with endophytic *Pseudomonas aeruginosa* PM389 resulted higher growth of wheat plants infected with pathogen (*E. carotovora*) than that of plants treated only with pathogen in both *in vitro* and in pot studies. Endophytic bacterial treatment was found to be more effective against fungal pathogen than the bacterial one in development of ISR. This investigation confirmed that *Pseudomonas aeruginosa* PM389 can be considered as a promising biocontrol and biofertilizer agent.
5. To understand compatible behaviour for endophytic bacteria, immune response of host plants to endophytic bacteria and pathogenic bacteria was compared by measuring level

of important defense enzymes. *Pseudomonas aeruginosa* PM389 and *Erwinia caratovora* were used as endophytic and pathogenic bacteria respectively. Level of all the defense enzymes namely (PO, PAL, ß-glucanase, PPO) produced in plants on endophytic bacterial inoculation was significantly lower than that of pathogen-treated plants. It indicates attenuation of immune response in plants on treatment with endophytic bacteria. To corroborate the data of biochemical assays, expression of pathogenesis related (PR) genes were analyzed against endophytic bacteria *Pseudomonas aeruginosa* PM389 employing qPCR. Suppression of PR genes on the endophytic challenge further supported the results obtained in biochemical assays inferring that endophytes establish inside the plant through attenuated immune response. In order to correlate levels of defense enzyme with bacterial colonization in root, bacterial population was estimated at various time intervals after inoculation. Endophytic bacterial population was found to be lower initially, but increased after 24 HAI (hour after inoculation) and remained almost constant for rest of the study period. However, population of pathogenic bacteria was higher initially but the population size kept fluctuating for rest of the study period from 24 HAI. Results of the comparative analyses indicated attenuated defense response in challenged host plants towards endophytic bacteria when compared to that of pathogenic bacteria, an important feature that helps endophytes establish inside endosphere of roots. Similar kind of results were obtained in qPCR analysis, confirming that attenuated immune response led to successful endophytic establishment inside the plant.

Future scope of work

Screening of potential PGPR and biocontrol endophytic agent as well as scaling of these PGPR and mixed inoculants (Microcosm studies) to actual farming conditions can provide future biofertilizer and biocontrol agents. Endophytic bacterial strains obtained in the present study can be tested in diverse environment and on different crop plants to extend its application as a biofertilizer agent. The industrial development of biofertilizer and biocontrol products can be a better alternative to chemical fertilizer and pesticides, and this eco-friendly approach can lead to sustainable agriculture.

Despite advancement in the field of genomics, transcriptomics and proteomics, several aspects related to endophyte-plant interactions are poorly understood. These include identification of factors determining plant-endophyte compatibility and molecular mechanism of endophytic colonization. *P. aeruginosa* PM389 and other biofertilizer strains obtained in this study can be further investigated to characterize various bacterial and plant factors, which are critical for establishment of plant-microbe association and optimization of conditions to attain higher plant growth and yield in natural farming conditions. We identified that immune response in host plant is weaker to endophytic bacteria which is important for establishment of bacteria in plants. In the future, in-depth study is required to investigate bacterial or plant factors responsible for attenuated immune response to endophytic bacteria.

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APPENDIX

Appendix I. Reagents for Lowry's method

- BSA stock 1 mg/ml.
- Reagent A: 50 ml of 2% Na_2CO_3 + 50 ml of 0.1 N NaOH (a)
- Reagent B: 10 ml of 1.56% $CuSO_4$ + 10 ml of 2.37% Sodium Potassium Tartarate (b)
- Reagent C: 2 ml of Reagent B + 100 ml of Reagent A
- Folin Ciocalteau reagent (2 N), diluted with water 1:1 to make 1 N working solution.

Appendix II. Plant growth promoting traits of various isolates obtained in this study.

Isolates	JNFB$^-$	LGI	IAA	Phosphate solubilization	Siderophore
PM01	+	+	-	-	-
PM02	+	+	-	-	-
PM03	+	-	-	++	-
PM05	+++	+++	+	-	++
PM16	+	+	-	+	+
PM219	+	++	+	-	-
PM220	+	++	-	-	-
PM221	+	+++	+++	+	+
PM222	+	++	-	-	-
PM223	+	+	-	-	+
PM224	-	++	+++	+	-
PM226	++	+	-	-	-
PM228	++	-	±	-	-
PM229	-	+	-	-	-
PM231	+	+	-	-	-
PM232	++	+	+	+	±
PM233	+++	-	+	++	+
PM241	+	++	+++	+	±
PM242	+	++	+	+	+
PM243	-	++	-	-	+
PM244	-	++	-	-	-
PM245	+	++	+	+	+
PM246	+	++	+	+	++
PM248	+	+	++	++	+
PM249	++	+	+	+	+
PM250	++	+	+++	+	+
PM251	++	-	±	-	-
PM252	++	-	-	+	-
PM253	+++	+	++	+	+
PM366	+	-	-	-	+
PM367	++	-	+	+	++
PM368	++	-	-	-	+
PM370	+	+	-	++	+++
PM371	+	-	-	++	++
PM372	+	+	++	++	++
PM374	-	-	-	-	-

PM376	+	++	++	-	++
PM377	+	++	-	+	+
PM378	+	++	-	++	++
PM379	+	+	-	-	-
PM380	+	+	-	-	-
PM381	+	++	-	-	-
PM385	+	++	-	++	++
PM386	+	-	+++	++	+
PM387	+	-	++	+	++
PM388	+	-	+++	+	++
PM389	+	++	-	++	+++
PM391	+	-	±	-	+
PM392	+	+	-	-	+
PM393	+	-	-	-	+
PM394	+	-	-	-	+
PM395	+	-	-	-	+
PM396	+	-	-	-	+
PM397	+	-	++	-	+
PM4104	+	-	±	-	-
PM4105	++	-	±	-	+
PM4106	+	-	++	-	-
PM4107	++	-	-	-	+
PM4108	+	+	+	±	+
PM4109	+	++	+	-	+
PM4110	+	++	+	-	+
PM4111	+	++	+	-	+
PM4112	+	++	+	++	++
PM4113	+	++	-	++	++
PM4114	+	+	+	-	+
PM4120	++	+	+	++	++
PM4121	+	+	±	++	++
PM4122	++	+	+	++	++
PM4123	++	+	+	++	++
PM4124	++	-	+	++	++
PM4126	-	++	+	++	+++
PM4127	-	++	+	++	+++

PM5140	+	++	+	++	++
PM5141	+	++	-	++	++
PM5142	-	+	+	-	-
PM5143	+	+	+	-	-
PM5146	++	+	-	-	-
PM5147	++	+	+	++	+
PM5148	++	+	-	++	++
PM5149	++	+	+	++	++
PM5150	+	+	+	++	+++
PM5153	+	+	+	-	+
PM5154	++	+	+	++	+++
PM5156	++	+	+	++	++
PM5157	++	+	+	-	-
PM5163	++	+	+	++	++
PM5164	++	+	+	-	-
PM5165	++	+	+	++	+++
PM5166	+	+	+	-	-
PM5167	+	++	+	+	+
PM5168	+	++	-	-	-
PM5169	+	++	+	++	++
PM6182	++	+	+	+	+
PM6183	+	+	+	++	+
PM6184	++	+	+	±	+
PM6185	++	+	+	++	+
PM6186	++	+	+	±	+
PM6187	+	++	+	+	++
PM6188	+	++	+	-	++
PM6189	+	++	+	+	++
PM6190	+	++	+	+++	++
PM6191	+	+++	-	-	+
PM6208	++	+	+	++	++
PM6209	+	++	+	++	++

PM6210	+	++	+	++	+
PM7220	-	+	±	-	-
PM7221	+	++	±	-	-
PM7222	+	+	-	++	++
PM7223	+	++	±	-	++
PM7224	-	+	±	-	-
PM7228	++	+	-	++	++
PM7229	++	+	-	++	++
PM7230	+	+	±	++	+
PM7232	+	+	-	+++	+
PM7233	++	+	-	-	++
PM7234	++	+	-	-	+
PM7245	-	+	-	-	-
PM7247	-	±	±	-	-
PM7248	-	+	-	-	-
PM7249	+++	++	±	-	+
PM7250	+++	+++	-	-	-
PM7252	+++	+++	-	-	+
PM7253	+++	+++	±	-	+
PM7254	+++	+	-	-	+
PM7257	++	+++	-	-	+
PM7258	++	+++	-	-	+
PM7263	+++	++	-	+	+
PM7264	+++	+	-	+	+
PM7265	+++	+++	-	±	+
PM7266	+++	++	-	++	+
PM7267	+++	++	-	++	+
PM7269	+++	+	-	++	+
PM7270	++	+	-	++	+
PM8279	+++	+++	-	±	+
PM8280	+++	++	-	++	+
PM8282	+	+++	-	++	++
PM8283	+	++	-	++	++
PM8284	+	++	-	++	++
PM8286	++	+++	-	++	++
PM8287	++	+	-	++	±
PM8302	+++	+	-	+++	-
PM8303	+++	++	±	-	-
PM8304	+++	++	+	+	-
PM8305	+	+++	-	-	-

PM8306	+++	++	-	++	+++
PM8307	+	++	-	++	+++
PM8308	++	+++	-	++	+++
PM8309	++	+++	-	+	++
PM8311	+++	+++	+	-	-
PM8335	+++	+++	-	-	-
PM8337	+++	+++	+	++	+
PM8342	+++	+++	++	++	+
PM8343	+++	+++	-	-	-
PM8347	+++	+++	-	-	-
PM9363	+++	+++	-	-	-
PM9364	+++	+++	-	-	-
PM9365	++	+	±	-	-
PM9366	+	-	±	+	+
PM9367	+	-	±	-	-
PM9368	+	+	-	-	-
PM9369	+	++	±	-	-
PM9370	+	++	+	++	-
PM9375	++	-	-	+	+
PM9376	+	-	±	-	-
PM9378	++	-	±	++	++
PM9382	-	++	-	++	++
PM9383	-	+	-	+	-
PM9396	+	+	-	-	-
PM9398	+	++	-	-	++
PM9399	+	+	-	-	-
PM9400	+	++	++	-	-
PM9401	+	++	++	-	-
PM9402	+	++	-	-	-
PM9403	+++	++	-	+	-
PM9404	++	+++	-	+	+++
PM9405	++	+	++	-	-
PM9406	+	+++	-	-	-
PM9408	+	+++	+	-	++
PM9409	+	+	-	-	-
PM9410	+++	+	±	-	-
PM9411	++	+++	++	-	-

PM9424	+	++	±	+	-
PM9426	+	++	++	-	-
PM9427	+	++	++	-	-
PM9429	+	+	-	-	-
PM9430	+	+	-	-	-
PM9432	+	+	±	-	-
PM9433	++	+++	-	-	-
PM9435	+	+++	+	-	-
PM9436	+	+	++	-	-
PM9437	+	+++	+++	-	-
PM9438	+	+++	++	-	-
PM10455	+++	++	+++	+	-
PM10458	+	++	-	-	-
PM10459	+	++	±	-	-
PM10461	++	+++	+++	-	-
PM10462	+	+++	+	+	-
PM10463	+	+	-	-	-
PM10464	++	+	+	-	-
PM10466	+	+	-	-	-
PM10475	+	+	-	+	-
PM10476	++	+	-	-	-
PM10478	++	+++	±	-	-
PM10489	+	+	±	++	+++
PM10490	+	++	±	++	++
PM10491	+	++	±	++	++
PM10501	+++	+	++	-	+
PM10503	+	+	-	-	-
PM10508	+	+	-	-	-
PM10509	-	+++	-	-	-
PM10510	++	+	-	-	-

- = absent, + = poor, ++ = average, +++ = good, ++++-excellent

Appendix III. Hoagland's Solution (Plant nutrient solution)

Component	**Stock solution (**g/l)	**ml Stock solution (per liter)**
2 M KNO_3	202	2.5
2 M $Ca(NO_3)_2 \times 4H_2O$	118	2.5
Iron (Sprint 138 iron chelate)	15	1.5
2 M $MgSO_4 \times 7H_2O$	493l	1
1 M NH_4NO_3	80	1
Minors:		1
H_3BO_3	2.86	
$MnCl_2 \times 4H_2O$	1.81	
$ZnSO_4 \times 7H_2O$	0.22	
$CuSO_4$	0.051	
$H_3MoO_4 \times H_2O$ or	0.09	
$Na_2MoO_4 \times 2H_2O$	0.12	
1 M KH_2PO_4 (pH to 6.0	136	0.5
with 3 M KOH)		

1) Stock solutions were stored in separate bottles with appropriate label.

2) Each component was added to 800 ml deionized water then volume was adjusted to 1L.

3) After the solution is mixed, it was ready to water plants.

Appendix IV. SDS PAGE

12% Resolving gel – 10 ml

MilliQ H_2O	-	3.2 ml
Acrylamide Mix	-	4.0 ml (30% acrylamide/bisacrylamide (37.5:1) aqueous solution (stored in the dark, at 4°C only for one month)
Tris 1.5 M (pH8.8)	-	2.5 ml
SDS (10%)	-	100 µl
APS (10%)	-	100 µl
TEMED	-	3.5 µl

Stacking gel – 5 ml

Milli Q H_2O	-	2.8 ml
Acrylamide Mix	-	850 µl
Tris 1.5 M (pH8.8)	-	1.25 ml
SDS (10%)	-	50 µl
APS (10%)	-	50 µl
TEMED	-	5.0 µl

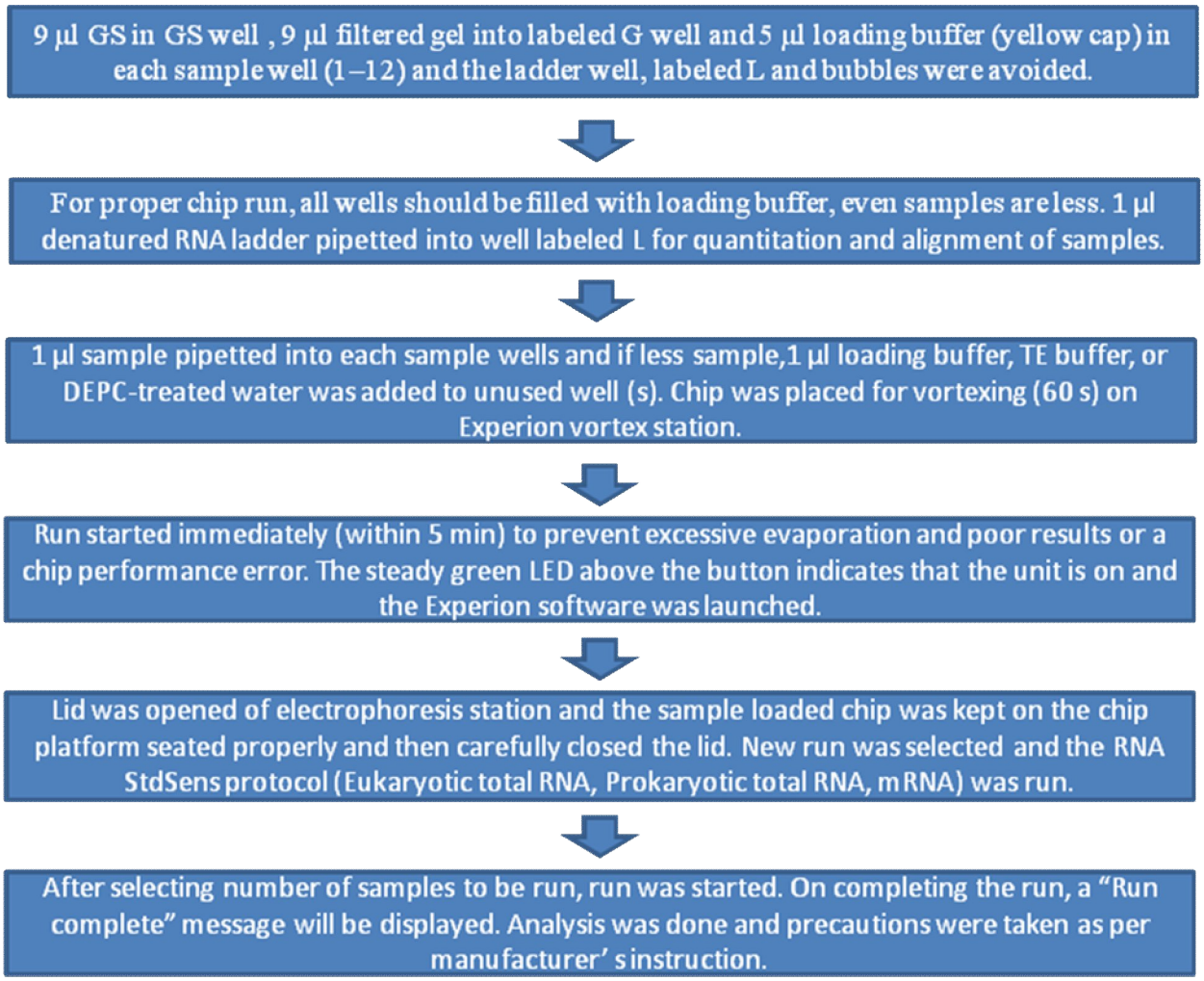
Electrophoresis buffer-Tris (125 mM), glycine (170 mM) buffer (pH 8.3) containing 0.15% SDS.

Staining solution – CBB G-250

The colloidal "blue silver" G-250 dye solution was prepared by adding 0.12% Coomassie Brilliant Blue G-250 (w/v), 10% ammonium sulfate and 10% phosphoric acid sequentially to a water solution (1/10 of the final volume). Further, water was added to attain 80% of the final volume. Finally, , anhydrous methanol (20% of final volume) was dissolved to this solution with stirring to attain final volume. This stock dye solution was kept in a brown bottle. It remains stable at room temperature for 6 months.

