Chapter 3

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

"To succeed in your mission, you must have single-minded devotion to your goal."

-Dr. Abdul Kalam

3.1 Introduction

The underlying mechanisms by which beneficial endophytes promote plant growth are similar to those adopted by free-living plant growth promoting (PGP) microbes and involve both direct growth enhancement as well as indirect benefit to the plants (Ryan et al. 2008). Direct enhancement of plant growth by endophytes is largely based on the ability of the microorganism to produce phytohormones, to increase availability of nutrients such as phosphate and nitrogen or to enable the plant to cope up with environmental stress. Many endophytic fungi and bacteria produce a repertoire of secondary metabolites that act as antimicrobials (Brader et al. 2014) and have an indirect beneficial effect on plants by antagonizing phytopathogens.

Nitrogen fixing (diazotrophic) endophytes have been the focus of attention for past two decades for providing biologically fixed nitrogen (BNF) to cereals and other non-leguminous plants (Baldani et al. 1997). Endophytic nitrogen fixing strains of *Gluconacetobacter diazotrophicus*, *Azoarcus* spp. and *Herbaspirillum* spp. are most well-studied for providing benefit to non-nodulating plants (Carvalho et al. 2014). These bacteria have a disadvantage that they do not survive well in the soil and are for this reason considered as "obligate endophytes" although they grow well in laboratory conditions (Baldani et al. 1997). On the other hand, bacteria belonging to *Firmicutes* and *Actinobacteria* thrive well in soil and are more robust in their survival skills. However, both are rarely found as endophytes from cultural (Rosenblueth and Martínez-Romero 2006) as well as molecular (Ikeda et al. 2010; Bai and Vorholt 2016) studies, which typically show that a large majority of endophytes (90%) belong to *Proteobacteria*. *Actinobacteria* emerges as the second most abundant group of endophytes (Ikeda et al 2010). Many *Actinobacteria* (other than the most well studied *Frankia*) are recently being reported to be nitrogen fixing (Gtari et al. 2012; Dahal et al. 2017). *Microbacterium, Corynebacterium, Micromonospora* and *Arthrobacter* species have been reported to fix nitrogen and many others have *nifH* gene, but these reports require further confirmation and also lack systematic study of their effect on plants (Gtari et al. 2012). Endophytic *Actinobacteria* on the other hand are well-characterized producers of diverse secondary metabolites influencing the survival of other organisms (Qin et al. 2011; Masand et al. 2015) and several are reported for the control of diseases in plants

In addition to direct inhibition of phytopathogens, plant disease can also be suppressed by triggering the plant's own defense response. Rhizosphere-dwelling *Pseudomonas* spp. are most well-characterized for the induced systemic response (ISR) that is triggered by elicitors such as bacterial lipopolysaccharides, siderophores, signaling molecules like acyl homoserine lactones and antibiotics such as 2,4-Diacetylphloroglucinol (Van Loon 2007). This phenomenon, known as "priming effect" leads to an enhanced defense repose upon pathogen attack. Endophytes are considered particularly important as elicitors of systemic resistance in plants because they are in a more intimate interaction with the plants and may be viewed by the plant as potential pathogens in response to which the plant mounts a weak defense response resulting in a compatible endophyte-plant interaction (Reinhold-Hurek and Hurek 2011). It is generally accepted that ISR is mediated by signaling molecules jasmonic acid (JA) and ethylene (ET), whereas salicylic acid (SA) pathway is the predominant mechanism by which pathogens induce systemic resistance (Pieterse et al. 2014).

In Chapter 2 several diazotrophic endophytic *Actinobacteria* amounting to 37% of the cultured endophytic isolates were obtained from different cereal plants and were identified as *Arthrobacter*, *Aeromicrobium*, *Microbacterium* and *Streptomyces* species. Their diazotrophic nature was based on repeated growth on nitrogen free medium and the detection of *nifH* gene by PCR amplification. Among these, *Streptomyces* isolates obtained from the stem tissue of sorghum were particularly interesting since they were the only members that also showed antifungal activity against the phytopathogen *Rhizoctonia solani*. In the present Chapter, the endophytic *Streptomyces* spp. are studied for their ability to inhibit rice pathogen *Magnaporthe oryzae*. EGFP tagging was done to study colonization and localization in sorghum as well as other cereal crops such as rice and wheat. The ability of these strains to promote growth and suppress disease in all three cereals is now documented. Defense gene expression of bacterized rice plants under normal and pathogen challenge suggested novel mechanism of defense response modulation.

3.2 Materials and Methods

3.2.1 Microbial strains and culture conditions

Bacterial and fungal strains used in this study are listed in Table 3.1. *Streptomyces* cultures were routinely grown on Mannitol soy agar (Fiedler et al. 2005) and nitrogen free (NFb) medium (Döbereiner et al. 1995) at 30 °C. Potato dextrose broth (PDB) was used as the growth medium for the production and extraction of antifungal compounds. *Streptomyces* spore suspensions were prepared by growing cultures on mannitol soy agar till the sporulation stage. Five ml of sterile 0.9% NaCl (N saline) solution was added on to the plate and spores were suspended into the solution using glass spreader. For the preparation of fungal spore suspension, *Magnaporthe oryzae* B157 was grown on oatmeal agar at 30 °C for 10-12 d and *Rhizoctonia solani* was grown on potato dextrose agar (PDA) for 5 d at 30 °C. Spores were harvested by flooding the plates with sterile distilled water, the spores were scraped off and resulting suspension was filtered through muslin cloth to remove the mycelia from suspension. The spore counts were determined using haemocytometer.

Table 3.1 Bacterial and fungal strains and PCR primers used in this study

Bacterial and fungal strains/ Primers	Description	Source
Bacterial strains		
Streptomyces sp. SS1 (KR921495) *Streptomyces* sp. SS5 (KR921497) *Streptomyces* sp. SS8 (KR921498)	Diazotrophic endophytic actinobacteria isolated from sorghum stem	Chapter 2
Escherichia coli DH5α	Used as an intermediate cloning host	(Sambrook and Russell 2001)
E. coli S17.1	Used for biparental mating to transfer plasmid in to *Streptomyces* strains	(Flett et al. 1997; Simon et al. 1983)

Enterobacter sp. C1D (JN936958)	Heavy metal resistant, plant growth promoting, rhizosphere colonizing strain	Lab isolate
Fungal strains		
Magnaporthe oryzae B157	Rice plant pathogen	(MTCC 12236)
Rhizoctonia solani	Plant pathogen	Lab isolate
PCR Primers		
Phl2a	5'- GAGGACGTCGAAGACCACCA- 3'	Raaijmakers et al. 1997
Phl2b	5'- ACCGCAGCATCGTGTATGAG- 3'	
qPCR primers		
Actin-F	5'GAGCTACGAGCTTCCTGATGGA3'	Hao et al. 2012
Actin-R	5'CCTCAGGGCAGCGGAAA3'	
PR10a-F	5'ACACTCGACGGAGACGAAGC3'	
PR10a-R	5'CAGGGTGAGCGACGAGGTA3'	
PAL-F	5'GGACTACGGGTTCAAGGGC3'	
PAL-R	5'ACGAGACCCAGCGAGTTCA3'	
NPR1-F	5'TTTCCGATGGAGGCAAGAG3'	Duan et al. 2014
NPR1-R	5'GCTGTCATCCGAGCTAAGTGTT3'	
LOX-F	5'GCATCCCCAACAGCACATC3'	
LOX-R	5'AATAAAGATTTGGGAGTGACATA3'	

3.2.2 Antifungal activity assay

The antagonistic effect of bioactive molecules produced by *Streptomyces* strains was checked against *M. oryzae* B157 and *R. solani* by dual culture method (Chapter 2, Section 2.2.6.6) as well as by agar diffusion assay. The ethyl acetate extracts (100 µl) obtained from endophytic *Streptomyces* spp. were added into wells bored on media 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.

