

RESEARCH ARTICLE

Bacillus spp., a bio-control agent enhances the activity of antioxidant defense enzymes in rice against *Pyricularia oryzae*

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Abstract

Plant growth promoting rhizobacteria (PGPR) are found to control the plant diseases by adopting various mechanisms. Induced systemic resistance (ISR) is an important defensive strategy manifested by plants against numerous pathogens especially infecting at aerial parts. Rhizobacteria elicit ISR by inducing different pathways in plants through production of various metabolites. In the present study, potential of *Bacillus* spp. KFP-5, KFP-7, KFP-17 was assessed to induce antioxidant enzymes against *Pyricularia oryzae* infection in rice. The antagonistic *Bacillus* spp. significantly induced antioxidant defense enzymes i.e superoxide dismutase (1.7–1.9-fold), peroxidase (3.5–4.1-fold), polyphenol oxidase (3.0–3.8-fold), phenylalanine ammonia-lyase (3.9–4.4-fold), in rice leaves and roots under hydroponic and soil conditions respectively. Furthermore, the antagonistic *Bacillus* spp significantly colonized the rice plants ($2.0E+00$ – $9.1E+08$) and secreted multiple biocontrol determinants like protease (1.1–5.5 U/mg of soil or U/mL of hydroponic solution), glucanase, (1.0–1.3 U/mg of soil or U/mL of hydroponic solution), siderophores (6.5–42.8 µg/mL or mg) in the rhizosphere of different rice varieties. The results showed that treatment with *Bacillus* spp. enhanced the antioxidant defense activities in infected rice, thus alleviating *P. oryzae* induced oxidative damage and suppressing blast disease incidence.



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Introduction

Plant growth promoting rhizobacteria (PGPR) are being widely used as an alternative to chemical fungicides and fertilizers due to their eco- friendly nature [1–4]. They suppress phytopathogens by utilizing various mechanisms in their habitation. Enhanced plant immunity referred as “induced systemic resistance” (ISR) is one of the important mechanism used by various PGPR to secure the plant against extensive range of fungal, bacterial and viral pathogens [5, 6].

ISR is an effective defensive mechanism which is manifested as a result of certain physiological changes in the plant, such as modification in cell wall structure and *de novo* synthesis of antimicrobial compounds like pathogenesis-related (PR) proteins and phytoalexins, that prevent the dispersion of pathogens [7–11]. The antioxidant enzymes peroxidase (PO), phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) might be elicitors of ISR as their activity in plant has been highly correlated with disease suppression [12].

The activities of various antioxidant enzymes help the plants to mitigate the reactive oxygen species (ROS) level which is source of oxidative stress during pathogen infection [13, 14]. The PO, and PPO enzymes are responsible for the production of phenolic compounds which contribute to the reinforcement of cell barriers [15–18]. PAL initiates the phenylpropanoid pathway, resulting in the biosynthesis of phytoalexins and/or phenolic compounds [19–22]. The phenolic compounds and specific flavonoids induce resistance in host plants after challenge inoculation of pathogens [23–26].

ISR in plants is induced by a number of biotic and abiotic agents. The biotic agents elicit responses around the infected plant cells which cause the cell death through incompatible interactions. Therefore, the pathogen can be trapped in dead cells and seems to be restricted to the initial infection site [27–29].

Several studies show the ability of beneficial microbes to stimulate variety of defense reactions in host plants, in response to pathogen infection, particularly, activity of antioxidant defense enzymes [30–33]. The PGPR bioformulation suppressed the early blight disease in tomato by elevating the activity of antioxidant enzymes such as PAL, PO, PPO, chitinase, β -1,3-glucanase, superoxide dismutase [34], catalase, lipoxygenase, and phenolics [31].

Bacillus spp. are recognized as valuable biocontrol agents against diverse phytopathogens through induction of systemic resistance as well as improve efficacy through consistency under field conditions [35, 36]. *Bacillus* spp. produce variable metabolites such as antibiotics, siderophores, salicylic acid (SA), lipopolysaccharides (LPS) and hydrolytic enzymes [37–39] to suppress pathogen either directly or through enhancing the plant defense mechanisms. The involvement of particular strain or/and its metabolites in regulating host defensive ability is scantily understood and needs to be explored. In our previous study, *Bacillus* spp. capable to produce qualitatively higher amounts of siderophores, protease and glucanase *in vitro* significantly reduced the blast disease caused by foliar pathogen *Pyricularia oryzae* [40]. The present study aims to gain an insight into the induction of activity of defense related enzymes in rice and secretion of their potential elicitor in rhizosphere during the *Bacillus* spp. *P. oryzae* interaction on different rice varieties.

Materials and methods

Microbes and culture conditions

Antagonistic *Bacillus* spp. strains KFP-5 (KT380825), KFP-7 (KT380826) and KFP-17 (KJ719446) capable to control the rice blast caused by virulent strain of *P. oryzae* [40] were obtained from Plant Microbe Interaction Laboratory, COMSATS Institute of Information Technology (CIIT) Islamabad, Pakistan. Routinely, the bacterial and fungal strains were grown on Luria-Bertani (LB) broth/agar and potato dextrose (PD) broth/agar respectively.

Quantification of rhizobacterial bio control determinants

The biocontrol determinates viz siderophores, protease and glucanase produced by *B. subtilis* KFP-5, KFP-7 and KFP-17 [40] were quantified by growing them in respective medium as described below:

Siderophores. Siderophores produced by the *Bacillus* spp. were quantified [41]. The bacterial strains were grown in the presence and absence of *P. oryzae* in nutrient broth at 37°C, 160 rpm for 96 h [42]. The cell free supernatant was collected by centrifugation at 10,000 rpm for 10 min followed by passing through 0.25 μ m filter and acidified (pH = 2.0) with 1M HCl. The siderophores were extracted with twice volume of ethyl acetate and dissolved in 5 mL of 50% ethanol. The absorbance was recorded spectrophotometrically at 700 nm. 2, 3- dihydroxy benzoic acid (Alfa Aesar) was used to plot the standard curve. The quantity of siderophore synthesized

was expressed as mmol of benzoic acid mL of culture filtrate. The experiment was repeated thrice to avoid experimental errors.

