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PGPM-induced defense-related enzymes in aerobic rice against rice leaf blast caused by *Pyricularia oryzae*

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Abstract Rice blast caused by *Pyricularia oryzae* is the most devastative disease especially under aerobic cultivation systems. The bio-efficacy of plant growth-promoting microorganisms: *Pseudomonas aeruginosa* (UPMP1), *Corynebacterium agropyri* (UPMP7), *Enterobacter gergoviae* (UPMP9) and *Bacillus amyloliquefaciens* (UPMS3), *Trichoderma harzianum* (UPMT1) and *Trichoderma virens* (UPMT2) in induction of defense-related enzymes against *Pyricularia oryzae* was evaluated in rice cultivated under aerobic conditions. Under dual culture plate testing, all PGPMs indicated antagonism against *P. oryzae* with percentage inhibition radial growth (PIRG) which ranged from 51.69–81.97 %. The bio-efficacy of the respective PGPM in induction of defense-related enzymes in rice seedlings was evaluated based on individual inoculation before challenged inoculation with *P. oryzae* under greenhouse conditions. Inoculation of all PGPMs significantly reduced rice leaf blast severity at day eight after *P. oryzae* inoculation. The reduction in rice leaf blast disease severity was associated to the increase of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities in rice seedlings when pre-inoculated with PGPMs. The highest leaf blast disease reduction (59.17 %) occurred with rice seedlings pre-inoculated with *C. agropyri* (UPMP9), followed by *P. aeruginosa* (UPMP1) (40.65 %), *T. harzianum* (UPMT1) (42.23 %), *T. virens* (UPMT2) (20.85 %), *E. gergoviae* (UPMP9) (17.84 %) and *B. amyloliquefaciens* (UPMS3). The high efficiency of PGPM in leaf blast disease suppression was associated with significant increase in total microbial activity (FDA hydrolysis) in rhizosphera soil (4.80–7.86 μg/g/0.5 h) compared to the control (2.25 μg/g/0.5 h). Thus, the application of PGPM is a potential alternative approach in rice leaf blast disease management of aerobic rice.

Keywords Plant growth-promoting microorganism · Aerobic rice · Rice leaf blast · Defense-related enzymes · *Pyricularia oryzae*

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Introduction

Rice diseases have always threatened the production to meet consumer demand. Rice blast caused by *Pyricularia grisea* (Cooke) Sacc., teleomorph *Magnaporthe grisea* (T. T. Hebert) Barr, is the most destructive rice (*Oryza sativa* L.) disease worldwide, especially under low water management systems. According to Scardaci et al. (1997), rice plants cultivated under drought-stressed conditions are more susceptible to blast. For instance, 100 % grain yield loss was reported in Brazil in a newly released upland rice cultivar (Prabhu et al. 2009). Currently, tricyclazole application was reported effective in reducing neck blast disease severity and multiple application was suggested for moderately susceptible or susceptible Italian rice cultivar in order to control the disease severity (Titone et al. 2015).

The growing concern and awareness to protect the agricultural environment and food safety have generated the desire to reduce pesticide usage in agriculture. Biological control through application of plant growth-promoting bacteria was highly suggested as a potential alternative in crop disease management (Nelson 2004). The direct application of PGPMs to the soil or through seed inoculation has shown to improve plant productivity, quality, health, and also help to minimize the dependence on of chemical pesticides and fertilization (Basja 2013). A number of studies confirmed that microbial inoculants lead to higher microbial populations in the soil and promote plant growth through improved nutrient acquisition, increased levels of phytohormones and other growth metabolites, suppression of plant diseases (Nelson 2004; Rodriguez et al. 2007; Umashankari and Sekar 2011; Yadav et al. 2011) and induced systemic resistance (ISR) (Van Loon 2007) in various crops including cereals (Raj et al. 2005). The PGPM-mediated ISR is important for disease control under conditions where the PGPM and pathogens are spatially separated (De Meyer et al. 1998). The systemic resistance induction process leads to increases in peroxidase (PO) and phenoloxidase (PPO) activities, which are involved in catalyzing lignin formation, and phenyl ammonia lyase (PAL), for the biosynthesis of phytoalexin and phenol (Filippi et al. 2011).