3.2.3 Extraction and identification of antifungal metabolite using GC-MS analysis

Extraction of the antifungal compound from the 5 d old culture supernatant, obtained by centrifugation of PDB grown cultures at 21690 xg for 5 min, was carried out by mixing with double the volume of ethyl acetate under shaking conditions for 1 h. The solvent layers were separated by centrifugation at 21690 xg for 5 min, the ethyl acetate fraction collected and evaporated till dryness. The yellow to dark brown colored powdery residue obtained was dissolved in HPLC grade methanol and filtered through 0.2 µm nylon membrane. The extracts were tested for antifungal activity by cup borer method. *M. oryzae* B157 containing plates were allowed to grow for 4-5 d and *R. solani* for 1 d after which antifungal extracts were added (150 µl) into the well made in agar and incubated for 5 d at 30°C. The extracts were also subjected to GC-MS (Single Quadrupole with prefilter, electro ionization, Perkin Elmer, USA) analysis at Sophisticated instrumentation centre for applied research and testing, Anand, India. Mass spectra were analyzed using National Institute of Standards and Technology (NIST) Library.

3.2.4 Detection of polyketide synthase gene in *Streptomyces* spp.

PCR reaction mixture was prepared in 25 µl system as mentioned in (Chapter 2, Section 2.2.2) using primers phl2a and 2b (Table 3.1). The PCR reaction was carried out at initial denaturation at 95 ºC for 5 min, followed by 35 cycles consisting of initial denaturation 94 ºC for 45 s, annealing temperature 58 ºC for 40 s, elongation at 72 ºC for 1 min, final elongation was carried out at 72 ºC for 10 min. The expected band size of *phl* gene was 726 bp.

3.2.5 EGFP tagging of *Streptomyces* spp.

Plasmid pIJ8660 (Sun et al. 1999) carrying constitutively expressed gene for enhanced green fluorescent protein (EGFP) was used for EGFP-tagging of the *Streptomyces* strains. Apramycin (25 µg ml^{-1}) was used for selection of *Escherichia coli* as well as *Streptomyces* transformants. Plasmid transformation in *E. coli* was performed using $CaCl_2$ method (Sambrook and Russell 2001). Plasmid pIJ8660 was introduced into *Streptomyces* spp. by biparental conjugation with *E. coli* S17.1 strain harboring pIJ8660 using the protocol of Flett et al. (1997). Briefly, *Streptomyces* spore suspension (approximately 10^8 spores ml^{-1}) was washed with Luria-Bertani (LB) broth, resuspended in 500 µl LB medium (instead of S medium mentioned in the original protocol) and incubated at 50 °C for 10 min to activate the germination process. The activated spore suspension

was centrifuged at 1677 xg for 3 min, supernatant discarded by leaving approximately 100 µl of it. An aliquot (100 µl) containing approximately 10^8 CFU *E. coli* S17.1 harboring the pIJ8660 plasmid was added to the actively germinating spore suspension. The mixture was spread on mannitol soy agar plates containing 10 mM $MgCl_2$ and incubated for 24 h after which the plates were overlaid with the 500 µg ml^{-1} nalidixic acid and 25 µg ml^{-1} of apramycin and incubated further for 5 d at 30 °C (Flett et al. 1997). *Streptomyces* transformant colonies were subcultured on apramycin containing NFb medium. Fluorescence of the EGFP tagged *Streptomyces* spp. was confirmed microscopically. Control plates processed similarly without the addition of donor *E. coli* S17.1 showed no growth.

3.2.6 Plant inoculation and visualization of colonization

Endophytic bacterial colonization of *Streptomyces* spp. in rice, sorghum and wheat was studied using hydroponics system. Surface sterilization of seeds was carried out as mentioned earlier (Chapter 2, Section 2.2.8). Wheat and sorghum seeds were transferred on 0.8% agar plates. Rice seeds were soaked in sterile distilled water for 2 h and then transferred on sterilized moistened paper in petri dish. Seeds were germinated in dark at 25 °C for 2-4 d. Seedlings with similar radicle length were transferred into the hydroponics jar containing 200 ml Murashige-Skoog medium (Himedia, India) to which *Streptomyces* spore suspension (1 ml containing 10^8 spores) was added and incubated under gnotobiotic conditions at 25 °C in greenhouse with natural daylight. *Enterobacter* sp. C1D (Subrahmanyam and Archana 2011) transformant carrying GFP containing plasmid pHC60 (Sharaff and Archana 2016) was used here as a rhizosphere colonizing strain for comparison and was inoculated in a similar manner on all three plants. On 8^{th} d, plants were harvested and processed for viewing under confocal scanning laser microscope (CSLM, LSM 700 Carl Zeiss, GmbH) with excitation at 488 nm. Endophytic colonization was also assessed by monitoring CFU g^{-1} of tissue fresh weight on NFb medium.

3.2.7 Plant growth promotion and plant protection assays

Plant growth promotion by endophytic *Streptomyces* was monitored in absence of fungal pathogens and plant protection was monitored by challenging pre-grown, endophytically colonized plants with the fungal pathogen. Rice, wheat and sorghum seeds were surface sterilized and germinated as mentioned in the (Chapter 2, Section 2.2.8). Seedlings were bacterized individually

with native *Streptomyces* spp. (strains SS1, SS5 and SS8) by mixing the spore suspension (containing approximately 10^8 spores ml^{-1}) with seedlings. Pots containing 2 kg sterile farm soil (obtained from Pulse Research Station, Anand Agricultural University, Model Farm, Vadodara, Gujarat) were sown with five seedlings each. Control pots received N-saline (0.9%) treated seedlings. Each treatment was given to plants in three independent pots. Two identical sets were allowed to grow for 30 d after which one set (of each treatment with three pots replicate) of plants was challenged with the fungal pathogens (for rice plants with *M. oryzae* B157 while wheat and sorghum plants were challenged with *R. solani*) by spraying 5 ml of spore suspension (10^8 spores ml^{-1}) on aerial parts of the bacterially treated as well as unbacterized control plants. All pots were continuously maintained in a totally random arrangement (maintaining the infected and healthy well separated) in the greenhouse with a relative humidity of 75% at 25 °C, under natural daylight (approximate 12 h of photoperiod). Plants were harvested after 60 d and analyzed for their plant growth parameters such as root and shoot length, wet weight of root and stem. Vigour index was calculated using following formula: (Root length + Shoot length) x 100%.

3.2.8 Study of defense related gene expression in rice

Rice plants were bacterized, allowed to grow for 30 d and challenged with *M. oryzae* B157 as mentioned above. On 8th day of infection, plants were uprooted, surface sterilized (Chapter 2, Section 2.2.4) and immediately processed for qPCR analysis. Total RNA was extracted from aerial parts of rice plants using the method described by Chomczynski and Sacchi (2006). Plant RNA was reverse transcribed to cDNA with 1 µg of RNA as template using PrimeScript reverse transcriptase kit and random hexamer primers as per instruction by the manufacturer (Takara, India) (Table 3.2). For qPCR analysis 0.5 µl of cDNA was added to 10 µl reaction mixtures in Step One Real-Time PCR machine (Applied Biosystems, USA). Reactions were carried out in duplicates using SYBR-master mix as per manufacturer's instructions (Takara, India) using the following PCR protocol: 95 °C for 30 s followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s. PCR products were immediately analyzed for melt curve. Actin was used as housekeeping gene (Hao et al. 2012) to normalize the expression of genes used in this experiment. Primers used for quantitative analysis targeted the genes encoding following defense related proteins: non-expressor of PR genes 1 (NPR1), Lipoxygenase-2 (LOX2) (Duan et al. 2014), phenylalanine ammonia-lyase (PAL) and pathogenesis-related (PR) 10a gene (Hao et al. 2012).

