Chapter 4

Engineered production of 2,4-Diacetylphloroglucinol in diazotrophic endophytic bacteria and its beneficial effect in multiple plant-pathogen systems

Fill the brain with high thoughts, highest ideals places them day and night before you and out of that will come great work.

–Swami Vivekanand

4.1 Introduction

Crop losses by disease causing phytopathogens are a major concern for sustainable food production. Plants can be protected against pathogen attack by biocontrol agents, which are plant growth promoting bacteria (PGPB) or fungi that either directly inhibit the growth of pathogens by the production of antagonistic compounds and enzymes, or trigger the natural defense responses of plants against the pathogen (Lugtenberg and Kamilova 2009). Majority of the biocontrol agents studied are free living rhizospheric fungi and bacteria which exert their effect by residing in the exterior environment of the root (Lucy et al. 2004).Virtually every plant studied has a population of microbes living in the internal tissues of the plant, known as endophytes, which like the free living PGPB also show plant growth promotion and biocontrol activity (Compant et al. 2005; Ryan et al. 2008). Plant-endophyte partnerships have been exploited for improvement of plant growth particularly by nitrogen fixation and induction of defense responses against phytopathogens (Wu et al. 2009) as well as for bioremediation of polluted soils (Afzal et al. 2014). Endophytic bacteria that have been shown to be efficient as biocontrol agents include those reducing the wilt disease in eggplant (Ramesh et al. 2009), damping-off disease in tomato (Goudjal et al. 2014; Zouari et al. 2016), yellow sigatoka disease in banana (Aman and Rai 2016), bacterial kiwi canker (Tontou et al. 2016), early blight and grey mold disease in tomato (Gao et al. 2017).

Nitrogen fixing (diazotrophic) endophytes are an important group of bacteria because endophytes offer a potentially significantly higher biological nitrogen fixation potential than associative diazotrophs (Baldani et al. 2005). The entry of endophytic bacteria in interior parts of the plants occurs through natural opening, wounds or root hairs from where they colonize the interior of roots and enter in to aerial parts of the plants (Cocking 2003; Monteiro et al. 2012). Colonization in interior parts of the plant by nonpathogenic PGPB often leads to activation of induced systemic resistance (ISR) in plants, which is mediated by jasmonic acid /ethylene (JA/ET) signaling pathway (Pieterse et al. 2014). The activation of ISR by PGPB is also known as the priming process which enables the plant to fight against the pathogen with advanced response within a shorter time (Haas and Défago 2005).

Pseudomonas spp. are well-studied biocontrol agents (Walsh et al. 2001) known for production of various antimicrobial metabolites such as 2,4-diacetylphloroglucinol (2,4-DAPG),

phenazines, pyrrolnitrin, and pyoluteorin (Dwivedi and Johri 2003). Among these, 2,4-DAPG plays a major role in pathogen inhibition as well as induction of host defense system (Iavicoli et al. 2003). Biosynthesis of 2,4-DAPG is carried out by condensation of three molecules of malonyl-CoA to give rise to phloroglucinol by a special type III polyketide synthase, the product of the *phlD* gene (Fig. 4.1). Further modification occurs by the transfer of acetyl groups at the second and fourth position to convert phloroglucinol into 2,4-DAPG ($C_{10}H_{10}O_5$). The acetyl group is transferred by acetyltransferase enzyme encoded by *phlACB* gene cluster (Yang and Cao 2012).

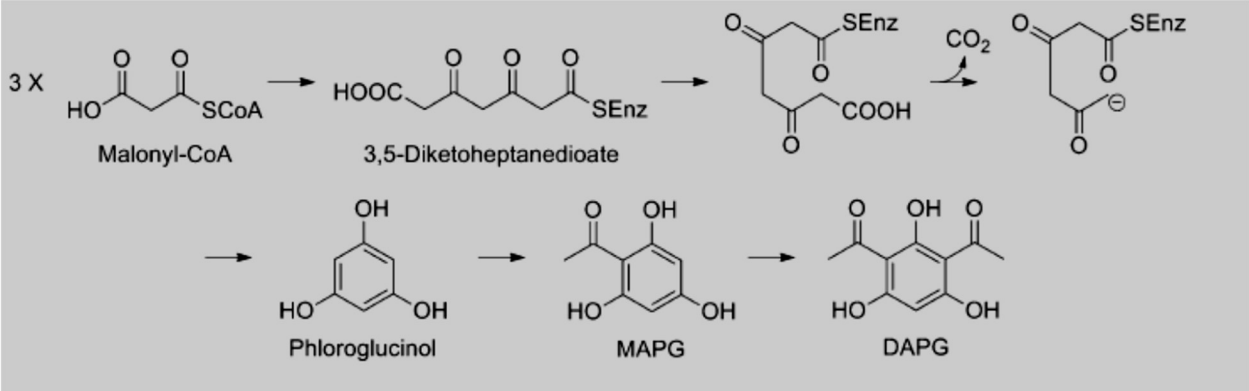


Fig. 4.1 Biosynthesis pathway of DAPG (Yang and Cao 2012)

2,4-DAPG gene cluster comprises of four genes (*phlACBD*) of which *phlACB* gene cluster encoding acetyltransferease is situated upstream to the *phlD* gene responsible for synthesis of phloroglucinol. *phlA* gene is proximal to the promoter region and separated from *phlC* gene by 31bp, similarly *phlC* and *phlB* genes are separated by 10 bp (Fig. 4.2).

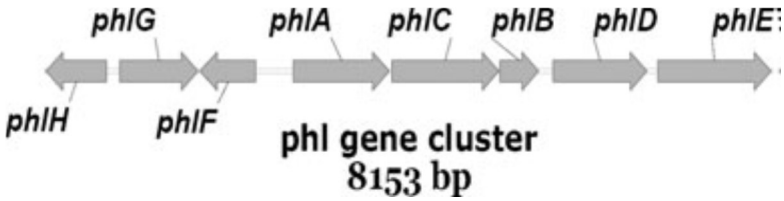


Fig. 4.2 Gene cluster for biosynthesis of 2, 4-DAPG from *P. fluorescens* (Yang and Cao 2012)

Present work deals with the heterologous expression of the 2,4-DAPG gene cluster from two well-characterized 2,4-DAPG producing rhizospheric strains *P. protegens* Pf-5 and *Pseudomonas* sp. G22 (GenBank Accession no. KY206885) in the endophytic bacterium *Pseudomonas* sp. WS5. Both strains are rhizosphere colonizing produce multiple antibiotics. The

diazotrophic endophytic *Pseudomonas* sp. WS5 (Chapter 2) is able to colonize the interior tissues of root and aerial parts of the plants but lack the antagonistic activity against phytopathogenic fungi. This study shows the 2,4-DAPG producing recombinant strain of *Pseudomonas* sp. WS5 improved growth and protection against two phytopathogenic fungi *Magnaporthe oryzae* B157 and *Rhizoctonia solani* in three important cereal crops.

4.2 Materials and Methods

4.2.1. Bacterial and fungal strains and plasmids used in this study

Bacterial and fungal strains used in this study are listed in Table 1 along with plasmids used. Wild type and plasmid carrying derivatives of *Pseudomonas* spp. were grown in Luria-Bertani (LB) broth amended with 2% of glucose for 2,4-DAPG production at 28 °C. *Escherichia coli* DH5α and its plasmid bearing transformants were maintained in LB at 37 °C. For the strains carrying pUCPM18Gm and its recombinant derivatives, the medium was supplemented with gentamicin (40 μg ml^{-1}). Bacterial strains carrying the plasmid pJBA28 and pJET1.2 (Fig. 4.3) were grown in the presence of kanamycin (50 μg ml^{-1}), and ampicillin (100 μg ml^{-1}) respectively. Plasmid maps were constructed using Snap Gene Viewer (http://www.snapgene.com/products/snapgene_viewer). Phytopathogenic fungi *Magnaporthe oryzae* B157 and *Rhizoctonia solani* were grown and maintained as mentioned in (Chapter 3, Section 3.2.1).

Table 4.1 Bacterial and fungal strains/plasmids used in this study

Bacterial and fungal strains/Plasmids	Description	Source/Reference
Bacterial strains		
Escherichia coli DH5*α*	Used as a cloning host	(Sambrook and Russell 2001)
E. coli S17.1	Used for biparental mating to transfer plasmids into gram-negative bacterial strains	(Simon et al. 1983)
Staphylococcus aureus	Used as indicator strain for bioassay 2,4-DAPG	(ATCC 6538)
Pseudomonas protegens Pf-5	Used as source of 2,4-DAPG gene cluster	(Paulsen et al. 2005)
Pseudomonas sp. G22	Used as source of 2,4-DAPG gene cluster	Lab isolate
Pseudomonas sp. WS5, *Pantoea* sp. MS3, *Herbaspirillum Seropedicae* Z67	Diazotrophic bacteria isolated from *Poaceae* plants, used for transformation of pAJK1.1 and pAJK1.2 plasmid construct	(Chapter 2)
Enterobacter sp. C1D	Rhizospheric bacterium used for transformation of pAJK1.1 and pAJK1.2 plasmid construct	Subrahmanyam and Archana 2011
Fungal strains		
Magnaporthe oryzae B157	Rice plant pathogen	(MTCC 12236)
Rhizoctonia solani	Plant pathogen	Lab isolate
Plasmids		
pJET1.2	Blunt ended cloning vector, Ap^r and a	MBI Fermentas (Fig.

	lethal gene eco471	4.3)
pUCPM18Gm	Broad-host-range mobilizable vector, Ap^r, Gm^r, derived by inserting Gm^r cassette at the HindIII site of pUCPM18	(Hester et al. 2000; Joshi et. al. 2008)
pAJK 1.1a	pUCPM18Gm vector harboring *phlD* gene of *P. protegens* Pf-5	This study
pAJK1.1b	pUCPM18Gm vector harboring *phlD* gene of *Pseudomonas* sp. G22	This study
pAJK1.2a	pUCPM18Gm vector harboring *phlDACB* gene cluster of *P. protegens* Pf-5	This study
pAJK1.2b	pUCPM18Gm vector harboring *phlDACB* gene cluster of *Pseudomonas* sp. G22	This study
pJBA28Kn	Constitutively expresses *gfp* under promoter PA1/04/03	(Rothballer et al. 2008)

Gm^r-Gentamicin resistance, Ap^r-ampicillin resistance, Kn^r- Kanamycin resistance