Protease. Protease activity was quantified by the modified method [43]. The bacteria were grown in LB broth [44] and cell free supernatant was obtained as described in section (Siderophores). The supernatant was precipitated by adding 50–55% ammonium sulphate and precipitate was used as crude enzyme extract. The crude enzyme extract and substrate (1.0% casein) solution were mixed in 1:1(V/V) ratio to set up reaction and incubated at 40°C for 20 min. The reaction was stopped by adding 3 mL of 10% tri-chloroacetic acid. The tyrosine released from casein was measured spectrophotometrically (Implen Pearl) at 280 nm and quantified by comparing the standard curve drawn between the absorbance and concentration of tyrosine. The protease activity was calculated by using the following formula [45].

Enzymes activity (Units/mL)

$$= \frac{\mu\text{mole tyrosine equivalent release} \times \text{Total Volume of Assay (mL)}}{\text{Volume of enzyme taken (mL)} \times \text{Incubation Time (min)} \times \text{Volume of sample taken in cuvette (mL)}}$$

Glucanase. Glucanase activity of the *Bacillus* spp. was quantified by DNS (Dinitro salicylic acid) method [46]. The DNS method is based on alkaline solution of 3, 5-dinitrosalicylic acid reacts with reducing sugars (eg. Glucose, lactose.) and is converted into 3-amino-5-nitrosalicylic acid with orange color. The bacteria were grown in selective media consisting of different ingredients (g/L) i.e. K_2HPO_4 (0.065), KH_2PO_4 117 (0.25), $(\text{NH}_4)_2\text{SO}_4$ (0.05), NaCl (0.25), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.012), yeast extract (0.15), 1% Laminarian at 37°C, 130 rpm for 96 h [47]. The cell free supernatant was obtained as described in section 2.2.1 and used as crude enzyme extract. The reaction was set up by mixing crude enzyme extract and DNS reagent in 1:2 (V/V) followed by incubation in boiling water for 15min. The reaction was stopped by cooling down the mixture. The absorbance was recorded at 500 nm in the concentration of glucose was computed through the standard curve plotted between absorbance and glucose concentration. The glucanase activity was measured by using the formula mentioned in section (Protease) except μmole glucose was used instead of tyrosine [46].

In planta experiments

Ability of *Bacillus* spp. to induce activity of defense related enzymes and produce biocontrol determinants *in situ* was assessed on three rice varieties viz Basmati-515, Basmati-super and Basmati-385 under hydroponic and soil conditions. The seeds were surface sterilized with 0.1% mercuric chloride (HgCl_2) and soaked in sterile distilled water for 12–16 h. The imbibed seeds were placed in rows on sterilized sand in a tray for germination and growth until 12–15 days.

Experimental design and microbial inoculation. The experiment was designed in complete randomized design (CRD) with three replications and six treatments viz *P. oryzae* (T_1 = Negative control), = Fungicide + *P. oryzae* (T_2 = Chemical control), *Bacillus* sp KFP-5 + *P. oryzae* (T_3), *Bacillus* sp KFP-7 + *P. oryzae* (T_4), *Bacillus* sp KFP-17 + *P. oryzae* (T_5), Untreated (T_6). The bacterial and fungal inoculum was applied as described in our previous study [40].

Growth of rice plants in hydroponic culture. Twelve days old seedling roots were sterilized with mercuric chloride (0.1%), dipped in bacterial suspension (10^9 CFU/mL) for 2 h and transplanted in hydroponic tray filled with the nutrient solutions consisting of $(\text{NH}_4)_2$ (48.2 mg/L), MgSO_4 (154.88 mg/L), K_2SO_4 (15.9 mg/L), KNO_3 (18.5 mg/L), KH_2PO_4 (24.8 mg/L), $\text{Ca}(\text{NO}_3)_2$ (86.17 mg/L), Fe- citrate (7 mg/L), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.9 mg/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.11 mg/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.04 mg/L) and H_2MoO_4 (0.01 mg/L)[48]. Fungicide was applied by

dipping the seedling roots in solution consisting of 1.5g Thiophanate Methyl dissolved per liter of water. Hydroponic solution was replaced after every four days. The cells present in nutrient solution were harvested by centrifugation and re dissolved in the fresh nutrient solution every time.

Growth of rice plants in soil. The earthen pots (20x 30 cm²) were obtained from local Nursery and filled with sterilized soil. The soil has clay loam texture with total nitrogen (N = 0.04%), phosphorous (P = 15.7 mg/Kg), sodium (Na = 4.6 meq/L), calcium magnesium (CaMg = 7.4meq/L), zinc (Zn = 0.76), iron (Fe = 4.73 mg/Kg), electric conductivity (1.3 dms⁻¹), pH (7.81), organic matter (0.72%), sodium adsorption ratio (SAR 2.6), exchangeable sodium percentage (ESP 2.5). The rice plants were grown by following the recommended agronomic practices as describe in our pervious study. [40].

Quantification of antioxidant defense related enzymes activity

The antioxidant defense related enzymes viz superoxide dismutase [34], peroxidase (PO) [49], polyphenol oxidase (PPO) and phenylalanine ammonialyas (PAL) were quantified from rice plants grown in hydroponic and pot conditions after 96 h of fungal inoculation.

