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Effect of bacterial endophyte on expression of defense genes in Indian popcorn against *Fusarium moniliforme*

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Abstract An endophytic *Bacillus amyloliquefaciens* subsp. *subtilis* was isolated from surface sterilized seedlings of tropical corn. To evaluate the hypothesis that this endophyte is defensive in nature and determine the mechanisms of host defense we examined the effects of the endophyte on pathogens and host disease resistance genes. The bacterial endophyte showed antifungal activity against fungal pathogens *Fusarium moniliforme*, *Colletotrichum gloeosporioides* and *Aspergillus flavus*. Plant protection activity was also observed in Indian popcorn seedlings inoculated with the endophyte against *F. moniliforme*. Gene expression analysis was conducted and demonstrated that up-regulation of several defense genes in corn was greater after 48 hours of pathogen challenge than 24 hours. Plant defense pathways triggered by the endophytic bacterium appeared to be SA (salicylic acid) independent. The abundance of the *zm Jasmonic acid-induced* gene expression was greater in pathogen challenged plants pretreated with the endophytic bacterium than in the plants challenged only with pathogen. Pre-treatment with the endophytic bacterium resulted in a more intense induction of maize pathogenesis related protein genes *PR-1* and *PR-10* during interaction with *F. moniliforme* compared to plants that were treated only with the fungal pathogen.

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1 Introduction

Plants have multiple pathways that function in defense against pathogen attack (Uknes et al. 1992; Van Loon and Bakker 2006). The systemic acquired resistance (SAR) pathway may be triggered by induction of plant defense through inoculation with a necrotizing pathogen or application of chemical agents such as salicylic acid (SA), 2–6-dichloroisonicotinic acid (INA), and benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Uknes et al. 1992). Salicylic acid is a signalling molecule involved in both locally- and systemically-induced disease resistances. SAR is characterized by an early increase of endogenously synthesized SA and enhanced production of pathogenesis related (PR) proteins, specifically *PR-1*, *PR-2*, and *PR-5*, not only at the site of primary infection but also systemically in the uninfected plant tissues (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1992). The expression of PR proteins is reported to stimulate pathogens to avoid further infection. Plant growth regulators jasmonic acid and ethylene also play an important role in defense mechanisms. This jasmonic acid/ethylene (JA/ET) is independent of the SA pathway. The exogenous application of these signalling molecules (JA/ET) induces synthesis of antimicrobial proteins such as defensins and thionins (Epple et al.1997). The signalling of JA and ET is also involved in induction of the PR proteins like PR-3, a basic chitinase; PR-4, a chitin-binding protein; and PDF1.2, a member of plant defensins (van Loon and van Stein 1999).

Some non-pathogenic plant-associated bacteria like *Pseudomonas* spp. and *Bacillus* spp. induce plant defense pathways that render the host more resistant to further pathogen attack (Ongena and Thonart 2006; Van Loon and Bakker

2006). This pathway of host defense is termed induced systemic resistance (ISR). It is reported that plant growth promoting *B. mycoides* strain Bac J and *B. pumilus* strains 203–6 and 203–7 provide ISR in sugar beet by enhanced peroxidase activity and increased production of one chitinase isozyme and two isozymes of β-1,3-glucanase (Bargabus et al. 2002; Bargabus et al. 2004). A non-pathogenic rhizobacterium *Pantoea agglomerans* isolated from Teosinte (maize ancestor) induces salt tolerance by triggering expression of salt resistance genes in modern maize (Gond et al. 2015a).

Endophytic bacteria colonize internal tissues of plants establishing symbiotic interactions with hosts (White et al. 2014; Hardoim et al. 2015). These microbes may promote plant growth by secreting anti-pest compounds, producing plant growth hormones, increasing nutrient supply, etc. (Ryan et al. 2008). Endophytic actinobacteria isolated from wheat tissues up-regulate defense genes like *PR-1* and *PR-4* of SAR as well as *PDF1.2* and *Hel* genes of the jasmonic acid/ethylene (JA/ET) pathway in *Arabidopsis thaliana* (Conn et al. 2008). In a recent study, endophytic *Bacillus* spp. were reported to produce antifungal lipopeptides and induce host defense gene expression like *PR-1* and *PR-4* in maize (Gond et al. 2015b). That study demonstrated that the endophytic bacterium which produces antifungal lipopeptides in vitro can also trigger expression of pathogenesis related genes of the host without interaction with pathogens. The aim of the current study is to evaluate the effect of the endophytic bacterium *Bacillus amyloliquefaciens* subsp. *subtilis* on salicylic acid dependent and salicylic acid independent pathways of Indian popcorn, a flint maize variety.