Table 3.2 Quantitative real time PCR reaction mixture

Reagents	Volume (µl)
Random hexamer (50 µM)	1
dNTP (10 mM)	0.4
RNA	1 µg
RNase-free dH_2O	Up to 10
The reaction mixture was incubated for 5 min at 65°C	
5X PrimeScript buffer	4
Rnase inhibitor (40 U/ml)	0.5
PrimeScript RTase	1
RNase-free dH_2O	Up to 20
Total	20

3.2.9 Data analysis

Data of plant inoculation experiments data were analyzed by Levene's test and Student's t-test using Microsoft Excel (Microsoft, 2007). 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.

3.3 Results

3.3.1 Inhibition of phytopathogenic fungi by diazotrophic endophytic *Streptomyces* spp.

All three *Streptomyces* spp. showed inhibition of *R. solani* (Chapter 2). In addition they also inhibited *M. oryzae* B157 on dual culture plate analysis (Fig.3.1). Secondary metabolites extracted from *Streptomyces* sp. SS1, SS5 and SS8 also showed growth inhibitory activity against the both *M. oryzae* B157 and *R. solani* (Figs. 3.2, 3.3). The control plates inoculated only with fungal pathogen shown the overgrowth of fungal pathogens within same period of incubation.

Fig. 3.1 Dual culture plate analysis of *Streptomyces* spp. against *M. oryzae* B157. (a) *Streptomyces* sp. SS1, (b) *Streptomyces* sp. SS5, (c) *Streptomyces* sp. SS8

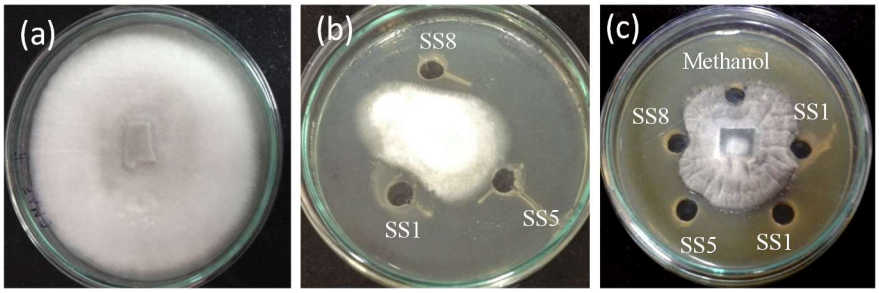


Fig. 3.2 Antifungal activity of *Streptomyces* spp. supernatant and ethyl acetate extract against the *M. oryzae* B157. (a) Control *Magnaporthe oryzae* B157 (b) Culture supernatant, (c) Antifungal metabolite crude extract in methanol

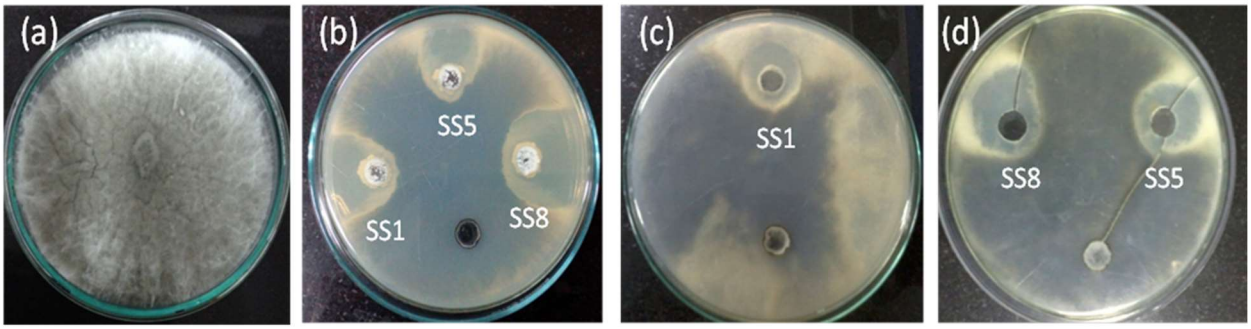


Fig. 3.3 Antifungal activity of *Streptomyces* spp. against fungal pathogen *R. solani*. (a) *R. solani* control, (b) Antifungal activity of culture supernatant, (c) Antifungal crude extract of *Streptomyces* sp. SS1, (d) Antifungal crude extract of *Streptomyces* sp. SS5 and SS8

3.3.2 Identification of antifungal metabolites produced by *Streptomyces* spp.

The bioactive metabolites extracted from *Streptomyces* strains were analyzed by GC-MS (Appendix I, Fig. A8). The mass spectrum was matched in the database of NIST library and Pubchem (https://pubchem.ncbi.nlm.nih.gov/compound). The peaks in the chromatogram was

identified in mass spectrum database and based on highest REV similarity index, it named: 2-(chloromethyl)-2-cyclopropyloxiran, 2, 4- ditert-Butylphenol, and 1□ethylthio□3□methyl□1, 3□butadiene produced by *Streptomyces* sp. SS1, SS5 and SS8 respectively.

3.3.3 Analysis of polyketide gene in diazotrophic endophytic *Streptomyces* spp.

All three *Streptomyces* spp. were evaluated for presence of *phl* gene by PCR amplification. Only *Streptomyces* sp. SS8 was showed the presence of *phl* gene (Fig. 3.4).

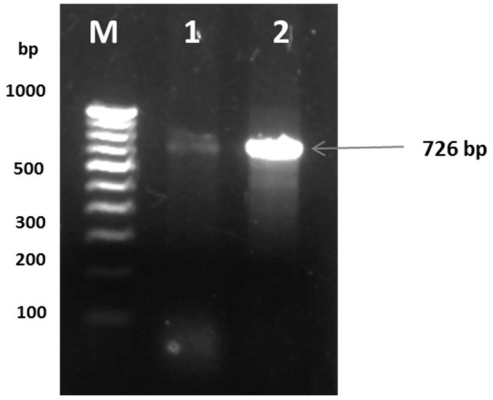
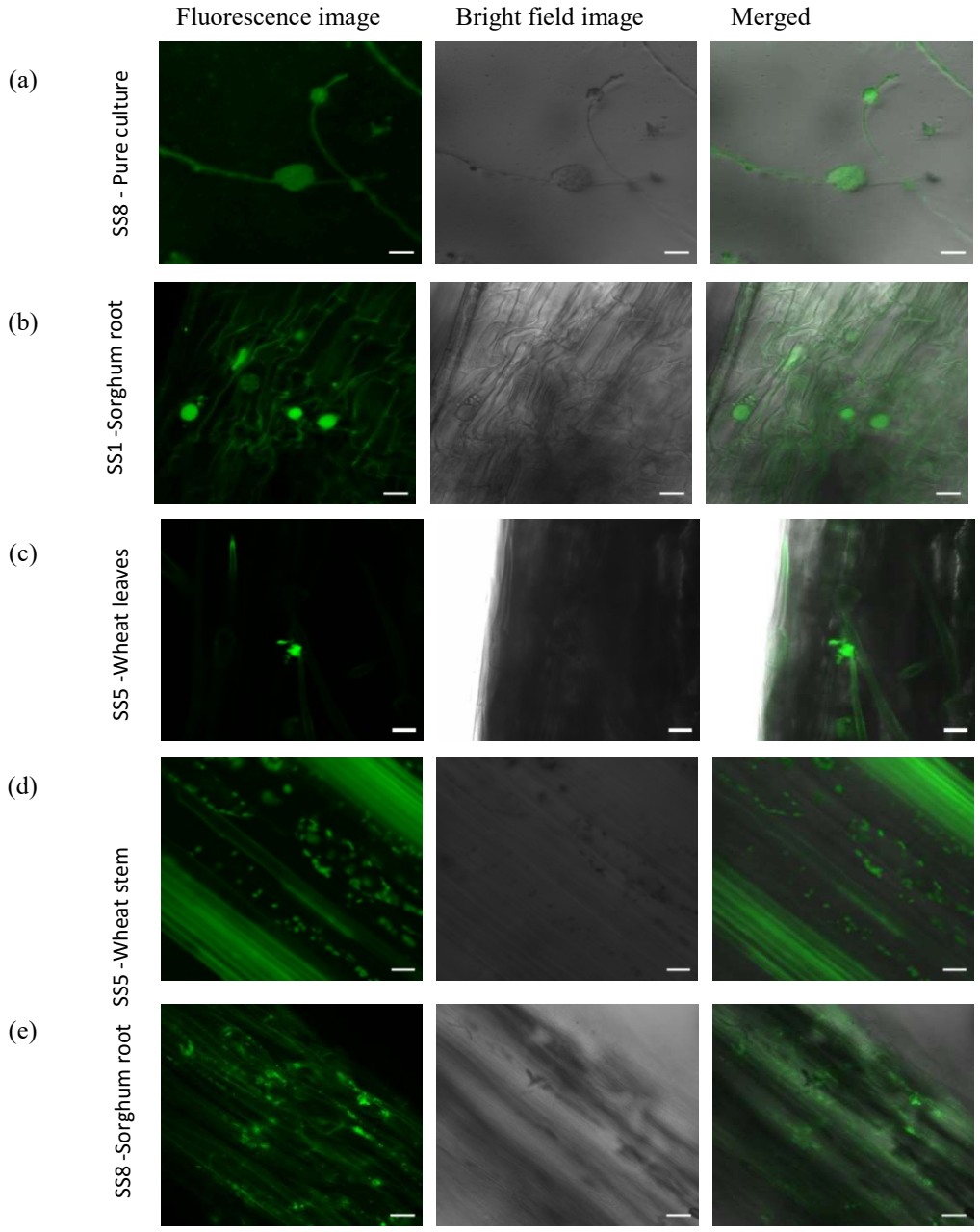


