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Seed priming with biotic agents invokes defense response and enhances plant growth in pearl millet upon infection with *Magnaporthe grisea*

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ABSTRACT

The induction of host-defense in plants is a reliable approach for the management of diseases in crop plants. In the present study, biotic agents like *Pseudomonas fluorescens, Trichoderma virens* and neem leaf extract have been evaluated for their efficacy to induce resistance against blast disease of pearl millet caused by *Magnaporthe grisea* and promote growth. Seeds treated with *P. fluorescens* exhibited maximum seed germination of 83.50% and seedling vigor of 1447.93 followed by seed treatment with *T. virens* and neem leaf extract. Seeds treated with *P. fluorescens* showed improved vegetative and reproductive parameters compared with other biotic agents. Under greenhouse conditions, *P. fluorescens* offered maximum disease protection of 62.25% against blast disease followed by *T. virens* with 58.25%. It was also found that the activity of the defense-related enzymes like Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Lipoxygenase (LOX) and β-1,3-glucanase was increased considerably in *P. fluorescens* treated and challenge inoculated seedlings thereby indicating that these defense enzymes play a pivotal role in induction of blast disease resistance in pearl millet.

1. Introduction

Apart from resistance breeding, a large number of methods like chemical control and somaclonal variation are in use to control the plant diseases, which have their limitations (Moffat, 2001; Abhayashree et al., 2017; Anil et al., 2018). The extensive use of chemicals has become a subject of severe concern, as they create an adverse effect on human health and also on the environment. Induced resistance is a potential disease management strategy due to its eco-friendly nature and can be used as an alternative to synthetic chemicals. Induced systemic resistance is one of the natural defense mechanism of the host plants, which is artificially stimulated by an inducer (Heil and Bostock, 2002; Murali and Amruthesh, 2015; Gowtham et al., 2018). Natural resistance mechanisms once induced are long-lasting and are effective against a wide range of plant pathogens (Vleesschauwer et al., 2008; Nagaraju et al., 2012). Pre-treatment of plants with biotic or abiotic inducers can enhance resistance to subsequent attack not only at the site of treatment but also in tissues far away from the initial infection sites (Peng et al., 2011; Murali et al., 2013).

Eliciting the host defense responses using biotic agents is one of the eco-friendly approaches invoked for plant disease management. An array of attempts has been made to induce systemic resistance in various crop plants using many biotic agents including microorganisms and plant extracts in various host-pathogen systems (Niranjanraj et al., 2003; Chowdappa et al., 2013; Shao et al., 2018). The mechanism of resistance offered by inducing agents has primarily expressed at the cellular level wherein the deposition of lignin, callose, phenols, etc., barricades the spread of the pathogen (Niranjan raj et al., 2012; Abhayashree et al., 2017). Further, biotic agents are also known to impart changes at biochemical level as the defense enzymes (PAL, POX, LOX, β-1,3-glucanase, etc.) gets activated upon the infection of the pathogen (Chowdappa et al., 2013; Manjula et al., 2015; Gowtham et al., 2018; Wang et al., 2019). These defense enzymes play a pivotal role in defense mechanism as they are known to increase antimicrobial activity, involve in the biochemical process of lignification, suberification, cell wall elongation, wound healing and may also directly participate in reduction in disease severity (Kombrink and Somssich, 1997; Belkhadir et al., 2004; Murali et al., 2013; Gowtham et al., 2018; Wang et al., 2019).

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is one of the earliest food known to man and major part of their diet from several hundred years in many parts of the world. A major portion of the pearl millet production is devoured as a staple food and about 5% is used as feed or fodder to animals (ICRISAT , 1997). Pearl millet is rich in essential nutrients and hence gained access to every household in all kinds of social strata. In recent years, pearl millet is suffered by blast disease

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caused by *Magnaporthe grisea* (Hebert) Barr. (Anamorph: *Pyricularia grisea* (Cooke) Sacc.). The pathogen attacks the aerial parts of the plant at all stages and has been reported from most of the pearl millet growing regions throughout the worldcausing enormous economic loss to the farmers (Wilson and Gates, 1993). Presently, blast disease of pearl millet is managed by the use of some chemical fungicides which have a deleterious effect on the environment (Naik and Jamadar, 2014). Hence the present investigation was aimed to assess the viability of some biotic elicitors to induce disease resistance in pearl millet against *Magnaporthe grisea*, which is the causal agent of blast disease.

2. Materials and methods

2.1. Seed sample

Pearl millet seeds of cv. ICMB95444 (susceptible) and cv. ICMR06222 (resistant) were obtained from ICAR-AICPMIP Mysore centre, University of Mysore, Mysuru. The seeds were stored in optimum conditions and used throughout the study.

2.2. Isolation and characterization of Magnaporthe grisea

Magnaporthe grisea (Hebert) Barr was isolated from pearl millet plants which were heavily infested with blast disease under field conditions. The infected leaves were washed under running tap water and surface sterilized with 0.5% NaOCl for 1 min and repeatedly rinsed with sterile distilled water (SDW). The surface-sterilized leaf bits were placed on three-layers of moistened blotter discs in Petri dishes which were previously autoclaved and incubated at 28 ± 2 °C for seven days. After incubation, each leaf bit was examined under a stereo binocular microscope. The colonies showing the typical sporulating structure of *M. grisea* were picked with a sterile inoculation needle and inoculated onto oatmeal agar (OMA) medium under aseptic conditions and identified based on their morphological, conidial, and culture characters (Mathur and Kongsdal, 2003). The genomic DNA of *M. grisea* was extracted from the lyophilized fungal mat by CTAB (cetyltrimethylammonium bromide) method (Weiland, 1997). The nuclear ribosomal DNA was amplified using 18S rRNA primer (Embong et al., 2008) and the amplicon obtained was sequenced. The obtained sequence was submitted to the GenBank of the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed by comparing *M. grisea* with the isolates showing highest similarities based on their 18S rRNA gene sequences available in the NCBI database. The phylogenetic tree was constructed using MEGA-X software by the neighbour-joining (NJ) method with the Kimura 2-parameter model.