2 Materials and methods

2.1 Isolation of endophytic bacteria

To obtain endophytic bacteria from the seedlings of tropical corn, seeds were soaked in 4 % NaOCl for 20 min and then washed three times with sterile distilled water and placed on sterile filter paper soaked with water in Petri dish for 5 days. The root and shoot parts of growing seedlings were dissected and again surface sterilized with 4 % NaOCl for 2 min and washed three times with sterile distilled water and placed on yeast extract (1 %) sucrose (1 %) agar (YESA) plates amended with antifungal cyclohexamide. The water after the last wash of the seedlings was also plated on separate culture plates to check the efficacy of surface sterilization. Plates were incubated at room temperature (~21 °C) for growth of bacteria. Based on appearance in culture and sequence analysis, a single bacterial species was obtained from seedlings. The bacterium was subcultured and purified using streak plating for identification. All isolates were stored at −80 °C in the Department of Plant Biology and Pathology, Rutgers University.

2.2 Identification of the endophytic bacterium

The bacterium was identified by 16 s rDNA sequencing from cultures. Total genomic DNA was extracted by the DNA extraction kit (Qiagen, Valencia, California, USA) and the 16 s rDNA sequence was amplified by primers 27f (AGAGTTTGATCMTGGCTCAG) and 1525r (AAGGAGGTGWTCCARCC) in a Biometra thermocycler (Biometra, Gottingen, Germany). The amplification program employed an initial denaturation at 95 °C for 5 min and then 30 cycles for 1 min at 95 °C, 1 min at 55 °C and 1.30 min at 72 °C. Final extension was at 72 °C for 10 min. The PCR product was purified by a PCR purification kit (Qiagen, Valencia, California, USA) and sent to Genewiz Inc. (South Plainfield, New Jersey, USA) for sequencing. The sequence was BLAST searched on the NCBI GenBank website to identify the closest matches.

2.3 Functional characterization of endophytic bacterium

2.3.1 Growth on N-free medium

The bacterium was streaked onto three plates of Norris Nitrogen-Free Agar and kept at room temperature for 48 h. For the control the bacterium was streaked onto three plates containing YESA.

2.3.2 Phosphate solubilisation

Phosphate solubilization capacity of the endophytic bacterium was determined by the plate assay method using Pikovskaya agar (Pikovskaya 1948). The bacterium was inoculated onto the centre of three Pikovskaya agar plates and incubated for one week. The clearing zones around the colonies were the positive indication of phosphate solubilization.

2.3.3 Auxin production

The bacterium was grown in LB broth with tryptophan (100 μg/ml) and inoculated at room temperature for two days at 100 rpm shaking. LB broth with tryptophan (100 μg/ml) was used as control. After incubation, cultures were centrifuged at 8000 rpm for 15 min at 4 °C. Two millilitres of freshly prepared Salkowski reagent (1 ml of 0.4 M $FeCl_3$ in 50 ml of 35 % sulphuric acid) was added to 4 ml of culture supernatant. The reaction mixture was incubated at room temperature for 25 min. Development of pink color indicated the production of IAA.

2.3.4 Chitinase production

The chitinase assay was done using colloidal chitin as a substrate in three plates of chitinase assay medium. Colloidal chitin was prepared according to the method of Berger and

Reynolds (1988). The composition of chitinase assay medium was Na_2HPO_4(6gL^{-1}); KH_2PO_4 (3 g L^{-1}); NH_4Cl (1 g L^{-1}); NaCl (0.5 g L^{-1}); yeast extract (0.05 g L^{-1}); agar (15 g L^{-1}) and colloidal chitin (1%*w*/*v*). Colonies showing clearing zones were considered to be chitinase-producers.