Destaining solution-Water:Methanol:Acetic Acid – 45:45:10.

Appendix V. Qualitative and quantitative estimation of RNA


9 µl GS in GS well , 9 µl filtered gel into labeled G well and 5 µl loading buffer (yellow cap) in each sample well (1–12) and the ladder well, labeled L and bubbles were avoided.
For proper chip run, all wells should be filled with loading buffer, even samples are less. 1 µl denatured RNA ladder pipetted into well labeled L for quantitation and alignment of samples.
1 µl sample pipetted into each sample wells and if less sample,1 µl loading buffer, TE buffer, or DEPC-treated water was added to unused well (s). Chip was placed for vortexing (60 s) on Experion vortex station.
Run started immediately (within 5 min) to prevent excessive evaporation and poor results or a chip performance error. The steady green LED above the button indicates that the unit is on and the Experion software was launched.
Lid was opened of electrophoresis station and the sample loaded chip was kept on the chip platform seated properly and then carefully closed the lid. New run was selected and the RNA StdSens protocol (Eukaryotic total RNA, Prokaryotic total RNA, mRNA) was run.
After selecting number of samples to be run, run was started. On completing the run, a "Run complete" message will be displayed. Analysis was done and precautions were taken as per manufacturer' s instruction.

List of Research publications

Papers published

1. Jha P.N., **Gupta G.**, Jha P., Mehrotra R. Association of rhizospheric/endophytic bacteria with plants: A potential gateway to sustainable agriculture. Greener journal of agricultural sciences 2013, 3, 73-84.
2. **Gupta G.**, Panwar J., Jha P.N. Temporal dynamics of *Pseudomonas aeruginosa*: A dominant diazotrophic endophytic bacterium of *Pennisetum glaucum* (L.) R. Br. Applied soil ecology 2013, 64, 252-261.
3. **Gupta G.**, Panwar J., Akhtar M.S., Jha P.N. *In*: Endophytic nitrogen-fixing bacteria as biofertilizer. In: E Lichtfouse (ed) Sustainable agriculture reviews. Springer Netherlands, pp183-221.
4. Misra N., **Gupta G.**, Aggarwal A., Jha P.N. Characterization of zinc tolerant mineral phosphate solubilizing bacteria isolated from zinc rich soil. Journal of basic microbiology 2012, 52, 1-10.

Manuscript submitted

1. **Gupta G.**, Paul S., Jha P.N. Cultivable diazotrophic endophytic community structure and diversity at various growth stages of field grown *Pennisetum glaucum* (L.) R. Br. (Plant and Soil).
2. **Gupta G.**, Das A., Jha P., Jha P.N. Attenuated immune response to endophytic bacteria favours successful interaction with host plant. (Journal of Applied Microbiology).

Papers published in conference/proceedings/symposium/Workshop

- Actively participated in the "Innovation and technology transfer to industries: Role of universities" held at Lucknow, U.P. (10^{th}, 11^{th} March'2014).
- Actively participated in the "International conference on health, environment and industrial biotechnology BIOSANGAM-2013" held at Allahabad, U.P. (21^{st}-23^{rd} November'2013).
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He is a recipient of Raman post-doctoral fellowship under India-US knowledge initiative 2013 programme (University Grant Commission, New Delhi), DST travel grant to attend international conference at Czeck-Republic, DBT- Postdoctoral fellowship, CSIR-SRF and K.C. Bose Young Scientist award (Gold medal). He was a Visiting Fellow in Department of Botany and Zoology, Institute of Life Sciences, Rajiv Gandhi University, Rono Hills, Doimukh, Itanagar, Arunachal Pradesh, India to teach Molecular Biology and Plant Biotechnology to post-graduate student. He has delivered motivational talk to students of Navodaya Vidhyalaya and scientific talks to research and academic institutes.

Biography of Garima Gupta

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Research Article

Association of Rhizospheric/Endophytic Bacteria with Plants: A Potential Gateway to Sustainable Agriculture

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ABSTRACT

Application of associative bacteria for sustainable agriculture holds immense potential. These bacteria are known to enhance growth and yield of plants by fixing atmospheric nitrogen, solubilization of phosphate, production of phytohormones and siderophores, possession of antagonistic activity as well as reducing the level of stress ethylene in host plants. Colonization of these bacteria can be tracked by tagging them with certain molecular markers such as β-glucuronidase (*gus*) or green fluorescent protein (*gfp*) followed by electron microscopy or laser scanning confocal microscopy. Associative bacteria and endophytes may express genes differentially to colonize and establish the plant interior. They may also use 'quorum sensing' molecules for colonization process. Present review aims to highlight various plant growth promoting properties, ecology and updates of molecular mechanisms involved in interaction between associative bacteria and plants as well as immune responses triggered by these bacteria in plants.

Keywords: Associative bacteria, endophyte, diazotrophy, biocontrol, induced systemic tolerance, induced systemic resistance.

INTRODUCTION

The over increasing population of the world has already touched the number of 6.8 billion. To feed this burgeoning population, farmers heavily rely on the use of chemical fertilizers especially inorganic nitrogen. Application of inorganic fertilizer has many repercussions, as it leads to ground and surface water contamination due to leaching and denitrification, which is detrimental for human and animal health. Secondly, manufacturing of industrial nitrogen fertilizer uses non-renewable resources like natural gas and coal and causes production of green house gases viz., CO_2 and NO_2 contributing to global warming (Bhattacharjee et al., 2008). Therefore, it's high time to opt for alternative fertilizers which can be used in sustainable agricultural practices without affecting the environment. Application of plant growth promoting associative bacteria can be a potential option for enhancing growth and yield of plant in sustainable manner.

On the basis of area of colonization, Plant Associated Bacteria (PAB) can be grouped into associative bacteria that include rhizospheric (in vicinity of root) and rhizoplanic (on surface of root) bacteria and, endophytic bacteria. Term 'endophytic bacteria' is referred to those bacteria, which colonizes in the interior of the plant parts, viz, root, stem or seeds without causing any harmful effect on host plant (Hallmann et al., 1997). These bacteria may promote plant growth in terms of increased germination rates, biomass, leaf area, chlorophyll content, nitrogen content, protein content, hydraulic activity, roots and shoot length, yield and tolerance to abiotic stresses like draught, flood, salinity etc. PAB can promote plant growth directly through Biological Nitrogen Fixation (BNF), phytohormone production, phosphate solubilization, inhibition of ethylene biosynthesis in response to biotic or abiotic stress (induced systemic tolerance) etc., or indirectly through inducing resistance to pathogen (Bhattacharya and Jha, 2012). Present review aims to focus on plant growth promoting abilities of rhizospheric and endophytic bacteria and their molecular aspects. PAB has been classified as the plant growth promoting bacteria on the basis of basic mechanisms through which it stimulates plant growth as PGPB, which induces plant growth directly and; bio-controller, which protects plants by inhibiting growth of pathogen and/or insect (Fig. 1) (Backman and

Sikora, 2008). In the present review, discussion regarding PGPB has excluded rhizobia associated with leguminous plants.

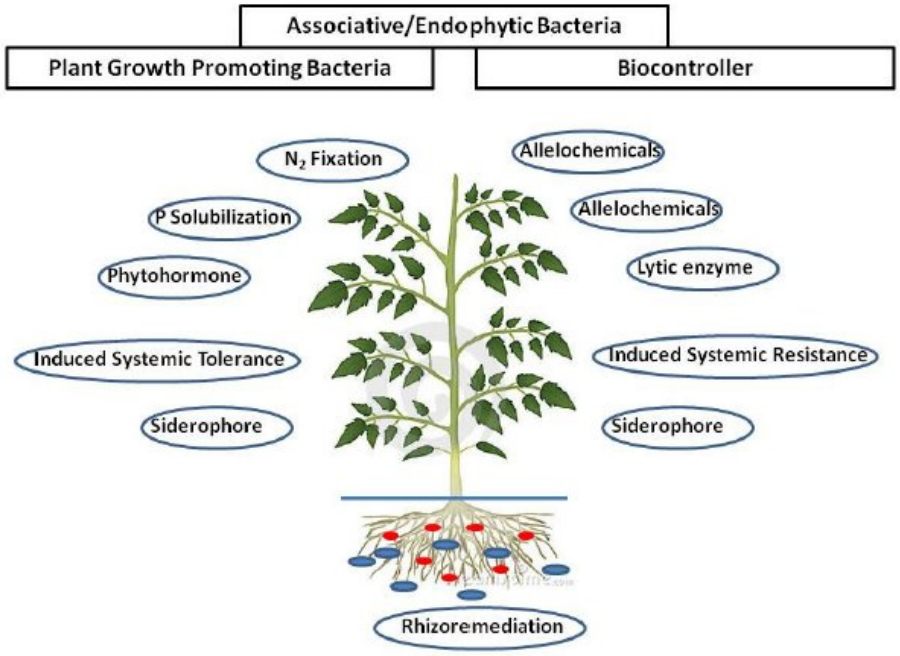

Figure 1: Properties of associative/endophytic bacteria for plant growth improvement. Based on the properties, associative/endophytic bacteria have been classified as Plant Growth Promoting Bacteria (PGPB) and biocontrol bacteria. PGPB may benefit associated plants through providing nutrition (nitrogen, phosphorous and iron), production of plant hormone and may enable plant tolerate abiotic stressors. Biocontrol bacteria (right panel in figure) protect plants from invasion of pathogenic microorganisms through antagonism and/or induced systemic resistance.

Plant Growth Promoting Bacteria

Associative bacteria as well as endophytic bacteria use same mechanisms to influence plant growth. However, they differ in efficiency through which they exert their beneficial effect. Based on various properties, plant growth promoting bacteria can be classified as biofertilizers, rhizoremediators, phytostimulators and stress controllers. Bacterial fertilizer is referred to the bacteria that supply nutrition to the associated plant. They may benefit plants by providing utilizable nitrogen through fixation of atmospheric nitrogen or they make free phosphate available from insoluble source of phosphate. Plant growth promotion due to solubilization of zinc compound driven by *Gluconoacetobacter* has also been reported Beneficial properties of these bacteria are described below in brief (Lugtenberg and Kamilova, 2009).

Biological Nitrogen Fixation: Many associative and endophytic bacteria are now known to fix atmospheric nitrogen and supply it to the associated host plants. A variety of nitrogen fixing bacteria like *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Derxia*, *Enterobacter*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, *Serratia* and *Zoogloea* have been isolated from the rhizosphere of various crops, which contribute fixed nitrogen to the associated plants. For instance, contribution of 20 Kg N ha^{-1} by *Azotobacter paspali* was demonstrated using ^{15}N dilution technique (Baldani and Baldani, 2005; Reinhold-Hurek and Hurek, 2011). In recent years, application of endophytic bacterial inoculants supplying N requirement efficiently to the various host

plants including cereal crops have drawn attention for increasing plant yield in sustainable manner. Additionally, some of the rhizobial isolates have also been found to colonize non-legume plant as an endophyte and benefit the associating host (Rothballer et al., 2008). In terms of benefiting through nitrogen fixation, endophytic bacteria are considered to be better than that of rhizospheric one as they provide fixed nitrogen directly to their host plant and fix nitrogen more efficiently due to lower oxygen pressure in the interior of plants than that of soil.

When diazotrophic bacteria establishes endophytic association with plants, total content of plant nitrogen rises which may be due to the biological nitrogen fixation or increased ability of nitrogen uptake from soil. In a well-organized study in Brazil suggested that 60-80% of the accumulated nitrogen in different varieties of sugarcane namely, CB45-3, SP70-1143 and Krakatau, was contributed by BNF (Boddey, 1995). Combination of nitrogen-fixing bacteria (viz.,*Rhizobium. trifolii* and *Burkholderia* MG43) and reduced amount of chemical fertilizer can achieve overall yield equivalent to the yield that was obtained from recommended full dose of chemical fertilizer (Bhattacharjee et al., 2008). *Gluconoacetobacter diazotrophicus* is the main contributor of endophytic BNF in sugarcane, which according to nitrogen balance studies fix as high as 150 Kg N $ha^{-1}yr^{-1}$ (Muthukumarasamy et al., 2005). However, contribution of BNF to host may vary with the genotype of host. Proteomic analyses of sugarcane variety SP70-1143 grown with *G. diazotrophicus* revealed up-regulated expression of ammonia lyase which indicates increased metabolism resulted from increased uptake of nitrogen contributed by bacteria (Lery et al., 2011). Up-regulation of genes for nitrogen metabolism during plant-bacteria interaction was also evident in differential gene expression studies carried out earlier (Nogueira et al., 2001). Another nitrogen-fixing endophyte of considerable interest is *Azoarcus*. This diazotroph inhabits the roots of kallar grass (*Leptochloa fusca*), which yields 20-40 t of hay ha^{-1} yr^{-1} without the addition of any N fertilizer in saline sodic, alkaline soils having low fertility (Ladha and Reddy, 2000). Percent contribution of plant nitrogen as a result of BNF by few associating endophytic bacteria has been given in table 1.

TABLE 1: Contribution of biological nitrogen fixation by associative/endophytic bacteria

Endophytic bacteria	Associating plant	% Ndfa*	Reference
Rhizobium leguminosarum bv. *trifolii*	Rice	19 to 28	Yanni et al., 1997; Biswas et al., 2000
Burkholderia	Rice	31	Baldani and Baldani, 2005
Herbaspirillum	Rice	19-47	Ladha and Reddy, 2000
Azospirillum	Rice	19-47	Ladha and Reddy, 2000
G. diazotrophicus, H. seropedicae, H. rubrisubalbicans, A. amazonense and Burkholderia sp	Sugarcane	29	Oliveira et al., 2002
K. pneumoniae 324	Rice	42	Iniguez et al., 2004
Burkholderia vietnamiensis	Rice	40-42	Govindrajan et al., 2008

*Nitrogen derived from air

At the molecular level, role of endophytic bacteria supplying fixed nitrogen to host was ascertained using non-nitrogen fixing *Klebsiella pneumoniae* where the rice plants inoculated with non- nitrogen fixing *K. pneumoniae* in nitrogen-deficient media showed signs of nitrogen deficiency on the contrary to the wild type counterpart (Iniguez et al., 2004). Nitrogen-fixation ability of endophytic bacteria *ex-planta* or *in-planta* is measured or detected on the basis of *nif* genes, encoding nitrogenase enzyme or by immunological detection of nitrogenase using antibody raised against nitrogenase enzyme (Nogueira et al., 2001). Presence of structural genes namely *nifH* or *nifD* in associative as well as endophytic bacteria have been detected by polymerase chain reaction using pair of universal primers (Jha and Kumar, 2009; Reinhold-Hurek and Hurek, 2011). Expression of *nif* genes has also been demonstrated by reverse transcription PCR (RT-PCR) from plants inoculated with *Azoarcus* BH72 and plants growing in field and in other associative diazotrophic bacteria (Terakado-Tonooka et al., 2008; You et al., 2005).

Phosphate Solubilization: Phosphate is known to be the second most limiting compound for plant growth. Although most of the soil is rich in phosphate but they are in insoluble form and cannot be utilized by plants or other soil organisms. A vast number of PGPB with phosphate solubilizing property have been reported which include members belonging to *Burkholderia*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Citrobacter* and *Azotobacter* (Park et al., 2010). Some plant growth promoting bacteria solubilize phosphate from organic or inorganic bound phosphates and

facilitate plant growth. Possible mechanisms for solubilization from organic bound phosphate involve either enzymes namely C-P lyase, non- specific phosphatases and phytases. However, most of the bacterial genera solubilize phosphate through the production of organic acids such as gluconate, ketogluconate, acetate, lactate, oxalate, tartarate, succinate, citrate and glycolate (Khan et al., 2009). Type of organic acid produced for P solubilization may depend upon the carbon source utilized as substrate. Highest P solubilization has been observed when glucose, sucrose or galactose has been used as sole carbon source in the medium (Khan et al., 2009; Park et al., 2010). Genetics and biochemical basis of acid secretion specifically gluconic acid in bacteria such as *Erwinia herbicola*, *Pseudomonas cepacia* and *Enterobacter asburiae* have been reviewed by Rodriguez *et al.* (Rodrıguez et al., 2006). Production of gluconic acid results from the conversion of glucose to gluconic acid by an enzyme glucose dehydrogenase (Gcd). Gcd is a cell-envelope bound enzyme which depends on cofactor pyrroloquinoline quinine (PQQ).

Production of Phytostimulating Compounds

PGPB exert its effects through the production of substances which stimulate plant growth. These substances include phytohormones namely auxins, cytokinins, gibberellins, certain volatiles and the cofactor pyrroquinoline quinine (PQQ). Several associative bacteria have been shown to produce auxins chiefly IAA, which enhances lateral root growth formation and thus increase nutrient uptake by plants and root exudation, which in turn stimulates bacterial colonization and thus amplify the inoculation effect. Plant growth promotion as a result of IAA has been documented in several plants in recent years (Spaepen et al., 2007). However, beneficial effects of bacterial IAA depend upon the optimum concentration, which may vary for different plants. The role of phytohormone produced by associative bacteria in the promotion of plant growth during stress conditions such as salinity or draught has also been demonstrated recently (Egamberdieva, 2009). Since, indigenously produced phytohormone in plants declines in salt stress condition, salt tolerant associative bacteria may enhance plant growth by supplying phytohormones synthesized by them. Similarly, IAA producing bacteria may enhance growth of plant in drought condition by stimulating formation of well- developed root system enough for providing sufficient water from soil. Moreover, the role of IAA in response to stress is evident from its increased production of IAA in *Azospirillum* sp. during carbon limitation and acidic pH (Spaepen et al., 2007).