Fig. 3.4 Amplification of *phl* gene in *Streptomyces* sp. SS8. Lane M-100 bp marker, Lane 1- *Streptomyces* sp. SS8, Lane 2- *Pseudomonas protegens* Pf-5

3.3.4 Evaluation of endophytic colonization by diazotrophic endophytic *Streptomyces* spp

The EGFP tagged strains of *Streptomyces* spp. were used to observe their ability to enter and colonize host tissue. All three diazotrophic endophytic *Streptomyces* spp. were successfully transformed with EGFP containing plasmid pIJ8660. The pIJ8660 transformed *Streptomyces* sp. SS8 pure culture was analyzed under CLSM for detection fluorescence [Fig. 3.5 (a)]. On 8^{th} d, plant parts observed under confocal laser scanning microscopy showed the microcolonies of endophytic *Streptomyces* spp. in interior parts of the root, stem and leaves wheat and sorghum plants. Fig. 3.5 shows representative images for some strains in specific plant parts. It was observed that all the three strains of *Streptomyces* colonized both the plants with highest colonization in stem, followed by roots and then leaves. The Z-stack analysis revealed that endophytic colonization of *Streptomyces* spp. was detected from 5 to 31 µm deep inside the root in wheat and sorghum plants while thickness of the roots varied from 45-111 µm. The unbacterized plants did not display any fluorescent cells. Rhizosphere colonizing *Enterobacter* sp.

C1D (pHC60) showed colonization on the root surface but not in the interior tissue neither in aerial parts (Fig. 3.5). In rice plants, high autofluorescence in tissues interfered in microscopic analysis of endophytic colonization. Therefore, confirmation of endophytic colonization was performed by CFU g^{-1} count of surface sterilized plant tissue. Overall CFU ranged from a range 10^2-10^4 CFU g^{-1} in wheat and rice and 10^5-10^6 CFU g^{-1} in sorghum plants. In agreement with microscopic observation, stem part showed highest CFU g^{-1} ($1.57x10^4$ and $1.20x10^6$) in wheat and sorghum respectively.



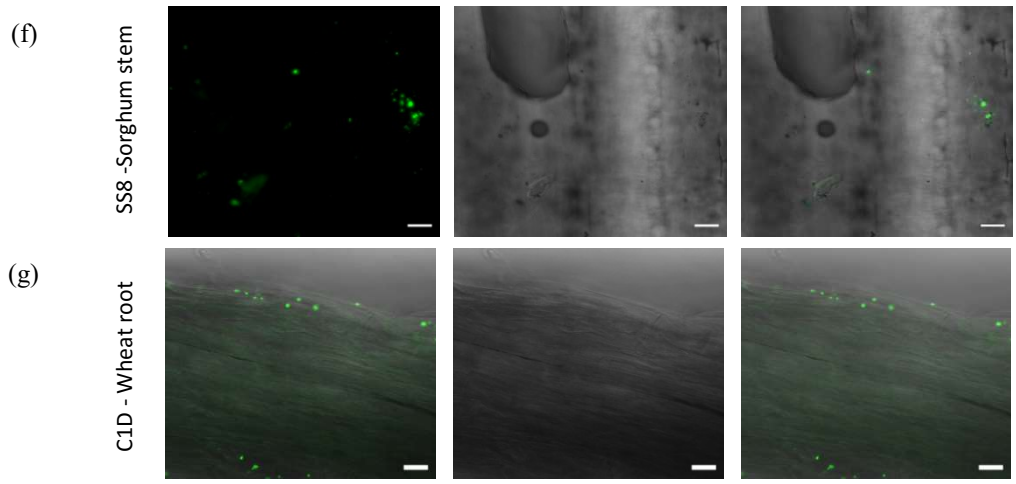


Fig. 3.5 Detection of *Streptomyces* spp. colonization in the interior tissue of wheat and sorghum plants. (a) Sorghum root colonization by *Streptomyces* sp. SS1, (b) Wheat leaves colonization by *Streptomyces* sp. SS5, (c) Wheat stem colonization by *Streptomyces* sp. SS5, (d) Sorghum root colonization by *Streptomyces* sp. SS8, (e) Sorghum Stem colonization by *Streptomyces* sp. SS8. Scale bar represents the 20 µm

3.3.5 Plant growth promotion by diazotrophic endophytic *Streptomyces* spp.

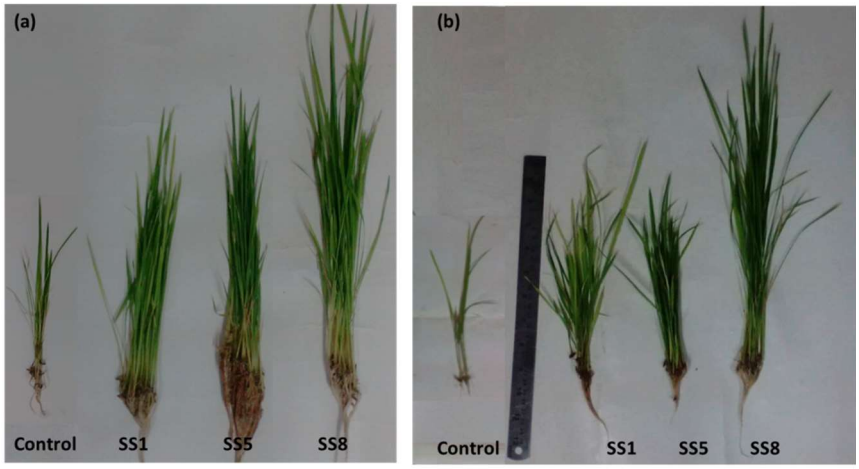
Streptomyces spp. inoculated on the plants at seedling level brought about plant growth promotion after 60 d incubation in the greenhouse (Fig. 3.6). All the three strains of *Streptomyces* spp. showed plant growth promotion in sorghum, wheat and rice plants (Table 3.3). Rice plants inoculated with *Streptomyces* spp. showed enhanced plant growth of aerial parts of the plants whereas in wheat and sorghum plants were found to be improved in both above and below ground parts of the plant as reflected in root:shoot ratios. Significant increase in vigour index was observed in bacterized plants. Rice plants bacterized with SS1 and SS8 showed an increase in wet weight of shoot by 187% and 518% respectively (Table 3.3). In sorghum plants SS5 and SS8 increase wet weight of shoot up to 205% and 210% respectively whereas in wheat plant SS8 showed increase by 127%. The root:shoot ratio of rice plants was found to decrease significantly upon bacterial colonization as compared to unbacterized control plants (Table 3.3).