2.3.5 Antifungal activity

The dual culture assay was done for antifungal activity of the bacterium. The fungal pathogens *Fusarium moniliforme*, *Colletotrichum gloeosporioides* and *Aspergillus flavus*were collected from stored samples in the Department of Plant Biology and Pathology, Rutgers University. The selection of these fungi was based on their pathogenicity against maize. A small disc of fungus was placed at the edge of each of three plates of potato dextrose agar (PDA), and after two days the bacterium on a 7 mm diam. Agar disc was placed opposite the growing fungal colony (Fig. 1). Inhibitory activity was determined after one week at lab ambient temperature (~21 °C) by assessing presence of radial growth inhibition. The radial growth inhibition was measured by subtracting the radial growth of mycelia towards bacterium side from radial growth of mycelia opposite to bacterium side from the centre of fungal disc. The data are averages of three replicates.

2.4 Corn seedling treatment with the endophytic bacterium and fungal pathogen

Roots of six 5-day-old Indian popcorn seedlings were dipped in 5-ml of bacterial suspension (~10^6 cells/ml) for 2.5 h and incubated for 24 h at room temperature under aseptic conditions. The controls were treated with sterile saline water. After 24 h, 1-ml of spore suspension of *F. moniliforme* (~10^4 spores/ml) was sprayed onto each seedling separately. Plant defense gene expression study was done after 24 and 48 h of *F. moniliforme* inoculations respectively.

2.5 RT-qPCR for expression of defense genes in corn seedlings by the endophytic bacterium

Induction of plant defense genes by the endophytic bacterium during interaction with the fungal pathogen was examined using relative quantification (RQ) in qPCR. Total RNA was extracted from 100 mg of fresh tissues after 24 and 48 h of the *F. moniliforme* inoculation of seedlings that were either pretreated with the endophytic bacterium or water. The 'only water' treatment was used as a control. RNA was extracted by RNeasy Plant Minikit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. The concentration and purity of total RNA was measured by a NanoDrop® ND1000 spectrophotometer. Real-Time One-Step RT-PCR was performed on Step One Plus Cycler (Applied Biosystems) by QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. The reverse transcription of RNA was set at 50 °C for 30 min. The PCR steps were initial activation of Taq polymerase at 95 °C for 15 min and cycling 94 °C for 15 s, annealing 60 °C for 30 s, extension 72 °C for 30 s for 40 cycles followed by melt curve analysis. The defense genes (*zmPR1*, *zmPR10*, *zm salicylic acid induced gene* and *zm jasmonic acid induced gene*) of the Indian popcorn were identified from the Maize genetics and genomics database (http://www.maizegdb.org/sequence.php). The selection of these genes was based on their expression against fungal pathogens and their role in SAR and ISR. The endogenous control gene was maize actin *zmAct* 1. The Primers for these genes were designed by 'Primer Express' software (Table 1). The significance of the differences in gene expression was calculated by 'Student's T-test'.

Fig. 1 Inhibitory activity of *Bacillus amyloliquefaciens* subsp. *subtilis* against fungal pathogens

2.6 Inoculation of bacterium and antifungal activity in *Arabidopsis* sp.

Seeds of a wild-type strain of *Arabidopsis thaliana* Col-0 were surface sterilized with 4 % NaOCl for 2 min and washed three times with sterile distilled water. The seeds were suspended in a one-day-old bacterial suspension for 2 h. The control seeds were treated with only the bacterial growth medium (LB broth). Seedlings were grown in sterile soil in magenta boxes under a 12-h alternating light/dark cycle (florescent lighting) at laboratory ambient temperature (~21 °C) and watered periodically using sterilized demineralized water to maintain soil moisture during the course of the experiment. After one week, seedlings were sprayed with a spore suspension of *F. moniliforme*. Seedlings were grown two additional weeks prior to comparing treatments.

3 Results

3.1 The endophytic bacterium and its functional characteristics

The endophytic bacterium isolated from tropical corn seedlings was rod-shaped, Gram-positive and identified as

Table 1 Primers used for RT-qPCR in the gene expression study

Accession No.	Gene name	Primers (5′-3′)
HQ834244	*zmPR1*	TCAGTCATGCCGTTCAGCTT
		TTGTCCGCGTCCAGGAA
FJ897503.1	*zmPR10*	CAACCCGGAAGCCTACAACTAG
		GAAATCCGTTCCCCATCGA
EU955244.1	*zm Salicylic Acid induced gene*	ACTGCATCGTTCTTGCGTGTA
		ACACAGAATTTACGGCTGTAGTAAGAAG
EU952909.1	*zm Jasmonic Acid induced gene*	CCTTACAGCGCATGATGTCAA
		GCAGAACGACGCGATACGAT
NM_001155179.1	*zmAct 1*	GGGATTGCCGATCGTATGAG
		GAGCCACCGATCCAGACACT