In addition to IAA, some of the associative bacteria have ability to produce other phytohormones such as cytokinin and gibberellin. Cytokinin produced by *Bacillus megatarium* UMCV1, a rhizospheric bacterium, was found to 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 (López-Bucio et al., 2007). Interestingly, few isolates are capable of producing more than one phytohormone. Moreover, few bacteria namely *B. subtilis*, *B. amyloliqufaciens* and *E. cloacae* promote plant growth through the production of volatile organic compounds (VOCs) such as acetoin and 2,3-butanediol. VOCs of PGPR were found to enhance plant growth by regulating auxin homeostasis in plants which was evident from induction of genes encoding enzymes of metabolism of IAA (Zhang et al., 2008).

Induced Systemic Tolerance

A few PGPB enable the associating plants to tolerate abiotic stresses such as drought, salt, nutrient deficiency or excess, extremes of temperature and, presence of toxic metals. Thus, physical and chemical changes in plants resulted from PGPB-induced tolerance to abiotic stresses has been termed recently as 'Induced Systemic Tolerance' (IST). IST is elicited through the production of bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase, antioxidants, cytokinin or VOCs (Fig. 2).

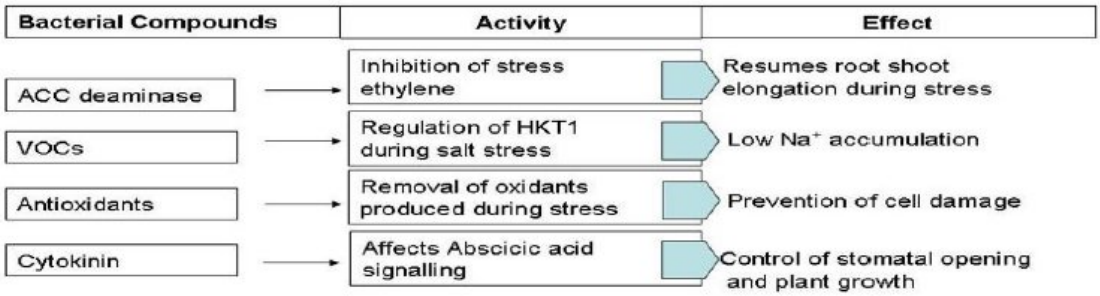

Figure 2: Various components of induced systemic tolerance. PGPB may help associated plant reduce effect of stressors through reducing level of stress ethylene due to presence of ACC deaminase activity, release of antioxidants, volatile organic compounds and plant hormone cytokinin.

PGPB equipped with ability to synthesize ACC deaminase (ACCD) reduce level of stress ethylene produced in plants in response to various biotic and abiotic stressors. ACCD degrades ACC, an immediate precursor of ethylene, to α-ketobutyrate and ammonia (Yang et al., 2009). In addition to ACC deaminase mediated IST, other mechanisms also exist to confer IST in response to stresses. In salt stress, level of Na^+ elevates, which decreases plant growth and productivity. The ion transporter high-affinity K^+ transporter 1 (HKT1) regulates Na^+ import in roots. VOC of *Bacillus subtilis* GB03 confer salt tolerance by down- and up-regulating HKT1 in roots and shoots respectively, and result in low Na^+ accumulation throughout the plant in comparison to control. Other PGPB mediated IST include production of cytokinin which affects abscicic acid (ABA) signaling of plants during stress and augmented production of antioxidant catalase (Yang et al., 2009).

Rhizoremediation

Bacteria with the ability to degrade organic pollutant can be used for remediation of soil. Although pollutant degrading bacteria characterized in laboratory environment may not thrive well in pollutant rich natural environment due to requirement of energy for primary metabolism. Aforementioned problem can be overcome with the use of associative and endophytic bacteria possessing ability to degrade soil pollutant. Since, PGPR colonizes in rhizosphere or rhizoplane; they obtain their source of energy from root exudates for primary metabolism and degrade efficiently organic xenobiotics present in the vicinity. For instance, *P. putida* PCL1444 effectively utilizes root exudates, degrades naphthalene around the root, protects seeds from being killed by naphthalene, and allows the plant to grow normally. Similarly, *in-situ* inoculation of *P. putida* W619-TCE reduced evapotranspiration of trichloroethylene by 90% under field condition (de Bashan et al., 2012). In a recent report, endophytic bacteria isolated from seeds of *Nicotiana tabacum* has been found to be potential candidate for reducing cadmium phytotoxicity (Mastretta et al., 2009). Application of endophytic bacteria for degrading the pollutants like petroleum, toluene and other organic solvent as well as protecting the plants from metals is of significant importance. In addition, endophytic bacteria engineered with genes encoding enzymes for degradation of pollutants can be better exploited for remediation of soil (de Bashan et al., 2012).

Biocontroller

World agriculture faces a great loss every year incurred from infection by pathogenic organisms. Application of microorganism for the control of diseases seems to be one of the most promising ways. Biocontrol systems are eco-friendly, cost-efficient and involved in improving the soil consistency and maintenance of natural soil flora. To act efficiently, the biocontrol agent should remain active under large range of conditions viz., varying pH, temperature and concentrations of different ions. Biocontrol agents limit growth of pathogen as well as few nematodes and insects. Biocontrol bacteria can limit pathogens directly by producing antagonistic substances, competition for iron, detoxification or degradation of virulence factors; or indirectly by inducing Systemic Resistance (ISR) in plants against certain diseases, signal interference, competition for nutrients and niches and interference with activity, survival, germination and sporulation of the pathogen (Lugtenberg and Kamilova, 2009).

Antagonism: Associative/endophytic bacterial biocontrol agents may inhibit growth of fungal pathogens by one or more of the several mechanisms, which include production of antibiotics, siderophore and lytic enzymes.
A vast array of antagonistic chemical compounds has been identified in bacterial biocontrol agents. Gram negative biocontrol agents such as *Pseudomonas* produce HCN, pyoleutorin (PLT), pyrrolnitrin (PRN), 2,4-diacetylphloroglucinol (2-DAPG) and phenazines (PHZ) chiefly phenazine-1-carboxylic acid and phenazine-1-carboxamide Lugtenberg and Kamilova, 2009). The Role of each antibiotic produced by bacterial biocontrol agent in conferring control of fungal pathogen may vary in different species. Control of *Sclerotinia sclerotiorum* by *P. chlororaphis* PA23 is primarily executed by PRN while PHZ (phenazine-1-carboxylic acid, 2-hydroxyphenazine) helps in the development of biofilm formation (Selin et al., 2010). On the contrary, PHZ (phenazine-1-carboxamide) produced by *P. chlororaphis* strain 1391 was identified to be responsible for controlling tomato fruit and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Few other biochemicals having pathogen inhibiting activity include gluconic acid, 2-hexyl-5-propyl resorcinol, munumbicin, and few VOCs (2,3-butanediol) produced by biocontrol agent (Backman and Sikora, 2008). The level of antibiotic synthesis depends upon nutritional factors viz., type of carbon source utilized, trace elements and availability of other nutrients as well as non-nutritional factors like environmental influences. Regulation of antibiotic production in biocontrol bacterial agents involves GacA/GacR or GrrA/GrrS, RpoD, and RpoS, N-acyl homoserine lactone (AHL) derivatives, and positive auto regulation (Compant et al., 2005).

Under iron-limiting condition, some of the biocontrollers secrete siderophore, which chelates available iron of the soil and sometime from cohabiting microorganism, and deprive pathogenic fungi from this element (Compant et al., 2005). In addition to the role of siderophore in biocontrol, bacterial siderophore has been implicated in iron nutrition of crop plants and heavy metal phytoextraction. Production of siderophore by diazotrophic bacteria seems physiologically more important since the role of catecholate type of siderophore has been implicated in transport of Mo under iron starved condition in *Azospirillum lipoferum*. Because nitrogen-fixing bacteria require both iron and Mo for the activity of nitrogenase, the role of siderophore seems pivotal for any diazotrophic bacteria especially under iron deficiency (Rajkumar et al., 2010).

Bacteria may limit growth of other microorganisms also through the production of hydrolytic enzymes such as chitinase, β-1, 3-glucanase, protease and, laminarinase etc. For instance, *Serratia marcescens* and *Paenibacillus* sp. secrete chitinase to exert antifungal activity against *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *cucumerinum* respectively. *Bacillus cepacia* destroys *Rhizoctonia solani*, *R. rolfsii*, and *Pythium ultimum* through the production of β-1, 3-glucanase. Secretion of protease and chitinase was found to be the possible mechanism for antagonistic activity of endophytic bacteria *Enterobacter* and *Pantoea* against fungal pathogen *Fusarium oxysporum* f.sp. *vasinfectum* (Backman and Sikora, 2008; Compant et al., 2005).

Induced Systemic Resistance: Certain bacterial interactions with root enables the associated plant to develop resistance against potent pathogens. This phenomenon is termed as Induced Systemic Resistance (ISR) and has been noted to be exhibited by both associative and endophytic bacteria (Table 2) (van Loon, 2007). It was first noticed in carnation and cucumber where inoculation with selected PGPB (rhizobacteria) reduced susceptibility to wilt and foliar disease respectively. In contrast to many biocontrol mechanisms, extensive colonization of the root system is not required for ISR to be exerted (Lugtenberg and Kamilova, 2009).

TABLE 2: Biocontrol activity of associative/endophytic bacteria

Endophytic Isolates	Plants	Pathogenic Fungi/Bacteria
P. fluorescens EP1	sugarcane	*Colletotrichum falcatum*
Burkholderia phytofirmans PsJN	Grapevine	*Botrytis cinerea*
Burkholderia phytofirmans PsJN	Tomato	*Verticllium dahlia*
P. Denitrificans 1-15	Oak	*Ceratocystis fagacearum*
P. putida 5-48	Oak	*Ceratocystis fagacearum*
P. fluorescens 63-28	tomato	*F. oxysporum* f. sp. *radicis-lycopersici*
P. fluorescens 63-28	pea	*Pythium ultimum* and *F. oxysporum* f. sp. *pisi*
Bacillus pumilus SE34	Pea	*F. oxysporum* f. sp. *Pisi*
Bacillus pumilus SE34	cotton	*F. oxysporum* f. sp. *Vasinfectum*
Bradyrhizobium Sp. Strain ORS278	*Arabidopsis thaliana*	transcriptome analysis based study
Paenibacillus alvei K165	*A. thaliana*	*Verticillium dahlia*
Actinobacteria	*A. thaliana*	Quantitative PCR analysis based study
Bacillus cereus AR156	*A. thaliana*	*Pseudomonas syringae*

The bacterial products that elicit induction of ISR are diverse and can induce in plants which possibly possess receptor for respective ligands. These elicitors may be lipopolysaccharides, flagella, siderophores, antibiotics, VOCs or quorum-sensing signals. Majority of ISR activated by PGPB is mediated by jasmonate or ethylene (van Loon, 2007). Mechanisms of ISR in Pseudomonas has been reviewed recently (Jankiewicz and Kołtonowicz, 2012). In a recent study, plant growth promoting *Bacillus cereus* AR156 was found to trigger ISR in *A. thaliana* through SA- and JA/ET-signaling pathways in an (Non-expressor of PR1) NPR1-dependent manner (Niu et al., 2011). Development of ISR may induce various genes to strengthen the host plant mechanically or metabolically. It involves fortification of plant cell wall strength, alteration of host physiology or metabolic responses and, 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 protect the host plant from future infections from pathogens. Local immune response induced by PGPR has also been demonstrated in few studies. However, pattern of local immune response depends on genotype of plants and respective bacterial species associated with them (Compant et al., 2005).

Biocontrol against Nematode: Few rhizobacteria acting as a biological control agent against plant-parasitic nematodes have also been reported (Tian et al., 2007). Antagonistic activity by aerobic endospore-forming bacteria (AEFB) (mainly *Bacillus* spp.) and *Pseudomonas* spp against nematodes is well known. It is mainly exerted by the means of metabolic by-products, enzymes and toxins including 2, 4-DAPG (*P. fluorescens*), hydrogen sulphide, chitinase, and hydrogen cyanide.

Colonization

Colonization of bacteria in rhizosphere or on plant surface is a complex process, which involve interplay between several bacterial traits and genes. The colonization is multi-step process and includes (a) migration towards root surface, (b) attachment, (c) distribution along root and (d) growth and survival of the population. For endophytic bacteria one additional step is required that is entry into root and formation of microcolonies inter-or intracellularly. Each trait may vary for different associative/endophytic bacteria. Colonization of bacteria is traced by tagging the putative colonizing bacteria with a molecular marker such as auto fluorescent marker (e.g., green fluorescent protein (*gfp*)) or β-glucosidase (*gus*) followed by microscopy (electron or confocal laser scanning microscopy) (Reinhold-Hurek and Hurek, 2011). Fluorescent in-situ hybridization with real time PCR analysis can also be used for tracking bacterial colonization and its quantification (Lacava et al., 2006). Understanding of molecular mechanism involved in associative or endophytic colonization process is not well understood. Recent reports based on the genomic data and other similar reports have suggested resemblance of colonization methods between pathogenic bacteria and PGPB (Hardoim et al., 2008).

Root Colonization

Root colonization is the first and the critical step in 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. In addition to providing nutritional substances, plants start cross-talk to microorganisms by secreting some signals which cause colonization by some bacteria while inhibits the other (Bais et al., 2006; Compant et al., 2011). The patterns of chemoattractant especially organic acids may vary in different isolates/strains. Malate, succinate and fructose are considered to be the strongest chemoattractants.
Exudate composition is in turn influenced by physiological status of plant, the presence of microbes and products from rhizobacteria such as phenazines, 2,4-DAPG, zearalenone and exopolysaccharide. Sloughed up root cap cells also have large impact on plant-microbe interaction. In addition to chemotaxis, electrotaxis (electrogenic ion transport at the root surface) is also considered as a possible mechanism for initiating rhizobacterial colonization. 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, and create 'biased' rhizosphere by exudating specific metabolic products. In some rhizospheric bacteria, root exudates induce flagellar motility that leads their colonization on plant surfaces. During root colonization process, movement of associative bacteria is followed by their adhesion on plant root which may be mediated by glycosylated polar flagellum, Role of bacterial major outer membrane protein (MOMP) in early host recognition has been recognized in earlier report, where MOMPs from *Azospirillum brasilense* showed stronger adhesion to extracts of cereals than extracts of legumes and tomatoes. It suggests involvement of MOMPs in adhesion, root adsorption and cell aggregation of the bacterium (Lugtenber and Kamilova, 2009). On the other hand, involvement of type IV pili and twitching motility has been identified in tomato root colonization by *Pseudomonas* using *pilA* and *pilT* mutant, *pilA* is the gene encoding prepilin, structural component of type IV pili and *pilT* encodes for protein required for pilus contraction that is responsible for twitching motility (Lugtenberg and Kamilova, 2009; Reinhold-Hurek and Hurek, 2011). Preston *et al.* (2001) identified SSIII secretion system III (SSIII) (hrp) in *P. fluorescens* SBW25 that is by *in-vitro* expression technology (IVET), a promoter trapping technique. Moreover, role of two component regulatory system ColR/ColS in competitive root colonization in *P. fluorescence* has been demonstrated. ColR/ColS system regulates methyltransferase/*WapQ* operon, and thus maintains the integrity of outer membrane for efficient colonization (de Weert et al., 2009).

Endophytic Colonization

Primary mechanism for colonization of endophytic bacteria is similar to that of associative one. Twitching motility and type IV pile were found to be essential for successful colonization of *Azoarcus*, obligate endophytic bacteria (Böhm et al., 2007; Reinhold-Hurek and Hurek, 2011). In addition, Bilal *et al*. (1993) suggested that cell-surface protein and Ca^{2+} dependent twitching motility may be implicated in specific interaction with plants. Chemical composition of lipopolysaccharides (LPS) present on the surface of bacteria might be determinative for successful colonization in host plants (Serrato et al., 2010). Requirement for plant signal such as flavonoid present in root exudates of host plant was also observed for stimulation of endophytic colonization of wheat and *Brassica napus* plants by *Azospirillum brsilense* and *A. caulinodans* respectively (Lugtenberg and Kamilova, 2009).

Majority of natural isolates associated with plants form biofilm in the rhizosphere, on the surface of plant as well as in the endorhizosphere. LapA (large adhesion protein A), a cell surface protein, or its homologue is supposed to be putative adhesion needed for the adhesion of *Pseudomonads* on plant roots (Lugtenberg and Kamilova, 2009). Entry of endophytic bacteria in plant roots is known to occur (a) through wounds particularly where lateral or adventitious roots occur; (b) through root hairs and (c) between undamaged epidermal cells (Harodoim et al., 2008). 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 (Preito et al., 2011; Reinhold-Hurek and Hurek, 2011).