3.3.6 Plant protection by endophytic *Streptomyces* spp. upon phytopathogen challenge

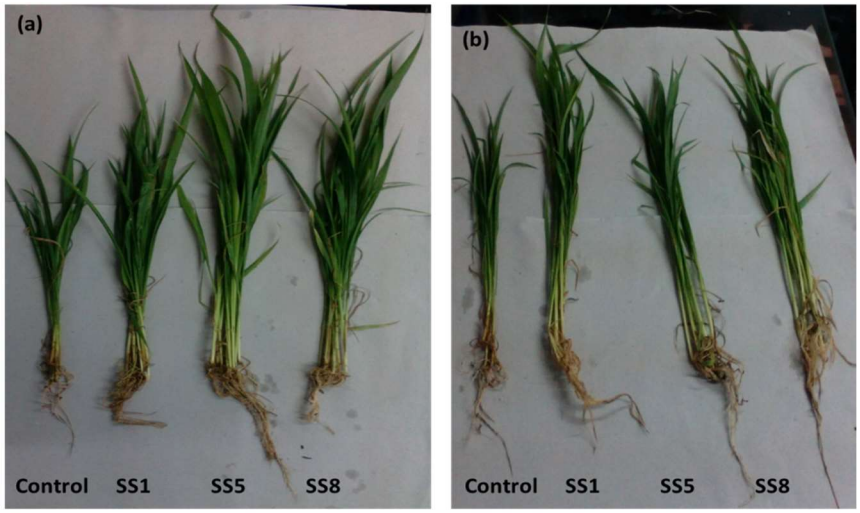
Plants challenged with the phytopathogens were analyzed after 60 d (30 d of healthy growth followed by 30 d of growth after pathogen challenge). Control plants without endophytic

colonization by the *Streptomyces* strains showed a reduction in various plant growth parameters due to disease condition (Fig. 3.6). The *Streptomyces* spp. inoculated plants under pathogen attack showed the enhanced growth of plants as compared to control (unbacterized) plants infected with fungal pathogens (Table 3.4). Rice plants infected with *M. oryzae* B157 showed a major effect on wet weight of shoot. *Streptomyces* sp. SS8 showed better protection in rice compared to other two strains. The wet weight of shoot in unbacterized plants was observed to be reduced by 61% compared to the unbacterized non-infected plants whereas the bacterized plants showed plant protection and increased shoot weight up to 132%, 178% and 501% in *Streptomyces* sp. SS1, SS5 and SS8 inoculated plants respectively as compared to the unbacterized fungal challenged control. In case of sorghum the highest plant protection was observed in *Streptomyces* sp. SS1 and SS5 inoculated plants (Table 3.4).

(A)



(B)



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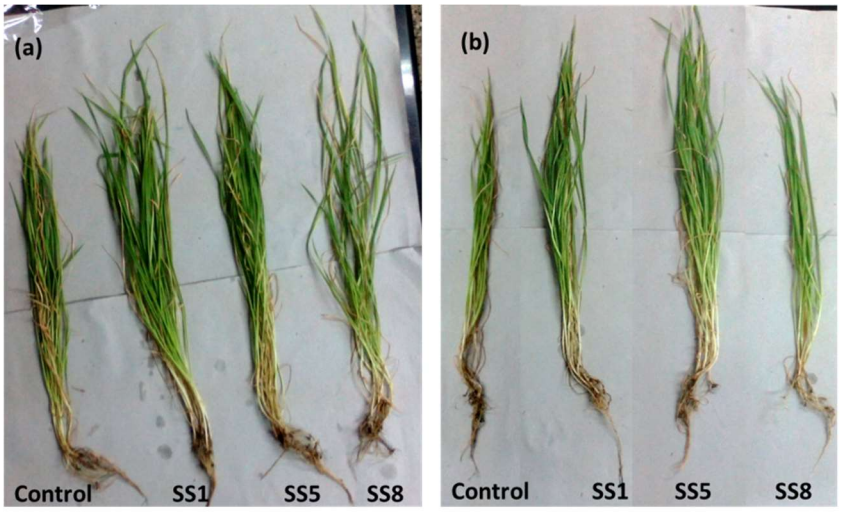


Fig. 3.6 Plant growth promotion and protection by *Streptomyces* spp. in rice (A), sorghum (B), wheat (C) plants. (a) Unbacterized and bacterized plants, (b) Unbacterized and bacterized plants challenged with *M. oryzae* B157 for rice and *R. solani* for wheat and sorghum. Control-unbacterized, SS1- *Streptomyces* sp. SS1, SS5- *Streptomyces* sp. SS5, SS8- *Streptomyces* sp. SS8

The wet weights of shoot in bacterized sorghum plants were observed to 222% and 115% in *Streptomyces* sp. SS1 and SS5 inoculated plants compared to fungal treated unbacterized plants. In wheat plants, overall plant protection was observed in case of plants inoculated with *Streptomyces* sp. SS1 and SS5. The vigour index of bacterized rice and sorghum plants was significantly increased compared to unbacterized fungal-challenged control. A wheat plants bacterization with *Streptomyces* spp. SS8 did not show any significant difference in vigour index whereas those colonized by SS1 and SS5 both were significant (Table 3.4).

Table 3.3 Plant growth promotion by diazotrophic, endophytic *Streptomyces* spp. inoculated 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	5.68±0.61	16.70±1.19	28.5±8.24	62.83±10.8	0.45±0.11	2238±135
SS1	5.28±0.79 (7% ↓)	40.57±4.89*** (143% ↑)	33±1.67 (16% ↑)	180.5±46.29*** (187% ↑)	0.19±0.04	4585±487*** (140% ↑)
SS5	5.62±0.38 (1% ↓)	43.30±1.16*** (159% ↑)	37.5±4.42* (32% ↑)	97.17±16.07*** (55% ↑)	0.39±0.06	4892±106*** (116% ↑)
SS8	5.47±0.55 (4% ↓)	45.33±4.50*** (171% ↑)	33.5±1.52 (18% ↑)	388.5±49.22*** (518% ↑)	0.09±0.01	5080±420*** (145% ↑)
	Sorghum plants					
Unbacterized	8.55±1.78	31.33±3.67	100.83±11.50	851.83±145.10	0.12±0.02	3988±477
SS1	11.82±1.94* (38% ↑)	39.83±1.83** (27% ↑)	186.67±57.05** (85% ↑)	1631±112.15*** (92% ↑)	0.12±0.04	5165±260*** (30% ↑)
SS5	18.08±1.36*** (112% ↑)	51.67±1.75*** (65% ↑)	343.17±49.52*** (240% ↑)	2598±206.24*** (205% ↑)	0.13±0.03	6975±154*** (75% ↑)
SS8	11.92±1.11** (39% ↑)	44.25±5.58** (41% ↑)	227.33±22.48*** (125% ↑)	2640±567.34*** (210% ↑)	0.09±0.02	5617±615*** (41% ↑)
	Wheat plants					
Unbacterized	6.83±0.98	44.33±3.14	18.83±1.33	693.33±126.44	0.03±0.003	5117±306
SS1	11.70±0.84*** (71% ↑)	44.87±0.92 (1% ↑)	68.17±9.77*** (262% ↑)	1235±24.36*** (78% ↑)	0.05±0.008	5657±112** (11% ↑)
SS5	10±0.64*** (46% ↑)	45.70±2.05 (3% ↑)	62.00±5.06*** (229% ↑)	1026±42.66*** (48% ↑)	0.06±0.006	5570±202* (9% ↑)
SS8	8.72±0.72** (28% ↑)	49.80±1.20** (12% ↑)	69.83±4.49*** (271%↑)	1580±157.43*** (127% ↑)	0.04±0.007	5852±162*** (14% ↑)

±, Standard deviation; 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 *Streptomyces* spp.; [a] Unbacterized fungal infected plant parameters are compared with the corresponding parameter of the respective unbacterized uninfected plants. SS1- *Streptomyces* sp. SS1; SS5- *Streptomyces* sp. SS5; SS8- *Streptomyces* sp. SS8