Bacillus amyloliquefaciens subsp. *subtilis* based on its 16 s rDNA sequence homology to strains from NCBI GenBank. The bacterium was able to grow on a nitrogen-free medium, indicating nitrogen fixing capacity. In dual culture assay, the bacterium was able to inhibit the growth of all filamentous fungi tested (Fig. 1, Table 2) while chitinase activity was not found on colloidal chitin agar (Table 2). Secretion of auxin was found in this bacterium while phosphate solubilisation capacity was not indicated on Pikovskaya agar.

3.2 Effect of the endophytic bacterium on the Indian popcorn defense against fungal pathogen

The Indian popcorn seedlings treated with *B. amyloliquefaciens* subsp. *subtilis* or water did not show any disease symptoms after 3 days of observations. The growth of the cottony mycelium of *F. moniliforme* was found on the seedlings pre-treated with only water (Fig. 2). The seedlings pre-treated with *B. amyloliquefaciens* subsp. *subtilis* appeared healthy after 48 h of the *F. moniliforme* treatment (Fig. 2).

3.3 Induction of defense gene expression in Indian popcorn by endophytic bacterium

The pattern of gene expression was determined after 24 and 48 h of pathogen treatment by transcript analysis using qRT-PCR. Among four genes studied, only *zmPR-10* was expressed in seedlings either treated with bacterium or pathogen after 24 h of treatment with greater expression in pathogen-treated seedlings pre-treated with the bacterium (Fig. 3). After 48 h from pathogen inoculation, the expression of *zmPR-1*, *zmPR-10* and *zm Jasmonic Acid induced* genes were significantly greater in bacterium pre-treated plants than 'only pathogen' treated plants (Fig. 4). The expression of *zmPR-1* was down-regulated in pathogen challenged plants and highly up-regulated in bacterium treated plants. The results show that the bacterium did not have any effect on the expression of the *zm Salicylic Acid induced* gene. The 'only fungal pathogen' treatment induced expression of the *zm Salicylic Acid* gene compared to the water treated control plants. The *zm Jasmonic Acid induced* gene was down regulated in 'only bacterium' treated plants compared to the water control plants but highly up-regulated in pathogen-treated plants pre-treated with the bacterium (Fig. 4).

3.4 Effect of endophytic bacterium on *Arabidopsis* defense against fungal pathogen

The *Arabidopsis* seedlings treated with *B. amyloliquefaciens* subsp. *subtilis* were free from fungal attack. The cottony mycelia of *F. moniliforme* were growing on the soil surface of both bacteria-treated and untreated *Arabidopsis* seedlings but the bacterium-treated seedlings were healthy and flowering (Fig. 5). All untreated control seedlings were infected with the fungal pathogen and exhibited necrosis symptoms.

Table 2 Characterization of endophytic bacterium isolated from seedlings of tropical corn

Endophytic bacteria	Growth on Norris Agar	Phosphate solubilisation	Auxin production	Chitinase production	Antifungal activity (radial growth inhibition in cm ± standard deviation)		
					Aspergillus flavus	*Colletotrichum gloeosporioides*	*Fusarium moniliforme*
Bacillus amyloliquefaciens subsp. *Subtilis*	+	-	+	-	1.70 ± 0.31	2.00 ± 0.30	2.00 ± 0.50

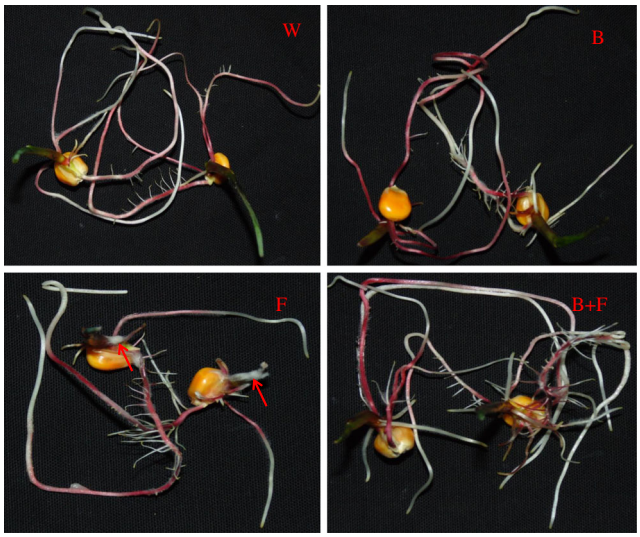

Fig. 2 Indian popcorn seedlings after 48 h of fungus inoculation (W-water; B-*Bacillus amyloliquefaciens* subsp. *subtilis*; F-*Fusarium moniliforme*; B + F-Bacterium and fungus); Arrow indicates fungal growth