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 *Azospirillum irakense*, *Azoarcus* sp. 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 plants. The endoglucanase loosen larger cellulose fibers, which may help entering to the plant. A homologue of endoglucanase gene has also been identified in *P. stutzeri* A1501, which

occasionally colonizes cortex of crop plants. In addition to endoglucanse, exoglucanases may also help in colonization process. An exoglucanase having cellobiohydrolase and β- glucosidase activity was identified to be key player in colonization process of *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek, 2011). In *Elaegnus* and *Mimosa*, the endophyte penetrates the radial walls presumably by digesting the middle lamella and then proceeds between cells and through intercellular spaces. In contrast to above examples, genes encoding plant cell wall degrading enzymes has not been found in endophytic bacteria *Herbaspirillum 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 (GUS) constitutively as a reporter gene (in *Azoarcus* sp. BH72). *Azoarcus* sp. BH72 colonize apical region of roots behind the meristem intensively and penetrate the rhizoplane preferentially in the zone of elongation and differentiation. They colonize in the cortex region both inter- and intracellularly. In older parts of the roots, they also occur in air spaces. *Azoarcus* sp. is capable of invading even the stele of rice and xylem vessels suggesting systemic spreading into shoots through the transport in vessels (Hurek and Reinhold-Hurek, 2003). On the contrary, shoot colonization of Gramineae appears to be more pronounced by *G. diazotrophicus* and *H. seropedicae* (Jha et al., 2004). Furthermore, Compant and associates reported colonization of endophytic bacteria *Burkholderia phytofirmans* in epidermis and xylem of even reproductive organ of grapevine. In another study Preito and associates suggested that endophytic bacteria are confined within an organelle most likely vacuole which arises by narrowing of an internal membranous structure in roots (Preito et al., 2011).

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 defense mechanism (Rosenblueth and Martínez-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. Influence of organic amendment on endophytic population has also been demonstrated (Hallman et al., 1997). Plant defense response plays critical role in regulating colonization of endophytic bacteria. In dicotyledonous plants, salicylic acid (SA) and ethylene restricts endophytic colonization. Ethylene, a signal molecule of ISR 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 colonization by bacteria indicated that jasmonic acid, not ethylene and SA, contribute in restricting endophytic colonization in grasses (Miché et al., 2006). Expression of jasmoic acid (JA) induced PR proteins (defense proteins) depends upon the compatibility of plant variety and endophytic bacteria. Antimicrobial peptides synthesized by some plants like rice and maize may reduce endophytic colonization (Hurek and Reinhold-Hurek, 2003). Understanding of molecular mechanism and conditions limiting the colonization process need to be elucidated for exploiting the beneficial endophytic or associative interaction with plants.

Future Prospects and Challenges

A thorough exploration of associative/endophytic bacteria and their obvious abilities to enhance plant growth and productivity indeed indicate the existence of natural associations of these bacteria and their 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 plant growth promoting properties at laboratory and green house level, these bacteria fail to exhibit consistent performance under natural conditions. The factors that affect colonization and thus PGPB derived benefit to plant may be soil type, nutritional status of soil, host plant genotype and age as well as climatic conditions (Bhattacharya and Jha, 2012). High amount of available utilizable nitrogen reduces colonization of PGPB in natural condition and it may also reduce the process of nitrogen fixation due to regulatory mechanism acting in the diazotrophic isolates. Therefore, a challenge is posed for systematic optimization for the application of suitable PGPB 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 nitrogen to support growth of microorganism and make the plant evade from negative effects of PGPB colonization on it.

One of the major challenges includes selection of plant genotype and age, and compatible associative bacteria. Understanding of this compatibility would help to enhance productivity by using specific strain for inoculation. Since, the colonization of associative bacteria also depends upon seasonal changes and soil hydric stress, multiples field trials are required to optimize parameters for obtaining the maximum output. Another factor which is to be studied in details is the plant defense response which may limit or reduce the colonization of associative bacteria. In addition, colonization mechanism is still not well understood. Intelligent analysis of genomic and functional genomics studies can help manipulate the conditions to enhance colonization process and increased plant growth properties.

Lastly and most importantly, extensive and intensive research on the understanding of associative and endophytic ecology will be major determinant to maximize benefit from these bacteria.

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Natural occurrence of *Pseudomonas aeruginosa*, a dominant cultivable diazotrophic endophytic bacterium colonizing *Pennisetum glaucum* (L.) R. Br.

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ABSTRACT

Endophytic microbial communities can have strong influence on the growth of their host plants. The present study reports a diazotrophic endophytic bacterial species that colonizes predominantly *Pennisetum glaucum* (pearl millet) and remains stabilized throughout the latter's various growth stages under field conditions. Qualitative and quantitative changes in the endophytic bacterial population structure were examined during the plant growth period at regular intervals of 7 days up to harvesting. DNA fingerprinting (ERIC-PCR) was used as a biomarker to track the identity of various isolates obtained at different time intervals. Identification of representative bacterial species corresponding to different ERIC types was made on the basis of 16S rRNA gene sequence analysis. Based on the colony forming unit (cfu) count of bacterial isolates observed at various growth stages, *Pseudomonas aeruginosa* PM389 was found to be the dominant diazotrophic species among the cultivable endophytes colonizing pearl millet. The population of *P. aeruginosa* was detected in the host 21 days after sowing (DAS), indicating its entry in plant roots from soil and suggesting its non-vertical transfer in pearl millet. Moreover, an upward migration of this dominant diazotrophic bacterial species to shoots was observed with the plant growth. Further evaluation of *P. aeruginosa* PM389 revealed its various plant growth promoting properties *viz.* nitrogen fixation, mineral phosphate solubilization, siderophore production and antagonistic properties.

1. Introduction

Cereals are the most consumed food worldwide. With the burgeoning population each year, world food production has to be increased to provide food security for the projected population in 2025 (www.nwf.org/population). Regular and excessive application of chemical fertilizers used for enhanced crop yield leads to damage of the chemical, physical and biological health of agriculture land (Rajak and Hasija, 2000; Upadhyay et al., 2001). Associative microorganisms which can promote plant growth are considered an excellent alternative to enhance agriculture in a sustainable manner. For last few decades, endophytic bacteria have drawn considerable attention of the scientific community for their ability to promote plant growth. The term endophytic bacteria refers to the bacteria which actively or passively colonize the internal plant parts (endosphere) without conferring deleterious effect to host plant (Gray and Smith, 2005). These bacteria enhance growth of their host plants through one or more ways that include nitrogen fixation, mineral phosphate solubilization, production of phytohormones and siderophores, induced systemic tolerance, antagonistic activities and/or induced systemic resistance (Ladha and Reddy, 2000; Rosenblueth and Martínez-Romero, 2006). They have a huge potential to be utilized as biofertilizer for a wide variety of crop plants in various climatic and edaphic conditions (Tilak and Subba Rao, 1987; Vessey, 2003).

A large number of endophytic bacterial species belonging to genera including *Achromobacter, Azoarcus, Burkholderia, Enterobacter, Gluconoacetobacter, Herbaspirillum, Klebsiella and Serratia* have been demonstrated to have influence on the plant growth due to their nitrogen fixing ability, among other beneficial attributes (Hallmann et al., 1997). In terms of benefiting plants by nitrogen fixation, endophytic bacteria are considered better than rhizospheric bacteria, as the former provide fixed nitrogen directly to host plants (Sturz et al., 2000). Moreover, the presence of relatively lower partial oxygen pressure in the plant interior as compared to rhizospheric soil is more amenable for nitrogen fixation (Ladha and Reddy, 2000).

Endophytic bacterial community can vary qualitatively and quantitatively across different plant species as well as within various growth stages of the same plant (Hallmann et al., 1997). These variations lead to difference in plant health and development. Moreover, soil factors and type have also been found to be critical in influencing endophytic bacterial community in plant endosphere (Long et al., 2010). While the population and diversity of endophytic bacteria have been studied in several staple crop plants including

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rice (Ladha and Reddy, 2000), wheat (Conn and Franco, 2004) and maize (Cavaglieri et al., 2009), fewer reports are found on studies analyzing periodical changes in the diversity of endophytic bacteria at various plant growth stages (Cavaglieri et al., 2009). Understanding of endophytic bacterial diversity and identification of dominant species inhabiting plants throughout the growth period can be helpful in formulation of suitable biofertilizer for a particular crop.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a staple cereal of the hottest, driest areas of tropics and subtropics that reliably produces both grain and forage. It is often referred as the *Camel*, because of its exceptional ability to tolerate drought. In the USA, Australia and South America, pearl millet is grown most extensively as a forage crop. It is the principal food cereal rain-fed crop cultivated in drought prone arid and semi-arid regions of Rajasthan, the largest state of India during '*Kharif*' (rainy to autumn) season (Manga and Yadav, 1995). The life cycle span of pearl millet is between 70 and 75 days. In earlier studies, rhizoplanic and endophytic association of diazotrophic *Azospirillum* sp. and their impact on growth of pearl millet plant has been observed (Tien et al., 1979; Rao and Venkateswarlu, 1983; Tilak and Subba Rao, 1987). During a field study, Umali-garcia et al. (1980) reported that pearl millet root hairs adsorb higher number of *Azospirillum* in comparison to other beneficial bacteria (*Azotobacter, Klebsiella* or *Pseudomonas*). Their study also demonstrated that release of mucigel helped the bacteria colonize rhizoplane of pearl millet plants. Moreover, their findings also suggested endophytic colonization of *Azospirillum* sp. To the best of our knowledge, so far no work has been carried out to systematically study the natural population dynamics of cultivable endophytic bacteria in pearl millet in order to use them as possible biofertilizer. The objectives of the present study were: (i) to examine the natural population dynamics of cultivable diazotrophic endophytic bacteria associated with pearl millet at various growth stages in field conditions, and (ii) to identify the dominant diazotrophic bacterial species among these and to check for its plant growth promoting activities.

2. Materials and methods

2.1. Study area and experimental design

The present study was conducted in a pearl millet field (200 × 500 ft) located in Pilani (28°37′N, 75°36′E), Rajasthan (India). The study area has an arid climate, uncertain and erratic rainfall, high wind velocity and nutrient deficient sandy clay loam soil belonging to Typic haplocamborthid class (USDA). The selected field was not supplemented with any kind of organic or inorganic fertilizers. Soil and plant samples were collected every week randomly, in triplicate, from the seed sowing stage to the harvesting stage of the crop. For plants taken 56 days after sowing (DAS), the aerial portion of plant was divided equally into lower (closer to root), middle and upper part each of 50–60 cm in length. These parts were processed separately for isolation and analysis of diazotrophic endophytic bacteria.

2.2. Chemicals

All reagents were of analytical grade and were prepared using distilled water obtained from a Milli-Q system. LGI media (Composition per liter: $CaCO_3$ 1.0 g, K_2HPO_4 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, $Na_2MoO_4{\cdot}2H_2O$ 5.0 mg, sucrose 5 g; pH 5.5) was used for growth and maintenance of diazotrophic bacteria (Tapia-Hernández et al., 2000). Nutrient agar (NA) medium (Himedia, India) was used to assess the sterilization effectiveness and MacConkey agar (MA) medium (Himedia, India) was used to test the fluorescent pigment production (Brodsky and Nixon, 1973).

2.3. Sampling and soil analysis

Rhizospheric soil samples were collected along with plant samples randomly from the experimental field in triplicate. The uppermost layer of soil (1–2 cm) was removed before taking the samples so as to avoid foreign particles and litter. Soil and plant samples were placed separately in self-sealing polythene bags inside an insulated container and transported immediately to the laboratory for analysis. Soil adhering to roots was collected carefully using fine brush and treated as rhizospheric soil. Before processing, soil samples were passed through a sieve (2 mm mess size) to remove coarse roots. One portion of each soil sample was air-dried and used for the estimation of various physico–chemical properties. Soil samples were analyzed for pH and electrical conductivity (EC) using digital pH and EC meter respectively, on a suspension of soil and water in the ratio 1:2.5. Available phosphorous (Olsen P) in soil was determined using chlorostannous-reduced molybdophosphoric blue color method by extraction with 0.5 M sodium bicarbonate for 30 min (Olsen et al., 1954). Estimation of organic carbon (OC) was done by the method of Walkley and Black (1934) using 1 N potassium dichromate and back titrated with 0.5 N ferrous ammonium sulphate solution. Total nitrogen was estimated by the method described by Jackson (1967). Available Fe, Mn, Cu and Zn were estimated as described by Lindsay and Norvell (1978).

2.4. Isolation of diazotrophic endophytic bacteria

Healthy pearl millet plants were uprooted and washed thoroughly under running tap water to remove soil particles. This was followed by vigorous washing with sterile water and glass beads to remove the surface adhered bacteria. Surface sterilization of seeds and plant samples was done by immersing them in 70% ethanol for 2 min followed by 4% sodium hypochlorite for 12 min. Finally, they were thoroughly rinsed with sterilized water to remove all traces of sterilants. A 100 μl aliquot of the last wash was collected and plated on NA media in order to check for effectiveness of sterilization. Surface sterilized seeds and plant parts (root and shoot) were homogenized separately in sterile phosphate-buffered saline (PBS) solution (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.4 g, and KH_2PO_4 0.24 g per liter; pH 7.4) using a mortar and pestle and serially diluted (decimally) up to 10^{-3} using PBS. A 100 μl sample of each dilution was spread on to LGI media in order to enrich the growth of diazotrophic bacteria. The plates were incubated at 30 °C for 4 days under dark conditions. Bacterial colonies obtained were counted and grouped on the basis of their morphological characteristics (size, shape and color). Quebec colony counter was used to magnify the colonies in plate. The most suitable dilution series was selected for counting the number of colony forming units (cfu). Each morphotype was purified and sub-cultured for several generations to ensure its diazotrophic nature. Isolates were subjected to acetylene reduction assay for confirmation of its nitrogen-fixing ability as described in Section 2.9.1 in the text. Glycerol stock (15%, w/v) of purified isolates were prepared and stored at −70 °C until further use.

2.5. Enterobacterial repetitive intergenic consensus sequences-PCR (ERIC-PCR)

ERIC-PCR was used as biomarker to track the identity of various diazotrophic isolates. DNA template for PCR was prepared by the boiling lysis method (Misra et al., 2012). Briefly, a 100 μl sample of overnight culture (in Nutrient broth) was harvested by centrifugation at 12,000 × *g* for 1 min, the pellet washed twice using 50 mM Tris–Cl (pH 8) and suspended in 100 μl Milli-Q water and the OD_{600} of culture was adjusted to 1.0. The sample was boiled in water bath for 15 min and then immediately placed

on ice. Then, the sample lysate was centrifuged at 16,000 × *g* for 15 min. The supernatant that contained the DNA was quantitated on a spectrophotometer. A 3 μl (50 ng DNA) aliquot was used as template for performing ERIC-PCR on a PTC thermal cycler (MJ Research, Inc., Walthon, MA, USA) as described by Versalovic et al. (1991). For a 50 μl PCR reaction mixture, 1.5 unit of Taq DNA polymerase, 125 μM each dNTPs (Bangalore Genei, Bangalore, India), 1.5 mM $MgCl_2$ and 30 pmol of each primer was used. The primers were suspended in molecular biology grade water (Genetix, India). The specific primers (Sigma–Aldrich, USA) used were 1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and 2R (5′-AAGTAAGTGACTGGGGTGAGC G-3′). The cycling conditions used were: 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The amplified products were analyzed on 2% agarose gel containing 0.5 μg ml^{-1} ethidium bromide using a gel documentation unit (Bio-Rad Laboratories, Hercules, CA, USA). The ERIC profiles were analyzed by studying gel pattern of all the isolates. Identification of representative bacterial species corresponding to different ERIC types was made on the basis of 16S rRNA gene sequence analysis.

2.6. Biochemical characterization of dominant diazotrophic endophytic bacteria

Biochemical tests such as Gram staining, Starch agar test, IMViC (Indole, Methyl Red, Voges Proskauer, Citrate utilization) test, carbohydrate utilization tests (Lactose, Sucrose, Dextrose), oxidase and catalase activity were performed for the dominant bacterial species (*Pseudomonas aeruginosa* PM389) obtained during the study period following standard protocols (Harley and Prescott, 2002). Production of fluorescent pigment was tested on MA media (Brodsky and Nixon, 1973).

2.7. Amplification and sequencing of 16S ribosomal RNA gene of dominant diazotrophic bacteria

The DNA template was obtained by boiling lysis procedure as described above. Specific primers (Sigma–Aldrich, USA) used for the amplification of 16S rRNA gene (Weisburg et al., 1991) were: 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1494R_C, 5′-TACGGCTACCTTGTTACGAC-3′; where M represents A (adenine) or C (cytosine). The PCR mixture contained 50 ng of template, 1.5 unit of Taq DNA polymerase, 125 μM each of dNTPs, 1.5 mM $MgCl_2$ and 30 pmol of each primer. Amplification was done by an initial denaturation at 94 °C for 5 min, 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. Amplified PCR products were visualized on 1% agarose gel.

Amplified PCR products (~1.5 kb) of 16S rRNA gene were purified using QIAquick PCR purification kit (Qiagen, USA) following manufacturer's instructions. The purity of DNA was assessed by taking ratio of absorbance at 260 and 280 nm. The 16S rRNA gene was sequenced by the dideoxy-chain termination method using an automated DNA sequencer (ABI Prism; Model 3100). The obtained sequence was compared with the GenBank database using BLAST algorithm to identify the dominant diazotrophic bacteria up to species level (Altschul et al., 1997). A 98% threshold of 16S rRNA gene sequence matching was used to assign the taxa of isolates. Phylogenetic tree was constructed using partial rRNA gene sequence of dominant bacterial species obtained in this study and some of the rRNA gene sequences of same species available in GenBank database using neighbor joining method in MEGA 4.0 software (Tamura et al., 2007).

2.8. Calculation of relative species abundance (RSA)

RSA was used to interpret the community shift occurring in bacterial populations with the growth of plant (Arnold et al., 2003). RSA was calculated by the following formula: Total cfu of ith species/total cfu of endophytic bacterial species, where ith species refers species of interest (*P. aeruginosa*), which was identified on the basis of 16S rRNA gene sequence analysis. Total cfu of endophytic bacterial species were calculated on the basis of ERIC profile results for that particular week of collection of bacterial isolates.

2.9. Estimation of plant growth promoting properties of dominant diazotrophic endophytic bacteria

Following parameters were determined to test the plant growth promoting activities of dominant diazotrophic endophytic bacterial strain. Each experiment was done in triplicate.

