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FOLIAR SPRAYED-SILICON TO INDUCE DEFENSE-RELATED ENZYMATIC ACTIVITY AGAINST *Pyricularia oryzae* INFECTION IN AEROBIC RICE

LEE CHUEN NG[1*], ZAHARI NURA ADILA[1], ELHAM MOHD SHAHRUL HAFIZ[1], AHMAD AZIZ[2] and MOHD RAZI ISMAIL[3]

[1]Laboratory for Pest, Disease, and Microbial Biotechnology (LAPDiM), Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Kuala Nerus, Terengganu, Malaysia
[2]School of Fundamental Science, Universiti Malaysia Terengganu, Kuala Nerus, Terengganu, Malaysia
[3]Institute of Tropical Agriculture and Food Security (ITAFoS), Universiti Putra Malaysia UPM, 43400 Serdang, Selangor, Malaysia
[*]E-mail: nglee@umt.edu.my

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ABSTRACT

Rice blast disease caused by *Pyricularia oryzae* is the most devastative disease. The alternative in rice blast disease management using foliar silicon (Si) application is gaining attention. The mechanism underlying defense-related enzyme induced through foliar Si application is still scarce. This research aimed to elucidate the bio-efficacy of foliar Si in inducing defense-related enzyme activity against *P. oryzae* in two aerobic rice cultivars: MR219-4 (blast-partially resistant) and MARDI Aerob 1 (resistant). Calcium silicate at 9 mg/L was foliar-sprayed and the disease severity index was evaluated and transformed to the area under disease progress curves (AUDPC). Foliar Si application significantly reduced rice blast disease severities in both cultivars tested. The AUDPC was reduced to 96.57 (MR219-4) and 21.90 (MARDI Aerob 1), from 148.57 (MR219-4) and 53.73 (MARDI Aerob 1). Plant defense-related enzymes: peroxidase (PO), polyphenol oxidases (PPO), and phenylalanine ammonia-lyase (PAL) were increased and might be associated to increase resistance. Also, there was a significant interaction (p=0.003) between rice cultivar and treatment to Si content in rice leaf. Thus, foliar application of Si in rice seedling underlined the important role of Si as a modulator in influencing plant defense-related enzymes with interacting with other stress signaling pathways leading to induce resistance.

Key words: Silicon, foliar application, defense-related enzyme, rice blast disease, *Pyricularia oryzae*, aerobic rice

INTRODUCTION

Rice blast disease is one of the major fungal diseases that seriously reduced the production of rice in Asia including Malaysia (Ashkani *et al.*, 2013). Several rice blast epidemics have occurred in different countries which resulting in heavy yield losses ranging from 50 to 90% (Agrios *et al.*, 2005). Rice blast disease is caused by the ascomycetous fungus the *Pyricularia oryzae* (Anamorph), or *Magnaporthe oryzae* (Teleomorph) and the disease occurrence and severity vary by year, location, and environmental conditions (Hosseini-Moghaddam & Soltani *et al.*, 2013). The chemical application was reported as the most effective measure in rice blast disease management. However, with the increasing concerns on food safety and environmental pollution, alternative control by using silicon (Si) is gaining huge attention.

Silicon application was reported effective to increase rice resistance to blast disease on both partially resistant and susceptible cultivars (Seebold *et al*., 2001) through stimulation of the defense-related enzymatic activities during plant-pathogen interactions (Fauteux *et al*., 2005; Datnoff *et al*., 2007; Van *et al*., 2013) including peroxidases, polyphenoloxidases, β-1,3-glucanase, phenylalanine ammonia-lyase, lipoxygenase and glucanase

* To whom correspondence should be addressed.

(Waewthongrak *et al.*, 2015). These are the key enzymes that regulating the production and accumulation of secondary metabolic compounds such as phenolics, phytoalexins, and momilactones (Gomes *et al*., 2005; Ye *et al*., 2013). Thus, impede *P. oryzae* penetration and decrease the number of blast lesions on leaf blades (Rodrigues & Datnoff *et al.*, 2005).

Foliar feeding entails the application of nutrients via spraying to plant leaves and stems and for absorption at those sites (Prakash *et al*., 2011) and was reported to be more economic and environmentally friendly compared with the ground application. Si is exported from the root to the shoot and unloaded into stems and leaves. A silicic acid transporter OLsi6 was found to be responsible for the redirection of Si at stem nodes (Yamaji & Ma *et al.*, 2007). However, under aerobic rice cultivation systems, with limited water conditions that cause low nutrients solubility and availability to plant uptake, the foliar application is the option to correct the deficiency. The cuticular pathway by diffusion through cuticle and uptake through stomata at the higher permeability at trichome base were explained as the major uptake mechanisms in the plant (Fernandez *et al*., 2016). The role of foliar Si application to induce plant physiological mechanism as a barrier to hinder pathogen penetration was reported by Ng *et al*. (2018) but not in defense-related enzyme activity.

Plant and pathogen interaction involved a complex network of interactions. The Si-mediated resistance mechanism in rice plants against fungal pathogens through the ground application of Si has been well document. However, the bio-efficacy of Si-induced through foliar application in enhancing defense-related enzyme activity in a plant infected with *P. oryzae* has yet to be investigated, especially under an aerobic cultivation system. Hence, this research aimed to elucidate the bio-efficacy of foliar Si in inducing defense-related enzyme activity against *P. oryzae* in two aerobic rice cultivars: MR219-4 (blast-partially resistant) and MARDI Aerob 1 (resistant).