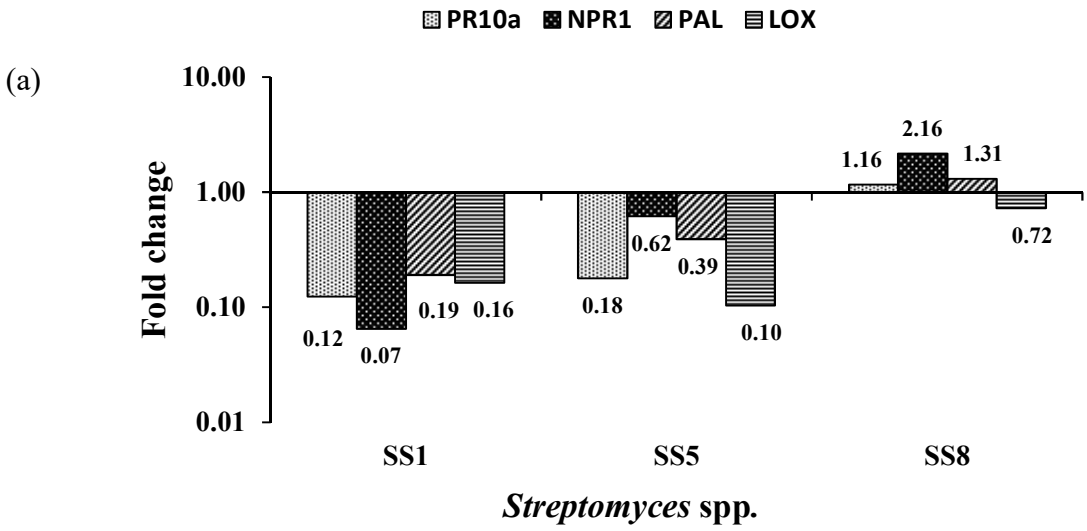
Table 3.4 Plant protection ability of diazotrophic endophytic *Streptomyces* spp. inoculated plants rice, sorghum and wheat upon fungal pathogen challenge

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[a]	*M. oryzae* B157	4.85±1.55 (15% ↓)	14.33±2.25 (14% ↓)	25±2.83 (12% ↓)	24.67±6.12 (61% ↓)	1.07±0.30	1918±196 (14% ↓)
SS1	*M. oryzae* B157	4.58±0.20 (5% ↓)	21.85±2*** (52% ↑)	29±2.19* (16% ↑)	57.17±1.17*** (132% ↑)	0.51±0.04	2643±213*** (38% ↑)
SS5	*M. oryzae* B157	5.42±0.66 (12% ↑)	18.67±1.51** (30% ↑)	34.5±1.64*** (38% ↑)	68.5±21.99** (178% ↑)	0.53±0.12	2408±147*** (26% ↑)
SS8	*M. oryzae* B157	5.93±0.96 (22% ↑)	46.17±2.56*** (222% ↑)	67±5.90*** (168% ↑)	148.17±14.99*** (501% ↑)	0.46±0.06	5210±198*** (63% ↑)
Sorghum plants							
Unbacterized[a]	*R. solani*	7.02±0.99 (18% ↓)	31.85±3.49	51.50±5.79 (49 % ↓)	515±57 (40% ↓)	0.10±0.01	3887±439 (3% ↓)
SS1	*R. solani*	11.40±2.53** (62% ↑)	40.57±4.89** (27% ↑)	172.33±37.28** (235% ↑)	1660±285*** (222% ↑)	0.10±0.02	5197±452*** (34% ↑)
SS5	*R. solani*	13.87±1.18*** (98% ↑)	43.30±1.16*** (36% ↑)	177.17±32.42*** (244% ↑)	1108±88*** (115% ↑)	0.16±0.04	5717±188*** (47% ↑)
SS8	*R. solani*	11.18±1.08** (59% ↑)	45.33±4.50** (42% ↑)	153±23.46*** (197% ↑)	915±88*** (78% ↑)	0.17±0.02	5652±451*** (45% ↑)
Wheat plants							
Unbacterized[a]	*R. solani*	6.28±0.53 (8% ↓)	38.67±2.34 (13% ↓)	48.83±20.47 (159% ↑)	330.33±29.45 (52% ↓)	0.15±0.06	4495±244 (12% ↓)
SS1	*R. solani*	11.38±1.02*** (81% ↑)	41.67±3.20 (8% ↑)	67±6.99 (37% ↑)	664.83±40.64*** (101% ↑)	0.10±0.02	5305±402*** (18% ↑)
SS5	*R. solani*	7.27±0.52** (16% ↑)	44.92±1.43*** (16% ↑)	37±3.90 (24% ↓)	905.50±40.47*** (174% ↑)	0.04±0.00	5218±148*** (16% ↑)
SS8	*R. solani*	5.92±0.73 (6% ↓)	40.5±2.35 (5% ↑)	45.50±4.89 (7% ↓)	749.67±19.29*** (128% ↑)	0.06±0.01	4642±180 (3% ↑)

Refer Table 3.3

3.3.7 Effect of endophytic colonization by *Streptomyces* spp. on defense gene expression in rice plants against blast pathogen

The plants treated with *Streptomyces* spp. at seedling stage, after 37 d of plant growth showed approximately 5-10 fold down-regulation of all the tested genes in case of isolates SS1 and SS5 whereas *Streptomyces* sp. SS8 did not lower the defense gene expression and in fact showed a 2.16 fold increase in *NPR1* gene expression (Fig. 3.7a). Plants colonized with *Streptomyces* spp. at the seedling stage when challenged with the fungal pathogen on 30th d of planting showed significantly higher induction of all the genes on day 7 post infection except *PAL* as compared to similarly treated unbacterized plants. Among the genes upregulated, the *NPR1* gene is induced maximally as compared to the unbacterized counterpart (Fig. 3.7b). The expression of *NPR1* gene in rice was found to be 51.85, 140.80 and 121.98 fold high in *Streptomyces* sp. SS1, SS5, SS8 respectively. *Streptomyces* sp. SS1 showed also showed an increase in the expression of genes *PR10a* and *LOX2* by 28.17 and 30.25 fold respectively while in *Streptomyces* sp. SS8 there was 11.08 fold higher *LOX2* gene expression. The unbacterized rice plants showed less survival and poor growth of the surviving plants. The leaves of unbacterized plants yellowed and dried up on 4th d of infection itself whereas endophytically colonized plants with all the three *Streptomyces* strains were comparatively healthy with appearance of tiny disease lesions after 3 d of infection which persisted up to 7 d and all plants survived (Fig. 3.8).



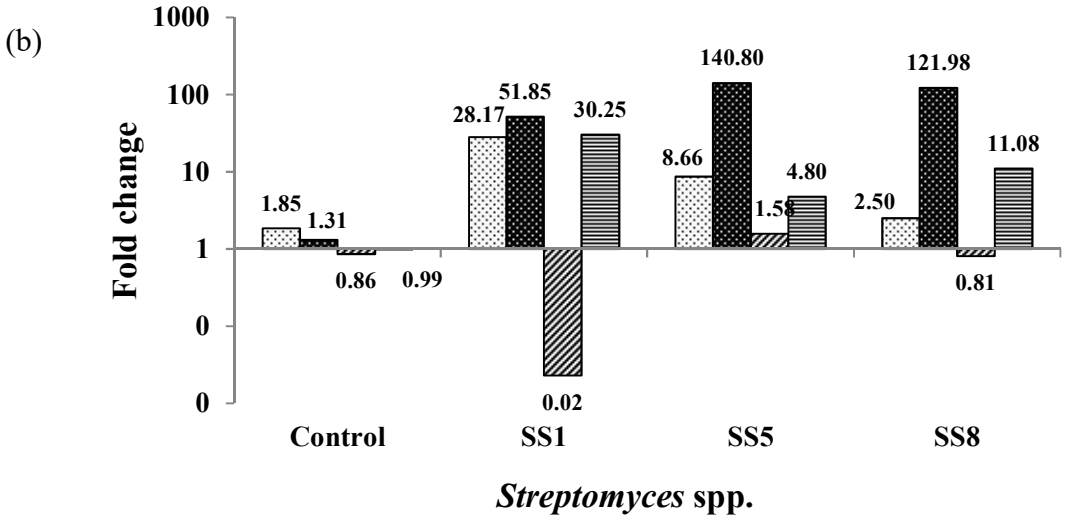


Fig. 3.7 Quantitative real time PCR analysis to determine the induction of plant defense gene expression by *Streptomyces* spp. in healthy and pathogen challenged. (a) Rice plant treated with only *Streptomyces* spp., (b) Rice plants inoculated with *Streptomyces* spp. and challenged with fungal pathogen *M. oryzae* B157