2.3. Preparation of inoculum

Pure colonies of *M. grisea* grown on OMA (10–15 days) were flooded with 5–10 mL of SDW for each plate and conidia were dislodged by shaking or by using sterile brush under aseptic conditions. The concentration of conidial suspension was adjusted to 1×10^5 conidia mL^{-1} using Haemocytometer and used throughout the study (Sharma et al., 2013).

2.4. Collection of biotic inducers

Biotic inducers such as *Trichoderma virens* (KF150219.1) and *Pseudomonas fluorescence* (HM229810) (were collected from the University of Mysore) and aqueous leaf extract of *Azadirachta indica* were used throughout the study.

2.5. Preparation of biotic inducers

Pseudomonas fluorescens: Twenty-four-hour old *P. fluorescens* culture grown in nutrient broth (NB) was centrifuged (8000 rpm for 10 min) and the resultant pellet was re-suspended in SDW to obtain 1×10^8 CFU mL^{-1} using Haemocytometer (Gowtham et al., 2016).

Trichoderma virens: Seven-day-old culture of *T. virens* grown on PDA was flooded with 5–10 mL of SDW and conidia were dislodged by shaking or by using sterile brush under aseptic conditions. The concentration of conidial suspension was adjusted to 1×10^8 conidia mL^{-1} using Haemocytometer (Murali et al., 2013).

Azadirachta indica: About 100 g of fresh leaves (washed under running tap water) were homogenized with 100 mL of sterile distilled water and kept on a rotary shaker at 200 rpm min^{-1} for 24 h. The obtained extract was filtered using Whatman Filter Paper No. 1 and was lyophilized to dryness. The extract was treated to seeds at 10% concentration (Meena, 2017).

2.6. Seed priming with biotic inducers

The susceptible pearl millet seeds were surface-sterilized with 0.02% sodium hypochlorite for 2 min and repeatedly rinsed with SDW (2–3 times). The surface-sterilized seeds were primed with all the biotic inducers by mixing seeds in 50 mL of each of the inducers and kept in a rotary shaker (25 ± 2 °C) for 3 h and 6 h independently. After treatment, the seeds were dried aseptically and used throughout the study. The SDW treated seeds served as a negative control.

2.7. Effect of seed priming with biotic elicitors on seed germination and seedling vigor of pearl millet

All the biotic inducers primed and respective control (four replicates of 100 seeds each for each treatment) were plated equidistantly on three layers of moistened blotter discs placed in Petri dishes to evaluate percent germination (Singh and Gopinath, 1985). The other set of seeds (treated and control) were subjected to between paper method to record seedling vigor (Abdul-baki and Anderson, 1973). All the samples were incubated for 7-days at 25 ± 2 °C. After incubation, percent seed germination and seedling vigor were calculated using the formula:

$$\text{Percent Seed Germination} = \frac{\text{Number of seeds germinated}}{\text{Total Number of seeds plated}} \times 100$$

Vigor index = % Seed Germination × (Mean Root Length + Mean Shoot Length)

2.8. Evaluation of seed priming with biotic inducers on plant growth parameters in pearl millet under greenhouse conditions

The pearl millet seeds treated with biotic elicitors and control (for 6 h) were sown in earthen pots (9 × 9 cm diameter) containing autoclaved potting medium (2:1:1 soil, sand and farmyard manure) and maintained under greenhouse conditions (25 ± 2 °C). Sixty-days-after sowing (DAS), vegetative and reproductive plant growth parameters *viz.*, plant height, days required for 50% flowering, length and girth of ear head and 1000 seed weight were recorded accordingly.

2.9. Evaluation of seed priming with biotic inducers on blast disease protection in pearl millet under greenhouse conditions

The pearl millet seeds primed with biotic elicitors and control (for 6 h) were sown in earthen pots (9 × 9 cm diameter) containing autoclaved potting medium (2:1:1 soil, sand and farmyard manure) and maintained under greenhouse conditions (25 ± 2 °C). The 14-day-old seedlings were challenge inoculated with *M. grisea* (1×10^5 conidia mL^{-1}) and observed daily for the typical symptoms of blast disease up to 30-days. Each treatment consisted of 10 pots, with ten seedlings per pot and the experiment was carried out in quadruplicates. At the end of 30-days-after challenge inoculation, disease protection was recorded

using the formula:

$$\text{Disease Protection (\%)} = \frac{\text{Number of infected plants}}{\text{Total Number of plants examined}} \times 100$$

2.10. Biochemical studies

2.10.1. Sampling of seedlings

Biotic inducer treated pearl millet seeds along with control sets (resistant and SDW treated) were subjected to between paper method and incubated at 25 ± 2 °C for seven days. After incubation, the seedlings were removed carefully without harming the roots and root dip inoculated with the conidial suspension of *M. grisea* (1×10^5 conidia mL^{-1}). The root dip inoculated and uninoculated pearl millet seedlings (susceptible inducer treated, susceptible and resistant) were harvested at 0, 3, 6, 12, 24, 36, 48 and 72 h after inoculation (h.a.i.) and were immediately stored at −80 °C until further use. The uninoculated susceptible inducer treated, susceptible and resistant pearl millet seedlings served as control.