The representative samples of leaves and roots were collected from rice plants. Two gram of each sample was crushed with 4 mL of 0.1M phosphate buffer (pH 7) in pre chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm, 4°C for 15 min and supernatant was used as crude enzyme extract [50]. Absorbance of PO, PPO and PAL, was observed on spectrophotometer at 280 nm, 450nm, 460 nm respectively [8, 38, 51–54]. Enzyme activity was expressed as change in absorbance min/ g fresh weight (FW).

For SOD activity the supernatants were obtained in Phosphate buffers (pH 7). Total SOD activity was assayed by the photochemical [55]. One unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of the rate of NBT reduction measured at 560 nm (U/ g FW) [56].

Colonization of *Bacillus* spp. with rice rhizosphere/rhizoplane/endosphere

The colonizing ability of *Bacillus* spp with root rhizosphere /rhizoplane/endosphere of rice was assessed at 96th h of pathogen inoculation. The rhizosphere/rhizoplane/endosphere sample of rice plants growing in hydroponic solution was obtained by collecting the different roots i.e. primary, secondary and lateral and 20 mL nutrient solution associated with these roots using sterile syringe [57, 58]. The roots were grinded, homogenized in nutrient solution and 1mL of this homogenate was used for further process. In pot experiment, the roots and adhering soil were collected and pooled. One g of homogenized soil was used to isolate the rhizobacteria. The *Bacillus* spp. were isolated by serial dilution as described in our previous studies [37, 38, 40].

Quantification of biocontrol determinants secreted *in situ*

Siderophores, protease and glucanase enzymes secreted by rice plants were extracted from the hydroponic solutions and soil. Hydroponic solution (50 mL) was centrifuged and supernatant was passed through 0.25µm syringe filter. Cell free solution was used for quantification of biocontrol determinants.

For extraction of siderophores, glucanase and protease from soil, One g soil was suspended in 100 mL of phosphate buffer (1 M, pH = 7.0), mixed well by shaking at 200 rpm for 10 min and kept at room temperature until settling down of the soil particles [59]. The mixture was filtered through Whatman filter paper three times. The filtrate was centrifuged at 13,000 rpm for

5 min at 4°C [60]. The biocontrol determinants were extracted and quantified from these supernatants as described in section (Siderophores)

Statistical analysis

The data was analyzed by variance analysis [61] using the statistical package Statistix 8.2. All the minimal data set has been included in supplementary material (S1 File).

Results

Quantity of biocontrol determinants produced by *Bacillus* spp

Bacillus spp. strains KFP-5, KFP-7 and KFP-17 produced variable quantities of siderophore, protease and glucanase in absence as well as presence of rice blast pathogen *P.oryzae*. The maximum biocontrol determinants were produced in presence of *P.oryzae* i-e., siderophore (29–43.3µg/mL), protease (20.9–29.3U/mL) and glucanase (0.73–1.24 U/mL). However in absence of *P.oryzae*, the production of biocontrol determinants was lower i-e., siderophores (20.5–27.0 µg/mL), protease (11.6–24 U/mL) and glucanase (0.3–0.6 U/mL). The *Bacillus* spp. showed same order of biocontrol determinants production in the absence or presence of *P. oryzae* i-e KFP-17>KFP-5>KFP-7 (Fig 1).

Effect of *Bacillus* spp. on the activity of antioxidant defense enzymes in rice

The antioxidant defense enzyme SOD, POD, PPO, PAL which act as first line of defense against the plant pathogens were elicited by the antagonistic *Bacillus* spp. The tested strains enhanced enzymatic activity in leaves and roots of three tested rice varieties viz—basmati super, basmati-515, basmati-385 grown in hydroponic solutions as well as soil.

Superoxide dismutase (SOD) activity. The antagonistic bacteria induced the SOD activity (1.7–1.9 fold) over negative control (*P. oryzae*) in both hydroponic and soil conditions. The induction of SOD activity in leaves was higher (1.3–1.9 fold) as compare to that of roots (1.1–1.7 fold) in all varieties. Maximum SOD activity was observed in basmati-385 (1.8–1.9 fold) followed by that of basmati-515 (1.2–1.6 fold) and basmati-super (1.2–1.4 fold). *Bacillus* sp KFP-

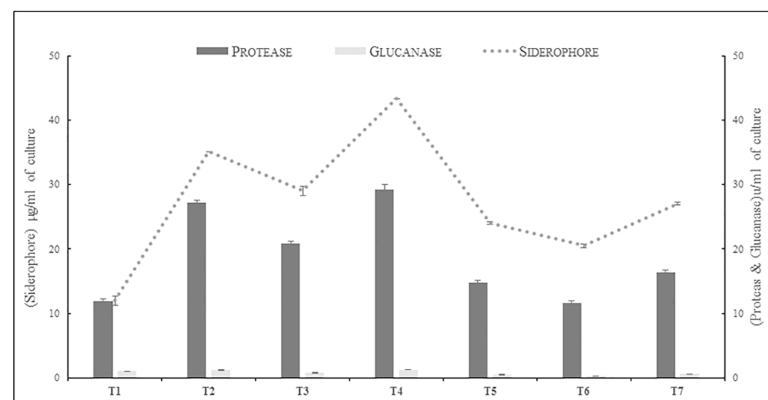


Fig 1. Production of biocontrol determinants by antagonistic bacteria in presence and absence of *P. oryzae*. T1 = *P.oryzae*, T2 = *Bacillus* sp KFP-5+*P.oryzae*, T3 = *Bacillus* sp KFP-7+*P.oryzae*, T4 = *Bacillus* sp KFP- 17+*P.oryzae*, T5 = *Bacillus* sp KFP-5, T6 = *Bacillus* sp KFP-7, T7 = KFP- *Bacillus* sp 17. Values are mean of three replicates Bars represent the standard error of means. All treatments are significantly different from each other at P<0.001.