4 Discussion

Endophytic bacteria may play roles in various plant growth promotion activities including plant protection against phytopathogens. The infection of plants by endophytic bacteria may occur in the rhizosphere or the phyllosphere (Rosenblueth and Martinez Romero, 2004). Some endophytic bacteria transmit through seeds of the host and form systemic infections that may colonize all parts of plants (Coombs and Franco 2003; Hallmann et al. 1997). The endophytic bacterium isolated in this study from tropical corn seedlings was identified as *Bacillus amyloliquefaciens* subsp. *subtilis* (Table 2). The most common genera reported as endophytic bacteria from maize varieties are *Bacillus* spp., *Burkholderia* spp., *Herbaspirillum* spp., *Enterobacter* spp., *Klebsiella* spp., and actinobacteria *Arthrobacter* spp. and *Microbacterium* spp. (Johnston-Monje and Raizada 2011; Zinniel et al. 2002; Chelius and Triplett 2000; McInroy and Kloepper 1995; Gond et al. 2015b). *Bacillus amyloliquefaciens* occurs as a common plant growth promoting rhizobacterium (PGPR) as well as an endophyte which secretes various kinds of antifungal molecules, including lipopeptides (White et al. 2014; Gond et al. 2015b). The strain of *B. amyloliquefaciens* subsp. *subtilis* isolated in this study showed strong activity against fungal pathogens and was able to grow on nitrogen-free media, and further was shown to reduce acetylene in culture (data not shown) indicating nitrogen fixing capacity (Table 2). This suggests that *B. amyloliquefaciens* subsp. *subtilis* promotes plant growth not only by secreting anti-pest compounds but also may increase a host's nitrogen supply (Szilagyi-Zecchin et al. 2014). This bacterium showed strong antifungal activity, but did not show chitinase secretion, suggesting that antifungal activity may be due to lipopeptides or similar inhibitors (Gond et al. 2015b). Phosphate solubilisation was not seen in this bacterium, perhaps because it grows within host tissues; populations of phosphate solubilising bacteria are frequently in greater abundance in rhizospheric soil than other habitats (Katznelson et al. 1962; Raghu and MacRae 1966).

We observed antifungal activity of *B. amyloliquefaciens* subsp. *subtilis* in dual culture assays; and further we demonstrated that the bacterium could protect plants of Indian popcorn from fungal pathogens (Fig. 2). The growth of fungal mycelia was not observed on bacterium treated seedlings. Similarly, an endophytic *Pseudomonas* sp. strain PsJN gave enhanced protection against *Verticillium* sp. in tomato (Sharma and Nowak 1998) and *Botrytis cinerea* in grapevine (Barka et al. 2000, 2002). The early response of plants in microbe-plant interactions is frequently release of signalling molecules including salicylic acid (SA) and jasmonic acid (JA). These signal molecules are involved in induction of expression of defense genes (Mur et al. 2006; Vlot et al.