An increasing number of PGPR-mediated ISR had been studied to prove the benefits of rhizobacteria associations to ISR in various plant disease management programs such as in cucumber against *Colletotrichum orbiculare* (Wei et al. 1996) and *Botrytis cinerea* in various crops (De Meyer et al. 1998). However, the application of PGPRs to control rice blast disease especially when cultivated under aerobic conditions is still very limited. Filippi et al. (2011), reported increases of peroxidase activities, ß-1, 3-glucanase and chitinase were associated with low leaf blast disease severity in aerobic rice pre-inoculated with potential rhizobacteria. In addition, the use of PGPM-fortified rice straw compost in aerobic rice was also proven to reduce the rice leaf blast severity, promote plant growth and productivity (Ng et al. 2011).

There is no detailed information on the bio-efficacy of PGPM in aerobic rice blast management, especially in understanding the PGPM-induced defense-related enzymes, such as PO, PPO and PAL. The present study evaluates the bio-efficacy of PGPM screened from rhizosphere soil of aerobic rice in the induction of systemic defense-related enzymes associated with rice leaf blast disease development in aerobic rice.

Materials and methods

PGPM isolation and selection

Six PGPMs: *Pseudomonas aeruginosa* (UPMP1), *Corynebacterium agropyri* (UPMP7), *Enterobacter gergoviae* (UPMP9), *Bacillus amyloliquefaciens* (UPMS3), *Trichoderma harzianum* (UPMT1) and *Trichoderma virens* (UPMT2) were previously isolated from disease-free aerobic rice fields from 10 different locality plots at Kepala Batas, Pulau Pinang, Malaysia. Ten plants were randomly sampled from each plot, the soil closely adhering to the root zones were defined as rhizosphere soils. Soil microorganisms were isolated using serial dilution technique. All the selected PGPM had the ability to produce indole-3-acetic acid (IAA) (Gorden and Weber 1951), siderophore (Alexander and Zuberer 1991), chitinase (Khoury et al. 1997) and solubilize phosphate (Mehta and nautiyal 1999) (our unpublished results) through in vitro screenings. The isolates were maintained in nutrient broth amended with glycerol (20 %) at −20 °C.

Pyricularia oryzae inoculation

Rice seeds of variety M4 were obtained from Malaysian Agricultural Research and Development Institute (MARDI) and were surface sterilized in 70 % ethanol,

followed by 5 % sodium hypochlorite before rinsed with sterilize water. The surface sterilized seeds were air-dried and inoculated by immersion in respective inocula of *P. aeruginosa*, *C. agropyri*, *E. gergoviae*, *B. amyloliquefaciens*, *T. harzianum* and *T. virens* at 10^8 cfu/ml for 45 min. Rice seeds immersed in distilled water served as control. Twenty PGPM-inoculated rice seeds were sown into 1 kg of sterilized mineral soil, (Oxisols, Prang series) in pots with holes at the bottom to allow for drainage. The rice seedlings were grown under an aerobic cultivation system (non-flooded soil) with drip irrigation twice daily (10 am and 6 pm) with an automatic intermittent stoppage after 30 min of irrigation under greenhouse conditions. At day seven after sowing (DAS), 5 ml of respective PGPM cell suspension (10^8 cfu/ml) was added into each pot to increase root and rhizosphere colonization (Filippi et al. 2011). Sterilized distilled water was applied to the control pots.

Rice leaf blast disease development

Fourteen day old rice seedlings were spray-inoculated with *P. oryzae* conidia (10^5 conidia/ml) suspension at 10 ml per pot. Uninoculated, with *P. oryzae*, rice seedlings served as control. *Pyricularia oryzae* inoculated rice seedlings were covered with plastic bags for 12 h to retain moisture. Mist sprinkle irrigation system was continued and applied at between 11 am to 3 pm for 15 min with every 30 min of stoppage for the following three days to maintain RH of 95–98 % and the temperature at 25–27 °C. Leaf blast rating was visually assessed from the 0 to 9th day after *P. oryzae* challenged inoculation on an individual plant basis using the standard evaluation system for rice (SESR) developed by International Rice Research Institute (2002). The disease severity index was calculated using the following formula by Shrestha and Mishra (1994), where:

$$\text{Disease severity index} = \sum \frac{(\text{number of plants in the rating} \times \text{severity rating})}{(\text{total number of plants observed} \times \text{maximum scale})} \times 100$$