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17 elicited highest SOD activity (1.2–1.9 fold) followed by that of *Bacillus* sp KFP5 and *Bacillus* sp KFP-7 (1.1–1.8 fold). The SOD activity was little induced in fungicide treatment (1.0–1.2 fold). The SOD activity was highly induced in pot experiments (1.9 fold) as compare to that of hydroponic (1.4 fold) experiment (Fig 2).

Peroxidase (PO) activity. In present investigation, the antagonistic bacteria induced the POD activity up to (3.5–4.1 fold) over negative control (*P. oryzae*) in both conditions. The expression level of POD activity was comparatively higher in leaves (3.5–4.2 fold) than roots (2.0–4.1 fold). The variety basmati-super showed the maximum response (3.5–4.1 fold) followed by that of basmati -515 (3.2–3.9 fold) and basmati -385 (2.8–3.6 fold). The maximum induction was showed in *Bacillus* sp KFP-17 treated plants (3.5–4.1 fold) followed by that of *Bacillus* sp KFP-5 (2.4–3.2 fold) and *Bacillus* sp KFP-7 (2.1–2.9 fold). POD activity of fungicide was observed (1.1–1.3 fold) over negative control. The POD activity was highly induced in pot experiments (4.2 fold) as compare to hydroponic experiment (3.6 fold) as shown in (Figs 3 and 4).

Polyphenol oxidase (PPO) activity. The antagonistic bacteria induced the maximum PPO activity (3.0–3.8 fold) over negative control (*P. oryzae*) in hydroponic and soil conditions. The bacteria enhanced the activity of PPO in roots (1.2–3.8 fold) as compared to leaves (1.5–2.9 fold) in all varieties. The PPO activity was highest in basmati -super (1.2–3.8 fold) followed by that of basmati-385 (1.5–3.0 fold) and basmati-515 (2.8–2.9 fold) while maximum in *Bacillus* sp KFP-17 (2.2–3.8 fold) followed by that of *Bacillus* sp KFP-5 (1.9–3.0 fold) and *Bacillus* sp KFP-7(1.2–1.7 fold). The PPO activity was observed in fungicide (1.1–1.3 fold) over negative control. The PPO activity was highly induced in pot experiments (3.8 fold) as compare to hydroponic experiment (2.9 fold) as shown in (Figs 3 and 4).

Phenylalanin ammonia-lyase (PAL) activity. In present analysis, the PAL activity was induced by the antagonistic bacteria (3.9–4.4 fold) in both conditions. The expression level of PAL activity was considerably higher in leaves (3.9–4.4 fold) as compared to roots (1.6–2.4

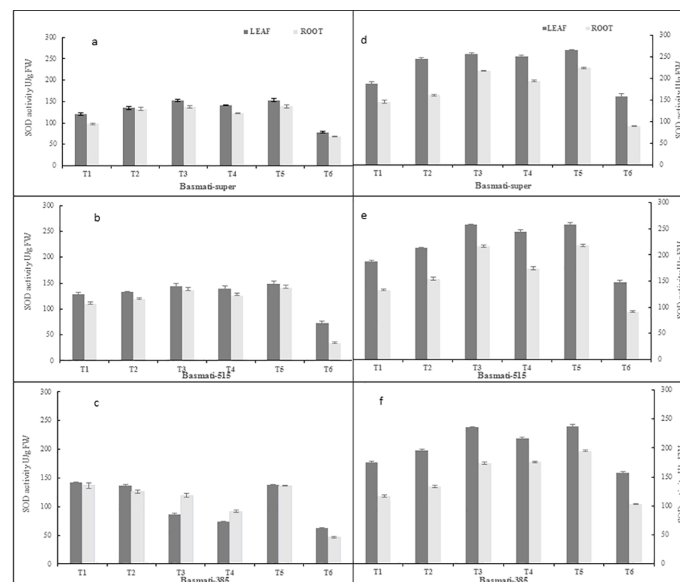


Fig 2. SOD contents in leaves and roots of different rice varieties grown hydroponically (left panel a, b, c and pot/soil experiment (right panel d, e, f). T1 = *P. oryzae*, T2 = Fungicide + *P. oryzae*, T3 = *Bacillus* sp KFP-5+ *P. oryzae*, T4= *Bacillus* sp KFP-7+ *P. oryzae* T5 = *Bacillus* sp KFP-17+ *P. oryzae* T6 = untreated. Values are means of three replicates and vertical bars represent the standard error. All treatments are significantly different from each other at P<0.001.

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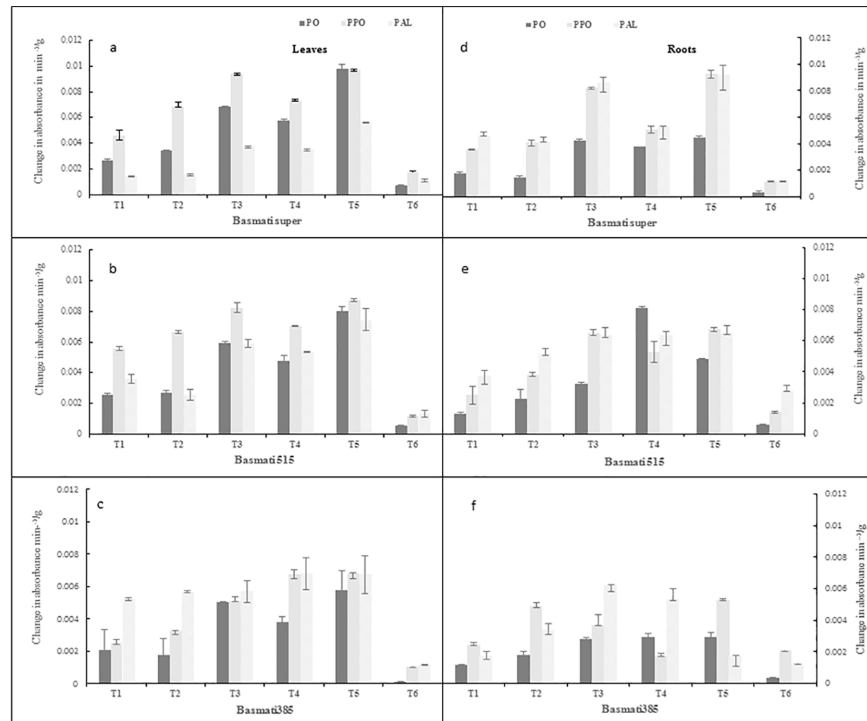