2.9.1. Nitrogenase activity assay

The nitrogenase activity was detected by acetylene reduction assay (Stewart et al., 1967). Briefly, 1 ml of overnight bacterial culture was washed and finally suspended in 1 ml of PBS. A 25 μl inoculum was transferred in 3 ml semi-solid (0.15% w/v) LGI medium in a 7 ml vacutainer tube (Becton-Dickinson, Rutherford, NJ, USA) and incubated for 96 h at 30 °C. Acetylene was injected into each tube using a hypodermic syringe to attain 10% final concentration and the tubes were incubated at 30 °C without shaking. After 24 h of incubation, 0.2 ml of gas phase was removed and the amount of ethylene produced from acetylene was analyzed using Nucon 5700 Gas Chromatograph (Nucon Engineers Ltd., New Delhi) equipped with a Porapak R column and flame ionization detector. Nitrogen was used as the carrier gas.

2.9.2. Amplification of *nifH* gene

nifH gene was amplified using specific primers (Poly et al., 2001): Pol F (5′-TGCGAYCCSAARGCBGACTC-3′) and Pol R (5′-ATSGCCATCATYTCRCCGGA-3′) (Sigma–Aldrich), where Y = C/T, S = G/C, R = A/G, B = G/T/C. Each PCR reaction mix contained 1× Taq DNA polymerase buffer, 50 pmol of each primer, 125 μM each dNTPs, 1 U Taq DNA polymerase and 50 ng of template DNA. The cycling conditions used were: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 30 s followed by extension at 72 °C for 5 min. The amplified product was analyzed on 2% agarose gel. Genomic DNA of *Escherichia coli* and *Azotobacter chroccum* were used as negative and positive control respectively.

2.9.3. Phosphate solubilization, IAA and siderophore production

Phosphate solubilization activity was measured as reported (Mehta and Nautiyal, 2001). Solubilized phosphate was quantified according to the method of Ames (1966) using K_2HPO_4 as standard. Production of indole-3-acetic acid (IAA) was tested by the colorimetric method of Gordon and Weber (1951). Ability of dominant isolate to synthesize siderophore was determined following the method of Schwyn and Neilands (1987). For this, bacterial isolate was grown on Chome Azurol-S (CAS) agar medium for 4 days. The efficiency of siderophore production was measured by calculating the siderophore index as per the following formula: $(D_s - D_c)/D_s$ where D_s and D_c referred to the diameter (in mm) of colony and of clear zone, respectively. Quantitative analysis of siderophore production was performed by CAS shuttle assay by calculating the percent siderophore units according to the following formula: $[(A_r - A_s)/A_r] \times 100$, where A_r and A_s were the absorbance after reaction and the absorbance of shuttle solution respectively. Siderophore typing was performed following the methods of Arnow (1937) and Csaky (1948) for catecholate- and hydroxamate type siderophores, respectively.

2.9.4. Antagonistic activity

The antagonistic activity of the dominant diazotrophic bacterial species was determined by the standard agar well diffusion

assay against important plant pathogenic fungal species to check its potential to be used as a biocontrol agent. The antagonistic activity assay was also carried out against other plant associated bacteria (both Gram positive and Gram negative) to understand the possible community shift of endophytes with the growth of pearl millet plants. Briefly, 6 mm diameter wells were prepared using sterile metallic borer in fresh lawn cultures of selected endophytic bacteria and fungal pathogens growing on NA and potato dextrose agar (PDA) plates respectively. Fresh broth culture (100 μl) of dominant endophyte was inoculated in wells. Boiled culture was used as controls. The plates were incubated at 30 °C (for bacteria) for 24 h and 28 °C (for fungi) for 96 h. The experiment was performed in triplicate. Antagonistic activity was determined by measuring the zone of inhibition for which following criteria were used: zone of inhibition <1 cm = poor (+), between 1 and 2 cm = average (++), >2 cm = good (+++).

2.10. Effect of dominant diazotrophic bacterial isolate on plant growth

On the basis of various growth properties shown by the dominant diazotrophic endophytic bacteria, their plant growth promoting activity as well as their ability to cross infect plants was tested using wheat (*Triticum aestivum* L.) plants under axenic conditions. Wheat was chosen being the most popular *Rabi* (autumn to winter season) crop cultivated in the study area. Surface sterilization of wheat seeds was done by treating with 70% ethanol for 2 min followed by three washings using sterilized water. After this, the seeds were exposed to 0.2% $HgCl_2$ solution for 3 min followed by three consecutive washing using sterile water to remove all traces of $HgCl_2$ and finally kept overnight for drying. For bacterization, 5 g of wheat seeds were treated with 25 ml of 1% carboxymethyl cellulose (CMC) solution prepared in PBS containing a final concentration of 10^8 cells ml^{-1} of dominant diazotrophic endophytic bacteria in a 250 ml conical flask under shaking condition (180 rpm) for 6 h at 30 °C. Control seeds were treated in similar manner but without bacteria. Inoculated seeds were air dried for two hours under aseptic conditions. Ten seeds were placed on sterile moist Whatman filter paper (No.1) in a petridish and incubated in dark for initial 4 days followed by 16:8 photoperiod up to 10 days at 28 ± 2 °C. The experiment was performed in triplicate. Sterile water was added to the petridishes as and when required to maintain the moisture. The seedlings were measured for shoot length after the incubation period using a measuring scale having 1 mm of spatial resolution. Root lengths were measured using grid line intersect method (Tennant, 1975). The vigor index was calculated using the following formula: (shoot length + root length) × percent seed germination.

2.11. Statistical analysis

Microsoft Excel 2007 was used for statistical processing of the data (standard deviation, correlation analysis and student's *t*-test). Least Significant Difference (LSD) (Quinn and Keough, 2002) and the Duncan's Multiple Range Test were used to compare the means wherever applicable.

3. Results

3.1. Soil properties

Soil analysis was done to understand the changes occurring in the nutritional status of rhizospheric soil during vegetative growth period of pearl millet plants. The physico–chemical properties of soil were determined weekly up to 35 DAS to record the changes in its nutritional status with the vegetative growth period of pearl millet plants (Table 1). Pre-sowing soil properties of selected field were as follows: pH 9.3, EC 0.43 dS m^{-1}, OC 0.06%, Olsen P 11.9 mg kg^{-1}, Total N 102.7 mg kg^{-1}, Zn 0.56 μg g^{-1}, Mn 13.63 μg g^{-1}, Fe 24.24 μg g^{-1} and Cu 1.09 μg g^{-1}. The soil pH ranged between 9.0 and 9.6, EC 0.24 and 0.56 dS m^{-1}, OC 0.08 and 0.21%, Olsen P 11.6 and 36.3 mg kg^{-1} and N 86.8 and 140 mg kg^{-1} at various DAS. No significant change was observed in the soil EC during the vegetative growth period. Change in % organic carbon at 35 DAS was found to be highly significant ($n = 3$, $p < 0.005$). The concentration of total nitrogen and soil micronutrients (Zn, Cu, Fe and Mn) decreased significantly with the growth of plants up to 21 DAS ($n = 3$, $p < 0.005$). A significant reduction in concentration of iron was observed as compared to other micronutrients ($n = 3$, $p = < 0.05$) with the growth of plants up to 21 DAS.

3.2. Total diazotrophic endophytic bacterial population

In order to study the colonization pattern, diazotrophic endophytic bacteria were isolated from healthy pearl millet plants at various growth stages in triplicate. To ensure that the isolates recovered from respective plant parts were endophytic in nature, the last wash of surface sterilization procedure was saved and plated on NA media. The absence of bacterial growth confirmed the 100% effectiveness of sterilization process used during the study. Different bacterial isolates were identified on the basis of morphological characteristics such as size, shape and color of the colonies. The pearl millet seeds used in this study were found to be colonized with five different diazotrophic morphotypes with a total population of 0.5×10^4 g^{-1} seed. However, out of these, only two morphotypes were observed in plant roots at 7 DAS. The overall total diazotrophic population ranged from 0.2×10^4 to 8.6×10^4 cfu g^{-1} fresh weight of plant material with a gradual increase up to 42 DAS (Table 2). In roots, the total population of diazotrophic endophytic bacteria significantly increased up to 42 DAS followed by subsequent reduction from 56 DAS ($n = 10$, $p < 0.05$). While, in shoots, the total diazotrophic population increased between 35 and 49 DAS ($n = 10$, $p < 0.05$). Due to the increase in length, the shoot was divided equally in lower (closer to root), middle and upper parts and analyzed separately from 56 DAS. At 63 and 70 DAS, a significantly higher population of diazotrophic bacteria was observed in the middle portion of shoot in comparison to that of lower and upper portion ($n = 10$, $p < 0.005$).

3.3. Tracking the isolates by ERIC-PCR

Various bacterial colonies recovered at different sampling period were subjected to ERIC-PCR based DNA fingerprinting. Out of the total 210 isolates, no amplification was obtained in 36 isolates. These 36 isolates were directly used for 16S rRNA gene sequence analysis. ERIC-profile of each isolate was used as marker to track the presence and abundance of diazotrophic endophytic bacterial species/strain (during cfu counting) at various growth periods of pearl millet plants. To ensure the similarity between members of each morphotypes, the bacterial colonies were picked up randomly for ERIC-PCR analysis. Isolates showing identical ERIC-PCR banding pattern were considered as same strain.

The ERIC-profile analysis suggests that strain PM389 was the dominant bacterial strain colonizing pearl millet plants (Fig. 1). ERIC profile specific to dominant bacterial strain appeared from 21 DAS. At 21 DAS, out of nine root isolates, three showed identical banding pattern corresponding to that of PM389. In shoot isolates, other ERIC-types having banding pattern similar to that of PM389 (40%) were also present. At 28 DAS, two ERIC-types were observed in root. Further, single morphotype was prevalent from 28 to 63 DAS in shoot and from 35 to 49 DAS in roots. ERIC results from randomly picked colonies (10 colonies from each sample) belonged

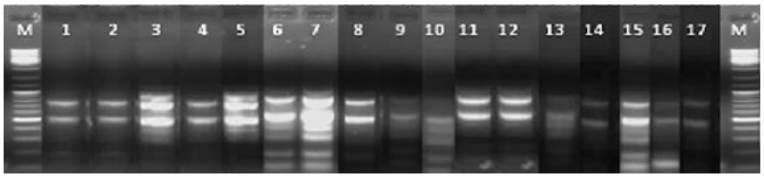

Fig. 1. ERIC-PCR profile of *P. aeruginosa* strains isolated from root and shoot of pearl millet at various growth stages. Each lane represents ERIC profile of isolates showing identical banding pattern at given sampling time. Lanes: M – molecular weight marker, 100-bp ladder from MBI fermentas; 1 – 3WR1; 2 – 3WS1; 3 – 3WS2; 4 – 4WR1; 5 – 4WR2; 6 – 4WS1; 7 – 4WS2; 8 – 5WR1; 9 – 5WS1; 10 – 6WR1; 11 – 6WS1; 12 – 7WR1; 13 – 7WR2; 14 – 7WS1; 15 – 8WR1; 16 – 8WLS1 and; 17 – 9WLS1. First numeral and W of each name refer to time of sampling week when bacterial samples were isolated from plant. W, R and S represent week, root isolate and shoot isolate respectively. Last numeral refers to types of ERIC-pattern obtained. In lanes 16 and 17, L denotes lower part of shoot from which isolation of bacteria was made.

to ERIC-type of dominant strain PM389. However, abundance of PM389 decreased in roots from 49 DAS. ERIC profile specific to PM389 was not obtained after 63 DAS in roots. On the other hand, it was present only in the lower part of shoot at 56 and 63 DAS and in the middle part of shoot at 70 DAS. In addition to PM389, one other strain showing similar DNA profile was also identified as *P. aeruginosa* on the basis of rRNA gene sequence. Moreover, out of 36 isolates showing no result in ERIC PCR, 3 more isolates were further identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence.

3.4. Identification of dominant bacterial species at biochemical and molecular level

The dominant bacterial species during various growth stages of pearl millet plants was subjected to various biochemical tests to confirm its species identity. The results revealed that it was a Gram negative bacillus showing positive reactions for catalase, citrate agar, oxidase, sucrose utilization test and negative reactions for Indole, Methyl Red, Voges Proskauer, carbohydrate utilization (dextrose and lactose) and starch agar test. This bacterial isolate showed fluorescence on MacConkey agar media. The isolate was primarily identified as *P. aeruginosa* on the basis of various biochemical tests. For further confirmation at molecular level, the bacterium was subjected to amplification and sequencing of full length 16S rRNA gene. On the basis of BLAST results, the dominant strain was identified and classified as *P. aeruginosa* PM389 having a closest match (99% sequence similarity of 92% sequence coverage) with strain NRRL B-14938 (Kuo et al., 2008). Identification on the basis of 16S rRNA gene sequence analysis had confirmed the biochemical test results. In addition to PM389, few other strains were also identified as *P. aeruginosa* on the basis of 16S rRNA gene sequence analysis. Furthermore, based on the percent divergence, PM389 shared significant similarity with other plant growth promoting *P. aeruginosa* strains for which sequences are available at GenBank database of NCBI (Fig. 2). The 16S rRNA gene sequences obtained during the present study were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/) with the accession number JF899310, JF899311, JF899312, JF899314 and JF899315 for bacterial isolate PM389, PM4107, PM7265, PM10490 and PM10491 respectively. Other bacterial species obtained in the present study belong to genera *Bacillus, Microbacterium, Pantoea, Enterobacter, Stenotrophomonas, Pseudomonas, Ochrobactrum, Arthrobacter, Rhodococcus, Mycobacterium, Curtobacterium, Nocardioides, Acinetobacter, Agrobacterium* and *Rhizobium.*

3.5. Relative species abundance

RSA represents the common or rareness of a species in relation to other species present in a community. Change in the relative abundance of dominant morphotype identified as *P. aeruginosa* was calculated on the basis of cfu count during the various growth

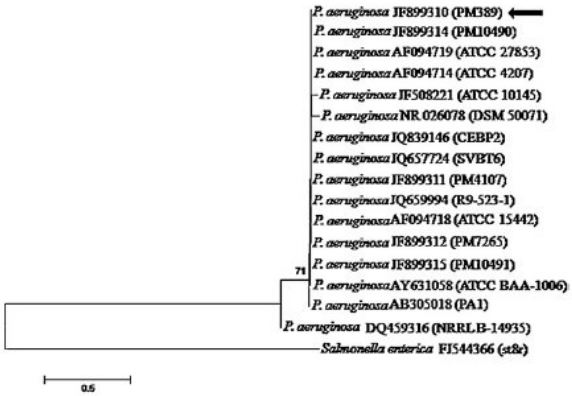

Fig. 2. Phylogenetic tree showing intra-species genetic relationship of *Pseudomonas aeruginosa* strains isolated from pearl millet and other plant growth promoting *P. aeruginosa* strains available in GenBank. *Salmonella enterica* strain st8r (FJ544366), a member of γ-proteobacteria was taken as an out group. Scale bar represents nucleotide substitution per site.

stages of pearl millet plants. The relative abundance of *P. aeruginosa* ranged from 0.48 to 1 (Fig. 3). It was found to be maximum at 42 DAS and was unrecoverable after 56 DAS in roots. Whereas, in shoot samples the relative abundance remain maintained at maximum level during 28 to 42 DAS followed by subsequent decrease. It is clearly evident from the results that with the maturity of plant, the relative abundance of *P. aeruginosa* became unrecoverable in the lower stem and increased to 1 in the middle portion of stem, suggesting an upward movement of bacterial colonization in pearl millet plants with time.

3.6. Population dynamics of P. aeruginosa PM389, the dominant diazotrophic endophyte

A gradual increase was observed in the cfu count of *P. aeruginosa* PM389 in root and shoot portion up to 42 DAS (8.6×10^4) and 35 DAS (1.4×10^4) respectively ($n = 10$, $p < 0.005$) (Fig. 4). However, a lesser colony count was observed during initial weeks (21 and 28 DAS) in shoot portion as compared to roots. A significant decrease in the cfu count of *P. aeruginosa* PM389 was observed from 42 DAS in roots and from 49 DAS in shoots ($n = 10$, $p < 0.005$). This diazotroph was not recovered in root samples from 63 DAS. The population of *P. aeruginosa* PM389 was found to be reduced in the lower shoot from 49 DAS with a significant increase in the middle shoot portion in the last week (70 DAS) ($n = 10$, $p < 0.005$). No diazotrophic bacterial population was observed in the upper shoot segments and seeds formed in the cob at crop maturity.

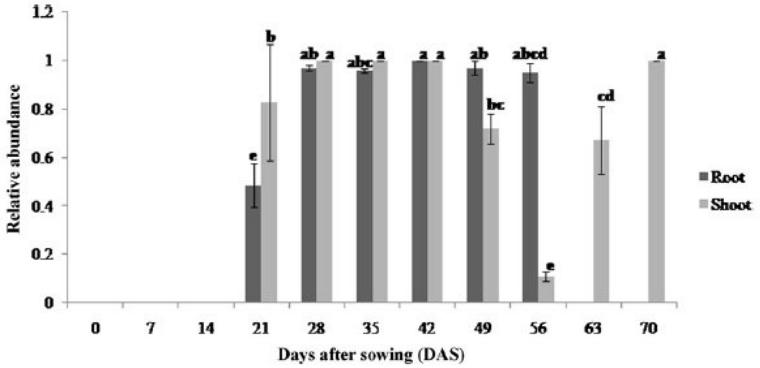

Fig. 3. Changes in the relative abundance of *Pseudomonas aeruginosa* PM389 in root and shoot of pearl millet plants at various growth stages. At 56 and 63 DAS, data for lower shoot and at 70 DAS, data for middle shoot population are represented (L – lower, M – middle, U – upper shoot). Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p < 0.05$. Values that are significantly different from each other are headed by different letters.