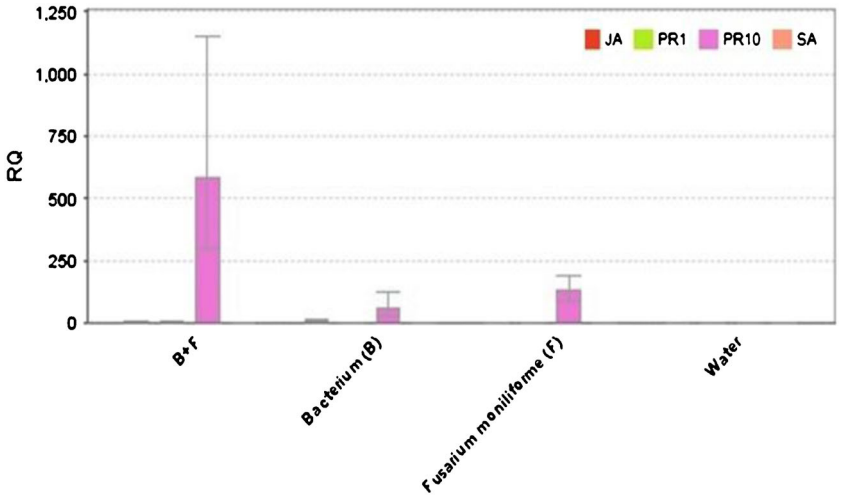

Fig. 3 Induction of defense gene expression in Indian popcorn seedlings by *Bacillus amyloliquefaciens* subsp. *subtilis* after 24 h of *F. moniliforme* inoculation showing up-regulation of *zmPR-10* gene in fungus treated plants pre-treated with bacterium (RQ-Relative quantification)

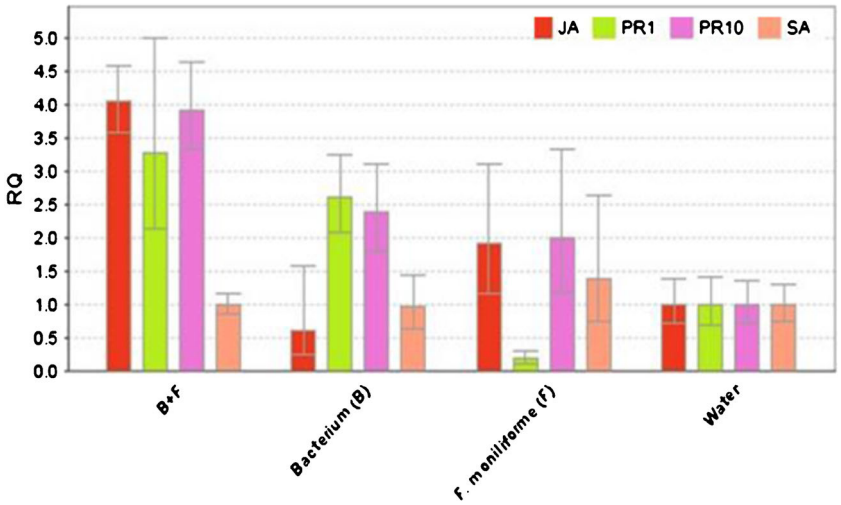

Fig. 4 Induction of defense gene expression in Indian popcorn seedlings by *Bacillus amyloliquefaciens* subsp. *subtilis* after 48 h of *F. moniliforme* inoculation showing up-regulation of *zmPR-1*, *zmPR-10* and *JA induced genes* in fungus treated plants pre-treated with bacterium (RQ-Relative quantification)

2009). The expression of signalling genes either for SA or JA was not detected after 24 h of pathogen challenge. It appears that *F. moniliforme* resistance generation in seedlings is SA independent because expression of the SA gene was down-regulated in 'bacterium treated' and 'bacterium and fungus treated' seedlings (Fig. 4). In contrast to this the abundance of the JA gene transcript was highest after 48 h of the pathogen challenged seedlings that were pre-treated with *B. amyloliquefaciens* subsp. *subtilis* (Fig. 4). In a similar study, endophytic *Streptomyces* sp. strain EN27mediated resistance in *Arabidopsis* seedlings against the fungal pathogen *F. oxysporum* via a NPR1-dependent pathway but also required SA and was JA/ET independent (Conn et al. 2008).