Fig 3. Defense enzyme (PO, PPO, PAL) contents in leaves (left panel a, b, c) and roots (right panel d, e, f) of rice varieties grown hydroponically. T1 = *P. oryzae*, T2 = Fungicide + *P. oryzae*, T3 = *Bacillus* sp KFP-5 + *P. oryzae*, T4- *Bacillus* sp KFP-7+ *P. oryzae* T5 = *Bacillus* sp KFP-17+ *P. oryzae* T6 = untreated. Values are means of three replicates and vertical bars represent the standard error. All treatments are significantly different from each other at $P < 0.001$.

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fold). Among rice varieties, highest response was observed in basmati-385 (3.9–4.4 folds) followed by that of basmati-515 (2.2–2.9 fold) and basmati-super (1.0–1.9fold). *Bacillus* sp KFP-17 induced highest PAL activity (1.9–4.4fold) followed by that of *Bacillus* sp KFP-5 (1.5–3.8 fold) and *Bacillus* sp KFP-7 (1.0–2.9 fold). The fungicide also induced PAL activity (1.0–2.1 fold) over negative control. The PAL activity was highly induced in pot experiments (4.4-fold) as compare to hydroponic experiment (1.9-fold; Figs 3 and 4).

In situ secretion of biocontrol determinants

Offensive PGPR colonization and defensive retention of rhizosphere niches are enabled by production of bacterial biocontrol determinants. The antagonistic bacteria secreted detectable quantities of siderophores, protease and glucanase in the rhizosphere of rice plants grown hydroponically and soil.

Protease. The antagonistic strains secreted protease in rhizosphere of all rice varieties. The maximum protease activity was recorded in rhizosphere of basmati -super (1.1–5.5 U/mg of soil or U/mL of hydroponic solution) followed by that of basmati-515 (1.0–4.9 U/mg or U/mL) and basmati-385 (0.8–4.2 U/mg or U/mL). The *Bacillus* sp KFP-17 secreted the maximum protease (1.1–5.5 U/mg or U/mL) followed by that of *Bacillus* sp KFP-5 (1.1–4.4 U/mg or U/mL) and *Bacillus* sp KFP-7(0.8–3.6 U/mg or U/mL). The minor quantity of protease was also recorded from the rhizosphere of fungicide treated plants (0.2–0.6 U/mg or U/mL). Higher amount of protease was recorded in the rhizosphere of soil grown rice plants (5.5 U/mg) as compare to that of grown hydroponically (1.9 U/mL) as shown in (Fig 5).

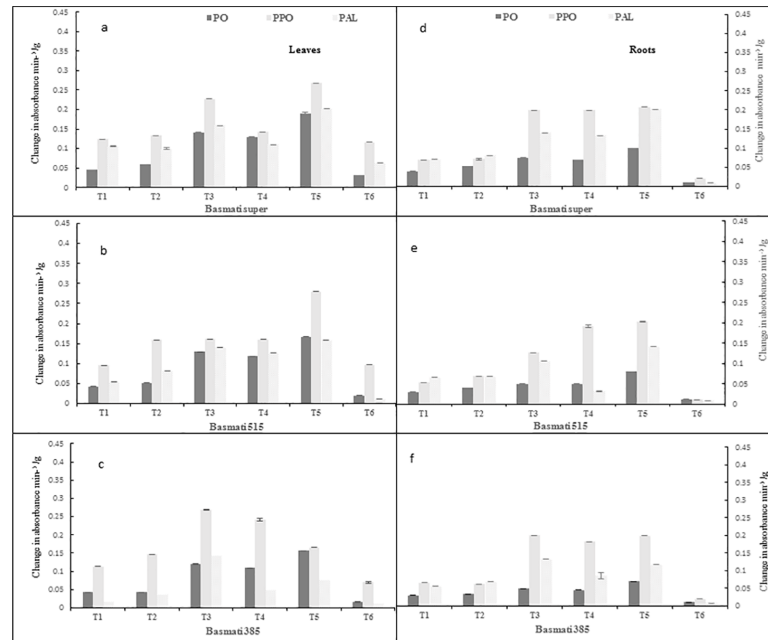


Fig 4. Defense enzyme (PO, PPO, PAL) contents in leaves (left panel a, b, c) and roots (right panel d, e, f) of rice varieties grown in pot/soil. T1 = *P. oryzae*, T2 = Fungicide + *P. oryzae*, T3 = *Bacillus* sp KFP-5+ *P. oryzae*, T4 = *Bacillus* sp KFP-7+ *P. oryzae*, T5 = *Bacillus* sp KFP-17+ *P. oryzae*, T6 = untreated. Values are means of three replicates and vertical bars represent the standard error. All treatments are significantly different from each other at $P < 0.001$.

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Glucanase. The glucanase was also secreted in the rhizosphere of all rice varieties. The maximum glucanase production was recorded in basmati-super (1.0–1.3 U/mg of soil or U/mL of hydroponic solution) followed by that of basmati-515 (0.8–1.3 U/mg or U/mL) and basmati-385 (0.7–1.3 U/mg or U/mL). The *Bacillus* sp KFP-17 secreted maximum glucanase (0.8–1.3 U/mg or U/mL) followed by that of *Bacillus* KFP-5 sp (0.7–1.1 U/mg or U/mL) and *Bacillus* sp KFP-7 (0.7–1.0 U/mg or U/mL). The production of glucanase (0.3–0.6 U/mg or U/mL) was observed in fungicide treated plants. Higher amount of glucanase (1.3 U/mg) was recovered from the rhizosphere of rice plants grown in soil as compare to that of grown in hydroponic (1.2 U/mL) (Fig 5).