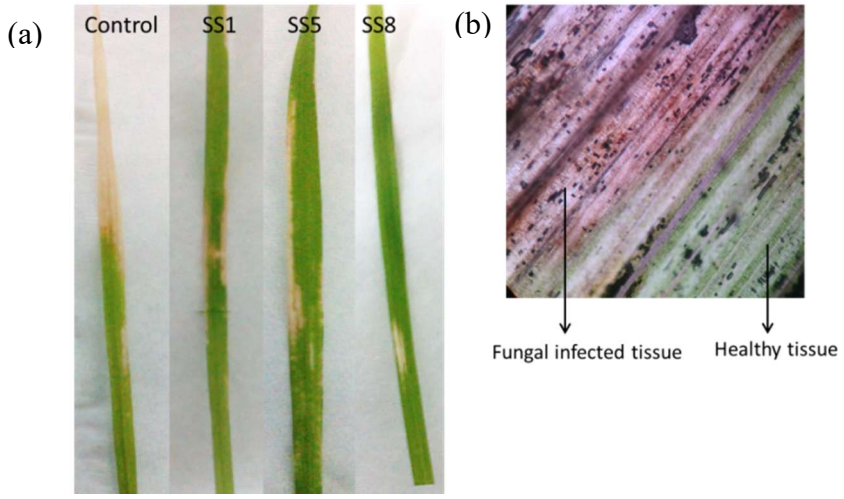


Fig. 3.8 Disease development in unbacterized and bacterized rice plants challenged with *M. oryzae* B157. (a) Disease spot progress in rice plant after various treatments; (b) Microscopic view of infected spot on leaf; Control- non-bacterized rice plant infected; SS1- *Streptomyces* sp. SS1; SS5- *Streptomyces* sp. SS5; SS8- *Streptomyces* sp. SS8

3.4 Discussion

In this study diazotrophic endophytic *Streptomyces* spp. isolated previously from sorghum aerial tissue were shown to produce antagonistic molecules that inhibit the phytopathogens *Magnaporthe oryzae* and *Rhizoctonia solani*. The latter fungus is one of the most devastating pathogens causing damping-off and root rot in a wide range of hosts including *Poaceae* plants (Paulitz et al. 2002), tomato (Goudjal et al. 2014), cucumber (Huang et al. 2012) and sugar beet (El-Tarabily 2004) while *M. oryzae* is the most important fungal pathogen of rice causing blast disease, a global threat to rice cultivation (Wilson and Talbot 2009). Many fungicides show the inhibition of phytopathogens at lab level but fail to produce similar effect during field application due to inability of fungicide to move into the root system of the plant (Paulitz et al. 2002). To address this problem, biocontrol agents comprising of endophytic bacteria are more beneficial because they are able to colonize the root as well as the aerial part of the plants and protect them against above as well as below ground infection. The *Streptomyces* spp. reported here are versatile in colonizing three cereal plants and protecting them from different fungal pathogens. Profuse colonization was observed in the roots and stem in wheat and sorghum plants by direct microscopic examination of EGFP tagged strains as well as by CFU counts in gnotobiotic hydroponics system. The experimental design suggests that the *Streptomyces* spp. entered through the root system and migrate upwards into the stems where they seem to proliferate and also reach leaves as seen by sparse but significant numbers in the leaf tissue. Coombs and Franco (2003) reported that EGFP labelled *Streptomyces* sp. EN27 showed the ability to enter inside the wheat embryo tissue at early phase of plant development and then into the emerging radicle. Bonaldi et al. (2015) concluded that the plasmid pIJ8641, belonging to the same series as plasmid pIJ8660 used here, does not confer any fitness disadvantage to *Streptomyces* spp. and also that it had no adverse effects on various PGP traits such as auxin, enzymes and siderophore production. The EGFP tagged endophytic bacteria were recovered from all the three plants and their different tissues indicating proficiency of the EGFP tagged strains to colonize. *Streptomyces* spp. were recovered even after 60 d of planting (data not shown) indicating the ability of the strains to thrive long term inside the plant. It remains to be seen whether the *Streptomyces* strains could be transmitted through the seeds into the next generation of plants.

Of total bioactive compounds known to be produced by microbes 70% are contributed by the single genus *Streptomyces* (de Jesus Sousa and Olivares 2016). The plant endo-microbiome being a novel niche for the hunt and discovery of new antimicrobials, endophytic *Streptomyces* spp. have been isolated from a variety of crops, medicinal plants and trees; they show variety of plant beneficial traits that are involved in direct growth promotion and protection against pathogen attack. The antifungal extract of endophytic *Streptomyces* spp. when analyzed by GC-MS showed that the three strains produced different sets of molecules even though they originated from the same plant. This is in accordance with the observation that *Streptomyces* spp. SS8 alone was positive for PCR amplification of *phl* gene (Raaijmakers et al. 1997) indicating genetic differences between the three strains. The compounds produced by *Streptomyces* sp. SS5 and SS8 are 2, 4- ditert-butylphenol (PubChem CID: 7311) and 1□ethylthio□3□methyl□1, 3□ butadiene (PubChem CID: 5369202) respectively satisfied Lipinski's rule of five which used to evaluate druglikeliness. The antifungal compound 2,4- ditert-butylphenol produced by *Streptomyces* sp. SS5 has been reported to be produced by *S. mutabilis, Lactococcus*, *Flavobacterium johnsoniae* and causes the inhibition of pathogenic fungi and yeast (Belghit et al. 2016; Varsha et al. 2015; Sang and Kim 2012). *Streptomyces* sp. SS1 produces 2□ (chloromethyl)□2□cyclopropyloxiran (PubChem CID: 548962) which likely to play a role in inhibiting the fungal pathogen. The oxirane (PubChem CID: 6354) moiety of this compound is known for its bactericidal, fungicidal, sporicidal and also shows its effectiveness against viruses.

The reported traits of endophytic PGP *Streptomyces* strains usually include auxin production, siderophore production, phosphate solubilization, antibiotic production and ISR response (Table 3.5). The *Streptomyces* strains described here are novel in that they are additionally nitrogen fixing. Recently Dahal et al. (2017) have found that *Streptomyces* spp. amounted to 50% of free living nitrogen fixing strains in badland soils (isolated using a method similar to that used here) however, they have not explored endophytic diazotrophic strains. The ability to carry out N fixation along with the other traits for direct plant growth promotion, accounts for the *Streptomyces* strains reported here it to promote growth of healthy (uninfected) plants. All the three cereal crops tested were positively affected by bacterization with the *Streptomyces* spp. Particularly noteworthy is the remarkable increase in shoot weight by strain SS8 in rice and sorghum. The increase in shoot far surpasses that of root in rice plants. While the significance of this is not clear, strain SS8 does not produce IAA, but yet shows higher

aboveground biomass as compared to other strains. It is well-known that root:shoot ratio, a good indicator of biomass allocation to aboveground or belowground parts, reflects the plants' nutritional status (Hermans et al. 2006). Of particular relevance is the observation by Reynolds and Antonio (1996) that among the 129 plant species they surveyed, the root:shoot weight ratio decreased with increased nitrogen availability in majority of cases. Based on this it may be summarized that lower root:shoot ratios with drastic increase in shoot biomass might reflect efficient nitrogen fixation by the diazotrophic strains. The pathogen challenged bacterized rice and wheat plants also showed the decrease in root:shoot ratio with concurrent increase in shoot weight, indicating that the overall health of the plant was improved along with the suppression of disease