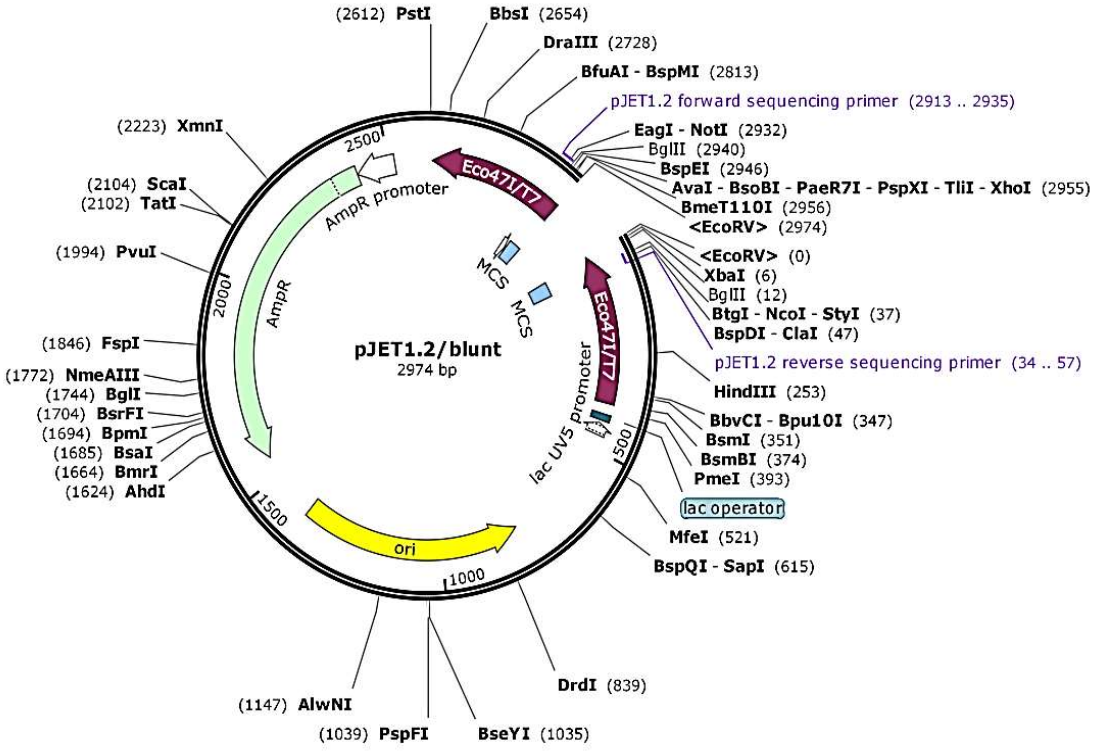


Fig. 4.3 Restriction map of pJET1.2 cloning vector map (MBI Fermentas)

Broad host range pUCPM18Gm vector is a derivative of the pUCP18 vector (Genebank accession number: U07164). This plasmid is stably maintained in *E. coli*, and *Pseudomonas* spp. Replication in *Escherichia coli* is uses origin of replication ColE1 (a high copy number origin of replication) whereas in *Pseudomonas* spp. replication carried out using *oriV* from *Pseudomonas aeruginosa* plasmid pRO1600 and replication controlling protein (West et al. 1994). The pUCPM18 was developed by adding mob region of 750 bp from pLAFRI and cloned at *SspI* restriction site of pUCP18 (Hester et al. 2000). Gentamicin gene cassette of 1612 bp was taken from pGEM160 by HindIII digestion and ligated pUCPM18 at HindIII site present in MCS region of the vector to obtain pUCPM18Gm (Joshi et al. 2008) (Fig. 4.4).

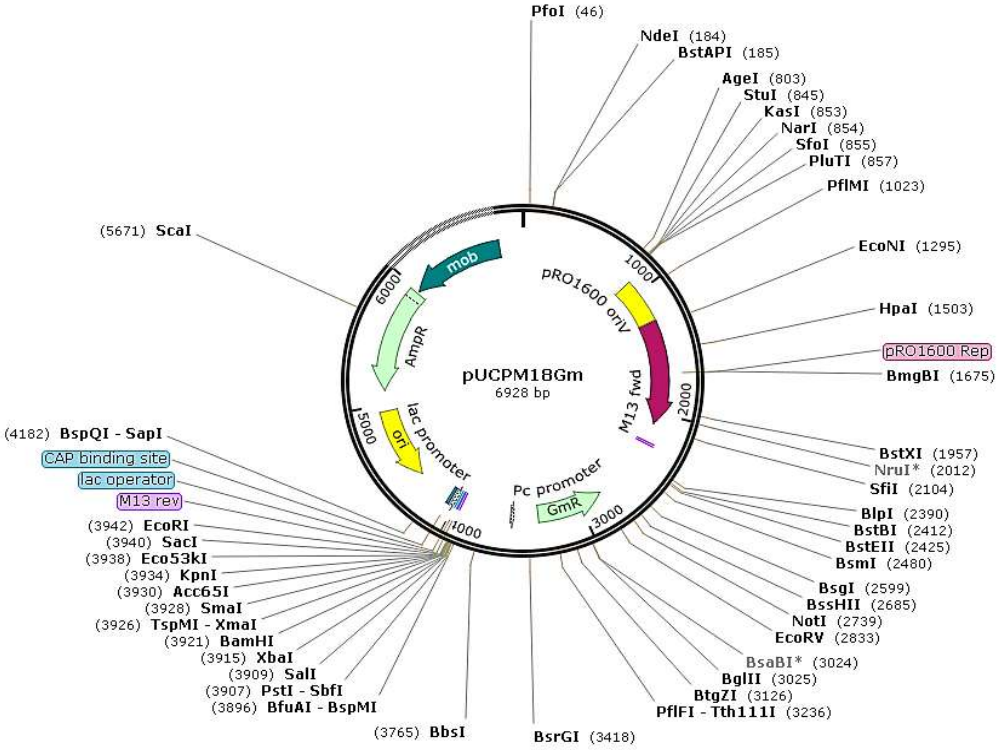


Fig. 4.4 Restriction map of pUCPM18Gm vector

4.2.2 *phlD* and *phlACB* gene amplification

Genomic DNA fragments encoding phloroglucinol synthase (*phlD*) and acetyltransferase (*phlACB* gene cluster) were amplified by PCR from *Pseudomonas protegens* Pf-5 and *Pseudomonas* sp. G22 (Lab isolate) by gene specific primers (*phlD* KpnI-F, *phlD* BamHI-R) with appropriate restriction sites and ribosome binding site (Table 4.1). PCRs were carried out in 25 µl reaction mixtures containing 50 ng of template DNA, 0.5 µl of 10 µM of each of the primers, 1 µl

of 2.5 mM dNTPs, 1 U of *Pfu* DNA polymerase, and 2.5 µl of 10X reaction buffer containing 1.5 mM $MgCl_2$. Amplification was carried out with an initial denaturation at 94 °C for 5 min, followed by 35 cycles, each consisting of denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s, elongation at 72 °C for 1-3 min with a final elongation at 72 °C for 10 min. Amplicons were detected by electrophoresis on 1% (w/v) agarose gels stained with ethidium bromide.

4.2.3 DNA manipulation and plasmid transformation

phlD and *phlACB* genes amplicons were cloned independently in pJET1.2 (Fig. 4.3) blunt ended cloning vector according to manufacturer's instruction (Thermo scientific, India). Blunt end products of PCR were cloned into blunt ended cloning vector. Amplicons were sub-cloned in the pUCPM18Gm vector using restriction sites presents in the primers. First the *phlD* genes of *P. protegens* Pf-5 and *Pseudomonas* sp. G22 were separately cloned using restriction sites KpnI and BamHI (giving rise to pAJK1.1a and pAJK1.1b, respectively), under the p*lac* promoter which is constitutively expressed in *Pseudomonas* spp. (Buch et al. 2009) (Fig. 4.4). Subsequently, *phlACB* genes of *P. protegens* Pf-5 and *Pseudomonas* sp. G22 were cloned downstream of the *phlD* genes into the BamHI and XbaI restriction sites in the respective plasmids. The resulting plasmids (pAJK1.2a and pAJK1.2b) were confirmed by PCR and restriction enzyme digestions. Ligation reaction and *E. coli* transformation were carried out using standard protocols described Sambrook and Russell 2001). Plasmid were subsequently transferred in to diazotrophic endophytes *Pantoea* sp. MS3, *Pseudomonas* sp. WS5, *Herbaspirillum seropedicae* Z67 and rhizosphere bacteria *P. protegens* Pf-5, *P. putida* G22, *Enterobacter* sp. C1D by bi-parental conjugation (Chapter 2, Section 2.2.7.2) by plasmid mobilizing strain *E. coli* S17.1 (Simon et al. 1983). Recombinant strains containing the pAJK1.2 plasmids were confirmed by plasmid isolation and PCR amplification of *phlD* and *phlACB* along with 16S rRNA gene amplification and restriction digestion (with HaeIII) patterns to confirm the host strain. All recombinant strains containing plasmids were grown in media containing 40 µg ml^{-1} gentamicin. Plasmid pJBA28 was also transferred to *Pseudomonas* sp. WS5 by bi-parental conjugation.

Plasmid isolation, restriction digestion and ligation reaction were carried out as described in Sambrook and Russell (2001). The enzymes and pJET cloning kit were procured from MBI Fermentas (Germany).

Table 4.2 PCR primer used in this study

PCR primers[a]	Sequence	Remark	Expected band size (bp)	Source
phlD *Kpn*I-F	5'C**GGTACC**AGAGGAGGA AAAATGTCTACACTTTGCC TTCCAC3'	Used to amplify *phlD* encoding type III polyketide synthase, a key biosynthetic gene for phloroglucinol	1080	This study
phlD *BamH*I-R	5'CTC**GGATCC**TTAGGCGG TCCACTCGC3'			This study
phlACB *BamH*I-F	5'C**GGATCC**AGAGGAGGA AAGTGAAAAAGATAGGTA TTGTCA3'	Used to amplify phlACB gene cluster encoding acetyltransferase, transfer the acetyl group to phloroglucinol at 2nd and 4th position to convert it 2,4-DAPG	2779	This study
phlACB *Xba*I–R	5'ACGGGC**TCTAGA**TTATAT ATCGAGTACGAACTTATAAG CGT3'			This study

[a] Nucleotides in bold represent the recognition sites for a restriction endonuclease and underlined sequence is the universal ribosomal binding site.

4.2.4 Phloroglucinol sensitivity test

Many bacterial strains were used initially as candidate for cloning 2,4-DAPG gene cluster. Strains were analyzed for the resistance to phloroglucinol (PG) and 2,4 –DAPG. Overnight grown cultures were inoculated in molten agar and different concentrations of PG and DAPG were added into the wells made in an agar plate. These plates were incubated at 30 °C except *E. coli* DH5α incubated at 37 °C.

4.2.5 Phloroglucinol Production

LB broth supplemented with 2% of glucose with gentamicin (40 μg ml^{-1}) was used for the production of PG for all recombinants except for *Pantoea* sp. MS3 which was grown in M9 minimal salt medium due to the interference of metabolites produced by this organism in rich medium. Cultures were grown in 50 ml broth incubated at 28 °C for 24 h at 180 rpm. *E. coli* DH5α was grown in similar medium but at 37 °C till it OD reached to 0.6 after which it was supplemented with 0.2 mM IPTG and further incubated at 28 °C for 24h.

For detection of PG produced, appropriate volume of the extract was spotted on TLC plate, allowed to dry and then sprayed with cinnamaldehyde-HCl [0.2% 4-hydroxy-3-methoxy-cinnamaldehyde in HCl:ethanol (v/v 1:3)] reagent for PG detection. For quantification of PG produced, the colorimetric method was used. An appropriate volume of the extract was made up to 300 µl with methanol, followed by addition of 100 µl of concentrated-HCL and 400 µl of the cinnamaldehyde-HCl solution as above. The reaction system was incubated for 5 min at room temperature and color developed was measured at 550 nm (Kidarsa et al. 2011). Pure PG was used for calibration. All the experiments replicated three times.