The disease severity in each treatment was further expressed as area under the disease progress curve (AUDPC) and the rate of disease development was expressed as epidemic rate (RL). AUDPC and epidemic rate were calculated by transforming the disease severity data using the Logit model described by Campbell and Madden (1990) in the Sigma Plot software (SPSS, Version 9.0. Systat Software Inc. (SSI), California, USA). The efficacy of treatments in rice blast disease reduction was calculated by the following formula:

$$\text{Disease reduction } (\%) = \frac{(\text{AUDPC in control-AUDPC in treatment})}{\text{AUDPC in control}} \times 100\%$$

Plant extraction

Peroxidase (PO), phenylalanine ammonine-lyase (PAL) and polyphenol oxidase (PPO) were analyzed using rice seedlings from each replication at 0th, 1st, 2nd, 3rd, 4th, 6th and 8th day after *P. oryzae* challenged inoculation. The extraction procedures for PO, PAL and PPO were as described by Mozzetti et al. (1995). All leaf samples for each replication were pooled and three sub-samples (2 g each) were randomly sampled and frozen in liquid nitrogen and ground to powder using a cold mortar and pestle. Ground leaves were re-suspended in cold 0.05 M phosphate buffer at pH 7.0 containing 0.5 g polyvinylpolypyrrolidone (PVP) in a 1:5 tissue to buffer ratio. The mixture was homogenized on a vortex mixer and the supernatant was separated by centrifuging at 14,000 × *g* for 20 min at 4 °C. The extracts obtained were used for PO and PPO assays.

Peroxidase activity

Peroxidase (PO) activity was determined by measuring the guaiacol oxidation in the presence of hydrogen peroxide (Merck, Germany) as described by Fecht-Christoffers et al. (2003). Leaf crude extract (100 μl) of each sample was added with 3 ml of mixture consisting of: 1 ml of 0.25 % guaiacol (O-methoxyphenol), 1 ml of 0.1 M hydrogen peroxide

and 1 ml of 0.01 M phosphate buffer. The changes in absorbance at 470 nm were monitored for 2 min using a spectrophotometer (Model UV-3600, Shimadzu). A mixture without leaf crude extract served as a blank. Peroxidase activity was measured using the Brandford method (1976) and expressed as change in absorbance (unit)/min/g of protein.

Polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was determined by adding the leaf crude extract to 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 μl of 0.1 M catechol. The value in absorbance at 410 nm was measured using a spectrophotometer (Model UV-3600, Shimadzu). Polyphenol oxidase activity was measured using the Brandford method (1976) and expressed as change in absorbance (unit)/min/g of protein.

Phenylalanine ammonine-lyase activity

Phenylalanine ammonine-lyase (PAL) activity was determined spectrophotometrically based on the production of trans-cinnamic acids from L-phenylalanine. The mixture contained 100 μl of the leaf crude extract, 1.15 ml of 0.1 M borate buffer (pH 8.8) and 1 ml of 10 mM L-phenylalanine. The mixture was incubated for 1 h at 40 °C in a water bath and the reaction was stopped by adding 250 μl of 5 N HCl. The amount of trans-cinnamic acid formed from L-phenylalanine was measured at a wavelength 290 nm using a spectrophotometer (Model UV-3600, Shimadzu). The phenylalanine ammonine-lyase activity was measured using the Brandford method (1976) and expressed in nmol of trans-cinnamic acids produced/min/g of protein.

Total microbial activity in rhizosphere soil

Total microbial activity in rhizosphere soil with 14 day old rice seedlings was determined based on total enzymatic activity using fluorescein diacetate (FDA) hydrolysis (Shaw and Burns 2005). The soil samples were mixed with 7.5 ml of potassium phosphate buffer (pH 7.6, 60 mM) and FDA solution. FDA solution was prepared by adding 25 mg fluorescein diacetate (3′ 6′- diacetatyl-fluorescein, Sigma-Aldrich) into 25 ml acetone. The reaction was started by adding 0.1 ml of FDA solution and incubated in a shaker for another 30 min at 25 °C. The substrate blank consisting of soil and buffer mixture with the FDA solution was replaced by 0.1 ml acetone. After 30 min of incubation, the reaction was stopped by added 7.5 ml of chloroform: methanol (2:1) and the suspension was measured for absorbance at 490 nm using a spectrophotometer (model UV-3600, Shimadzu) against the blank.