2.10.2. Time-course analysis of phenylalanine ammonia-lyase (PAL) assay

PAL activity was determined following the method of Geetha et al. (2005). About 1 g fresh weight of each of the harvested pearl millet seedlings at all the time intervals was frozen with liquid nitrogen and homogenized in a 25 mM Tris-HCl buffer, pH 8.8, containing 32 mM of β-mercaptoethanol in a pre-chilled mortar and pestle. The homogenate was centrifuged (10,000 rpm at 4 °C for 25 min) and the supernatant was collected (enzyme source). The reaction mixture containing 0.5 mL of enzyme extract was incubated with 1 mL of 25 mM Tris-HCl buffer (pH 8.8) and 1.5 mL of 10 mM L-phenylalanine in the same buffer for 2 h at 40 °C and the activity was stopped using 5 N HCl. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm. The enzyme activity was expressed as µmol of *trans*-cinnamic acid mg protein^{-1} h^{-1}. Each experiment was repeated thrice taking three replicates each time.

2.10.3. Time-course analysis of peroxidase (POX) assay

POX activity was determined following the method of Hammerschmidt et al. (1982). About 1 g fresh weight of each of the harvested pearl millet seedlings at all the time intervals was frozen with liquid nitrogen and homogenized with 0.2 M sodium phosphate buffer (pH 6.5) in a pre-chilled mortar and pestle. The homogenate was centrifuged (10,000 rpm at 4 °C for 15 min) and the supernatant was collected (enzyme source). The reaction mixture (3 mL) consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. To the reaction mixture, 5 µL of crude extract was added to initiate the reaction and the absorbance was measured at 470 nm. The enzyme activity was expressed as the increase in absorbance at 470 nm mg protein^{-1} min^{-1}. Each experiment was repeated thrice taking three replicates each time.

2.10.4. Time-course analysis of lipoxygenase (LOX) assay

Lipoxygenase activity was measured following the method of Borthakur et al. (1987). About 1 g fresh weight of each of the harvested pearl millet seedlings at all the time intervals was frozen with liquid nitrogen and homogenized with 0.2 M sodium phosphate buffer (pH 6.5) containing 1% polyvinylpyrrolidone (PVP), 0.1% TritonX-100 and 0.04% sodium metabisulfite in a pre-chilled mortar and pestle. The homogenate was centrifuged (9000 rpm for 20 min at 4 °C) and the supernatant served as an enzyme source. Enzyme activity was measured by monitoring the appearance of the conjugated diene hydroperoxides at 234 nm. Linoleic acid was used as a substrate, which was prepared according to the standard method (AxelrodCheesbroughLaaks, 1981). The activity was recorded for 3 min using a spectrophotometer. The enzyme activity was expressed in terms of mmol quinone formed min^{-1} mg protein^{-1}.

2.10.5. Time-course analysis of β-1,3-glucanase assay

The β-1,3-glucanase activity was assayed by the modified method of Pan et al. (1991). About 0.5 g fresh weight of each of the harvested pearl millet seedlings were frozen with liquid nitrogen and homogenized with 0.05 M of sodium acetate buffer (pH 4.6) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 23,000 rpm at 4 °C for 20 min and the resultant supernatant was used as an enzyme source. About 100 µL of p-nitrophenyl-β-D-glycopyranoside was added to 350 µL of 0.05 M sodium buffer (pH 4.6), followed by an initial incubation at 30 °C for 5 min and 50 µL of enzyme extract was added and incubated at 30 °C for 15 min. The reaction was stopped by adding 700 µL of 0.2 M sodium carbonate and the absorbance was determined using a spectrophotometer at 420 nm. Enzyme activity was calculated as µM of p-nitrophenol released min^{-1} mg^{-1} protein. The activity was calculated based on the molar extinction coefficient (U) = 1.12×10^4 $M^{-1}cm^{-1}$.

2.10.6. Protein estimation

Protein in the crude enzyme extracts was estimated by the dye-binding method (Bradford, 1976) using bovine serum albumin (BSA) (Sigma) as standard.

2.11. Statistical analysis

The mean data of all the experiments were subjected to analysis of variance (ANOVA) using SPSS Inc.16.0. Significant effects of treatments were determined by the magnitude of the F value ($p \leq 0.05$). Treatment means were separated by Tukey's HSD test.

3. Results

3.1. Isolation and characterization of Magnaporthe grisea

The PCR amplification with specific primers for the 18S rRNA primer region generated bands at ~1200 base pair (Suppl. Fig. 1). The nucleotide sequences obtained were analyzed (compared with GenBank database) to identify the similarity using the NCBI BLAST at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The partial 18S rRNA gene sequence of *M. grisea* was deposited to GenBank, NCBI with Accession No. MK610799 (https://www.ncbi.nlm.nih.gov/nuccore/MK610799). A phylogenetic tree was constructed to show the relationship between the *M. grisea* with the available taxa of same species with an out group species of the same genera (*M. oryzae*) (Fig. 1). The sequence of *M. grisea* MgPB1 showed 99% similarity with *M. grisea* strain 793. The phylogenetic tree represents the evolutionary relationship of *M. grisea* MgPB1 with the available taxa of the same species retrieved from NCBI.

3.2. Effect of seed priming with biotic elicitors on seed germination and seedling vigor of pearl millet

The pearl millet seeds primed with biotic inducers for two different time intervals (3 h and 6 h) were evaluated for their effect on seed germination and seedling vigor. It was observed that all the treatments were able to enhance seed germination and seedling vigor significantly at both the time periods evaluated with best results obtained in seeds treated for a period of 6 h (Table 1). Among the inducer evaluated, maximum seed germination and seedling vigor of 83.50% and 1447.93, respectively was observed upon treatment with *P. fluorescens* for 6 h followed by *T. virens* and neem leaf extract respectively at the same time interval of treatment. The control seedlings offered 70% and 834.5 of seed germination and seedling vigor and there was no significant difference upon 3 h and 6 h treatment with SDW. Further studies were carried out in seeds treated with biotic inducers for 6 h as they offered significant enhancement in seed germination and seedling vigor.