4.2.6 2,4-DAPG production, extraction and quantification

LB broth supplemented with 2% glucose and gentamicin (40 µg ml^{-1}) was used for the growth and production of 2,4-DAPG by the recombinant *Pseudomonas* strains carrying the pAJK1.2 plasmids and vector control. Cultures were grown in 50 ml broth incubated at 28 °C for 24 h to 120 h at 180 rpm. Cell-free supernatants obtained by centrifugation at 21,690xg for 10 min were acidified to pH 2.0 by the addition of 1N HCl and extracted twice with equal volume of ethyl acetate, evaporated till dryness and final content was dissolved in high performance liquid chromatophy (HPLC) grade methanol (1 ml). This extract, referred henceforth as ethyl acetate extract, was filtered through 0.2 µm nylon filter and used for bioassay. In experiments where 2,4-DAPG production was studied at different time points, the transformants were grown individually in 50 ml LB broth with gentamicin (40 µg ml^{-1}) till OD reached to 1.5 at 28 °C under shaking (180 rpm). Cultures washed twice with 0.9% NaCl (N saline) were used to inoculate fresh 50 ml LB amended with 2% glucose and gentamicin and incubated 28 °C under shaking (180 rpm) and every 2 h a flask was withdrawn and the culture supernatant extracted with ethyl acetate.

4.2.7 Quantification of 2,4- DAPG using HPLC

Quantitative analysis of 2,4-DAPG samples was carried out by injecting 20 µl of the ethyl acetate extract sample in an HPLC column run with the mobile phase consisting of 30% acetonitrile, 25% methanol, 45% double distilled water with the stationary phase C18 reverse phase silica column (Shimadzu, Japan). The flow rate was maintained 1 ml min^{-1} (Nowak-Thompson et al. 1994) and UV detector at 270 nm was used for monitoring the sample. Pure 2,4-DAPG was used for

standard curve preparation using peak area for calibration. All the estimations were replicated three times.

4.2.8 Antagonistic activity of ethyl acetate extracts

Antagonistic activity of ethyl acetate extracts was checked against *Staphylococcus aureus* (ATCC 6538), known to be specifically sensitive to 2,4-DAPG and two fungi *M. oryzae* B157 and *R. solani*. An overnight grown *S. aureus* culture in LB medium was seeded in top agar and poured on a pre-set LB agar plate. Wells were made by cup-borer (1 cm) and 100 µl ethyl acetate extracts were added to each well and incubated at 37 °C for 24 h after which zone of inhibition was observed. Each treatment was replicated three times and experiments performed twice.

The ethyl acetate extracts (100 µl) obtained from recombinant strains of endophytic bacteria were added into wells bored on PDA plates inoculated with *M. oryzae* B157 or *R. solani* and incubated for 10 d and 5 d respectively at 30 °C. The plates were observed for fungal growth inhibition. All the treatments were replicated three times and experiments repeated three times.

4.2.9 Surface sterilization and bacterization of seedlings

Surface sterilization and germination wheat, sorghum and rice seeds mentioned earlier (Chapter 3, Section 3.2.6). Germinated seedlings with similar radicle length were soaked in pure cultures (approximately 10^8 CFU ml^{-1}) of *Pseudomonas* sp. WS5 and recombinant strains harboring the empty vector and pAJK1.2 plasmid for 30 min.

4.2.10 Plant growth promotion and disease protection by recombinant endophytic bacteria

Plant growth promotion and disease protection was studied as described in (Chapter 3, Section 3.2.7). Only bacterial strains were replaced with wild type *Pseudomonas* sp. WS5, vector control (pUCPM18Gm), 2,4-DAPG producing recombinant strain of WS5 (pAJK1.2). All treatments were replicated three times and experiments repeated two times.

4.2.11 Defense gene expression analysis in rice plants

The wild type *Pseudomonas* sp. WS5 and its genetically modified DAPG producing recombinants were evaluated in a greenhouse pot inoculation experiment for their potential to induce defense related gene expression in rice plants. Pot experiment with rice plant was set up similar to that

mentioned in (Chapter 3, Section 3.2.8). On 8th d infection, plants were uprooted and processed for quantitative gene expression analysis. All qPCR reactions were carried out in duplicates

4.2.12 Statistical analysis

Microsoft Excel (Microsoft, 2007) were used for Levene's test and student's t-test analysis of the plant experiments. Each experimental data has been represented as mean ± SD in the table and number of replicates mentioned in each experiment. Quantitative real-time PCR calculation was performed as per instruction's mentioned in Applied Biosystems manual.

4.3 Results

4.3.1 Construction of plasmids containing *phlD* and *phlACB* genes

The pAJK1.2 plasmid construct was developed in two steps by firstly cloning the phloroglucinol synthase (*phlD*) gene under the constitutive p*lac* promoter in pUCPM18Gm expression vector. Downstream to *phlD* gene acetyltransferase (*phlACB*) gene cluster was added. The pAJK1.1 and pAJK1.2 constructs were confirmed by PCR amplification and restriction digestion. The pUCPM18Gm vector was taken as a control in this study. The intermediate cloning vector used in this study was pJET1.2 suicidal vector. The desired transformants carrying *phlD*, *phlACB* and *phlDACB* gene cluster were confirmed as shown in Figs. 4.5, 4.6, 4.7 and 4.8.

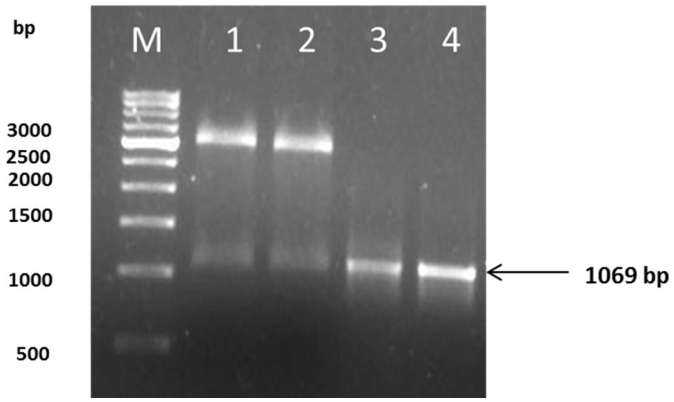


Fig. 4.5 Restriction endonuclease digestion of pJET 1.2 clone containing *phlD* gene with KpnI and BamHI and PCR amplification of *phlD* gene. Lane 1-Restriction digestion of pJET clone containing *phlD* gene from *P. protegens* Pf-5, Lane 2- Restriction digestion from pJET 1.2 clone containing *phlD* gene from *P. putida* G22, Lane 3- PCR amplification of *P. protegens* Pf-5 *phlD* gene from the clone, Lane 3- PCR amplification of *Pseudomonas* sp. G22 *phlD* gene from the clone Lane M- 500bp Marker

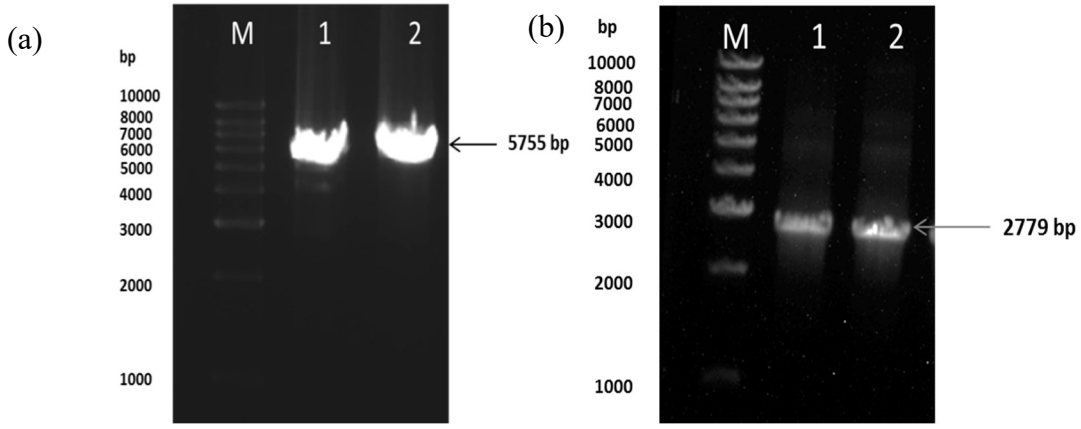


Fig. 4.6 Restriction endonuclease digestion of pJET 1.2 containing *phlACB* gene clusters with BamHI and PCR gene amplification. (a) Lane 1– Restriction digestion from pJET 1.2 clone containing *phlACB* from *P. protegens* Pf-5, Lane 2- Restriction digestion from pJET 1.2 clone containing *phlACB* from *P. putida* G22, (b) Lane 1 and 2- PCR amplification of *phlACB* from both clones, Lane M- 1kb ladder

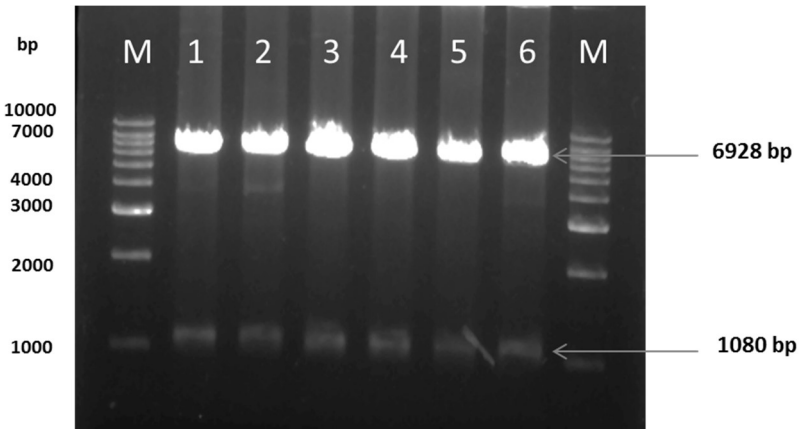


Fig. 4. 7 Restriction endonuclease digestion of pAJK1.1 plasmid with KpnI and BamHI to confirm the presence of *phlD* gene. Lane 1 to 3- pAJK1.1a transformants and Lane 4 to 5- pAJK1.1b transformants, Lane M- 1 kb ladder