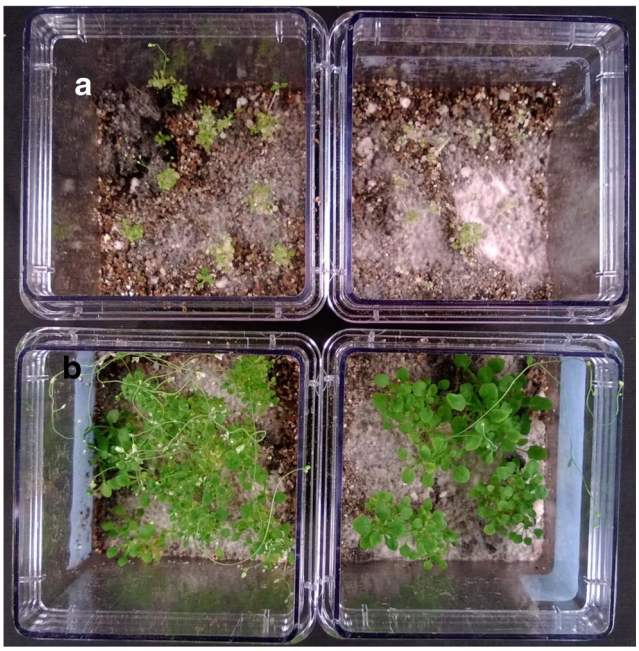

Fig. 5 Effect of *B. amyloliquefaciens* subsp. *subtilis* on pathogenesis of *F. moniliforme* on *Arabidopsis* seedlings (**a**- Control; **b**-Treated with *B. amyloliquefaciens* subsp. *subtilis*)

The expression of endogenous JA is involved in a variety of critical functions, including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation, and defense responses against pests and pathogens (Creelman and Mullet 1997). The over-expression of pathogenesis related genes *zmPR-1* and *zmPR-10* was also observed in pathogen challenged seedlings pre-treated with *B. amyloliquefaciens* subsp. *subtilis* (Fig. 4). Actinobacteria have been shown to up-regulate both SAR and JA/ET pathway defense genes, but defense responses by *P. fluorescens* WC417r or LSW17S and *Bacillus* spp. were found to be SA independent and were rather found to be associated with JA and ET (Kloepper et al. 2004; Pieterse et al. 1996 and 1998; Conn et al. 2008). The induction of ISR in *Arabidopsis thaliana* by root-colonizing strains of *Pseudomonas fluorescens* was shown to be ethylene- and JA-dependent but SA-independent (Knoester et al. 1999; Ton et al. 2002; Iavicoli et al. 2003).

In this study after 24 h of pathogen challenge, only the *zmPR-10* gene was induced in seedlings pre-treated with *B. amyloliquefaciens* subsp. *subtilis* (Fig. 3). The expression of *zmPR-10* was also greater in pathogen challenged seedlings pre-treated with bacterium compared to only pathogen challenged seedlings (Fig. 4). This suggests a role of the zmPR-10 protein in protection of corn seedlings against the fungal pathogen *F. moniliforme*. The expression of zmPR-10 protein is also induced during kernel development of maize and protects against *Aspergillus flavus* (Chen et al. 2006). The expression of PR-10 protein has also been reported in rice plants to protect against infection by the pathogen *Magnaporthe grisea* and in cowpea to protect against infection by the rust *Uromyces vignae* (McGee et al. 2001; Mould et al. 2003). The up-regulation of the *zmPR-1* gene was observed after 48 h in 'pathogen challenged seedlings pre-treated with bacterium' and seedlings 'treated with bacterium' while down-regulated in seedlings only challenged with the fungal pathogen (Fig. 4). The up-regulation of the *zmPR-1* gene was also

reported in maize seedlings treated with endophytic *B. subtilis* by Gond et al. (2015b).

Plant protection activity of *B. amyloliquefaciens* subsp. *subtilis* was also seen in the model plant *Arabidopsis thaliana* Col-0 against *F. moniliforme* (Fig. 5). The endophytic bacterium was able to protect *A. thaliana* Col-0 plants, while the fungal pathogen was seen to grow on the soil surface only (Fig. 5). In an earlier study an endophytic strain of *B. subtilis* isolated from tropical maize was found to suppress the growth of seed mycoflora of *Arabidopsis thaliana* (Gond et al. 2015b). Endophytic *Bacillus* species not only act against fungal pathogens but also protect plants against bacterial diseases. A strain of *Bacillus subtilis* (Lu144) was found to reduce incidence of bacterial wilt of mulberry (Ji et al. 2008).

5 Conclusions

Our results demonstrate the role of endophytic microorganisms in enhancing plant growth and development. *B. amyloliquefaciens* subsp. *subtilis* induces Indian popcorn plants to synthesize defense molecules against fungal pathogen *F. moniliforme*. The defense pathway triggered by *B. amyloliquefaciens* subsp. *subtilis* in Indian popcorn seedlings was jasmonic acid dependent. The expression of pathogenesis-related genes like *PR-1* and *PR-10* was highly induced by *B. amyloliquefaciens* subsp. *subtilis* in response to the pathogen. The capacity of *B. amyloliquefaciens* subsp. *subtilis* to protect plants from disease was also demonstrated in *Arabidopsis thaliana* Col-0 against the fungal pathogen *F. moniliforme.*

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