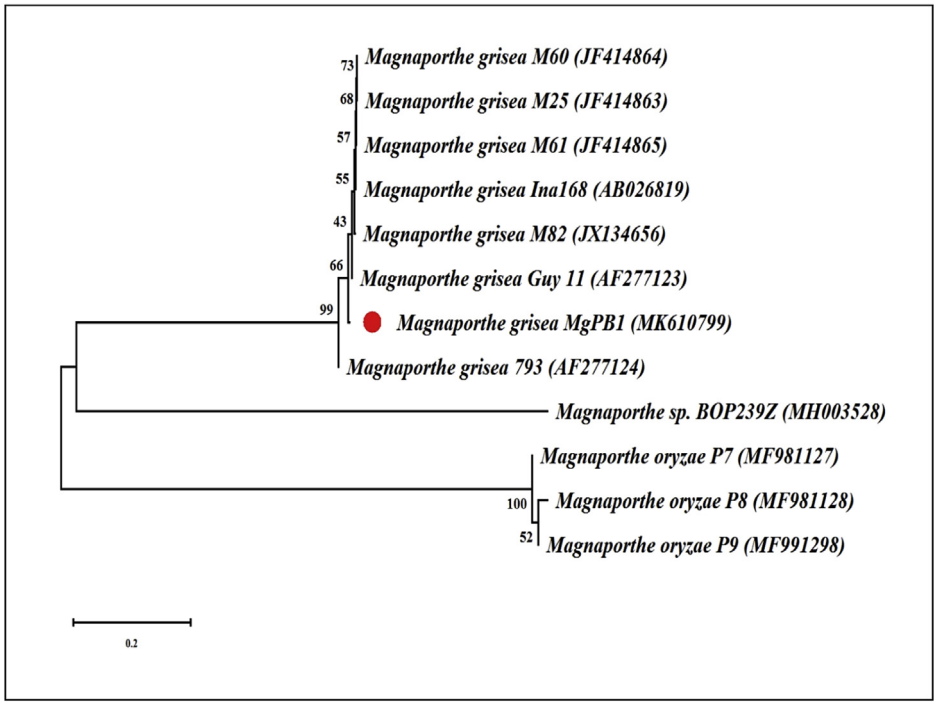

Fig. 1. Phylogenetic tree derived from Neighbour-Joining analysis illustrating the evolutionary relationship of *M. grisea* with their closest BLAST hits.

Table 1
Effect of biotic elicitor treatments for 3 h and 6 h on seed germination and seedling vigor of Pearl millet.

Treatments	Hours	Seed Germination (%)	Seedling Vigour
Control	3	70.25 ± 0.62^{e}	858.60 ± 16.30^{e}
	6	70.00 ± 0.70^{e}	834.50 ± 8.37^{e}
P. fluorescens	3	76.25 ± 0.62^{c}	1291.43 ± 6.92^{b}
	6	83.50 ± 0.64^{a}	1447.93 ± 18.82^{a}
T. virens	3	74.00 ± 0.40^{d}	1126.53 ± 12.51^{c}
	6	80.00 ± 0.40^{b}	1276.13 ± 13.40^{b}
Neem Leaf Extract	3	72.75 ± 0.47^{d}	923.60 ± 27.18^{d}
	6	78.50 ± 0.64^{b}	1133.83 ± 14.90^{c}

Values are means of four independent replicates. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD test ($p \leq 0.05$).

3.3. Evaluation of seed priming with biotic inducers on plant growth parameters in pearl millet under greenhouse conditions

The seedlings treated with biotic inducers were evaluated for their effect on plant growth (both vegetative and reproductive) parameters after 60 DAS under greenhouse conditions. The study showed a significant increase in plant growth parameters in biotic inducer treated seeds compared to control (Table 2). The seeds treated with *P. fluorescens* offered maximum increase in plant growth parameters [*viz.*, plant height (109.23 cm), 50% flowering (39 days), number of basal tillers (3.25 plant^{-1}), length of ear head (12.2 cm), girth of ear head (4.7 cm) and 1000 seed weight (11.52 g) among the treatments. There was about 20%–80% increase in plant growth parameters and the plants flowered about 5 days earlier upon treatment with *P. fluorescens* compared to control.

3.4. Evaluation of seed priming with biotic inducers on blast disease protection in pearl millet under greenhouse conditions

Pearl millet seeds treated with different biotic elicitors significantly induced blast disease resistance upon treatment (Fig. 2). Maximum disease protection of 62.25% was observed in seeds treated with *P. fluorescens* after 45 DAS followed by *T. virens* and neem leaf extract which offered disease protection of 58.25% and 51.75%, respectively (Fig. 3). The results confirm that the seed treatment with biotic elicitors was capable of inducing resistance in pearl millet to blast disease. Further studies on biochemical changes were carried out in seedlings treated with *P. fluorescens* as it offered maximum disease protection.

3.5. Biochemical studies

3.5.1. Time-course analysis of phenylalanine ammonia-lyase (PAL) assay

PAL enzyme activity was estimated in all the biotic inducer treated seedlings with and without inoculation along with control sets. Significant differences in PAL enzyme activity was observed in biotic inducer and control seedlings (Fig. 4). A progressive increase in PAL

Table 2
Effect of biotic elicitor on vegetative and reproductive growth parameters.

Treatments	Plant Height (cm plant^{-1})	Days required for 50% flowering (plant^{-1})	Number of basal tillers (plant^{-1})	Length of Ear head (cm)	Girth of Ear head (cm)	Weight Per 1000 seeds (g)
Control	78.475 ± 0.63^{d}	44.50 ± 0.28^{a}	1.75 ± 0.25^{b}	8.60 ± 0.12^{d}	3.45 ± 0.10^{d}	9.47 ± 0.12^{c}
P. fluorescens	109.23 ± 0.72^{a}	39.00 ± 0.40^{c}	3.25 ± 0.25^{a}	12.20 ± 0.12^{a}	4.70 ± 0.09^{a}	11.52 ± 0.14^{a}
T. virens	104.30 ± 0.41^{b}	40.75 ± 0.47^{b}	2.50 ± 0.28^{ab}	10.65 ± 0.18^{b}	4.27 ± 0.06^{b}	10.57 ± 0.17^{b}
Neem Leaf Extract	96.875 ± 0.71^{c}	42.00 ± 0.40^{b}	2.00 ± 0.40^{b}	9.77 ± 0.16^{c}	3.92 ± 0.10^{c}	9.97 ± 0.10^{c}

Values are means of four independent replicates. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD test ($p \leq 0.05$).