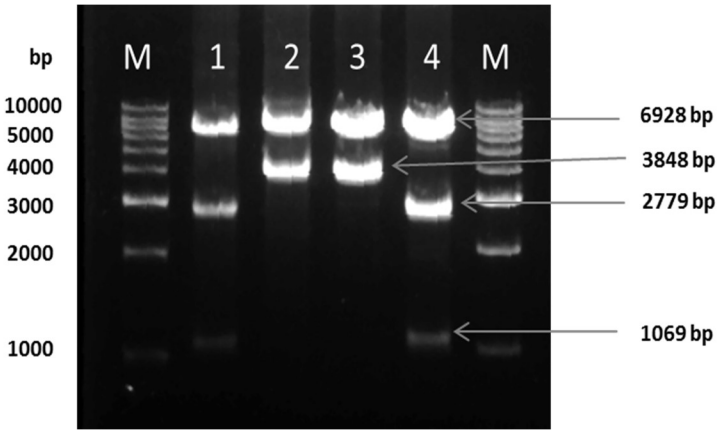


Fig. 4.8 Restriction endonuclease digestion patterns of pAJK1.2a and pAJK1.2b isolated from transformants of *Pseudomonas* sp. WS5. (Lanes 1 and 4) RE digestion with KpnI, BamHI, and XbaI to release the *phlD* (1.1 kb) and *phlACB* (2.8 kb) gene cluster; (Lanes 2 and 3) RE digestion with KpnI and XbaI to release the *phlDACB* (3.8 kb) gene cluster; (Lane M) 1 kb Ladder

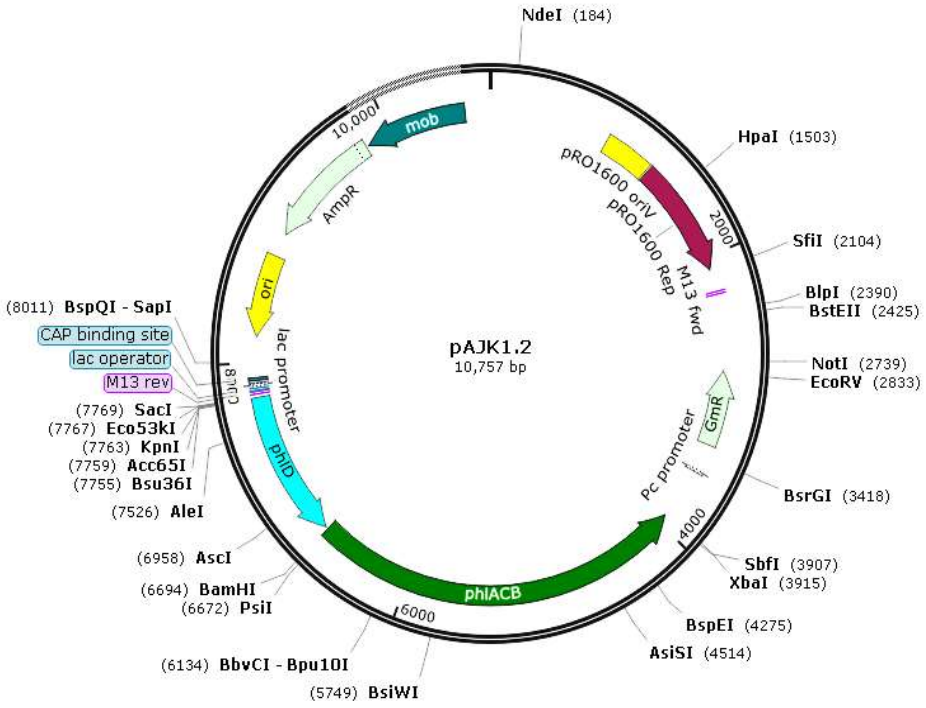


Fig. 4.9Restriction map of pAJK1.2 plasmid

4.3.2 Phloroglucinol tolerance

The selection of appropriate strains for the transformation of pAJK1.2 plasmids was done by checking for phloroglucinol sensitivity. The endophytic and rhizospheric strains showed growth till the 3 mg ml^{-1} concentration whereas *E. coli* DH5α tolerated only up to 1mg ml^{-1} concentration

of PG (Table 4.3) after that growth was inhibited. All the strains used in this study did not show inhibition to 2,4-DAPG till tested concentration of 10 µg ml^{-1} (data not shown) .

Table 4.3 Phloroglucinol tolerance of bacterial strains used in this study

Bacterial Strain	PG concentration (mg ml^{-1})				
	1	2	3	4	5
E. coli DH5α	+	-	-	-	-
Enterobacter sp. C1D	++	++	++	+	-
Pantoea sp. MS3	++	++	+	±	-
Pseudomonas sp. WS5	++	++	+	-	-
H. seropedicae Z67	++	+	-	-	-

[a] ++: Luxuriant growth, +: moderate growth, ±: weak growth, -: no growth

The pAJK1.1a and 1.1b plasmids were transformed in *E. coli* DH5α, *Enterobacter* sp. C1D, *Pantoea* sp. MS3, *Pseudomonas* sp. WS5 and *H. seropedicae* Z67. Phloroglucinol production was detected in diazotrophic endophyte *Pseudomonas* sp. WS5 among all the strains (Fig. 4.10). The other strains transformed with pAJK1.1 plasmid did not show the stable production of phloroglucinol and hence all further studies were done with *Pseudomonas* sp. WS5.

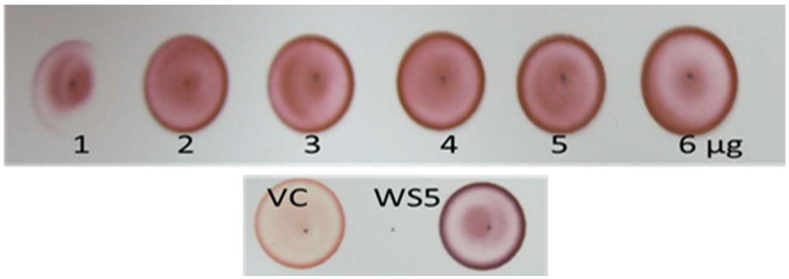


Fig. 4.10 Detection of PG production in *Pseudomonas* sp. WS5. Upper panel shows the different concentration of pure PG. WS5- *Pseudomonas* sp. WS5 harboring pAJK1.1 plasmid; VC - *Pseudomonas* sp. WS5 harboring pUCPM18Gm

4.3.3 2,4-DAPG produced by diazotrophic endophytic *Pseudomonas* sp. WS5 transformants

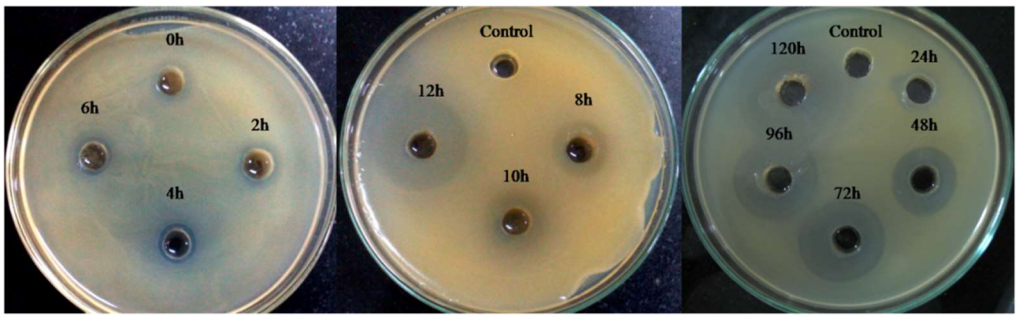
pAJK1.2 plasmids were transformed in diazotrophic endophytic bacterium *Pseudomonas* sp. WS5. The stability of the plasmid was checked on nitrogen-free medium for many generations without selective pressure and then analyzed for 2,4-DAPG production as well as presence

plasmid (Appendix I). A phenotypic character change was observed upon transfer of the pAJK1.2 plasmids in the endophytic system. There was the production of red pigment on King's B agar and broth observed in recombinant strains of *Pseudomonas* sp. WS5 (Fig. 4.11). For all purpose, the, plasmid pAJK1.2a and 1.2b were similar in the phenotype and when not specified it is implied for both the plasmids.

Fig. 4.11 Red color pigment production in plate and broth by *Pseudomonas* sp. WS5 transformants carrying pAJK1.2 plasmid

Qualitative analysis of DAPG obtains from genetically modified *Pseudomonas* sp. WS5 strains showed the increasing zone of inhibition with the time. In this study, wild-type *P. protegens* Pf-5 and lab isolate *Pseudomonas* sp. G22 were used to compare with the DAPG production by genetically modified strains. Bioassay with *S. aureus* showed the production of antimetabolite by the recombinant strains but not the vector control (Fig. 4.13). Standard strains showed significant amount of DAPG production starting at 10 h whereas the recombinant strains showed the production from 2 h (Fig. 4.12).

(a)