A calibration curve was constructed by using fluorescein solution (2000 μg/ml) which was added into fluorescein sodium salt (3′ 6′- diacetatyl-fluorescein, Sigma-Aldrich) and potassium phosphate buffer (pH 7.6, 60 mM). The standard curve was used to calculate the colour intensity of fluorescein produced in each assay and all results were expressed as μg fluorescein/g of dry sample/0.5 h of incubation with sodium salt as substrate.

Experimental design and data analysis

The experiments for rice leaf blast disease development evaluations were arranged in a randomized complete block design. All experiments were repeated three times with five replications per treatment. All data collected were subjected to analysis of variance and tested for significance using Fisher's Protected Least Significant Difference (LSD) Test at $P \leq 0.05$ with the SAS software (version 9.1).

Results

Rice leaf blast disease development

The onset of rice blast symptoms were observed as small brown specks of pinpoint size or large brown specks without a sporulating center, which enlarge to become spindle-shaped lesions with yellow and brown margins, later coalescing without a distinct margin.

There was a gradual increase in percentage of disease severity over time (Fig. 1). Rice seedlings pre-inoculated with *C. agropyri*, *P. aeruginosa* and *T. harzianum* showed significantly lower percentage of disease severity as compared to other treatments at all observation timings. On the 9th day after the *P. oryzae* challenged inoculation, percentages of disease severity on rice seedlings pre-inoculated with *E. gergoviae*, *T. virens* and *B. amyloliquefaciens* showed no significant differences with the control (Table 1). However, seedlings pre-inoculated with *C. agropyri* exhibited a significantly lower disease severity of 40.44 %,

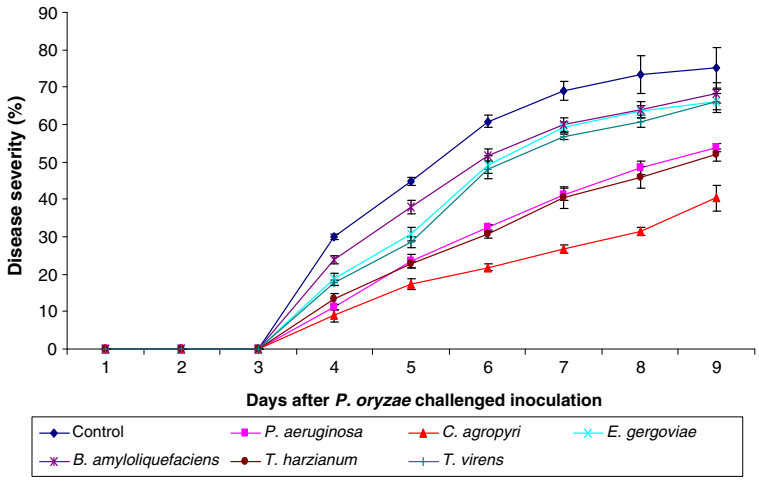

Fig. 1 Rice blast disease severity in rice seedlings pre-inoculated with PGPMs. (Vertical bars indicate standard error)

followed by *T. harzianum* (52.00 %) and *P. aeruginosa* (53.78 %) (Table 1).

Disease severity was further expressed as the area under the disease progress curve (AUDPC) and epidemic rate of disease development (R_L). Rice seedlings pre-inoculated with *C. agropyri* exhibited statistically lower ($P \leq 0.05$) AUDPC (126.44 unit^2), followed by *T. harzianum* and *P. aeruginosa* with 178.89 and 183.78 unit^2, respectively. Similarly, the epidemic rate was also significantly lower in plants pre-inoculated with *C. agropyri* with a value of 4.80 unit/day followed by *T. harzianum* (6.62 unit/day), *P. aeruginosa* (6.91 unit/day), *T. virens* (8.86 unit/day) and *E. gergoviae* (9.07 unit/day) (Table 1).

Rice seedlings pre-inoculated with *C. agropyri* exhibited the greatest reduction in rice blast development of 59.17 %, followed by *T. harzianum* (42.23 %), *P. aeruginosa* (40.65 %), *T. virens* (20.85 %), *E. gergoviae* (17.84 %) and *B. amyloliquefaciens* (12.16 %) (Table 1).