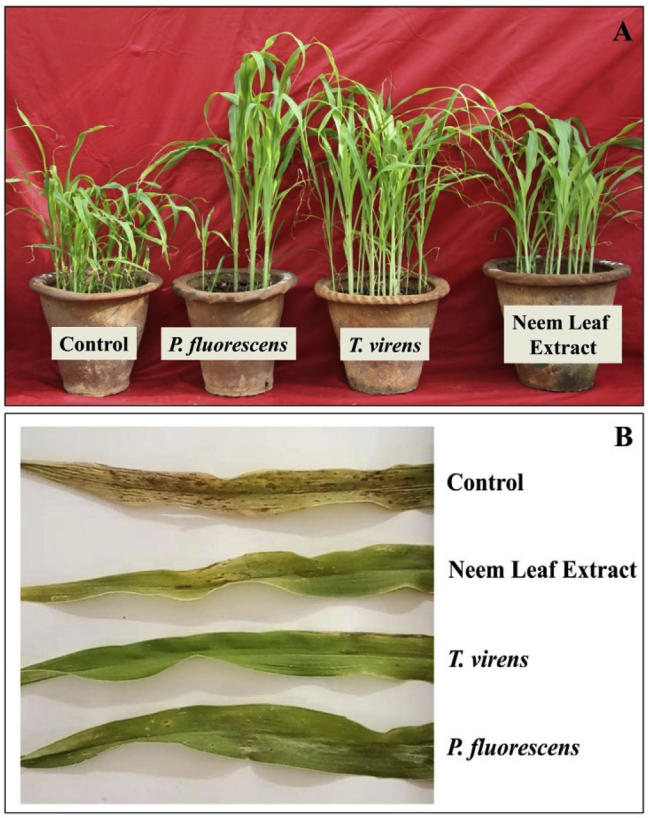

Fig. 2. Effect of biotic elicitors on pearl millet blast disease protection at the end of 45 DAS. A: Greenhouse studies; B: Leaf showing the symptoms of blast disease.

activity was observed from 0-9 h.a.i. and the activity was maintained thereafter in all the samples evaluated. The maximum PAL activity was observed in resistant challenge inoculated seedlings (35.63 U) followed by *P. fluorescens* treated inoculated seedlings (30.46 U) at 9 h.a.i. The susceptible inoculated seedlings showed a maximum PAL activity of 14.33 U at the same time interval. It was observed that a total of 1.4 and 1.2 fold increase in PAL activity was observed in resistant and inducer treated challenge inoculated seedlings over the susceptible inoculated seedlings, respectively. PAL enzyme activity was maximum at 48 h.a.i. in all the samples tested, but there was no significant increase in PAL activity in susceptible uninoculated seedlings.

3.5.2. Time-course analysis of peroxidase (POX) assay

The results of POX enzyme activity of inducer treated pearl millet seedlings showed significant enhancement in enzyme activity compared to control irrespective of challenge inoculation (Fig. 5). POX enzyme activities were higher in pathogen-inoculated seedlings when compared with uninoculated ones. The POX enzyme activity increased from 0 h.a.i. to 9 h.a.i. and decreased thereafter in resistant and inducer treated challenge inoculated and uninoculated seedlings, while susceptible inoculated and uninoculated seedlings offered maximum enzyme activity at 72 h.a.i. A maximum of 46.5 U of enzyme activity was observed in resistant challenge inoculated seedlings followed by inducer treated and challenge inoculated seedlings (46.33 U). It was observed that there was up to 1.7 fold increase was observed in POX activity in both resistant and *P. fluorescens* treated challenge inoculated seedlings over the susceptible inoculated control.

3.5.3. Time-course analysis of lipoxygenase (LOX) assay

The spectrophotometric analysis of LOX activity is depicted in Fig. 6. The results of the study revealed that LOX activity in all the samples evaluated was found maximum at 24 h.a.i. irrespective of treatment. Maximum enzyme activity was observed in resistant and challenge inoculated seedlings (8.66 U) followed by inducer treated challenge inoculated seedlings (7.53 U). LOX activity of 2.8 U and 4.06 U was observed with respect to susceptible and susceptible inoculated seedlings, respectively. There was an increase of 1.1 and 0.85 fold increase in enzyme activity was observed in resistant and *P. fluorescens* treated challenge inoculated seedlings, respectively over the susceptible inoculated control.