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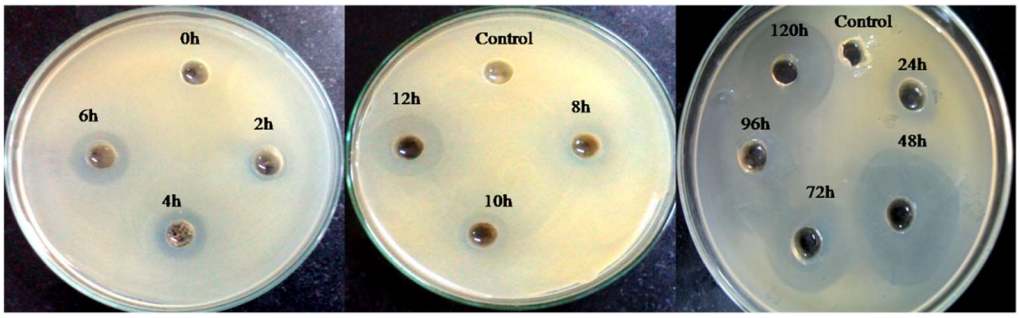
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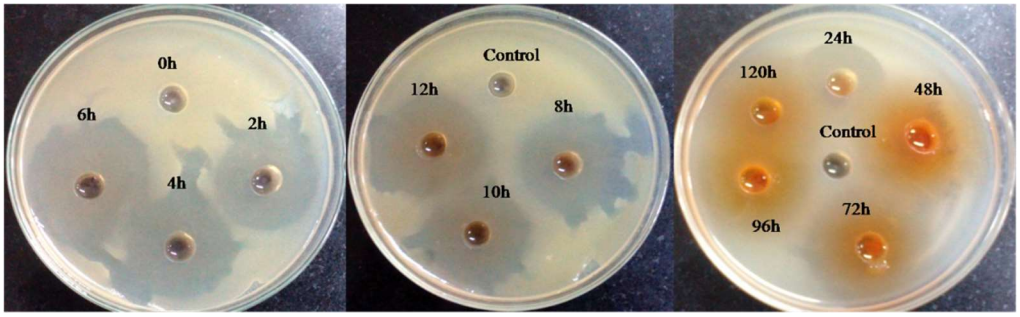
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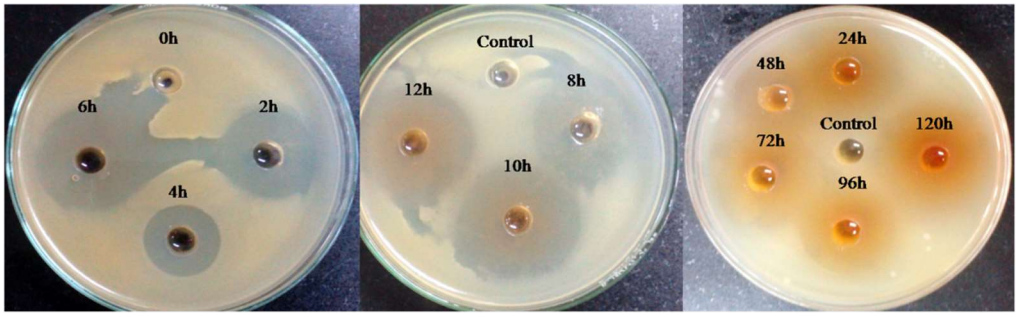
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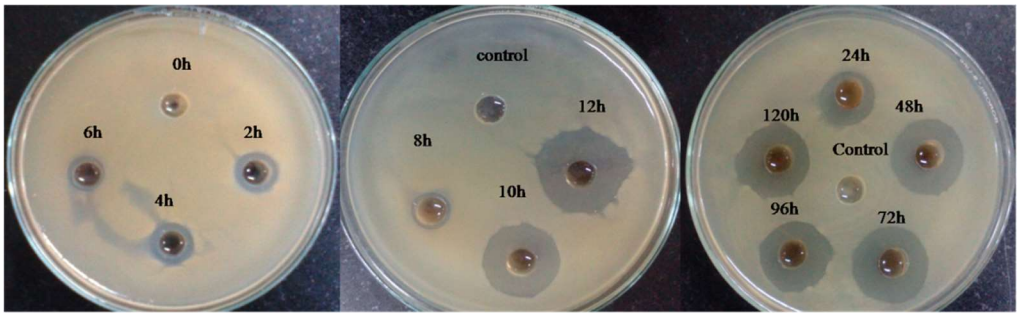
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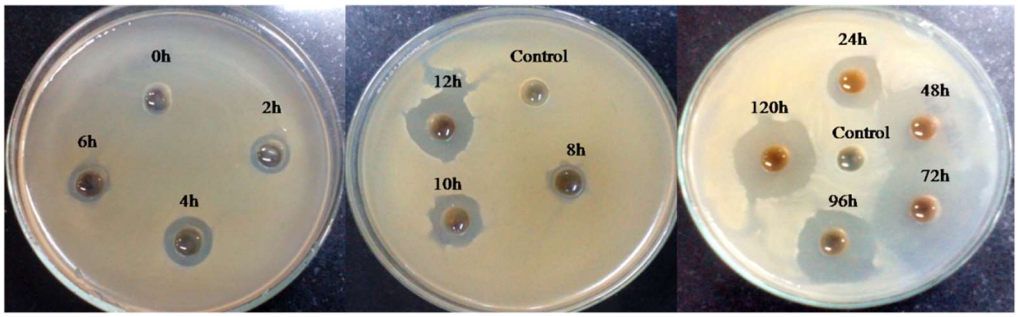
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Fig. 4.12 Detection of DAPG produced by wild type standard and recombinant strains. (a) *P. protegen* Pf-5, (b) *Pseudomonas* sp. G22, (c) *P. protegens* Pf-5 (pAJK1.1a), (d) *P. protegens* Pf-5 (pAJK1.2a), (e) *Pseudomonas* sp. WS5 (pAJK1.2a), (f) *Pseudomonas* sp. WS5 (pAJK1.2b). Control- ethylacetate extract of from wild type *Pseudomonas* sp. WS5 and Vector control

4.3.4 Heterologous 2,4-DAPG production in diazotrophic endophytic *Pseudomonas* sp. WS5

DAPG was extracted at every 24 h of incubation and analyzed for the production using HPLC technique. The highest production observed with the *P. protegens* Pf-5 and *Pseudomonas* sp. G22 strains was recorded after 120 h of incubation as 1.28 µg ml^{-1}, 1.53 µg ml^{-1} respectively (Fig. 4.13). A similar production achieved with the genetically modified strains of *Pseudomonas* sp. WS5 carrying pAJK1.2a and pAJK1.2b plasmids after 24 h of incubation was 1.66 µg ml^{-1}, 1.10 µg ml^{-1} respectively.

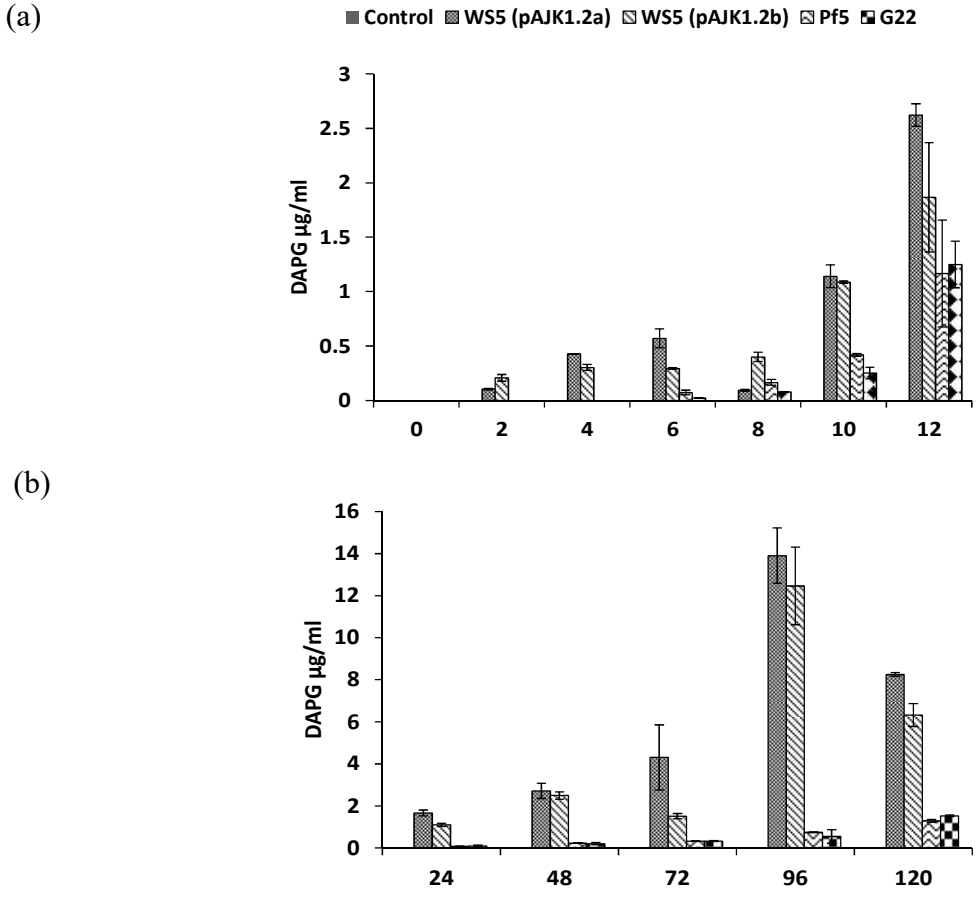


Fig. 4.13 Quantitative 2,4- Diacetylphloroglucinol production by wild type and recombinant strains of *Pseudomonas* spp. (a) Time course study from 0 to 12 h, (b) Time course study from 24 to 120 h. Control- uninoculated LB and wild type *Pseudomonas* sp. WS5 and vector control; WS5 (pAJK1.2a)-

Pseudomonas sp. WS5 harboring pAJK1.2a; WS5 (pAJK1.2b)- *Pseudomonas* sp. WS5 harboring pAJK1.2b; Pf-5- *Pseudomonas protegens* Pf-5, G22- *Pseudomonas* sp. G22

4.3.5 Antifungal activity of DAPG against fungal pathogen

Antifungal extracts obtained from the genetically modified diazotrophic endophytic *Pseudomonas* sp. WS5 were applied against the *M. oryzae* B157 and *R. solani* in plate assay and showed the significant growth inhibition whereas the extracts from vector control and the native strain not show the inhibition (Fig. 4.14).

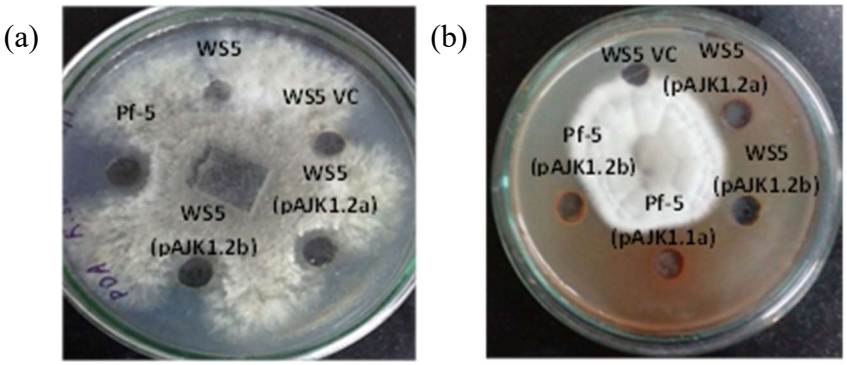


Fig. 4.14 Antagonistic effect of extracts from *phlDABC* overexpressing recombinant strains of *Pseudomonas* sp. WS5 and *Pseudomonas* protegens Pf-5 on fungal pathogens. (a) *R. solani*, (b) *M. oryzae* B157. Refer Fig. 4.13 legend for further strain denotation

4.2.6 Colonization of endophytic bacterium *Pseudomonas* sp. WS5 in various plants

Plant colonization by *Pseudomonas* sp. WS5 as observed by tagging the strain with *gfp.* Results showed that the bacteria colonized from root hair to colonize intracellular spaces of root in the wheat (Fig. 4.15). The endophytic colonization of interior tissues of sorghum and rice were analyzed by CFU count where wheat and sorghum roots showed comparatively higher colonization (10^6-10^7 CFU g^{-1} of fresh weight of tissue) and lesser colonization in the stem and leaves (10^2-10^3 CFU g^{-1} of fresh weight of tissue). In rice plants colonization in the root, stem and leaves (10^2- 10^4 CFU g^{-1} of fresh weight) was after 7 d of inoculation.