PGPM-induced defense-related enzymes

Peroxidase, PPO and PAL activities were detected at significantly higher levels in rice seedlings pre-inoculated with PGPM as compared to the control (uninoculated with PGPM) (Fig. 2). In all treatments, PO, PPO and PAL activities reached a maximum on the 3rd day after *P. oryzae* challenged inoculation and subsequently gradually decreased. The PO activity was significantly higher in rice seedlings pre-inoculated with *T. harzianum* with 1.65 unit/min/g of protein, while no significant differences were observed in seedlings pre-inoculated with *C. agropyri*, *E. gergoviae*, and *T. virens*.

Table 1 Rice blast disease severity, AUDPC, epidemic rates (R_L) and disease reduction on the 9th day after challenged inoculation with *P. oryzae* on rice (variety M4) pre-inoculated with PGPMs

Isolates	Disease severity (%)	AUDPC (unit^2)	Epidemic rate (R_L) (unit/day)	Disease reduction (%) (based on control)
Control	75.11 a	309.68 a	10.24 a	-
P. aeruginosa	53.78 b	183.78 d	6.91 c	40.65
C. agropyri	40.44 c	126.44 e	4.80 d	59.17
E. gergoviae	66.22 a	254.44 bc	9.07 b	17.84
B. amyloliquefaciens	68.40 a	272.02 b	9.29 ab	12.16
T. harzianum	52.00 b	178.89 d	6.62 d	42.23
T. virens	66.22 a	245.11 c	8.86 b	20.85

Values are means of three repeated experiments with five replications. Means within columns with the same letters are not significantly different by Fisher's Protected Least Significant Different test at $P \leq 0.05$