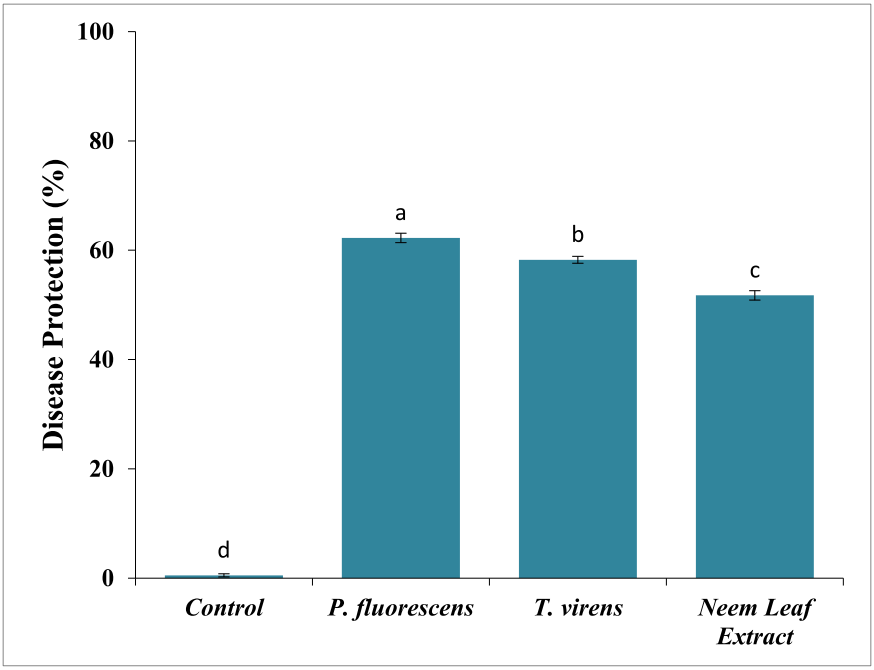

Fig. 3. Effect of biotic elicitors on disease protection under greenhouse conditions at the end of 45 DAS. Values are the means of four independent replicates.Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD test ($p \leq 0.05$).

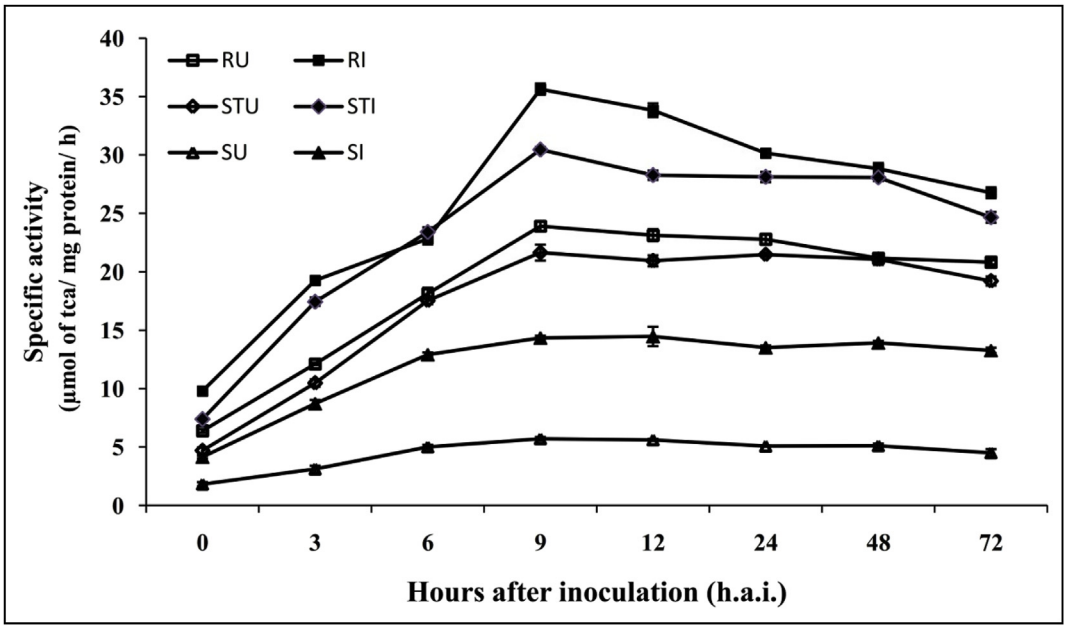

Fig. 4. Time course analysis of PAL enzyme activity in pearl millet seedlings upon *P. fluorescens* seed treatment. RU: Resistant uninoculated; RI: Resistant inoculated; STU: Susceptible inducer treated uninoculated; STI: Susceptible inducer treated inoculated; SU: Susceptible uninoculated; SI: Susceptible inoculated. Values are the mean for three replicates (n = 3) and vertical bar indicates the standard error.

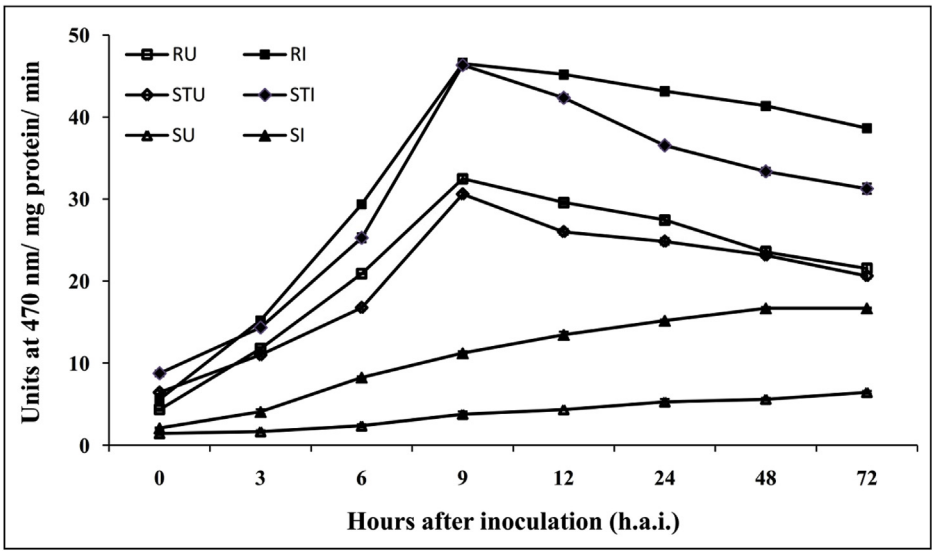

Fig. 5. Time course analysis of POX enzyme activity in pearl millet seedlings upon *P. fluorescens* seed treatment. RU: Resistant uninoculated; RI: Resistant inoculated; STU: Susceptible inducer treated uninoculated; STI: Susceptible inducer treated inoculated; SU: Susceptible uninoculated; SI: Susceptible inoculated. Values are the mean for three replicates (n = 3) and vertical bar indicates the standard error.