In addition to the direct antimicrobial effect on phytopathogens, the strains reported here also show modulation of defense response in rice plants. Interestingly bacterized plants did not show induction of the defense related genes tested on 37th d after planting. In case of two strains, a down-regulation was observed indicating that *Streptomyces* spp. subside the defense related gene expression in healthy plants. However, upon pathogen invasion plants bacterized with *Streptomyces* spp. showed the high expression of *NPR1* gene along with *PR10a* and *LOX2* genes. This is agreement with the general observation that plant colonization with ISR inducing bacteria does not result in induced expression of defense related genes when colonized in healthy plants, but rather results in a potentiated response when the colonized plants are under pathogen attack (Pieterse et al. 2014). A common observation in most plant-pathogen systems is that PR protein expression is activated by SA dependent pathogen induced systemic acquired resistance (SAR) response but not by the JA/ET pathway based ISR response induced by non-pathogenic microbes (Pieterse et al. 2014). The *PR10a* gene of rice however, is reported to be upregulated not only directly by *M. oryzae* infection but also by exogenous application of SA and JA to healthy plants (McGee et al. 2001). This suggests that *PR10a* is not exclusively over-expressed in SAR but might also be unregulated in the JA mediated ISR response. The observation that priming by *Streptomyces* species resulted in an enhanced expression of *PR10a* in rice upon pathogen challenge, clearly supports this view. The observed upregulation of the *LOX* gene, which is involved in JA biosynthesis pathway (Turner et al. 2002), during *M. oryzae* infection of bacterized plants indicates that the JA pathway is operative exclusively during fungal attack on plants colonized by *Streptomyces* spp. but not when unbacterized plants are infected with *M.*

oryzae. The upregulation of PAL gene, also known to be of SAR pathway, was observed only in rice plants bacterized with *Streptomyces* sp. SS5 upon pathogen attack. The most highly upregulated gene in case of all the three endophytic strains tested was *NPR1*, known to be a common regulator of defense induction by both the SA and JA/ET pathways (Spoel et al. 2003). In the SAR pathway, *NPR1* has a unique role that involves redox-mediated protein modification, nuclear translocation and protein-protein interaction ultimately culminating in transcriptional activation of *PR* genes (Pieterse et al. 2014). The role of *NPR1* in ISR is not well understood but is certainly different than in SAR. Nonetheless, transgenic *Arabidopsis* plants overexpressing the *NPR1* gene demonstrate enhanced plant protection against the infection by an accentuated expression of PR proteins upon pathogen attack indicating SAR induction (Cao et al. 1998). In transgenic strawberry plants ectopically expressing the *Arabidopsis* NPR1, the unchallenged plants demonstrated constitutive expression of PR proteins again indicating SAR pathway induction (Julliany et al. 2015). On the other hand, result reported here show that during priming by endophytic *Streptomyces*, upregulation of *NPR1* gene expression is associated with JA pathway being induced. In general, endophytic *Streptomyces* strains are able to induce SA (PR proteins) and JA/ET dependent [Plant Defensin 1.2 (*PDF1.2*); ERF transcription factor (*CaTF2*); Hevein-like protein (*HEL*); Lipoxygenase (*LOX*)] defense related genes dependent on specific pathogen (Table 3.5) suggesting both pathways may be operational. The co-expression of SAR and ISR pathway simultaneously helps in efficient plant protection against the pathogen infection (Van Wees et al. 2000; Yi et al. 2013). It is important to point out that ISR induction in monocot plants is relatively less well-studied (Balmer et al. 2012) and in general ISR induction and its mechanism are well-characterized in gram negative bacteria (Van Loon et al. 1998) but not in Gram positive bacteria. This work sheds light on mechanism of ISR induction in monocots by gram positive bacteria. To the best of our knowledge, this is the first report of an endophytic *Streptomyces* possessing nitrogen fixation and biocontrol properties effective in *Poaceae* plants.

In conclusion, the results of this Chapter show that diazotrophic endophytic *Streptomyces* spp. are able to inhibit the two important fungal pathogens in three cereal plants. Diazotrophic endophytic *Streptomyces* spp. colonized profusely in the roots as well as shoots of the cereal plants, promoted plant growth under healthy conditions and protected against pathogen attack by direct antagonism as well as induction of plant defenses. These characteristics make them a potential candidate for field application. Since the mode of fungal infection was spraying of

fungal spores on aerial tissue it may be concluded that the bacterized plants are able to withstand fungal entry through above-ground parts as well as curb intracellular growth in aerial tissues, a property that could be used in combination with rhizosphere biocontrol agents that prevent entry through the root system.

Table 3.5 Comparative analysis of properties of various *Streptomyces* spp. as biocontrol and plant growth promoter

Strain	Source	PGP traits					Plant-fungal system	Defense gene response	Reference
		N[a]	IAA[b]	Sid [c]	P[d]	Ab[e]			
Several *Streptomyces* spp.	Wheat root (Endophytic)	?	?	+	-	+	Wheat- *Rhizoctonia solani;* *Gaeumannomyces graminis var. tritici*	?	Coombs et al. 2004; Conn et al. 2008; Misk and Franco 2011
***Streptomyces* sp. EN27**		?	?	+	-	+	*Arabidopsis- Erwinia carotovora* subsp. *carotovora*	***PDF1.2*↑**	
							Arabidopsis- Fusarium oxysporum	*PR1*↑, ***PDF1.2*↑**	
***Streptomyces* sp. EN28**		?	?	+	-	+	*Arabidopsis- Erwinia carotovora* subsp. *carotovora*	***PDF1.2*↑**	
							Arabidopsis- Fusarium oxysporum	*PR1*↑, *PR5*↑, ***Hel*↑**	
Several *Streptomyces* spp.[f]	Chick pea, Faba Beans, Lentil	?	-	+	-	+	Chickpea- *Phytophthora medicaginis*	?	
***S. rochei* SM3**	Agricultural farm soil (Rhizosphere)	?	+	-	-	+	Chickpea- *Sclerotinia sclerotiorum*	***CaTF2*↑**	Srivastava et al. 2015
***Streptomyces* sp. EUSKR2S82**	Rhizosphere soil of *Eucalyptus* (Endophytic)	?	+	+	+	+	Eucalyptus- *Cylindrocladium* sp.	?	Himaman et al. 2016
***Streptomyces* sp. EUSNT1H43**	Rhizosphere soil of *Eucalyptus* (Rhizosphere)	?	+	+	+	+			
***Streptomyces* sp. EURKR1S17**	*Eucalyptus* root (Rhizosphere)	?	+	+	+	+			
***Streptomyces* sp. SS1**	Sorghum stem	+	+	+	+	+	Rice- *Magnaporthe oryzae* B157	*PR10a*↑, *NPR1*↑, ***LOX2*↑**, *PAL*↓	This study
***Streptomyces* sp. SS5**		+	+	+	+	+		*PR10a*↑, *NPR1*↑, ***LOX2*↑**, *PAL*↑	
***Streptomyces* sp. SS8**		+	-	+	-	+		*PR10a*↑, *NPR1*↑, ***LOX2*↑**, *PAL*↓	

[a]- Nitrogen fixation; [b]- Indole acetic acid; [c]- Siderophore production; [d]- Phosphate solubilization; [e]- *In vitro* antibiosis; [f]- Traits shown by majority of strains; +indicates positive and – indicates negative; ? indicates no data available; Up arrows and down arrows indicate up-regulation or down-regulation of gene expression of the specific genes mentioned. Bold form genes belong to JA/ET pathway, underlined genes are common for both the pathways and remaining genes (in regular font) belong to SA pathway.