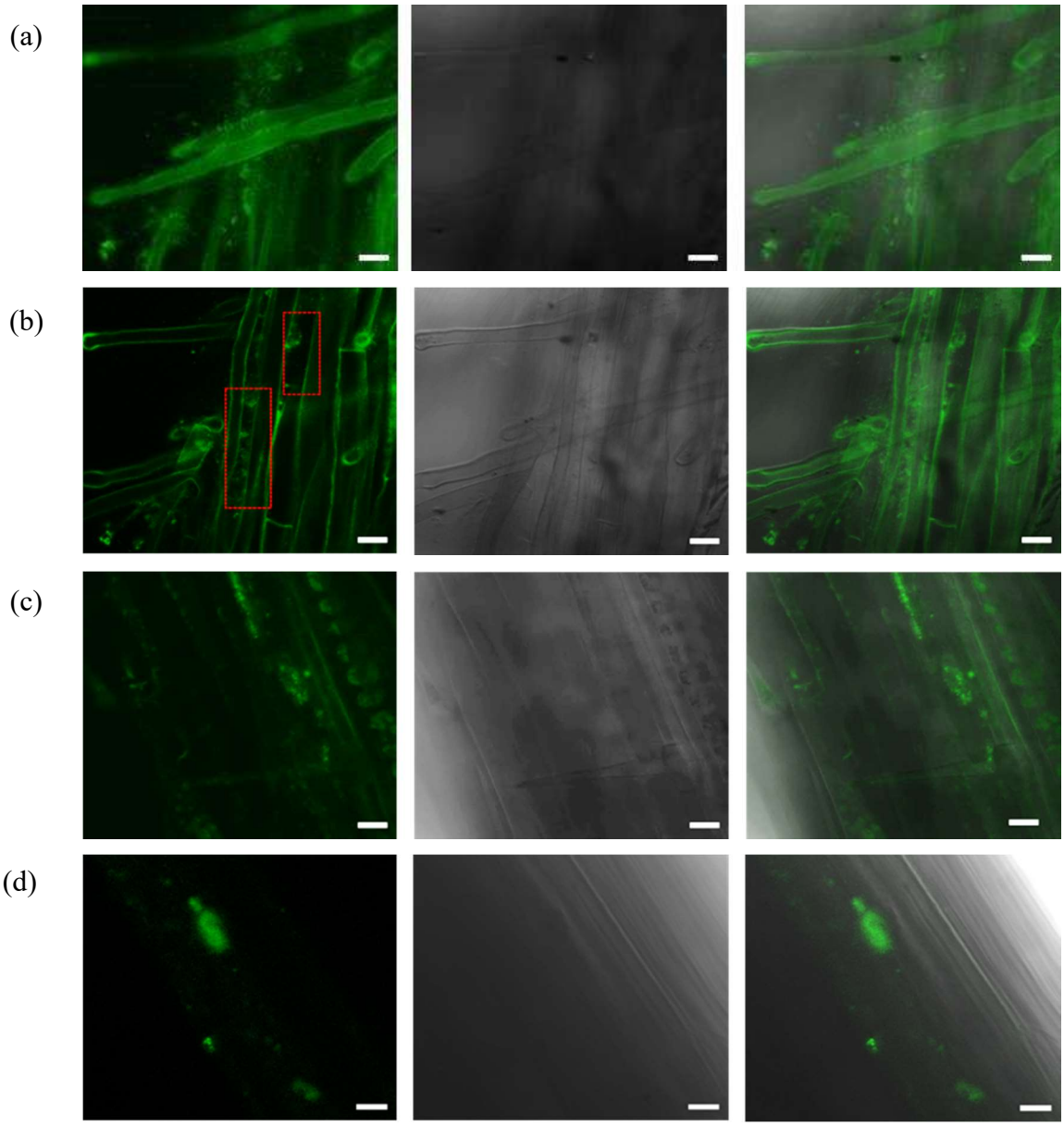


Fig. 4.15 Visualization of endophytic colonization of *gfp* tagged *Pseudomonas* sp. WS5 in wheat plants by confocal laser scanning microscopy. (a) Root hair surface interaction, (b) Root intracellular spaces colonization, (c) Stem intracellular colonization, (d) Leaf intracellular colonization. Rectangular box showing the endophytic colonization (b); Scale bar represent: (a-c) 20 µm, (d) 10µm

4.3.7 Plant growth promotion by 2,4-DAPG producing transformants of *Pseudomonas* sp. WS5 in cereals

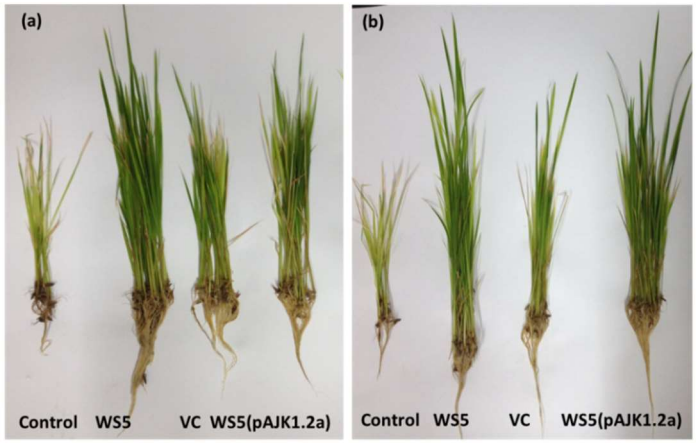
Plant growth promotion was observed in rice, sorghum and wheat plants upon bacterization with the native and recombinant strains at seedling level after 60 d of growth period in green house

(Fig. 4.16). All three plants showed enhanced growth of above as well as below ground parts of the plant which is also reflected in root:shoot ratios. Significant increase in vigour index was also observed in bacterized plants. Rice plants bacterized with WS5, VC and WS5 (pAJK1.2a) showed an increase in wet weight of shoot up to 261%, 242% and 198% respectively (Table 4.4). In sorghum plants WS5, VC and WS5 (pAJK1.2a) increase wet weight of shoot up to 17%, 13% and 38% respectively. Similarly, wheat plant WS5, VC and WS5 (pAJK1.2a) showed increase by 15%, 11% and 21%. Rice plants showed the significant improvement in wet weight of root as well in plants bacterized with WS5, VC and WS5 (pAJK1.2a) by increase 161%, 125% and 144%

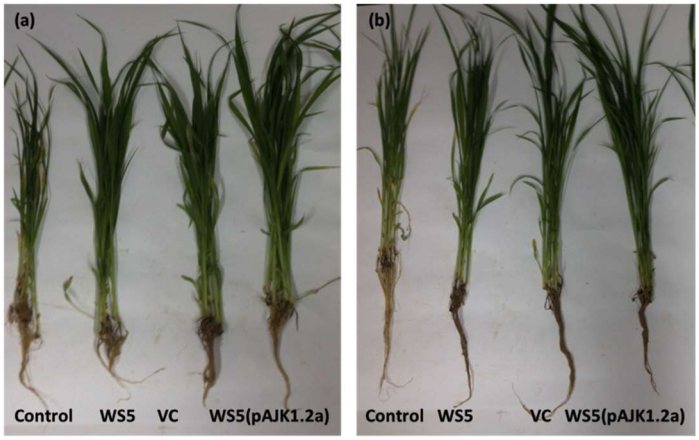
4.3.8 Plant protection upon pathogen challenge

Plants challenged with the phytopathogens analyzed after 60 d (30 d of healthy growth followed by 30 d of growth after pathogen challenge) showed the reduction in plant growth due to disease condition (Fig. 4.17). The plants inoculated with *Pseudomonas* sp. WS5, VC and WS5 (pAJK1.2a) strains showed the enhanced growth compared to control (unbacterized) plants under pathogen attack (Table 4.5). In rice plants bacterized with *Pseudomonas* sp. WS5 carrying the pAJK1.2a plasmid when infected with *M. oryzae* B157 showed the good maintenance of the above and belowground growth compared to those bacterized with *Pseudomonas* sp. WS5 and VC. The wet weight of shoot in unbacterized plants was observed to be reduced by 28% by fungal infection as compared to the unbacterized non-infected plants whereas the bacterized plants showed plant protection and increased shoot weight up to 283%, 204% and 321% in *Pseudomonas* sp.WS5, VC and WS5 (pAJK1.2a) inoculated plants respectively as compared to the unbacterized fungal challenged control. In case of sorghum plants, protection was observed in both WS5 and WS5 (pAJK1.2a) inoculated plants. The wet weight of shoot in bacterized sorghum plants were observed to be 215% and 193% in WS5 and WS5 (pAJK1.2a) inoculated plants compared to fungal treated, unbacterized plants. In wheat plants, overall plant protection was observed in case of plants inoculated with WS5 (pAJK1.2a). The vigour index of all bacterized rice, sorghum and wheat plants was significantly higher as compared to unbacterized fungal challenged control. The root:shoot ratio of all three plants suggest the all the bacterized plants promote the growth of aerial part of the plant (Table 4.5).

(A)



(B)



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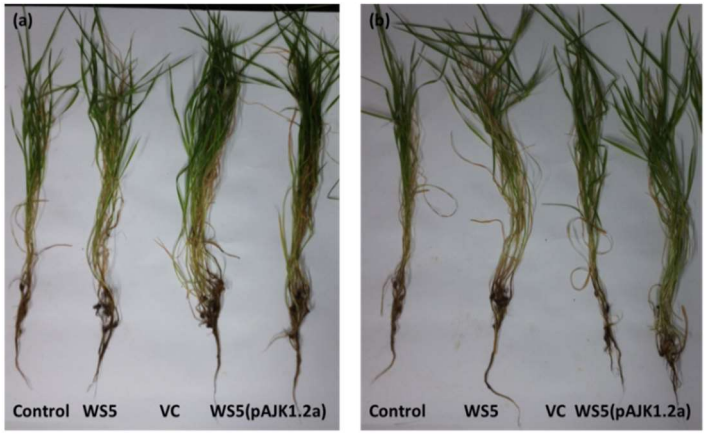


Fig. 4.16 Study of plant growth promotion and disease suppression by *Pseudomonas* sp. WS5 wild type and 2,4- DAPG producing recombinant strains inoculating in sorghum, wheat and rice plants. (A) Rice plants, (B) Sorghum plants, (C) Wheat plants- (a) unbacterized and bacterized plants, (b) unbacterized and bacterized plants challenged with fungal pathogen *R. solani* (wheat and sorghum) and *M. oryzae* (rice). Control-unbacterized, WS5- *Pseudomonas* sp. WS5 wild-type; VC- *Pseudomonas* sp. WS5 harboring pUCPM18Gm; WS5 (pAJK1.2a) - *Pseudomonas* sp. WS5 harboring pAJK1.2a

Table 4.4 Effect of 2,4-DAPG producing transformant of endophytic diazotrophic bacterium *Pseudomonas* sp. WS5 on rice, sorghum and wheat plants

Treatment	Root length (cm)	Shoot length (cm)	Wet weight of Root (mg)	Wet weight of Shoot (mg)	Root:shoot mass ratio	Vigour index
	Rice plants					
Unbacterized	6.45±0.54	19.88±0.65	27.50±1.38	45.83±2.32	0.60±0.05	2633±97.91
Pseudomonas sp. WS5	10.42±0.26*** (61% ↑)	23.63±2.05** (19% ↑)	71.83±10.83*** (161%)	165.33±17.41*** (261% ↑)	0.44±0.06	3405±218.24*** (29% ↑)
Pseudomonas sp. WS5 VC[a]	9.72±0.73*** (51% ↑)	19.67±0.72 (1% ↓)	62±6.23*** (125% ↑)	156.83±13.38*** (242% ↑)	0.40±0.06	2938±107.97*** (12% ↑)
Pseudomonas sp. WS5 (pAJK1.2a)	12.75±0.63*** (98% ↑)	23.13±2.87* (16% ↑)	67.17±3.19*** (144% ↑)	136.50±19.77*** (198% ↑)	0.50±0.09	3588±252.78*** (36% ↑)
	Sorghum plants					
Unbacterized	10±0.59	35.30±2.66	101.17±5.91	712.67±17.74	0.14±0.01	4530±257.60
Pseudomonas sp. WS5	15.32±0.77*** (53% ↑)	41.42±1.80** (17% ↑)	131.83±3.97*** (30% ↑)	836.83±143.74 (17% ↑)	0.16±0.03	5673±208.58*** (25% ↑)
Pseudomonas sp. WS5 VC[a]	14.98±0.66*** (50% ↑)	38.52±0.50* (9% ↑)	134.50±10.63*** (33% ↑)	802.83±8.93** (13% ↑)	0.17±0.01	5350±61.32*** (18% ↑)
Pseudomonas sp. WS5 (pAJK1.2a)	16.50±1.35*** (65% ↑)	45.78±1** (30% ↑)	135.33±8.89*** (34% ↑)	980.83±41.42*** (38% ↑)	0.14±0.01	6228±198.0*** (37% ↑)
	Wheat plants					
Unbacterized	11.78±0.70	47±3.10	54±7.92	585±21.97	0.09±0.02	5878±255.53
Pseudomonas sp. WS5	15.70±0.61*** (33% ↑)	56.63±1.06*** (20% ↑)	78.33±10.37** (45% ↑)	671.33±20.08*** (15% ↑)	0.12±0.02	7233±131.10*** (23% ↑)
Pseudomonas sp. WS5 VC	14.95±0.74*** (27% ↑)	55.63±1.63*** (18% ↑)	76.17±3.71*** (41% ↑)	649.67±35.54** (11% ↑)	0.12±0.01	7058±111.07*** (20% ↑)