3.7. Plant growth promoting properties of P. aeruginosa PM389

The growth of *P. aeruginosa* PM389 on LGI media, which lacks organic or inorganic nitrogen even after subculturing for several generations, indicates its diazotrophic nature. PM389 showed efficient reduction of acetylene (28.91 ± 3.5 nmol ethylene mg^{-1} protein h^{-1}) during the acetylene reduction assay, which is based on the activity of nitrogenase, a key enzyme responsible for biological nitrogen fixation. The rate of nitrogen fixation for other strains of *P. aeruginosa* namely PM4107, PM7265, PM10490 and PM10491 were found to be 25.68 ± 4.5, 26.00 ± 2.5, 23.34 ± 1.8 and 25.67 ± 1.9 nmol ethylene mg^{-1} protein h^{-1} respectively. Furthermore, the amplification of the *nifH* gene supports the nitrogen fixing potential of *P. aeruginosa* PM389 at the molecular level. The desired band of 342 bp corresponding to *nifH* gene was obtained by using universal primers for *nifH* gene. Positive results were also obtained for the mineral phosphate solubilization and siderophore production. *P. aeruginosa* PM389 mainly produced catecholate type of siderophore (63.5% units) which exhibited siderophore index of 0.711. The level of hydroxymate type siderophore production was found to be very low. However, negative results were obtained for IAA production.

P. aeruginosa PM389 showed considerably good antagonistic activity against the majority of bacterial and fungal species tested during the present study (Table 3). It showed strong antagonistic activity against *Arthrobacter woluwensis* (Gram positive) and *Enterobacter* sp. (Gram negative). In case of fungal species, highest antagonistic activity was observed against *Fusarium oxysporum* followed by *Verticillium* sp. However, antagonistic activity was not observed against *Aspergillus niger*.

3.8. Effect of P. aeruginosa PM389 on plant growth

Cross infection studies were conducted *in vitro* to study the effect of *P. aeruginosa* PM389 on seed germination and growth of wheat seedlings (Table 4). Significant increase ($p < 0.05$, $n = 3$) in root and shoot length as well as in vigor index was observed in wheat seedlings treated with *P. aeruginosa* PM389 as compared to control (Fig. 5).

3.9. Correlation analysis

Table 5 reveals the correlation analysis of various soil parameters with the population of *P. aeruginosa* PM389 and total diazotrophic bacterial isolates. The results clearly show that the population of *P. aeruginosa* PM389 and total diazotrophic bacterial isolates showed a strong positive correlation ($n = 4$, $p < 0.05$) with soil OC content.

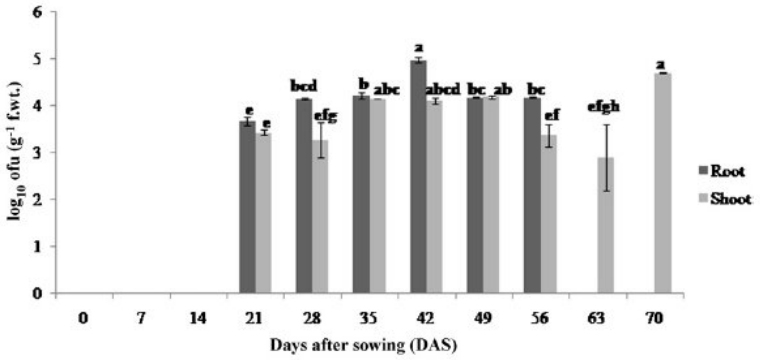

Fig. 4. Changes in the *Pseudomonas aeruginosa* PM389 population in root and shoot of pearl millet plants at various growth stages. At 56 and 63 DAS, data for lower shoot and at 70 DAS, data for middle shoot population are represented. Vertical bars represent standard deviation. The mean values were compared, using Duncan's multiple range test (DMRT) at $p < 0.05$. Values that are significantly different from each other are headed by different letters.

Table 1
Changes in physico–chemical properties in rhizosphere soils of *Pennisetum glaucum* during initial weeks of the study period.

Days after sowing (DAS)	pH	EC (dS m^{-1})	OC (%)	Olsen P (mg kg^{-1})	Total N (mg kg^{-1})	Zn (μg g^{-1})	Mn (μg g^{-1})	Fe (μg g^{-1})	Cu (μg g^{-1})
7	9.0 ± 0.02^{d}	0.41 ± 0.03^{ab}	0.08 ± 0.02^{bc}	12.10 ± 0.15^{e}	100.80 ± 1.40^{e}	0.50 ± 0.01^{ab}	13.56 ± 1.60^{a}	22.68 ± 1.10^{a}	1.02 ± 0.25^{a}
21	9.5 ± 0.10^{ab}	0.36 ± 0.02^{abc}	0.09 ± 0.01^{b}	36.30 ± 0.37^{a}	86.80 ± 1.31^{d}	0.25 ± 0.03^{cd}	7.66 ± 1.00^{bcd}	3.39 ± 0.56^{bcd}	0.32 ± 0.11^{bc}
28	9.6 ± 0.17^{a}	0.56 ± 0.03^{a}	0.07 ± 0.01^{bcd}	11.60 ± 0.32^{cd}	140.00 ± 1.00^{a}	0.53 ± 0.01^{a}	8.56 ± 1.50^{b}	4.66 ± 0.63^{b}	0.64 ± 0.01^{b}
35	9.3 ± 0.03^{bc}	0.24 ± 0.02^{abcd}	0.21 ± 0.03^{a}	16.50 ± 0.23^{b}	120.40 ± 0.96^{b}	0.35 ± 0.01^{c}	8.31 ± 0.95^{bc}	3.53 ± 0.52^{c}	0.32 ± 0.34^{abc}

± represents standard deviation. Letters in common are not significantly different as per Fisher's LSD test.

Table 2
Changes in total diazotrophic endophytic bacterial population in root and shoot of pearl millet at various growth stages. L, M and U represents lower, middle and upper segment of shoot respectively.

DAS	Log_{10} CFU of FW of root	Log_{10} CFU of FW of shoot
7	3.47 ± 0.01^{i}	0.00^{p}
14	3.99 ± 0.06^{bcdefg}	3.72 ± 0.03^{gh}
21	4.02 ± 0.02^{bcdef}	3.43 ± 0.05^{hijk}
28	4.20 ± 0.01^{bcd}	3.27 ± 0.38^{klmn}
35	4.29 ± 0.00^{b}	4.11 ± 0.07^{bcdef}
42	4.98 ± 0.06^{a}	4.15 ± 0.01^{bcde}
49	4.19 ± 0.00^{bcde}	4.34 ± 0.06^{bc}
56	4.21 ± 0.04^{bc}	–
56 (L)	–	4.31 ± 0.06^{bcd}
56 (U)	–	3.67 ± 0.02^{ghi}
63	3.89 ± 0.06^{defgh}	–
63 (L)	–	3.63 ± 0.12^{ghij}
63 (M)	–	4.43 ± 0.09^{ab}
63 (U)	–	3.32 ± 0.02^{ijklm}
70	3.40 ± 0.33^{ij}	–
70 (L)	–	3.40 ± 0.33^{ijkl}
70 (M)	–	4.71 ± 0.01^{a}
70 (U)	–	3.81 ± 0.14^{efg}

± represents standard deviation. Letters in common are not significantly different as per Fisher's LSD test.

Table 3
Antagonistic activity of *Pseudomonas aeruginosa* PM389 against plant associated bacterial species and plant pathogenic fungal species.

Organisms tested	Zone of inhibition (mm)	Results
Gram positive bacteria		
Arthrobacter woluwensis	39.0 ± 0.49	+++
Bacillus sp.	8.0 ± 0.11	+
Bacillus subtilis	33.0 ± 0.14	+++
Microbacterium sp.	NA	–
Gram negative bacteria		
Ochrobactrum sp.	28.0 ± 0.07	++
Pseudomonas oryzihabitans	NA	–
Enterobacter sp.		
Fungal species		
Verticillium sp.	28.0 ± 0.19	+++
Aspergillus flavus	25.0 ± 0.00	+++
Aspergillus niger	NA	–
Fusarium oxysporum	38.0 ± 0.23	+++
Colletotrichum caspasci	20.6 ± 2.50	+++
Colletotrichum acutatum	5.3 ± 2.50	+

–, absent; +, poor; ++, average; +++, good; ± represents standard deviation; NA, no activity.

Fig. 5. Effect of *P. aeruginosa* PM389 on the growth of wheat plants. (A) Seedlings developed from bacterized seeds. (B) Control seedlings.

Table 4
Effect of *P. aeruginosa* PM389 inoculation on growth of wheat plants.

Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Vigor Index
Control (uninoculated media)	100	10.3 ± 0.5	8.9 ± 0.3	1920.0
Inoculated seeds (PM389)	100	11.3 ± 0.7*	9.4 ± 0.5*	2070.0

± represents standard deviation of mean.
* $p < 0.05$, $n = 3$ (Student's *t*-test).

4. Discussion

A systematic study to examine the population dynamics of diazotrophic endophytic bacterial species at different growth stages of the host plant can provide a sustainable way to use the dominant one as possible biofertilizer. To the best of our knowledge, this is the first report on the exclusive dominance of *P. aeruginosa* at various growth stages of pearl millet. The present study was based exclusively on growth of cultivable bacteria.

In general, the physicochemical properties of soil get altered during the vegetative growth period of the plant and its associated microflora, due to their enhanced metabolic activities. Initial increase in Olsen P content during the present study (Table 1) indicates mobilization of phosphates from inorganic or organic insoluble form of phosphate either by the colonizing bacteria having phosphate-solubilizing property or by the plant itself. A reduction in Olsen P content at 28 DAS was probably due to its uptake by the actively growing pearl millet plants. Moreover, a strong positive correlation ($n = 4$, $p < 0.05$) was observed between organic carbon and increased population of *P. aeruginosa* PM389 in soil. This may be due the release of root exudates and exo-polysaccharides by the actively growing plants that provide nutrition and attract diazotrophic endophytic bacteria through chemotaxis to colonize the plant roots.

Increase in nitrogen content of rhizospheric soil at 28 DAS suggests the possibility of nitrogen fixation by associated bacteria as well as release of nitrogenous substances in root exudates by the host plant. On the other hand, intermittent decrease in nitrogen content during the initial plant growth period might be due to increased consumption of available organic nitrogen. A recent study (Koranda et al., 2011) has demonstrated that nutrients provided by the plants through rhizo-deposition influence the microbial process and community composition. Similarly, the initial decrease observed in the concentration of soil micronutrients (Zn, Cu, Fe, Mn) may be due to their enhanced uptake by associated microorganisms and plants during the period of maximum growth (Liu et al., 2000). Reduction of iron concentration in soil that we observed might be due to the bacteria-released siderophore that chelates the iron present in soil to make it available for the growth of bacteria and host plants, as previously reported (Fgaier and Eberl, 2011).

Since ERIC sequences are distributed throughout the genome, their analysis covers the whole genome and enables differentiating bacteria even at strain level (Li et al., 2009). ERIC-PCR based DNA fingerprinting approach has been used for *P. aeruginosa* in earlier studies (Syrmis et al., 2004). In our study, the presence and dominance of the banding pattern corresponding to PM389 at various stages of plant growth clearly demonstrates that *P. aeruginosa* was an active colonizer of pearl millet plant. Germida et al. (1998) also reported Pseudomonads as more diverse, more numerous, dominating, and aggressive colonizers. However, more studies are required to see whether the dominance of culturable *Pseudomonas* in pearl millet is a general phenomenon or it was only the case on studied field.

The colonization of *P. aeruginosa* PM389 in different plant parts progressed with the maturity of crop. It is further supported by the relative species abundance (RSA) analysis that suggests that this is the dominant strain of diazotrophic endophytic bacterial population observed in pearl millet plant. Absence of *P. aeruginosa* PM389 in the pearl millet seeds used for sowing during the present study showed its non-vertical transfer in plants. It indicates that seed-borne endophytes were replaced by soil-borne *P. aeruginosa* and other bacteria during plant growth. Predominance of PM389 and elimination of other bacteria during the active growth period of plants could have occurred due to the production of siderophores or antagonistic compounds (Muñoz-Rojas et al., 2005; Fgaier and Eberl, 2011) by PM389. Antagonistic activity of PM389 against various bacterial species (Table 3) indicates its ability to compete for the given niche over other bacterial species (Viebahn et al., 2005; Ikeda et al., 2010). Contribution of antagonistic activity in modulation of community structure has also been demonstrated in previous reports (Long and Azam, 2001; Grossart et al., 2004). Predominance of one species over others has also been demonstrated in earlier studies where *Pantoea* sp. was found to severely inhibit the colonization of *Ochrobactrum* sp. co-inoculated in rice plants (Verma et al., 2004).

A significant increase in population of *P. aeruginosa* PM389 in the middle portion of shoot at 70 DAS suggests the upward migration of endophyte possibly through xylem vessels of stem under the influence of transpiration stream, passively (Chatelet et al., 2006; Thorne et al., 2006). In the later phase of plant growth, the *P. aeruginosa* PM389 population reduced to a drastic low, in a gradual manner (Figs. 3 and 4). This decline in the population might be due to the intra-species competition for food and space (Bacon and Hinton, 2006). Qualitative changes in plant metabolism and various plant associated bacterial communities can vary during various plant growth phases (Andreote et al., 2010). Several factors may contribute towards altered endophytic bacterial population at later stages of plant growth. These include development of various biotic and abiotic stresses within plant during growth which affect the endophytic bacterial population (Miller and Woods, 1996), change in the plant metabolism with age (Andreote et al., 2010), shifting of sink and changes in the nutritional condition (Compant et al., 2005). Moreover, the absence of *P. aeruginosa* PM389 in the upper part of stem may be due to the slow systemic spreading, problems in xylem connectivity or occluded pits in tracheary elements, which restrict the passage

Table 5
Correlation analysis of *P. aeruginosa* PM389 and total diazotrophic population with various soil parameters.

Soil parameters	*P. aeruginosa* PM389 population		Total diazotrophic population	
	Root	Shoot	Root	Shoot
pH	0.49	−0.04	0.50	−0.03
EC ($dS\,m^{-1}$)	−0.12	−0.73	−0.31	−0.74
OC (%)	0.61	0.98*	0.70	0.98*
Olsen P ($mg\,kg^{-1}$)	−0.24	−0.07	−0.01	−0.03
N ($mg\,kg^{-1}$)	0.75	0.28	0.56	0.25
Zn ($\mu g\,g^{-1}$)	−0.01	−0.35	−0.26	−0.39
Mn ($\mu g\,g^{-1}$)	−0.67	−0.45	−0.78	−0.47
Fe ($\mu g\,g^{-1}$)	−0.74	−0.50	−0.84	−0.52
Cu ($\mu g\,g^{-1}$)	−0.59	−0.63	−0.77	−0.65

* $p < 0.05$, $n = 4$.

of the bacteria within xylem elements and increase the time taken by it to reach the uppermost part of stem (Compant et al., 2008).

Diazotrophic nature of PM389 is evident from the presence of *nifH* gene and positive results for nitrogenase activity. The presence of *nifH* in associative as well as endophytic bacteria has been detected by PCR using pair of universal primers in several earlier studies (Ueda et al., 1995; Desnoues et al., 2003; Jha and Kumar, 2009). Similar to other plant growth promoting bacteria (Vessey, 2003; Berg et al., 2005; Tian et al., 2007), *P. aeruginosa* PM389 also showed phosphate solubilization activity, siderophore production and strong antagonistic activity against pathogenic fungal and plant associated bacterial species.

In vitro cross-infection studies on the effect of *P. aeruginosa* PM389 on wheat plants showed an increased plant growth as compared to controls, suggesting that PM389 can also cross-infect other plant species and can have broad application as a plant growth promoting agent. The antagonistic activity against plant pathogens and enhancement of host plant growth opens up the prospects of *P. aeruginosa* PM389 being utilized as a biofertilizer in the future. Similar to our results, inoculation of associative bacteria *Azospirillum* was shown to increase in dry matter of pearl millet plant growth in both laboratory and field experiments (Umali-garcia et al., 1980; Tien et al., 1979; Rao and Venkateswarlu, 1983; Tilak and Subba Rao, 1987).

5. Conclusion

To the best of our knowledge, this study is the first systematic report on changes in diazotrophic endophytic bacterial population at various growth stages of pearl millet plants in field conditions. Our results indicated a non-vertical transmission of diazotrophic endophytic bacteria in pearl millet plants. *P. aeruginosa* PM389 was found to be the most dominant endophytic diazotrophic bacteria colonizing pearl millet. Results for the plant growth promoting as well as antagonistic activities suggest its application as potential biofertilizer for sustainable crop production under arid environment. However, more detailed studies are required to understand the mode of infection and subsequent transfer of endophytic bacteria in plant systems.