Siderophores. The antagonistic strains also secreted siderophores in the rice rhizosphere. Among rice varieties, maximum siderophore activity was observed in basmati-super (6.5–42.8 µg/mL) followed by that of basmati-515 (6.7–28.4 µg/mL) and basmati-385 (3.6–25.8 µg/mL). The *Bacillus* sp KFP-17 secreted highest amount of siderophore (5.53–42.5 µg/mL) followed by that of *Bacillus* sp KFP-5 (4.4–42.0 µg/mL) and *Bacillus* sp KFP-7 (3.6–34.2 µg/mL). The siderophores (2.9–29.2 µg/mL) were recovered in fungicide treatment. Higher quantity of protease (42.5 µg/mL) was secreted in soil as compared to hydroponic (6.7 µg/mL) (Fig 5).

Root colonization by antagonistic bacteria

The antagonistic strains significantly colonized the rice plants grown either hydroponically or soil. The maximum root colonization ($2.0E+00$ – $9.1E+08$) was observed in *Bacillus* sp KFP-17 colonized ($6.3E+07$ – $9.13E+08$) followed by *Bacillus* sp KFP-5 ($4.8E+07$ – $8.4E+08$) and *Bacillus* sp KFP-7 ($4.3E+07$ – $6.13E+08$). Furthermore the rice varieties basmati-super ($8.6E+08$ – $9.1E+08$) followed by that of basmati-515 ($7.4E+07$ – $8.4E+08$) and basmati-385 ($6.6E+06$ – $7.8E+08$). The root colonization is shown in (Tables 1 and 2).

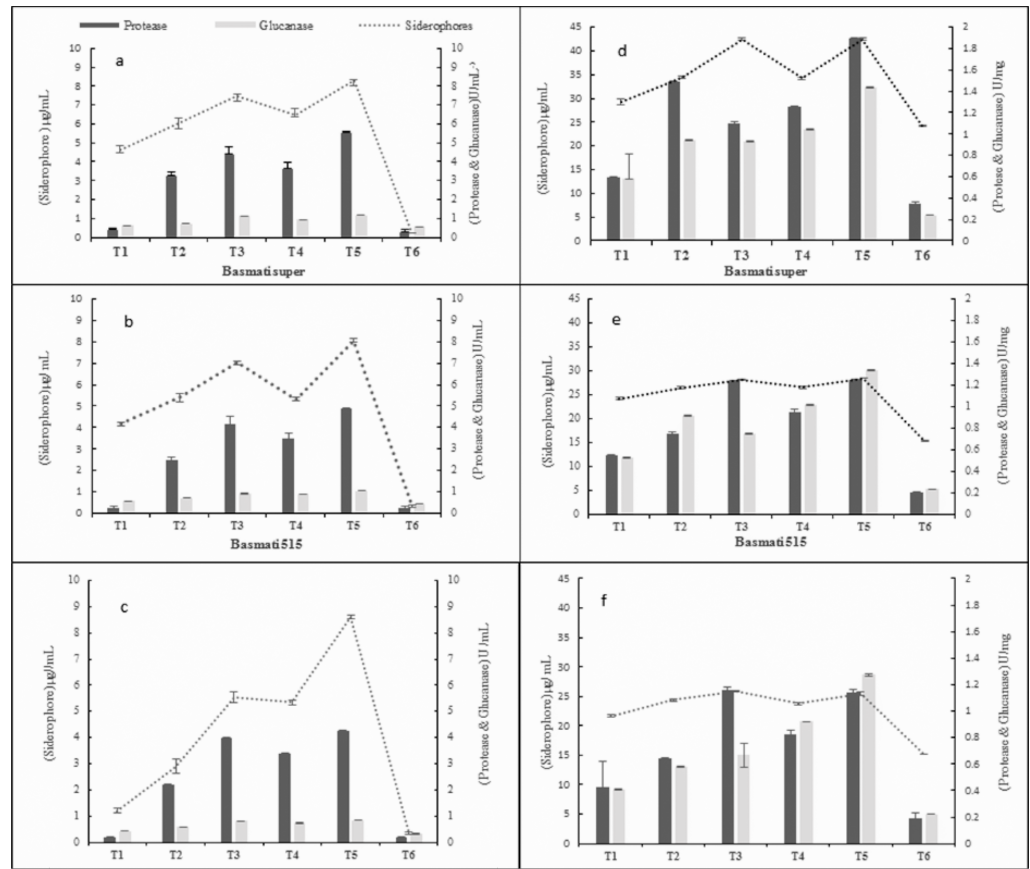


Fig 5. Secretion of biocontrol determinants in rhizosphere of different rice varieties grown hydroponically (left panel a, b, c) and pot/ soil (right panel d, e, f). T1 = *P. oryzae*, T2 = Fungicide + *P. oryzae*, T3 = *Bacillus* sp KFP-5+ *P. oryzae*, T4- *Bacillus* sp KFP-7+ *P. oryzae* T5 = *Bacillus* sp KFP-17+ *P. oryzae* T6 = untreated. Values are means of three replicates and vertical bars represent the standard error. All treatments are significantly different from each other at $P < 0.001$.

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Discussion

Use of antioxidant mechanism by plants in protection from aerial phytopathogens has been well documented [62–65]. Several PGPR stimulate the antioxidant machinery in plants but whether they secrete the eliciting determinants *in situ* has not yet been reported. In this study,

Table 1. Potential of *Bacillus* spp to colonize root under hydroponic conditions.