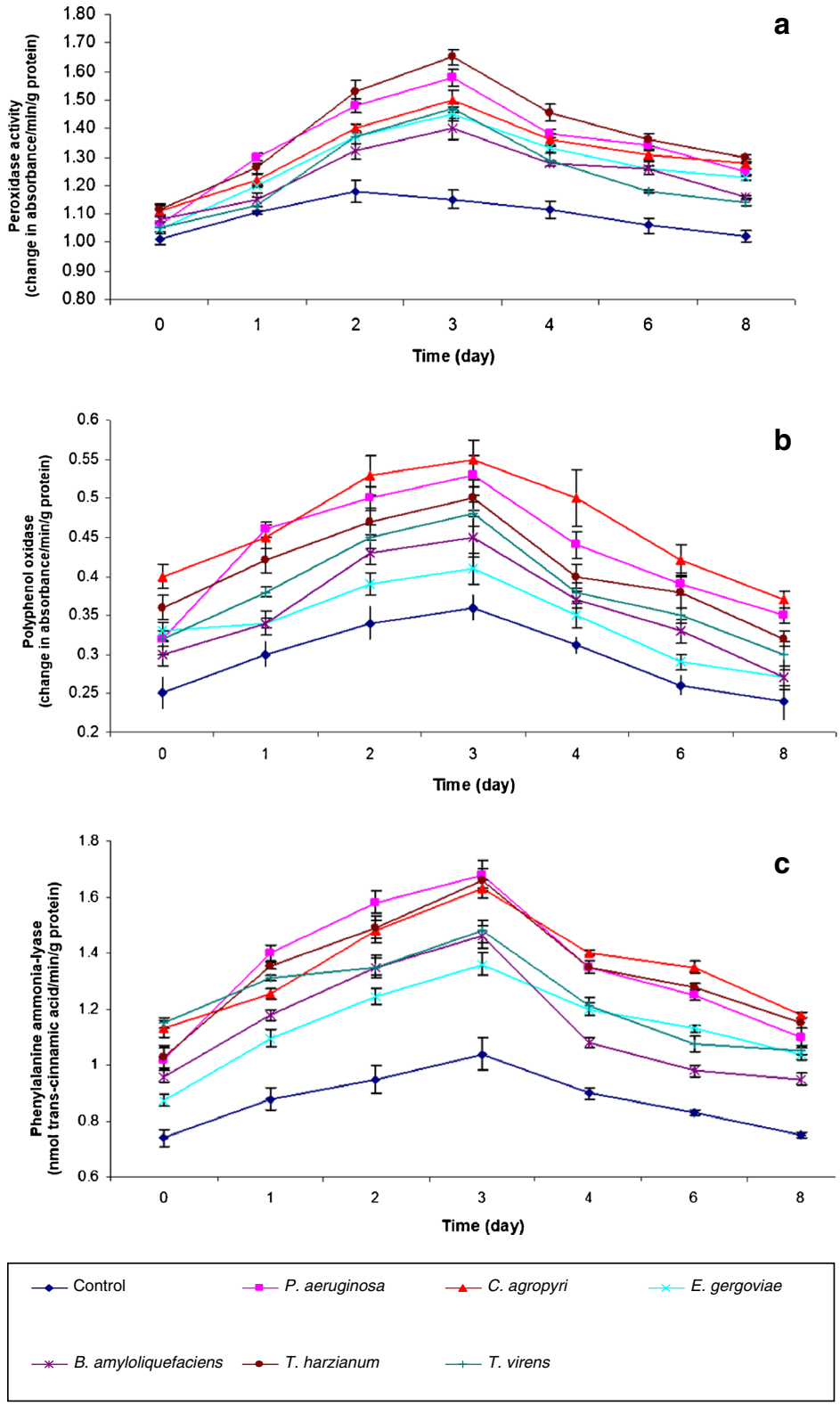

Fig. 2 Peroxidase (**a**), polyphenol oxidase (**b**) and phenylalanine ammonia-lyase (**c**) activity in rice seedlings pre-inoculated with PGPMs. (Vertical bars indicate standard error)

A similar trend was observed for PPO and PAL activity with a gradual increase during the initial three days after introducing *P. oryzae* and subsequently followed by a gradual decline. Polyphenol oxidase activity reached significantly higher levels on day three in seedlings pre-inoculated with *C. agropyri* (0.55 unit/min/g of protein), followed by *P. aeruginosa* (0.53 unit/min/g of protein), *T. harzianum* (0.50 unit/min/g of protein), *T. virens* (0.48 unit/min/g of protein), *B. amyloliquefaciens* (0.45 unit/min/g of protein) and *E. gergoviae* (0.41 unit/min/g of protein) (Fig. 2). In rice seedlings pre-inoculated with *P. aeruginosa*, *T. harzianum* and *C. agropyri* highly significant PAL activity (1.68, 1.66 and 1.63 nmol trans-cinnamic acid/min/g of protein, respectively) was detected on day three after *P. oryzae* inoculation (Fig. 2).

Total microbial activity in rice rhizosphere soil

The colonization and the viability of the inoculated isolates in rhizosphera soil was associated to the total microbial activity based on the FDA hydrolysis value. Rice seeds pre-inoculated with PGPM followed by soil inoculation significantly enhanced the total microbial activity (FDA hydrolysis value) in rhizosphere soil as compared to the control (Table 2). The highest FDA value was scored by *T. virens* (7.86 μg/g/0.5 h), followed by *T. harzianum* (7.55 μg/g/0.5 h), *B. amyloliquefaciens* (5.42 μg/g/0.5 h), *E. gergoviae* (4.99 μg/g/0.5 h), *C. agropyri* (4.80 μg/g/0.5 h) and *P. aeruginosa* (4.73 μg/g/0.5 h) suggesting the ability of the isolates to colonize and proliferate in the rice rhizosphere.

Table 2 Total microbial activity (based on fluorescein diacetate hydrolysis) in rhizosphere soil of aerobic rice

Treatment	FDA (μg/g/0.5 h)
Control	2.25 (± 0.20) d
P. aeruginosa	4.73 (± 0.24) c
C. agropyri	4.80 (± 0.22) c
E. gergoviae	4.99 (± 0.10) bc
B. amyloliquefaciens	5.42 (± 0.19) b
T. harzianum	7.55 (± 0.18) a
T. virens	7.86 (± 0.19) a

Values are means of three repeated experiments with five replications. Means with the same letters are not significantly different by Fisher's Protected Least Significant Different test at $P \leq 0.05$ (± values indicate standard errors)