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Research Paper

Assessment of mineral phosphate-solubilizing properties and molecular characterization of zinc-tolerant bacteria

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Plant growth-promoting bacteria with the ability to tolerate heavy metals have importance both in sustainable agriculture and phytoremediation. The present study reports on the isolation and characterization of mineral phosphate-solubilizing (MPS) bacteria associated with the *Achyranthes aspera* L. plant (prickly chaff, flower plant). Out of 35 bacterial isolates, 6 isolates, namely RS7, RP23, EPR1, RS5, RP11 and RP19, with high MPS activity were selected and subjected to the assessment of MPS activity under various stress conditions, *viz.* $ZnSO_4$ (0.30–1.5 M), NaCl and temperature. MPS activity by the selected isolates was observed at concentrations of as high as >1.2 M $ZnSO_4$. Significant improvement in plant growth was observed on bacterization of seeds (pearl millet) with all of the six selected isolates. Plant growth was measured in terms of root length, shoot length, fresh weight and % increase in root biomass. The molecular diversity among the phosphate-solubilizing bacteria was studied employing enterobacterial repetitive intergenic sequence-PCR (ERIC-PCR). Representative strains from each ERIC type were identified, on the basis of a partial sequence of the 16S rRNA gene, as members of the genera *Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Serratia*, and *Enterobacter*. Among all the isolates, RP19 was the best in terms of phosphate-solubizing activity and its response to various stresses. The ability of RP19 and other isolates to exhibit MPS activity at high $ZnSO_4$ concentrations suggests their potential as efficient biofertilizer for growing plants in metal ($ZnSO_4$)-contaminated soil.

Abbreviations: MPS – mineral phosphate-solubilizing; PGP – plant growth-promoting; NA – (nutrient agar); P – phosphate; PSB – phosphate-solubilizing bacteria; MPS – mineral phosphate solubilization; IAA – indole-3-acetic acid; ACC – 1-aminocyclopropane-1-carboxylate; ERIC-PCR – enterobacterial repetitive intergenic sequence-PCR

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Introduction

Phosphorus is the second most limiting nutrient for plant growth. A major portion of the phosphorus present in soil is usually in the immobilized form and thus unavailable for assimilation by plants [1]. Only 1–5% of the total soil phosphorus is available in a soluble form that can be utilized by plants [2]. This necessitates the application of phosphate (P) fertilizer for attaining high growth and yield of plants. However, extraneously added phosphorous fertilizers get converted into salts in soils and become insoluble by forming a complex with cations like Ca, Al and Fe [3]. Moreover, excessive application of fertilizers leads to economic and environmental problems. Therefore, application of microorganisms capable of mobilizing P from the bound form is considered to be a potential biofertilizer for sustainable agriculture in a cost-effective and ecofriendly manner [4–6]. Several bacterial genera including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobac-*

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terium, *Erwinia* and *Klebsiella* are known to possess P solubilization activity [7]. The basic mechanisms considered for P solubilization are mediated by organic acid (chiefly gluconic acid) production, ammonium ion assimilation or phytase activity [7]. The P solubilization ability of microorganisms has been employed for improving crop yield in agriculture and horticulture [8]. In order to exploit P-solubilizing bacteria as biofertilizer, bacteria should possess the ability to compete with other members of the soil microflora and to tolerate local stress. Exploration of P-solubilizing bacteria with the above properties will be of immense importance in sustainable agriculture practice.

The composition of soil and other environmental factors can severely affect plant growth-promoting (PGP) bacteria [9]. Toxic levels of heavy metals including zinc in the soil restrict the growth of both plants and associated bacteria by altering their cellular metabolism. Although the availability of zinc in the soil is essential for supporting cellular growth, high concentrations of zinc can lead to an inhibition of the growth and maintenance of microbial cells. A decrease in the metabolic activity of bacteria and other organisms in the presence of zinc has been reported in previous studies [10]. However, certain bacteria have evolved one or several mechanisms to tolerate the uptake of heavy metal ions, which enables them to survive in metal-contaminated area. The role of PGP bacteria capable of showing enhancement of plant growth in certain metal-contaminated soils has been demonstrated in the recent years [11].

Many areas in Rajasthan and other states of India are heavily contaminated with toxic amounts of zinc due to mining and other human activities, which affects the soil microflora as well as plants growing in that region [10]. Moreover, the soil of Rajasthan faces other abiotic stresses like salt and high temperature, which restrict vegetation in these areas. In the present study, we report on the isolation and characterization of P-solubilizing bacteria associated with the *Achyranthes aspera* L. plant (prickly chaff, flower plant), a member of the family Amaranthaceae, which grows in soil rich in zinc compounds. Application of such bacteria will be of great importance in agricultural practice and also in bioremediation of soil.

Materials and methods

Isolation of bacteria

Plants (*Achyranthes aspera*) and soils associated with plants were collected from the location of Hindustan Zinc Ltd., Udaipur, Rajasthan, India (24°58° N 73°68 E, 598 m above sea level). The soil samples were analyzed for their zinc concentration by atomic absorption spectroscopy (AAS). Rhizospheric, rhizoplanic and endophytic bacteria were isolated separately following standard methods. Rhizospheric bacteria were isolated from soil adhering to the roots of the plants. Of rhizospheric soil, 1 g was suspended in 10 ml sterilized water, diluted to 10^{-5} and 100 µl of each dilution was spread on nutrient agar (NA) medium. Similarly, soil-free roots (2–5 g) were shaken vigorously with glass beads in 250 ml flasks containing sterilized distilled water for 30 min; the water was diluted and spread on NA medium for the isolation of rhizoplanic bacteria. Isolation of endophytic bacteria from surface-sterilized roots and culms was done according to Jha and Kumar [28]. Briefly, surface sterilization of plant parts was done by 70% ethanol for 90 s, followed by treatment with 4% sodium hypochlorite solution for 5 min. To confirm efficiency of the sterilization procedure, the last wash was spread on NA medium. After incubation of the plates for 4 d at 30 °C, the growth of bacterial colonies was recorded.

Screening and analysis of P solubilization activity

Bacterial isolates were spot-inoculated on National Botanical Research Institute's phosphate (NBRIP) growth medium containing (g/l): glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2 \cdot 6\,H_2O$, 5; $MgSO_4 \cdot 7\,H_2O$, 0.25; KCl, 0.2, $(NH_4)_2SO_4$, 0.1; and agar-agar, 20 [12]. They were grown at 30 °C for 4 d, followed by observation for the appearance of a clear zone around the colonies. A clear halo zone was scored as positive test for MPS. Quantitative estimation of P released by bacterial isolates was made by the *ortho*-phosphomolybdate method as described by Ames [13]. Different concentrations of K_2HPO_4 were used to prepare a standard. P solubilization was measured in terms of mg/ml/OD. The optical density (OD_{600}) of the cultures was measured after adding 0.5 volumes of 1 N HCl to dissolve the residual insoluble P against a blank identically treated. The pH of the medium after P solubilization was measured after 4 d of incubation. The initial pH of the medium was adjusted to 7.0.

Evaluation of the minimum inhibitory concentration of $ZnSO_4$

In order to test the toxicity of various concentrations of $ZnSO_4$ on the growth of six selected isolates, the minimum inhibitory concentration (MIC) for each isolate was tested prior to the test for P solubilization activity in the presence of $ZnSO_4$. The standard strain of *Escherichia coli* was subjected to the above test as ne-

gative control. For testing toxicity, selected isolates were grown in nutrient broth medium supplemented with 0.1–1.5 M $ZnSO_4$ for 48 h and thereafter the OD of the culture was read at 600 nm in a spectrophotometer.

Effects of $ZnSO_4$, NaCl and temperature stress on P solubilization

The effects of various stresses, *viz.* $ZnSO_4$, NaCl and temperature, on the P-solubilizing activity were tested in six selected isolates. With a view to test the effect of $ZnSO_4$ and NaCl on MPS activity, bacterial cultures were grown in NBRIP broth medium supplemented with 0.3–1.5 M $ZnSO_4$ or 0.25–2.0 M NaCl. To evaluate the effect of temperature stress, the cultures were grown at 30, 37 and 45 °C in a water bath. Estimation of P was done as mentioned above.

Test for PGP activity

The production of indole-3-acetic acid (IAA) was determined by the colorimetric method of Gordon and Weber [14]. Cultures grown for 4 d were harvested and a reagent containing $FeCl_2$ and perchloric acid was added to the supernatant. The OD of pink color appearing after 25 min of incubation was measured at 530 nm in a UV/Vis spectrophotometer (Jasco).

ERIC-PCR

With a view to study the molecular diversity among P-solubilizing bacteria, all the isolates were subjected to ERIC-PCR. Accordingly, 250 µl of overnight-grown cultures were harvested and washed twice with 50 mM Tris-Cl (pH 8.0). The pellet was suspended in 500 µl water, boiled for 5 min and centrifuged at 12,000 rpm for 1 min. Of the resulting supernatant, 4 µl was used for PCR as template. Sequences of the primers used for ERIC-PCR were: ERIC-1R, 5′-ATG TAA GCT CCT GGG GAT TCA C-3′ and ERIC-2-5′-AAG TAA GTG ACT GGG GTG AGC G-3′. ERIC-PCR was performed as per the method of Versalovic *et al.* [15]. The reaction cycle included an initial denaturation of 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The amplified products were analyzed on 2% agarose gels using a gel documentation unit (Bio-Rad Laboratories, USA). The DNA profile was compared by feeding 1 and 0 for the presence and absence of a band, respectively, into a matrix, and a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm with the help of NTSYSpc 2.1 software to determine the genetic diversity among the P-solubilizing bacteria.

16S rRNA gene amplification and sequence analysis

Amplification of the 16S rRNA gene was performed in a PTC-100 thermal cycler (MJ Research, Inc., USA). DNA templates were prepared as described above. The PCR mix included 1.5 U Taq DNA polymerase, 1 × PCR buffer, 1.5 mM of $MgCl_2$, 125 µM of each dNTP (Bangalore Genei, Bangalore, India), 20 pmol of each of the primers (8f 5′-AGA GTT TGA TYM TGG CTC AG-3′ and 1495r-5′-CTA CGG CTA CCT TGT TAC GA-3′ (Sigma, USA)) in a total volume of 50 µl [16]. Thermal cycles for the amplification were set at initial denaturation for 3 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C for 1.5 min, followed by final extension of 5 min at 72 °C. The amplified PCR product (1.5 kb) was visualized on 1% agarose gels using a gel documentation unit (Bio-Rad Laboratories, USA).

The 16S rRNA gene (1.5 kb) amplified by PCR was purified using a QIAquick PCR purification kit (Qiagen), following the manufacturer's instructions. The 16S rRNA gene was sequenced by the dideoxy chain termination method using an automated DNA sequencer (ABI Prism; model 3100). The 16S rRNA gene sequence was compared with the GenBank database by using the BLASTN algorithm [17] to identify the most similar 16S rRNA gene. A phylogenetic tree was constructed by performing multiple DNA sequence alignment based on the neighbor-joining method using MEGA 4.0 software [18]. The evolutionary distances were computed using the maximum composite likelihood method and are given in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset using the complete deletion option. There were a total of 367 positions in the final dataset.

Effect of bacterial inoculation on plant growth

Surface-sterilized seeds of pearl millet were subjected to treatment with 20% gum acacia for 20 min, air-dried and soaked in bacterial suspension (10^8 cells/ml) for 1 h. Seeds were bacterized separately for each selected isolate. Bacterized seeds were sown in pots containing sterilized soil supplemented with 0.1% (wt/wt) tricalcium phosphate and were allowed to germinate. Seedlings were grown at 25 ± 2 °C with a light/dark cycle of 16/8 h. Experiments were conducted in triplicate with 10 seeds in each pot. The pots were irrigated intermittently with sterile water. After 15 d, plant growth was measured in terms of length of roots and shoots, fresh weight, and root biomass content of the test plants.

Statistical analysis

The data of quantitative estimation of P solubilization in different experimental conditions as well as of effects of bacterial inoculation on plant growth were analyzed statistically for standard deviation by using Sigma Plot software (2004). The mean values were compared, using Duncan's multiple range test at $p < 0.05$ [19].

Nucleotide sequence accession numbers

The 16S rRNA nucleotide sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI). The accession numbers of the 16S rRNA nucleotide sequences of the strains RP23, EPR13, EPS11, RP1, RP8, RP11, RP19, RP24, RS7, EPR2, EPR1 and RS5 are HM042254, HM042255, HM042256, HM042257, HM042258, HM042259, HM042260, HM042261 HM042262, HQ224512, HQ224513 and HQ224514, respectively.

Results

Isolation of plant-associated P-solubilizing bacteria

Before isolation and characterization of bacteria, a physicochemical analysis of the rhizospheric soil was made. Analysis of the soil showed a pH at around 5.7 and the value of the Zn content was 8.2 μg/g soil. Based on morphological features, 64 morphotypes of bacterial isolates were recovered, which included rhizoplanic (24), rhizospheric (10) and endophytic (30) isolates. The populations of rhizospheric, rhizoplanic and endophytic bacteria were found to contain 5.7×10^6, 3×10^6 and 1.4×10^4 CFU/g soil or root, respectively (CFU = colony forming unit). All 64 isolates were screened for P-solubilizing activity, of which 35 isolates including 4 rhizospheric, 14 rhizoplanic and 17 endophytic were found to be positive for P solubilization activity. The results of the quantitative estimation of the P solubilization activity ranged between 0.619 and 4.59 mg P/ml in all the 35 isolates. The highest activity was recorded in isolate RP19 (4.59 mg P/ml) followed by RS5 (3.4 mg P/ml of culture) (Table 1).

Six isolates exhibiting high MPS activity, namely RS7, EPR1, RP11, RS5, RP19 and RP23, were selected for further studies. Assuming P solubilization to result from the production of organic acid by the bacterial isolates, the pH of the medium was measured after growing the selected isolates in NBRIP medium for 4 d. The pH of the medium after growth in medium supplemented with tricalcium phosphate ranged between 2.7 and 4.4. Isolate EPR1 showed the highest drop in the pH of the medium; the lowest drop in pH was observed for RP19.

Table 1. Test for P solubilization and IAA production in different isolates.

Bacterial isolates[a]	P solubilization (μg/ml)[b]	IAA production[c] (μg/ml)
RP23	2103.00 ± 0.78	–
EPS7	775.75 ± 0.08	38.5 ± 1.22
RP4	654.66 ± 0.57	29.0 ± 0.55
RP22	950.56 ± 0.13	35.4 ± 0.80
RP11	1975.88 ± 0.06	150.0 ± 2.33
EPS3	683.29 ± 0.10	126.5 ± 1.98
RP8	974.40 ± 0.08	65.6 ± 0.90
RP24	983.00 ± 0.08	–
RP19	4590.00 ± 0.32	37.6 ± 0.86
RS5	3417.45 ± 0.23	–
EPR4	619.49 ± 0.05	–
RP1	942.76 ± 0.13	35.0 ± 0.50
EPR1	2214.26 ± 0.01	–
RS7	1894.18 ± 0.16	55.8 ± 0.76
EPR2	855.67 ± 0.02	–
RS3	863.27 ± 0.06	–
EPS5	1696.29 ± 0.25	–
RS1	922.86 ± 0.05	–
EPS8	922.76 ± 0.07	30.9 ± 0.42
EPR15	302.01 ± 0.17	–
EPR10	756.08 ± 0.20	30.4 ± 0.95
EPS10	603.75 ± 0.57	30.3 ± 0.44
EPR11	730.39 ± 1.56	34.7 ± 0.63
RP9	928.00 ± 0.68	–
RP3	941.00 ± 0.98	–
RP13	618.44 ± 0.93	48.3 ± 1.05
EPR13	952.89 ± 0.02	–
EPR17	716.47 ± 0.67	29.0 ± 0.38
RP16	736.45 ± 0.46	–
RP17	699.12 ± 0.22	–
RP18	813.16 ± 0.20	29.8 ± 0.66
EPR12	536.17 ± 0.24	28.5 ± 0.35
EPR18	676.06 ± 0.56	28.0 ± 0.54
EPS6	721.43 ± 0.57	125.6 ± 0.96
EPS11	1527.63 ± 0.06	–

[a] RS, RP, EPR and EPS denote rhizospheric, rhizoplanic, root-endophytic and shoot-endophytic isolates, respectively.
[b] The P solubilization test was made following growth of cultures in NBRIP medium. Free phosphorous was estimated after 4 d of growth. For test of IAA production, growth media were supplemented with 100 μg/ml tryptophan.
[c] IAA estimation was made at 4 d of incubation. The experiment was done in triplicate. Values given are means ± SD of experiments performed in triplicate.

Effect of stress conditions on P-solubilizing activity

The P-solubilizing activity of the selected isolates was assessed in the presence of $ZnSO_4$, at different temperatures and varying NaCl stress. Before conducting the test for MPS activity, the toxicity of $ZnSO_4$ to various isolates was tested. The MIC of $ZnSO_4$ for all the isolates ranged between 1.3 and 1.5 M. Increasing concentrations of $ZnSO_4$ in the medium affected P solubilization to varying degrees. There was 30–73% inhibition of the MPS activity in different isolates (Fig. 1). Out of six iso-

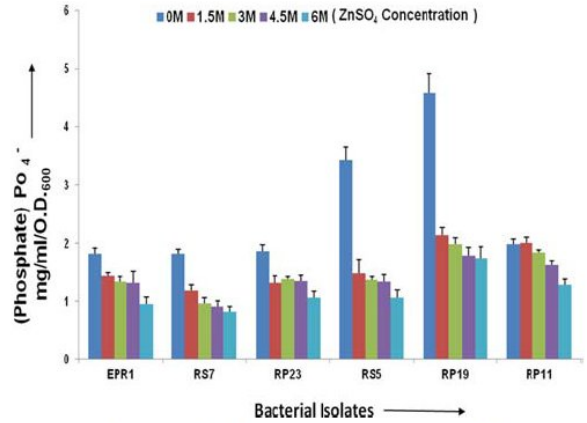

Figure 1. Effect of $ZnSO_4$ on P solubilization (MPS) activity. Release of phosphate was estimated after 4 d of growth at 30 °C. Results are means ± SD of three experiments conducted separately in triplicate.

lates, RP19 showed the highest P solubilization at a concentration of 1.2 M $ZnSO_4$.