Treatment	Bas-super	Bas-515	Bas-385
<i>P. oryzae</i>	3.3E+01 ^c	2.6E+03 ^d	2.0E+00 ^c
Fungicide + <i>P. oryzae</i>	3.5E+01 ^{bc}	2.67E+02 ^d	7.67E+01 ^b
<i>Bacillus</i> sp KFP-5+ <i>P. oryzae</i>	8.2 E+08 ^b	6.3E+07 ^b	4.8E+07 ^b
<i>Bacillus</i> sp KFP-7+ <i>P. oryzae</i>	6.1E+08 ^b	4.3E+07 ^c	4.5E+07 ^c
<i>Bacillus</i> sp KFP-17+ <i>P. oryzae</i>	8.6E+08 ^a	7.4E+07 ^a	6.3E+07 ^a
Untreated	6.67E-01 ^c	6.67E-01 ^d	6.67E-01 ^c

Values are means of three replicates and values bearing different letters in the same column are significantly different from each other according to the Fisher's least significant difference (LSD) test at $p < 0.001$.

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Table 2. Potential of *Bacillus spp* to colonize root under pot/soil conditions.

Treatment	Bas-super	Bas-515	Bas-385
<i>P.oryzae</i>	4.67E+01 ^c	3.33E+02 ^c	7.00E+01 ^b
Fungicide + <i>P.oryzae</i>	6.00E+02 ^c	4.00E+02 ^c	7.00E+01 ^b
<i>Bacillus</i> sp KFP-5+ <i>P.oryzae</i>	8.4E+08 ^{ab}	7.8E+08 ^a	5.2E+08 ^{ab}
<i>Bacillus</i> sp KFP-7+ <i>P.oryzae</i>	5.0E+08 ^b	4.7E+08 ^b	4.7E+08 ^{ab}
<i>Bacillus</i> sp KFP-17+ <i>P.oryzae</i>	9.13E+08 ^a	8.47E+08 ^a	7.8E+08 ^a
Untreated	1.67E+00 ^c	1.33E+00 ^c	1.33E+00 ^b

Values are means of three replicates and values bearing different letters in the same column are significantly different from each other according to the Fisher's least significant difference (LSD) test at $p < 0.001$.

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we unveiled the activation of antioxidant system in rice by the antagonistic *Bacillus* sp in response to blast pathogen *P. oryzae* through the quantification of defense enzymes involved in induction of systemic resistance in the host. These findings clearly demonstrate that *Bacillus* sp enhanced the activity of peroxidase [49], polyphenol oxidase (PPO), peroxidase, phenylalanine ammonia lyase (PAL) and superoxide dismutase [34] by secreting protease, siderophores and glucanase near rice rhizosphere grown in hydroponic as well as soil.

P. oryzae infection in rice generates reactive oxygen species (ROS), such as radicals of superoxide ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$) and molecules of hydrogen per oxide (H_2O_2) [66–68]. The accumulation of ROS cause damage in the infected plant cells [69, 70]. The effect of ROS molecules are neutralized by an effective ROS-scavenging system which majorly comprise of antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, Ascorbate peroxidase (APX) Guaiacol peroxidase (GPX) Monodehydroascorbate reductase (MDHAR) Dehydroascorbate reductase (DHAR) Glutathione reductase (GR) and polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) [34].

Our results depict that plants inoculated with *P. oryzae* in absence of biocontrol agents showed a decreased activity of PO, SOD, PPO and PAL, confirming that oxidative damage is associated with the ROS scavenging system. Low amounts of antioxidant enzymes are produced under high level of ROS [71, 72].

Nevertheless, the antagonistic *Bacillus* sp significantly enhanced the activity of antioxidant enzymes by approximately 2–5 fold in shoots and roots of rice in response to *P. oryzae* infection. These findings are in consistent with the earlier reports in which potential of antagonistic bacteria to enhance activities of antioxidant enzymes in rice as defensive mechanism to multiple pathogens has been reported [12, 73–75]. However, in our study, strain KFP-17 was best in eliciting the activity of PO, PPO and SOD. This enhanced activity may be correlated with its best ability to suppress the blast disease as reported in our previous studies [40]. Moreover, a positive correlation between production of antifungal metabolites; siderophores, protease and glucanase by the *Bacillus* sp. and activity of antioxidant enzymes was observed (Tables 3 and 4). These findings are further supported by the earlier reports in which siderophores and hydrolytic enzymes production by rhizobacteria significantly enhance the activity of antioxidant enzymes. A significant decrease in disease incidence/progression of *Sclerotium rolfsii* pathogen was observed attributable to high activity of PO, PAL and PPO in rice plants treated with siderophore producing rhizobacteria/*Streptomyces* spp.[76]. Similar findings have been documented by Naureen et al [77] in which hydrolytic enzymes producing rhizobacteria induced systemic resistance against rice sheath blight disease in rice caused by the causative agent *Rhizoctonia solani*. Similarly, there is increasing evidence depicting that siderophores,

Table 3. Pearson's correlation among antioxidant enzyme content and biocontrol determinants secreted in rice rhizosphere grown hydroponically.