Pseudomonas sp. WS5 (pAJK1.2a)	17.72±0.62*** (50% ↑)	48.17±4.17 (2% ↑)	76.83±3.54*** (42% ↑)	710.17±35.47*** (21% ↑)	0.11±0.01	6588±397.76** (12% ↑)

±, Standard deviation; [a] VC indicates vector control i.e. *Pseudomonas* sp.WS5 carrying pUCPM18Gm; Asterisks indicate the significant differences at *P<0.05, **P<0.01, ***P<0.001 for the same plant parameter compared between respective unbacterized plants and plants treated with *Pseudomonas* sp. WS5 or its transformants; Values in brackets are the percentage increase (↑) or decrease (↓) in the particular plant parameter compared to the respective unbacterized plants.

Table 4.5 Effect of 2,4-DAPG producing transformant of endophytic diazotrophic bacterium *Pseudomonas* sp. WS5 on rice, sorghum and wheat plants infected with *Magnaporthe oryzae* B157 or *Rhizoctonia solani*

Treatment	Root length (cm)	Shoot length (cm)	Wet weight of Root (mg)	Wet weight of Shoot (mg)	Root:Shoot mass ratio	Vigour index
	Rice plants infected with *M. oryzae* B157					
Unbacterized[b]	5.48±1.45 (15% ↓)	10.15±1.59 (49% ↓)	19.33±4.59 (30% ↓)	33.17±5.60 (28% ↓)	0.59±0.15	1563±187 (41% ↓)
Pseudomonas sp. WS5	5.17±0.42 (6% ↓)	20.10±1.71*** (98% ↑)	43.50±2.74*** (125% ↑)	127.17±13.27*** (283% ↑)	0.34±0.04	2527±201*** (62% ↑)
Pseudomonas sp. WS5 VC[a]	5.12±0.20 (7% ↓)	16.92±1.17*** (67% ↑)	39.33±6.86*** (103% ↑)	100.67±8.33*** (204% ↑)	0.39±0.07	2203±125*** (41% ↑)
Pseudomonas sp. WS5 (pAJK1.2a)	9.57±0.37*** (74% ↑)	22.83±3.69*** (125% ↑)	56±2.76*** (190% ↑)	139.5±14.86*** (321% ↑)	0.41±0.05	3240±361*** (107% ↑)
	Sorghum plants infected with *R. solani*					
Unbacterized[b]	6.88±0.15 (31% ↓)	29.03±1.96 (18% ↓)	44.33±4.18 (56% ↓)	397.67±26.66 (44% ↓)	0.11±0.01	3592±188.83 (21% ↓)
Pseudomonas sp. WS5	10.52±1.12*** (53% ↑)	41.78±0.53*** (44% ↑)	93±5.02*** (110% ↑)	1253.33±92.01*** (215% ↑)	0.07±0.01	5230±84.62*** (46% ↑)
Pseudomonas sp. WS5 VC[a]	11.43±0.41*** (50% ↑)	34±3.35* (17% ↑)	74±3.95*** (67% ↑)	770.3±47.13*** (94% ↑)	0.10±0.01	4543±313.86*** (26% ↑)
Pseudomonas sp. WS5 (pAJK1.2a)	12.10±0.32*** (65% ↑)	41.65±0.69*** (43% ↑)	86.33±4.80*** (95% ↑)	1166.67±44.57*** (193% ↑)	0.07±0.01	5375±76.62*** (50% ↑)

	Wheat plant infected with *R. solani*					
Unbacterized[b]	7.20±0.31 (39% ↓)	34.33±3.83 (27% ↓)	45.83±5.34 (15% ↓)	338±37.62 (42% ↓)	0.14±0.02	4153±392.46 (29% ↓)
Pseudomonas sp. WS5	10.45±2.41* (45% ↑)	45.70±2.15*** (33% ↑)	60±7.04** (31% ↑)	535.67±21.44*** (59% ↑)	0.11±0.01	5615±423.21*** (35% ↑)
Pseudomonas sp. WS5 VC[a]	8.05±0.68* (12% ↑)	38.50±3.62 (12% ↑)	62.67±7.09*** (37% ↑)	498.33±40.03*** (48% ↑)	0.13±0.02	4655±300.45* (12% ↑)
Pseudomonas sp. WS5 (pAJK1.2a)	11.68±0.64*** (62% ↑)	50.42±1.01*** (47% ↑)	77±7.43*** (68% ↑)	540±29.14*** (60% ↑)	0.14±0.01	6210±121.16*** (50% ↑)

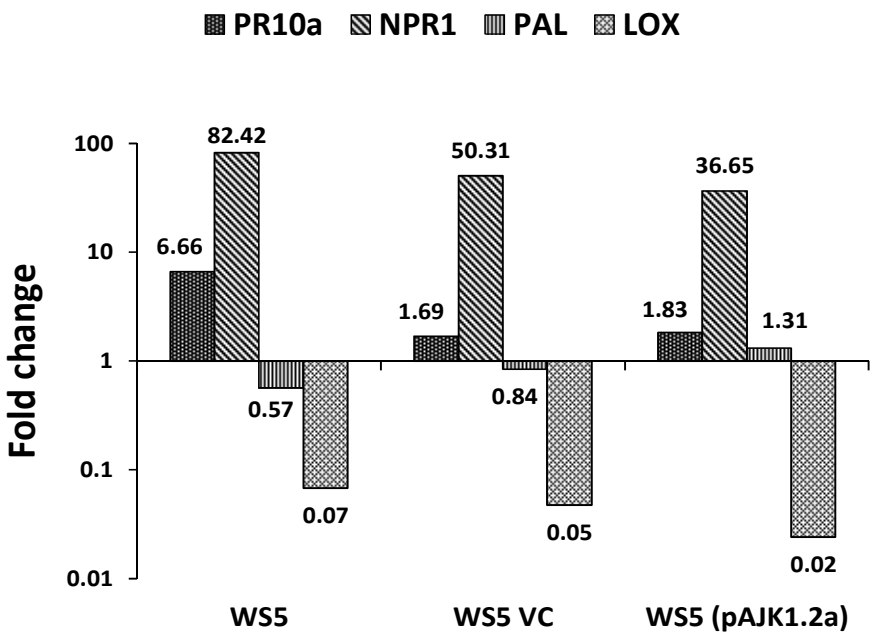
[b] Unbacterized fungal infected plant parameters are compared with the corresponding parameter of the respective unbacterized uninfected plants.

Please refer table 4.4 for further symbols information.

4.3.9 Defense gene expression in rice upon bacterization with 2,4-DAPG producing recombinant strains of *Pseudomonas* sp. WS5

In order to check whether the wild type and recombinant 2,4-DAPG producing strains are proficient at inducing systemic resistance in plants against fungal attack, bacterized rice plants were challenged with the fungal pathogen *M. oryzae* B157 after 30 d growth period and after 30 d of fungal challenge key marker genes of JA and SA defense pathways were analyzed for quantitative gene expression and compared with unchallenged bacterized rice plants. As seen (Fig. 4.17a), bacterized plants showed the high expression of *NPR1* genes as compared to unbacterized rice plants, with 82.42 and 36.65 fold increase in plants inoculated with the wild type and 2,4-DAPG producing strain respectively. Upon the blast fungal attack, the expression of *NPR1* gene in rice plants was found to be higher by 58.63 and 32.34 fold in wild type and 2,4-DAPG producing strain respectively as compared to the fungal treated unbacterized plants (Fig. 4.17b). Along with this, expression of *PR10a* gene was also observed to be increased by 9.10 fold in plants inoculated with the recombinant strains (Fig. 4.17b). Lesions of infection were observed as spot development after 4-5 d on bacterized plants whereas the uninoculated control showed patches of drying on leaf due to spread of infection (Fig. 4.18). All the bacterized plants were able to survive during pathogen challenge.

(a)



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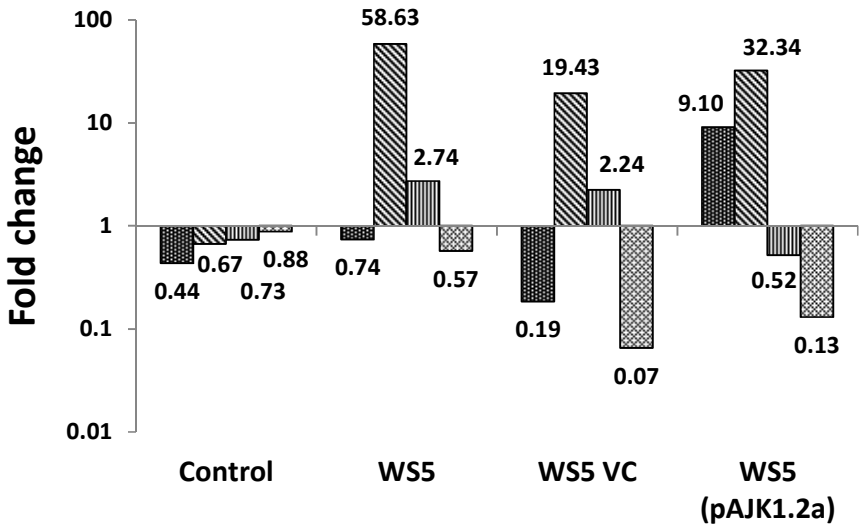

Fig. 4. 17 Quantitative real time PCR analysis of defense related genes in rice plants colonized by endophytic *Pseudomonas* sp. WS5 and its 2,4-DAPG producing recombinant derivative against rice pathogen *M. oryzae* B157. (a) Without fungal infection, (b) Upon challenge with the blast fungus. Standard deviation values are incorporated into the data. Fold change calculated by comparing with gene expression in the unbacterized plants. Strain denotation is as mentioned in Fig. 4.16