3.5.4. Time-course analysis of β-1,3-glucanase assay

The study revealed that the resistant and inducer treated pearl millet seedlings showed elevated levels of β-1,3-glucanase activity over susceptible seedlings with higher activity at 24 h.a.i. in all the samples which constantly decreased thereafter (Fig. 7). A maximum of 4.53 µmol min^{-1} mg $protein^{-1}$ of β-1,3-glucanase activity was observed in resistant challenge inoculated seedlings followed by 4 µmol min^{-1} mg $protein^{-1}$ in *P. fluorescens* treated challenge inoculated seedlings at 24 h.a.i. The enzyme activity in susceptible uninoculated seedlings was 1.10 µmol min^{-1} mg $protein^{-1}$, while it increased upon pathogen inoculation (1.93 µmol min^{-1} mg $protein^{-1}$) at the same time interval (24 h.a.i.). There was an increase of 1.3 and 0.5 fold increase in enzyme activity was observed in resistant and *P. fluorescens* treated challenge inoculated seedlings, respectively over the susceptible inoculated control.

4. Discussion

Management of plant diseases has reached new heights in the last few decades. Many novel strategies have been adopted into this area of crop protection due to their eco-friendly attributes. In the present study, selected biotic-agents were evaluated for their effect on plant growth promotion and induction of blast resistance in pearl millet. The biotic agents are known to activate many signalling pathways at histological, biochemical and molecular levels that will help the plants to overcome the infection process caused by the pathogens (Zimmerli et al., 2000; Murali et al., 2013; Abhayashree et al., 2017). Various workers have reported growth promotion and induction of systemic resistance in a variety of crops using different biotic elicitors like rhizobacteria, fungi and plant extracts (Vleesschauwer et al., 2008; Gowtham et al., 2018; Brijesh Singh et al., 2019).

The pearl millet seeds were treated with selected biotic agents for 3 h and 6 h to study their effect on seed growth parameters. The results

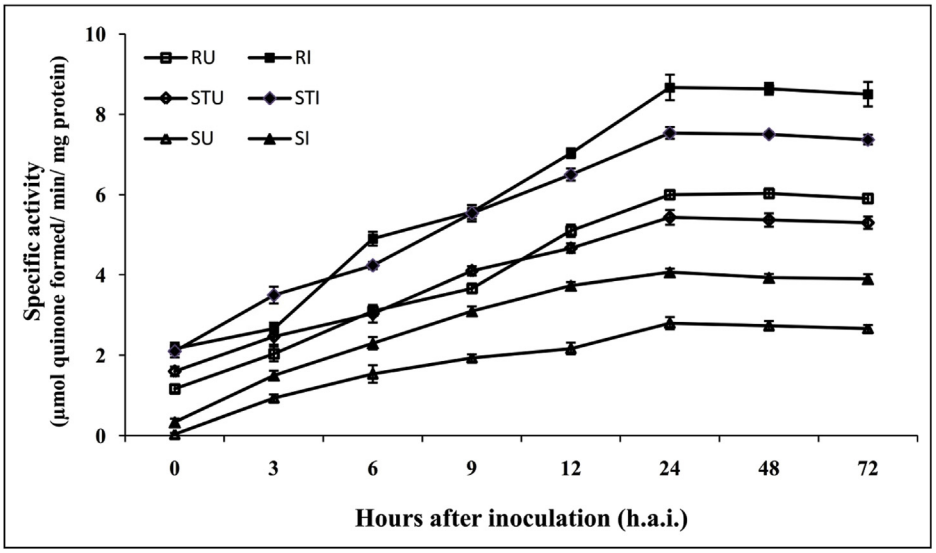

Fig. 6. Time course analysis of LOX enzyme activity in pearl millet seedlings upon *P. fluorescens* seed treatment. RU: Resistant uninoculated; RI: Resistant inoculated; STU: Susceptible inducer treated uninoculated; STI: Susceptible inducer treated inoculated; SU: Susceptible uninoculated; SI: Susceptible inoculated. Values are the mean for three replicates (n = 3) and vertical bar indicates the standard error.

revealed that among the different time points of treatment, 6 h treatment with biotic inducers were found to be more effective than the 3 h treatments with maximum germination of 83.50% and seedling vigor of 1447.93 offered by *P. fluorescens* followed by *T. virens*. There was a significant increase in seed growth parameters in seeds treated with biotic agents compared to control. Similar observations on the enhancement of seed growth parameters were noticed in pearl millet seeds treated with *P. fluorescens*, *T. virens* and neem leaf extract from previous reports (Mondal et al., 2009; Niranjan raj et al., 2012). In addition, there are also reports on the use of plant extracts, plant growth promoting rhizobacteria (PGPR) and fungi (PGPF) as seed treatment which have resulted in the enhancement of seed growth parameters in many crop plants (Nagaraju et al., 2012; Niranjan raj et al., 2012; Murali and Amruthesh, 2015; Abhayashree et al., 2017; Adebola et al., 2017; Gowtham et al., 2018). Apart from increasing the seed growth parameters, the biotic-agents used in the study also enhanced vegetative and reproductive plant growth parameters significantly compared to control plants. In accordance with the results obtained, Nagaraju et al. (2012) have reported the improvement of seed and plant growth parameters (both vegetative and reproductive) when the sunflower seeds were treated with *Trichoderma* spp. The results are also in confirmation with the findings of Mythrashree et al. (2013) and Hariprasad et al. (2014), wherein seed treatment with PGPR and plant extracts enhanced both seed and plant growth parameters in tomato and pearl millet, respectively.