Regarding NaCl stress, all the selected isolates showed MPS activity in the presence of as high as 0.8 M NaCl, although a significant decline in the MPS activity was noted with increasing concentrations of NaCl in the medium. Among all the six isolates, RS7 and RP23 showed more tolerance to NaCl stress (Fig. 2). Similar to the data of $ZnSO_4$ and NaCl stress, a sharp decline in MPS activity (20–75%) was noticed with increasing growth temperature. However, the isolates RP19 and RS5 showed significant levels of MPS activity even at 42 °C (Fig. 3).

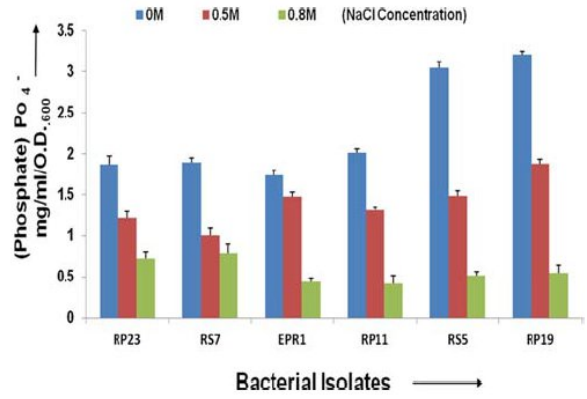

Figure 2. Effect of NaCl stress on MPS activity. All other conditions were identical to those in Fig. 1.

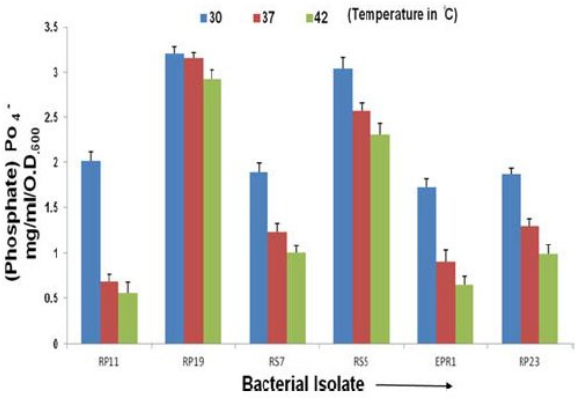

Figure 3. Effect of temperature stress on MPS activity. All other conditions were identical to those in Fig. 1.

PGP properties

With a view to evaluate certain other PGP activities of the test isolates, the test for indole-3-acetic acid (IAA), a phytohormone that stimulates root proliferation, was made. Nineteen isolates were found positive for the production of IAA, which included one rhizospheric, eight rhizoplanic and ten endophytic isolates. In general, the production of IAA ranged from 28 to 150 μg/ml/OD 600 nm (Table 1). Out of six efficient P-solubilizing isolates, three isolates, namely RP19, RP11 and RS5, were found to be positive for the production of IAA. The highest production of IAA was observed in the rhizoplanic isolate RP11 (150 μg/ml) (Table 1).

Molecular diversity among the P-solubilizing bacteria

For understanding the molecular diversity among MPS bacteria associated with *Achyranthes aspera* plants growing in a zinc mine, DNA fingerprinting employing the ERIC-PCR technique was performed. Notable differences in the electrophoretic banding patterns of DNA fragments were evident in the different isolates. ERIC-PCR generated two to seven bands ranging between ~160 bp and 1.5 kb. On the basis of similarities in the DNA banding pattern, a phylogenetic tree was constructed using the neighbor-joining approach (Fig. 4). From the 35 isolates, 26 different electrophoretic patterns were observed. The highest diversity was observed for the rhizoplanic bacteria (Fig. 4). Strains showing more than 90% similarity were grouped together, resulting in 12 groups. Isolate RP11 showed a completely different ERIC profile from all other isolates. Representatives of each group that showed higher P-solubilizing activity were selected for partial 16S rRNA gene sequence analysis.

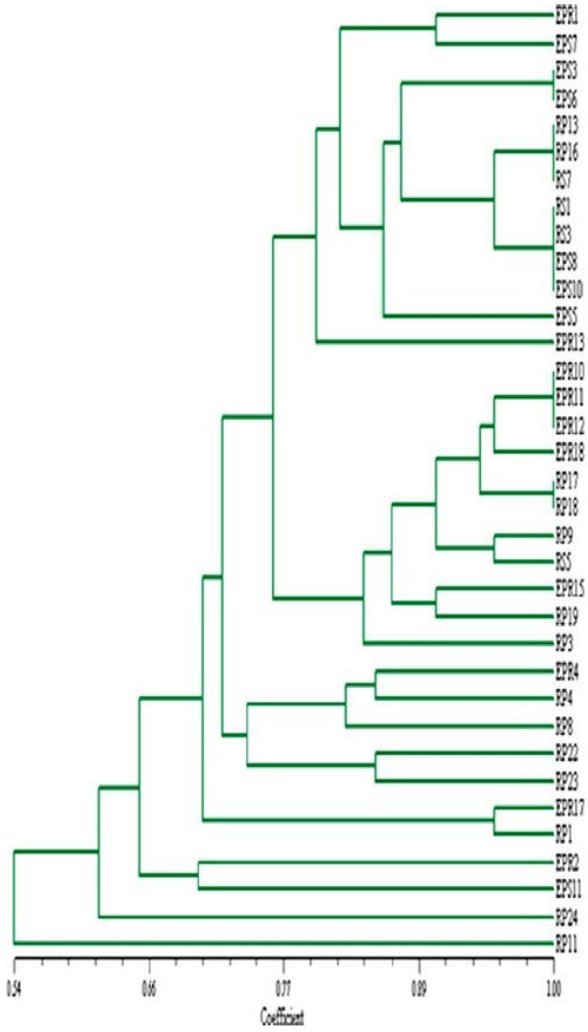

Figure 4. Dendrogram constructed on the basis of the ERIC-PCR profiles.

16S rRNA gene analysis and study of molecular diversity

On the basis of sequence similarities, representative isolates from each kind of ERIC cluster were identified as members of the genera *Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Serratia* and *Enterobacter* (Table 2). All isolates belonged to the γ-proteobacteria. The 16S rRNA gene sequences of all the above bacteria, along with sequences of related bacteria, were used to construct a phylogenetic tree, which showed close affinity (Fig. 5). Branches corresponding to partitions reproduced have more than 50% bootstrap value, which indicated that the tree and the phylogenetic relations are indeed significant.

Effect of P-solubilizing bacteria on plant growth

In order to study the effects of the bacterial isolates on plant growth, a pot experiment was performed. All the isolates significantly stimulated plant growth as compared to the growth of a non-inoculated control plant. Increases of 45–75% and 5–68% in root and shoot length, respectively, were noted in bacterized plants. Additionally, 64–88% increases in fresh weight and up to 100% increase in lateral root formation were observed. Out of the six isolates tested, the highest stimulation of plant growth was observed with RP19 (Table 3).

Discussion

In the present study, the bacterial populations of the rhizosphere and the rhizoplane were comparable, but the diversity of the bacteria in terms of different morphotypes in the rhizoplane and the endosphere was much higher than that in the rhizosphere. This result, to a certain extent, contradicts earlier studies suggest-

Table 2. Identification of selected isolates representing each group based on ERIC profiles on the basis of partial sequences of 16S rDNA.

Bacterial isolates	Taxonomic affiliation	Reference strain at NCBI[a]
EPR13, RP24, RS7	*Pseudomonas aeruginosa*	HQ143666, HQ143666, HQ143666
EPS11	*Pseudomonas* sp.	EF565941
RP23, EPR2,	*Citrobacter freundii*	HQ170626
RP1, RP19	*Acinetobacter haemolyticus*	GU991530, HM629451
RP8	*Serratia marcescens*	HQ143657
EPR1, RS5	*Serratia* sp.	AB560622, GU270568
RP11	*Enterobacter cloacae*	HQ701802

[a] The strain showing closest similarity to the 16S rRNA gene sequence of the test isolate was used as reference strain.

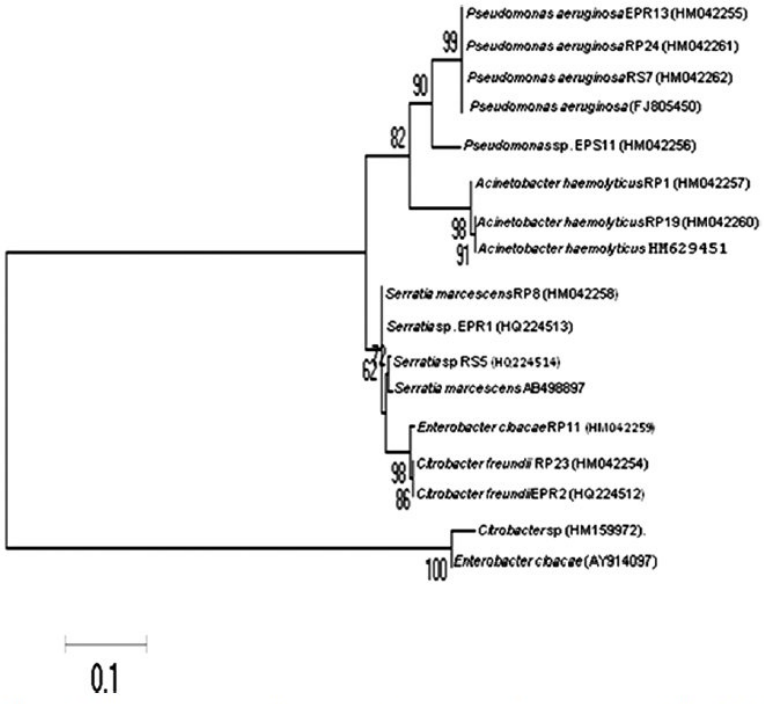

Figure 5. Phenogram showing the genetic relationship of 12 representatives of the ERIC-PCR groups. Partial sequences of the 16S rRNA genes of related species were included for establishing the phylogenetic relationship. The GenBank accession number of each isolate is given together with the name of the bacterial species. Multiple sequence alignment was done in the CLUSTAL W program and the data were used to construct a phylogenetic tree using MEGA 4.0. The phylogenetic tree was analyzed for 1000 boot strap values. The bar indicates 0.1 substitutions per nucleotide site.

ing that bacterial populations as well as diversity are higher in the rhizosphere than in the rhizoplane and the endosphere [20]. Knowing that a quantitative estimation of the P solubilization activity by using the plate method is not accurate [4, 8], we used the phosphomolybdate method to avoid any ambiguity. From the results, it is evident that the MPS activity of the selected six isolates is very high (up to 459 mg/ml). In an earlier report, a maximum P solubilization of ca. 2.7 mg/ml (2700 mg/l) by the mutant strain GPS5 of *Serratia marcesens* was observed. However, the culture OD of the bacterial isolates in the medium was not represented in the above study [21]. The lower activity in the endophytic isolates may be due to the fact that these bacteria are not exposed directly to insoluble phosphates and thus have less expression of enzymes responsible for the P solubilization process.

Since most of the P-solubilizing bacteria exhibit MPS activity through the production of organic acids or an efflux of H^+, a decline in the pH of the medium follow-

Table 3. Test for the effect of bacterial inoculation on plant growth by selected bacterial isolates.

Bacterial isolate	Shoot length (cm)	Root length (cm)	Fresh weight (g)	% Increase in root biomass
Control	12.9 ± 0.09	2.6 ± 0.10	0.11 ± 0.002	–
RP19	22.5 ± 0.02 (74.96)	4.3 ± 0.02 (68.0)	0.28 ± 0.002 (88.18)	100.0
EPR1	17.9 ± 0.06 (39.19)	3.2 ± 0.14 (25.0)	0.19 ± 0.002 (72.72)	61.0
RP23	20.0 ± 0.19 (55.90)	3.2 ± 0.24 (25.0)	0.18 ± 0.003 (63.63)	44.0
RP11	18.6 ± 0.07 (44.63)	2.7 ± 0.31 (05.5)	0.18 ± 0.002 (63.63)	38.5
RS7	19.9 ± 0.12 (54.74)	3.5 ± 0.21 (37.5)	0.20 ± 0.005 (81.81)	77.5
RS5	18.1 ± 0.05 (40.74)	2.7 ± 0.09 (05.5)	0.18 ± 0.006 (63.63)	50.0

* Values in parentheses denote % increase over the non-inoculated control. Results are means ±SD of three experiments conducted separately with at least six plants under identical conditions.

ing P mobilization is expected to occur. This fact is supported by our results as a pH decrease was evident for all the isolates. The role of organic acids in MPS has been demonstrated in earlier studies [4]. However, a direct correlation between the rate of P solubilization activity and the drop in pH has not been reported. We also did not find a sharp decrease in the pH of the medium in the case of one isolate, i.e. RP19. This indicates the possibility of additional or alternative mechanism(s) of MPS activity, such as ammonium ion assimilation or phytase activity [7].

The importance of PGP bacteria in phytoremediation deserves special attention for reclamation of soil [22]. Among several heavy metals, zinc toxicity in soil is of great concern due to its toxic effect at higher concentrations [10]. In this study, there was a decrease in the level of P solubilization activity, but significant activity was present even at a very high concentration of $ZnSO_4$ (1.2 M). Detailed studies are required for understanding the mechanism(s) involved in conferring resistance to zinc in these isolates. The effect of zinc on P solubilization has been demonstrated in a few studies. Rajkumar *et al.* [23] reported P solubilization by *Bacillus weihenstephanensis* at 200 mg/l $ZnSO_4$. In the present study, we, for the first time, report resistance (tolerance) to $ZnSO_4$ of P-solubilizing *Acinetobacter haemolyticus*, *Enterobacter cloacae*, *Serratia marcescens* and *Citrobacter freundii*. It is presumed that zinc-tolerant phosphate-solubilizing bacteria will have the potential to stimulate plant growth in regions contaminated with zinc. Additionally, bacteria tolerant to zinc may also show resistance to other heavy metals, such as Pb, Cd and Ni, as reported in some recent studies [24, 25].

Bacteria growing in alkaline soil in India are subjected to high NaCl, pH and temperature stress, especially during the summer season. Frequently, the temperature rises to above 45 °C. Under such stress, growth and metabolic processes of bacteria may be severely affected, but species that can overcome the stress may grow and survive. Henceforth, the utility of bacteria exhibiting P solubilization under salt and temperature stress has been acknowledged as these strains may successfully colonize plants growing in saline soil. Similar to our results, P solubilization at as high as 10% NaCl has been reported in several studies [4, 8, 26]. Nautiyal *et al.* [26] have reported P solubilization at 37–45 °C in certain bacteria isolated from alkaline soil.

Production of IAA in moderate amounts by phosphate-solubilizing bacteria may show an additional influence by enhancing plant growth. IAA stimulates plant growth by enhancing lateral root formation, which in turn increases the root surface area for absorbing more nutrients from the soil [20]. Moreover, IAA production by phosphate-solubilizing bacteria is considered to play an important role in phytostabilization [27]. IAA production by P-solubilizing *Achromobacter xylosoxidans*, *Pseudomonas*, and *Azospirillum brasilense* has been reported in previous studies [4, 28–30].

ERIC-PCR used for the study of molecular diversity is primarily based on the presence of repetitive DNA (124–127 bp) sequences interspersed in multiple copies throughout the genome. The number and distribution of ERIC segments across the genome vary in different species/strains of bacteria. This technique is reproducible and useful in differentiating various bacterial isolates at the species/strain level [31]. A number of researchers have employed ERIC- and BOX-PCR for studying the bacterial diversity of rice-associated diazotrophic bacteria [32, 33]. In this study, a greater diversity in rhizoplanic bacteria was noted; this may be due to the availability of higher amounts of nutrients on the root surface. Most probably, readily available sources of nutrients on the root surface support the growth of diverse types of bacteria. It is intriguing and interesting to note that the result of the 16S rRNA gene analysis does not match with the results of the ERIC-PCR profile, to a certain extent. This difference may be due to the fact that the rRNA gene sequence analysis represents a small segment of the genome whereas the ERIC profile is representative of the whole genome. As such, the ERIC profile seems to be more reliable for depicting molecular diversity among the bacterial isolates [31]. To our knowledge, two isolates, namely RP19 and RP1, which were identified as *Acinetobacter haemolyticus*, have not been reported to possess P-solubilizing ability as well as zinc tolerance. However, other species of *Acinetobacter*, i.e. *A. calcoaceticus* and *A. baumanii*, have been reported to solubilize mineral phosphate [19, 33].

In the bacterization experiments, inoculation of selected isolates remarkably increased the plant growth. As the soil was supplemented with tricalcium phosphate, the enhanced plant growth might have resulted chiefly due to mobilization of utilizable P from the tricalcium phosphate. However, a role of other PGP attributes cannot be ruled out. The effect of PGP bacterial inoculation on plant growth has been demonstrated in earlier studies. Poonguzhali *et al.* [29] reported that inoculation of P-solubilizing *Pseudomonas poae* CPBE37 resulted in an about 9-fold increase in cabbage plant biomass in comparison to the untreated control. However, no relationship between the P solubilization ability of bacteria and plant growth promotion was observed [30].

Conclusion

In conclusion, the present study reports the isolation of certain novel bacteria with PGP properties. P solubilization activity by certain isolates at high concentrations of $ZnSO_4$ is a new finding of this study. The ability of these bacteria to tolerate metal contamination will be useful for growing plants at metal-contaminated sites. The efficiency of these bacteria to colonize roots would be beneficial for plant health as well as in phytoremediation of soil. Further studies are required to study the mechanism of zinc tolerance in these isolates. The stress-tolerant strains may serve as suitable candidates for developing microbial formulations for the growth of plants in desert-like areas that experience diverse types of abiotic stress.

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