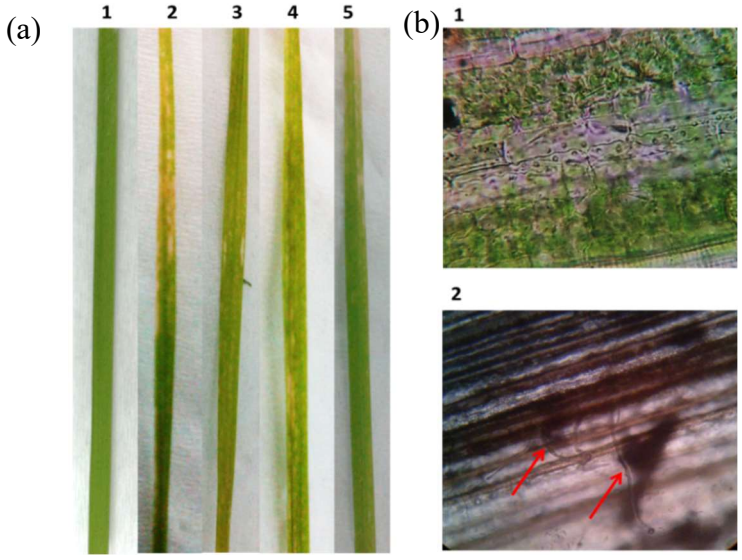

Fig. 4.18 Study of the disease development in rice plants during *M. oryzae* B157 infection in control plants and plants bacterized with *Pseudomonas* sp. WS5 or its 2,4-DAPG producing transformants. (a) Disease spot development in rice plants after various treatments; (1) *Pseudomonas* sp. WS5, (2) *M. oryzae* B157, (3) *Pseudomonas* sp. WS5 and *M. oryzae* B157 (4) *Pseudomonas* sp. WS5 harboring empty vector and *M.*

oryzae B157, (5) *Pseudomonas* sp. WS5 (pAJK1.2a) and *M. oryzae* B157; (b) Microscopic view (1) Control leaf, (2) Fungal invasion (arrows) in interior tissues of unbacterized leaf.

4.4 Discussion

Endophytic bacteria promote plant growth by similar mechanisms as the rhizospheric (root associated) bacteria and can also prevent pathogen growth by production of antimicrobial compounds. However, they are envisaged as more effective biocontrol agents than rhizosphere bacteria due to their sharing the same niche as pathogens inside the plant parts and thus have the potential to provide systemic protection to the plants. The characteristics of several diazotrophic endophytic plant growth promoting bacteria showed that most of them did not possess any antagonistic activity against the phytopathogenic fungi. Present work deals with the incorporation of the gene cluster directing the biosynthetic pathway for a potent antifungal compound, 2,4-DAPG in the endophytic bacterium *Pseudomonas* sp. WS5 that was isolated from wheat plant and has inherently other plant growth promoting traits such as nitrogen fixation ability, siderophore production and IAA production (Chapter 2).

The 2,4-DAPG biosynthetic gene cluster comprising of 4 cistrons, *phlDABC* was constructed by PCR cloning from two well characterized *Pseudomonas* strains, *P. protegens* Pf-5 (Paulsen et al. 2005), *Pseudomonas* sp. G22 (Lab isolate) and both were found to be equally effective for the production of 2,4-DAPG. The expression of the cloned genes in *Pseudomonas* sp. WS5 resulted in significantly high productivity of not only 2,4-DAPG but also its non-acetylated precursor phloroglucinol (Achkar et al. 2005) as indicated by the presence of red pigment only in the recombinant strains. Such pigment formation was not seen in case of the native strains which were the original source of the genes. The higher production by heterologous cloning in recombinant strain as compared to the native strains could be due to several reasons; one of them being the use of *E. coli lac* promoter to drive the expression, as this is known to be a strong and constitutively expressed promoter in pseudomonads (Buch et al. 2009). Besides, in the native state, the *phl* genes are under the positive as well as negative feedback regulatory loops mediated through the repressors encoded by *phlF* and *phlH* genes in different strains (Haas and Keel 2003), both of which have been excluded in the artificial construct used in this study. *Pseudomonas* spp. WS5 is phylogenetically affiliated to *P. putida* while the native strains are *P. protegens.* This difference in the host background might have also a role in the higher production

through appropriate metabolic support by a higher supply of the precursor malonyl-CoA (Zha et al. 2009). Another contributory factor to the early and higher productivity in the recombinant strains might be gene order, which in in native strains it is *phlACBD* while in the artificial construct is *phlDACB,* where the location of the key biosynthetic gene *phlD*, encoding the polyketide synthase catalyzing phloroglucinol synthesis is immediately adjoining the promoter. In accordance with the observation of Achkar et al. (2005) for *E. coli*, we too found that the absence of *phlE* gene, implicated in the export of 2,4-DAPG, did not occlude its extracellular secretion in the recombinant strain.

In Chapter 2, endophytic bacteria isolated from different cereal plants such as rice, pearl millet maize etc. were monitored for colonization in wheat plants and showed efficient colonization regardless of which crop they were isolated from. Since *Pseudomonas* spp. WS5 was itself a wheat isolate, its colonization of other plants was not reported in Chapter 2. The present study showed that *Pseudomonas* spp. WS5 was promiscuous in its colonization and entered into the above-ground parts of rice and sorghum. Bacterial attachment to the root hair was noticed which may the possible way of entry inside the host plants by this endophytic bacterium and subsequent travel to the aerial parts of the plant (Compant et al. 2010).

Since the recombinant strains produced high and uncontrolled levels of bioactive 2,4-DAPG, and the strain could colonize at least three important cereal plants, it was of interest to study the effectiveness of recombinant endophytic bacterium in plant growth promotion and combating pathogen attack with the hypothesis that it would possess a broad spectrum of pathogen control capacity. Indeed both the native as well as recombinant strains promoted growth of all three plants under healthy (disease-free) conditions implying that the inherent plant growth promoting attributes of the native strain were not compromised by the presence of the plasmid. This is remarkable since earlier work showed that presence of plasmid imparts metabolic load on the bacterium and reduces its plant growth promoting phenotypes such as gluconic acid mediated P-solubilization (Sharma et al. 2011). *Pseudomonas* sp. WS5 does not display P-solubilization but possesses other plant growth promoting traits which apparently are not adversely affected by the plasmid maintenance. The difference in the wild-type and recombinant derivatives of *Pseudomonas* sp. WS5 was clearly seen when the plants were challenged with the phytopathogenic fungi. The 2,4-DAPG producing recombinant strain had clearly better protective ability than the wild-type. That the wild-type strain also offered plant protection against fungal

attack was an interesting observation. Since the strain does not display any antagonistic activity against the fungi it was surmised that it might induce plant defenses. It is known that endophytic bacteria act as moderate pathogens while entering inside the plant, and often trigger the plant's own systemic resistance (Ryan et al. 2008). The wild-type strain was more effective in controlling *R. solani* infection of wheat and sorghum but not *M. oryzae* infection of rice. The 2,4-DAPG producing strain was effective against both the pathogens. Iavicoli et al. (2003) had concluded that the 2,4- DAPG molecule could act as a trigger to induce the defense responses in plants. Thus the 2,4- DAPG producing recombinant strain showed greater plant protection than the native strain because of the direct antagonistic activity of 2,4- DAPG as well as indirect effects by inducing the plant defense machinery. It should also be pointed out that the fungal infection in present work was carried out by spraying fungal spore suspension on above-ground parts; hence the endophytic strain was able to combat fungal attack through aerial mode.

Rice plants inoculated with wild type and vector control strain showed the upregulation of defense related gene *PR10a* and *NPR1* whereas the recombinant strain producing 2,4-DAPG also showed the modest upregulation of *PAL* gene. But when plants were challenged with *M. oryzae* B157 fungal pathogen, upregulation of NPR1 and PAL genes was observed in wild type and vector control strain inoculated rice plants. Plants bacterized with 2,4-DAPG producing recombinant *Pseudomonas* sp. WS5 challenged with *M. oryzae* B157 showed the upregulation of *PR10a* and *NPR1* and none of the rice plants showed upregulation of *LOX2* gene in any condition which associated with JA pathway. Interestingly, NPR1 gene was commonly upregulated in fungal non-challenged and challenged plants. The overexpression of NPR1 gene in transgenic plants is associate a with the high expression of PR genes under pathogen attack (Cao et al. 1998) is modulated by salicylic acid (SA) changes the redox potential converts the NPR1 protein from oligomeric to monomeric. The active monomeric form of *NPR1* enters the nucleus and triggers the transcription of PR genes (Pieterse et al. 2014). PR10a gene expression was observed in case of plants challenged with the *M. oryzae* as well as by application SA and JA inoculation to the healthy rice plants (McGee et al. 2001). PAL gene involved in SA synthesis which ultimately leads to SAR pathway activation. The results from this study demonstrate the induction of SAR pathway in plants challenged with *M. oryzae* B157 and bacterized with *Pseudomonas* sp. WS5, vector control and 2,4-DAPG producing *Pseudomonas* sp. WS5. The recombinant strain carrying the empty vector showed a lesser induction of defense related gene expression compared to the

wild type strain showing that ability to induce defense responses may be compromised due to plasmid load. This is reminiscent of a similar earlier observation wherein this vector was found cause the metabolic perturbation in *P. fluorescens* 13525 (Buch et al. 2010). The 2,4-DAPG producing recombinant strain showed additionally an induction of the PR gene when plants were challenged with fungus. This demonstrates that 2,4-DAPG induces a stronger defense response against the pathogen attack and the JA pathway does not seem to play role in this case.

It has long been suggested that endophytic bacteria with the multiple plant growth promoting traits can be further improved by genetic modification to enhance their benefits to plants (Compant et al. 2005). The present study exemplifies such a case in which the genetic modification of a plant growth promoting endophytic bacterium was carried out to further build upon its plant beneficial traits. The results provide useful insight in to the incremental benefit that can be provided to plants by heterologous gene expression leading to 2,4- DAPG production. The native as well as 2,4-DAPG producing recombinants induced plant defenses but 2,4-DAPG strains further accentuated the defense system. Another significant aspect of this study is the utility of this strain for disease control in three different plant-pathogen systems albeit with some differences. The variation in the outcomes of the application of the same PGPB to different host-pathogens is an interesting aspect for further study. The recombinant biocontrol strain developed here could be used in combination with rhizosphere-dwelling biocontrol agents as this could be expected to ward off both aerial and soil- borne infection simultaneously.

To summarize, 2,4-DAPG producing genetically modified derivative of the endophytic diazotrophic bacterium *Pseudomonas* sp. WS5 was found to secrete high amounts of 2,4-DAPG as compared to the native *Pseudomonas* strains. The native as well as recombinant strains were able to promote growth of rice, wheat and sorghum under healthy conditions. During pathogen attack plant survival was improved upon bacterization with the wild type but the recombinant strain was more effective particularly in rice plants infected with *M. oryzae*. The 2,4-DAPG producing recombinant strains were effective in plant protection not only due to the direct antimicrobial effect but also owing to indirect effect by induction of host defense responses. This was evident from the higher induction of salicylic acid dependent defense pathway in the plants colonized by the recombinant strain. This study demonstrated the usefulness of the genetically modified strain for plant growth promotion in both healthy and infected plants in multiple plant-pathogen systems.