Discussion and conclusions

The bio-efficacy of individual PGPMs for the production of defense-related enzymes were compared in rice seedlings after challenged inoculation with *P. oryzae*. Several studies have demonstrated positive results utilizing PGPMs for controlling rice leaf blast disease under irrigated (Vleesschauwer et al. 2008; Lucas et al. 2009) and aerobic conditions (Filippi et al. 2011). In the current study, rice seedlings pre-inoculated with PGPMs had higher levels of PO, PPO and PAL after challenged inoculation with *P. oryzae*. These secondary biosynthetic pathways have been reported to respond to *P. oryzae* and are associated with plant induced resistance (Smith and Metraux 1991; Melvina Joe and Sivakumar 2010; Umashankari and Sekar 2011). For instance, enhancement of the phenylpropanoid pathway (PAL) in rice plants was reported as evidence of induced resistance in rice plants against *P. oryzae* (Ouyang et al. 1987). Besides, PO was also reported to have several functions in plant resistance, including the oxidative polymerization of hydroxycinnamyl alcohols to form lignin in plant defense mechanisms (Vance et al. 1980) and making cell walls more resistant to microbial enzyme degradation by oxidative cross-linking of pre-existing hydroxyproline-rich structural proteins (Bradley et al. 1992) and providing a physical barrier to the plant. The disease progress evaluated during the nine consecutive days showed that rice seeds pre-inoculated with PGPMs significantly reduced AUDPC and were hence related to ISR mediated by PO, PPO and PAL.

Rice seeds pre-inoculated with *C. agropyri*, *P. aeruginosa*, *T. harzianum* and *T. virens* resulted in rice blast disease reductions of 59.17 %, 40.65 %, 42.23 % and 20.85 %, respectively. Besides, rice leaf blast disease severity, epidemic rate and AUDPC were also significantly lower in seedlings pre-inoculated with *C. agropyri*, *P. aeruginosa* and *T. harzianum*. Interestingly, the high efficacy of *C. agropyri*, *P. aeruginosa* and *T. harzianum* in controlling rice leaf blast was associated with high levels of PO, PPO and PAL activity at the third day after *P. oryzae* challenged inoculation. This explains the involvement of PGPM-mediated ISR as the leaf blast disease was controlled under conditions where the PGPM and the pathogens are spatially separated (De Meyer et al. 1998). Our results explain the mechanism involved in the study conducted by Ng et al. (2011) where the application of the same PGPM-fortified rice straw compost reduced rice leaf blast disease

severity. Similarly, PGPR-mediated ISR was also proven in disease management of various crops (Wei et al. 1996; De Meyer et al. 1998; Bharathi et al. 2004; Saravanakumar et al. 2007).

Enhancement of rice seedling resistant against *P. oryzae* infection was closely related with the beneficial interaction between rhizosphere microbes and plant roots. This was evident with the high total microbial activity detected in rhizosphere soil of PGPM inoculated rice seedlings. A possible mechanism involved for increasing seedling resistant against *P. oryzae* was related to the ability of the PGPM to colonize and proliferate in the rhizosphere and roots. The total microbial activity measured using FDA hydrolysis provides a general indicator of different enzymatic activities such as proteases, lipases and esterases (Schnurer and Rosswall 1982) in rhizosphere soil which relatively indicates the activity and density of the introduced PGPM. Thus, rice seed pre-inoculated with *C. agropyri*, *T. harzianum*, *P. aeruginosa and T. virens* improved total microbial activity in the rhizosphere soil and consequently induced defense-related enzymes in rice seedlings after challenged inoculation with *P. oryzae* under the aerobic cultivation system. It is scientifically proven that inoculation of microorganisms in the rhizosphere helps to increase the microbial diversity and leads to significantly improved soil and plant health (Glick 1995; Rodriguez et al., 2007).

Rice seeds pre-inoculated with *C. agropyri*, *T. harzianum*, *P. aeruginosa and T. virens* reduced rice leaf blast severity with an associated increase in PO, PPO and PAL activities. This suggests the possible involvement of ISR as a mechanism in the suppression of *P. oryzae*. The significant differences in the efficacy of individual PGPM applications, point to the need for development of a consortium PGPM formulation for sustainable effect. A further detailed study is required to find the potential combination and to understand the exact mechanisms involved. Our investigations reveal that the selected PGPM have the potential to be developed as biological control agents in rice leaf blast management under aerobic cultivation systems.

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