Pathogen control by biotic inducers occurs through antibiosis, predation, myco-parasitism, ISR and SAR induction (Chowdappa et al., 2013; Niranjan raj et al., 2012; Murali and Amruthesh, 2015; Gowtham et al., 2018). Induced resistance is based on multiple defense mechanisms which are often characterized by a broad spectrum of protection and relatively long duration of expression, which makes it less likely to be overcome by the pathogens (Kessmann et al., 1994; Walters et al., 2013). In the present study, among the three biotic inducers evaluated, *P. fluorescens* offered maximum disease protection of 62.25%, followed by *T. virens* and neem leaf extract. The results are in line with many researchers, wherein pre-treatment of seeds with biotic inducers (PGPR, PGPF and plant extracts) of many crop plants suppressed both bacterial and fungal diseases effectively and induced disease resistance through the activation of plant defense mechanisms (Djonovic et al., 2006; Chowdappa et al., 2013; Gowtham et al., 2018).

Disease resistance mechanisms in plants include pre-existing physical and chemical barriers as well as inducible defense responses in the form of induction of defense-related enzymes that are activated upon pathogen infection (Kombrink and Somssich, 1997; Manjula et al., 2015).The host-pathogen interaction results in biochemical changes, primarily the defense enzymes viz. PAL, POX, LOX (JA and/or ET

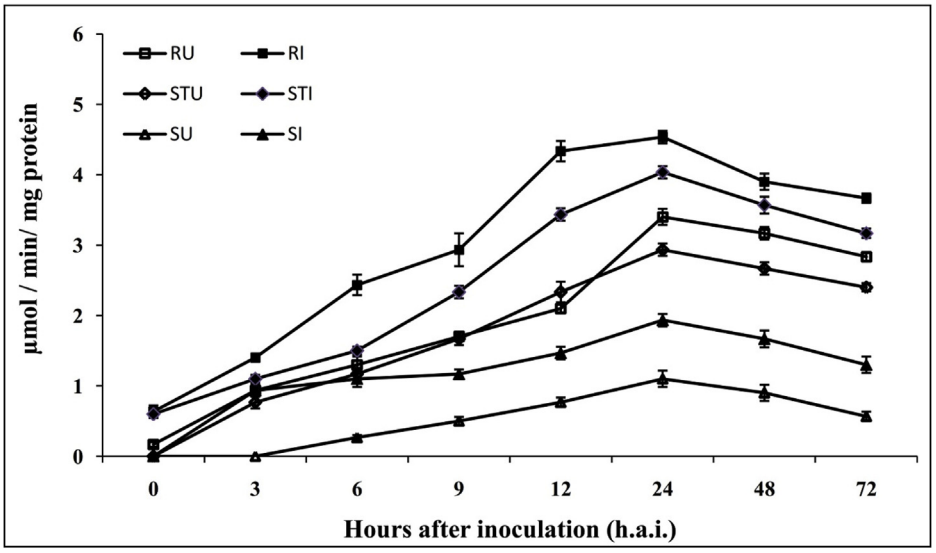

Fig. 7. Time course analysis of β-1,3-glucanase enzyme activity in pearl millet seedlings upon *P. fluorescens* seed treatment. RU: Resistant uninoculated; RI: Resistant inoculated; STU: Susceptible inducer treated uninoculated; STI: Susceptible inducer treated inoculated; SU: Susceptible uninoculated; SI: Susceptible inoculated. Values are the mean for three replicates (n = 3) and vertical bar indicates the standard error.

dependent pathway) and Glucanase (SA dependent pathway)which are normally up regulated in infected plant tissues upon biotic stress (Chen et al., 2000; Murali and Amruthesh, 2015; Gowtham et al., 2018). It has been reported that, when the rice plants were infected with *M. oryzae* these enzymes (PAL, POX, LOX, Glucanase, etc.) were upregulated indicating there importance in disease protection (Anushree et al., 2016) and hence based on the previous reports these enzymes were selected as indicators of blast resistance. These enzymes possess multifaceted activities acting in both biotic and abiotic stress responses, thereby effectively induce resistance in plants to pathogen infection (Takahashi et al., 2007; Gao et al., 2012). In the present study, a total of four defense enzymes were evaluated for their response in pearl millet upon challenge inoculation with *M. grisea*. The results of the study revealed enhanced defense enzymes in pearl millet seedlings treated with biotic inducers which were in line with resistant seedlings thereby indicating the effective role of these biotic inducers to induce resistance to pathogen infection. The results revealed that maximum enzyme activity was observed at the 9 h.a.i. in PAL and POX, while 24 h.a.i. for LOX and β-1,3-glucanase. There was an increase of 0.5–1.7 fold increase in all the defense enzymes in biotic inducer treated challenge inoculated seedlings compared to susceptible inoculated seedlings. The results are in confirmation with previous studies wherein researchers have demonstrated the defense responses (PAL, POX, LOX, β-1,3-glucanase, etc.) in primed plants are not activated directly but are accelerated upon attack by pathogens or insects, resulting in faster and stronger resistance to the attacker encountered (Chen et al., 2000; Van Wees et al., 2008; Abhayashree et al., 2017; Gowtham et al., 2018; Wang et al., 2019). The results confirm the effectiveness of *P. fluorescens* treatment to pearl millet seedlings in inducing resistance through activation of defense enzymes (act as biochemical markers) thereby directly inhibiting the furtherance of infection to host plant which is in agreement with the findings of Lebeda et al. (2001) and Gowtham et al. (2018).

5. Conclusions

The present study has validated the efficiency of seed priming with *P. fluorescens*, *T. virens* and neem leaf extract in promoting plant growth and protection against blast disease of pearl millet. The disease protection studies were validated by increased activity of defense enzymes (PAL, POX, LOX and β-1,3-glucanase). The results strongly back the application of *P. fluorescens* as an efficient inducer of systemic resistance in pearl millet against blast disease caused by *M. grisea*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101279.

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