Basmati Super							
	Glucanase	PAL	PO	PPO	Protease	Siderophores	SOD
Glucanase	1.00**						
PAL	0.940**	1.00					
PO	0.988**	0.910**	1.00				
PPO	0.984**	0.955**	0.956**	1.00			
Protease	0.972**	0.937**	0.966**	0.944**	1.00		
Siderophores	0.959**	0.969**	0.949**	0.947**	0.939**	1.00	
SOD	0.996**	0.921**	0.987**	0.977**	0.956**	0.946**	1.00
Basmati 515							
Glucanase	1.00						
PAL	0.927**	1.00					
PO	0.893**	0.990**	1.00				
PPO	0.940**	0.965**	0.919**	1.00			
Protease	0.989**	0.943**	0.902**	0.972**	1.00		
Siderophores	0.878**	0.802**	0.801**	0.763*	0.825**	1.00	
SOD	0.917**	0.971**	0.937**	0.981**	0.957**	0.720*	1.00
Basmti 385							
Glucanase	1.00						
PAL	0.914**	1.00					
PO	0.227	0.178	1.00				
PPO	0.36	0.507*	0.066	1.00			
Protease	0.995**	0.906**	0.252	0.329	1.00		
Siderophores	0.984**	0.899**	0.324	0.415	0.979**	1.00	
SOD	0.991**	0.894**	0.231	0.296	0.996**	0.964**	1.00

Asterisks indicate significance as follows p< 0.05

**Highly significant

* Significant

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protease and cellulase producing microbes protect the plants from pathogen stress by eliciting the ROS scavenging system [63, 72, 78].

Our findings firstly present the secretion of siderophores, protease and glucanase in the rhizosphere of rice. A significant correlation between the quantities of protease, siderophores, glucanase recovered from rhizosphere and activities of antioxidant enzymes indicate the role of these metabolites in activation of antioxidant enzyme system under *P. oryzae* infection. The role of these metabolites in eliciting systemic resistance against *Fusarium oxysporum*, *Pyricularia oryzae*, *Alternaria* sp. and *Sclerotium* sp. has been established earlier [79, 80]. It has been reported that strain *P. fluorescens* 3551, B224 was unable to activate defense enzymes /induce SR/ suppress pathogen, when it was mutated to enable its siderophore producing ability. Similarly, mutated *B. subtilis* strain deficient in chitinase production showed less antagonistic and hydrolytic activity [80, 81].

Our findings indicate their role in disease management not only by suppressing the pathogen but also through modulating the ROS scavenging system in plants. This mechanism of defense is highly worth full for the control of diseases caused by the aerial pathogens where the chances of direct contact between the pathogen and biocontrol agent are very rare. Furthermore, studies indicate that activities of enzymes involved in the biosynthesis of either

Table 4. Pearson's correlation among antioxidant enzyme content and biocontrol determinants secreted in rice rhizosphere grown in pot/soil.

Basmati Super	Glucanase	PAL	PO	PPO	Protease	SOD	Siderophores
Glucanase	1.00						
PAL	0.933**	1.00					
PO	0.969**	0.936**	1.00				
PPO	0.923**	0.985**	0.965**	1.00			
Protease	0.868**	0.916**	0.957**	0.970**	1.00		
SOD	0.886**	0.839**	0.968**	0.910**	0.962**	1.00	
Siderophores	0.827**	0.943**	0.909**	0.977**	0.972**	0.883**	1.00
Basmati 515							
Glucanase	1.00						
PAL	0.687	1.00					
PO	0.797**	0.829**	1.00				
PPO	0.718**	0.903**	0.979**	1.00			
Protease	0.968**	0.837**	0.832**	0.801**	1.00		
SOD	0.657	0.977**	0.897**	0.964**	0.794**	1.00	
Siderophores	0.658	0.978**	0.847**	0.922**	0.798**	0.982**	1.00
Basmati 385							
Glucanase	1.00						
PAL	0.660*	1.00					
PO	0.739*	0.948**	1.00				
PPO	0.735*	0.934**	0.999**	1.00			
Protease	0.579	0.986**	0.922**	0.907**	1.00		
SOD	0.740*	0.985**	0.986**	0.978**	0.959**	1.00	
Siderophores	0.556	0.977**	0.904**	0.889**	0.958**	0.948**	1.00

Asterisks indicate significance as follows $p \leq 0.05$

**Highly significant

* Significant

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flavonoids or structural polyphenols such as lignin have also been induced by the application of PGPR [82–85]. Here, we observed that application of antagonistic *Bacillus* sp. (especially the strain KFP-17) enhanced the PPO and PAL activity in rice plants. PAL is a first enzyme of phenylpropanoid pathway and involved in the biosynthesis of lignin, phenolics, phytoalexins and salicylic acid which serve as first defense line against phytopathogens [86–89].

Differential changes in the activity of POD and SOD, as a consequence of *Bacillus* spp. treatments, were also observed. SOD constitutes the first line of defense in the enzymatic antioxidant responses by catalyzing the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 [90, 91]. The H_2O_2 is further scavenged by the POD. Major differences were detected in the antioxidant enzyme of roots/shoots and treatments (bacteria) under *P. oryzae* stress (Fig 5D and 5F).

There are numerous studies indicating the ability of PGPR to induce antioxidant activity in plants but the exact mechanism by which they induce is still a question. It could be speculated that PGPR, being microbes, contain conserved signatures on their cell wall or motility organs. Plant recognizes them through its efficient immune system consisting of pattern recognition receptors (PRRs) and microbe associated molecular patterns (MAMPs). This PRR-mediated microbe sensing induces various defense responses including activation of antioxidant enzymes [92].

Microbes secrete different secondary metabolites such as effectors, proteases or other metabolites to colonize the host [93]. The PGPR metabolites especially hydrolytic enzymes cleave their substrates yielding oligosaccharides/ sugars which act as signal to induce antioxidant enzymes [94]. The siderophores also trigger signaling mechanism by altering the iron status in plant vascular system leading to the higher induction of antioxidant enzymes [95].

The different trends in soil and hydroponic treatments suggest that there are fundamental differences in the nature of these two systems that influence the responses of defense enzymes. The production of antioxidant enzyme and biochemical determinants was lower in hydroponic conditions as compared to pot experiments. Selected rice variety basmati-super showed maximum activity of antioxidant enzymes and biochemical determinants with respect to all treatments as compared to the basmati -515 and basmati- 385. Accordingly, further work should focus on the exploration of physiological mechanisms in this variety responsible for higher response to antagonistic bacteria inoculation.

Supporting information

S1 File.
(XLSX)

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