MATERIALS AND METHODS

Planting material and inoculation

Two aerobic rice cultivars MR219-4 (rice blast-partially resistant) and MARDI Aerob 1 (resistant) were cultivated using topsoil and maintain under greenhouse conditions. At 14 days after sowing (DAS) were foliar sprayed with calcium silicate (200 mesh, 99%, Sigma/Aldrich) at 9 mg/L, with 15 mL per pot for each treatment. Surfactant *Tween 20* at the concentration of 0.05% (v/v) was added into the Si solution to improve adhesion on the plant surface (Hayasaka *et al*., 2008). The treatments (T1, T2, T3, and T4) were tabulated in Table 1 with three replications for each treatment.

Ten days after Si foliar application, rice seedlings were inoculated with *Pyricularia oryzae* conidia suspensions (1×10^6 conidia/mL) added with *Tween20* (0.05%) at 15 mL/pot using a hand sprayer. The inoculated rice seedlings were covered with a black plastic bag for 24 hr after inoculation to retain humidity for effective infection. Non-inoculated seedlings (T2 and T4) were sprayed with sterile distilled water containing only *Tween20* (0.05%).

Rice blast disease evaluation

The rice leaves from each of the treatment were observed at 0, 2, 4, 6, and 8 days after *P. oryzae* inoculation for both inoculated and non-inoculated rice plants to evaluate the disease severity index based on visual assessment of lesions caused by *P. oryzae* on a nine-grade scale (Table 2) as according to IRRI standards (2002). Disease severity index for each replication was calculated according to the equation below (Cai *et al.,* 2008):

$$\text{Disease severity (\%)} = [\Sigma(r \times nr)/ (9 \times Nr)] \times 100$$

r indicates rating value (0–9)
nr indicates the number of infected leaves with a rating of r
Nr indicates the total number of leaves tested for each replication

The disease severity index data were transformed into the area under the disease progressive curve (AUDPC) for a disease progress curve. AUDPC was calculated using the equation as below:

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(X_{i+1} + X_i)/2][t_{i+1} - t_i],$$

where:
X_i = the proportion of host tissue damaged at the i^{th} day
t_i = the time in days after the appearance of the disease at the i^{th} day
n = the total number of observations

Table 1. The treatment conducted on rice cultivars MARDI Aerob 1 and MR219-4

Treatment	Calcium silicate (9 mg/L)	*Pyricularia oryzae* inoculation
T1	Si+	Po+
T2	Si+	Po-
T3	Si-	Po+
T4	Si-	Po-

+ = Present; - = Absent.

Table 2. The Rice Blast Disease Severity Scale

Scale	Description	Host behavior
0	No lesion observed	Highly Resistant
1	Small brown specks of pinpoint size Resistant	Resistant
2	Small roundish to slightly elongated, necrotic gray spots, about 1–2 mm in diameter, with a distinct brown margin. Lesions are mostly found on the lower leaves	Moderately resistant
3	Lesion type same as in 2, but a significant number of lesions on the upper leaves	Moderately resistant
4	Typical susceptible blast lesions, 3 mm or longer infecting less than 4% of leaf area	Moderately susceptible
5	Typical susceptible blast lesions of 3mm or longer infecting 4–10% of the leaf area	Moderately susceptible
6	Typical susceptible blast lesions of 3 mm or longer infecting 11–25% of the leaf area	Susceptible
7	Typical susceptible blast lesions of 3 mm or longer infecting 26–50% of the leaf area	Susceptible
8	Typical susceptible blast lesions of 3 mm or longer infecting 51–75% of the leaf area many leaves are dead	Highly susceptible
9	Typical susceptible blast lesions of 3 mm or longer infecting more than 75% of leaf area affected	Highly susceptible

Determination of defense-related enzymatic activity

The leaf samples were randomly collected at 0, 2, 4, 6, and 8 days after *P. oryzae* inoculation for plant defense-related enzymatic activity. The enzyme analyzed were polyphenol oxidases (PPO), peroxidase (PO), and phenylalanine ammonia-lyase (PAL).

Protein extraction from leaves

The rice leaf proteins were extracted following the procedure as described by Mozzetti *et al*. (1995). The fresh rice leaves samples (3 g) were randomly collected and added with liquid nitrogen and ground to powder by using a sterilized mortar and pestle. For the PPO and PO assays, ground rice leaves were added with 0.05 M phosphate buffer at pH 7.0 containing 0.5 g polyvinylpolypyrrolidone (PVP) at 1:5 (tissue to buffer) ratio and homogenized using a vortex. The supernatant was obtained by centrifugation at 14,000 × *g* for 20 min at 4°C.

Polyphenol oxidases (PPO) activity assay

The polyphenol oxidase reaction mixture consisted of 100 µL of supernatant, 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5), and 200 µL of 0.1 M catechol. The PPO activity was determined by measuring the change in absorbance at 410 nm using a UV spectrophotometer (Model UV-1800, Shimadzu, Japan) at every 30 sec interval for 2 min. Polyphenol oxidase activity was expressed as a change in absorbance (unit)/min/g of protein (Mozzetti *et al*., 1995).

Peroxidase (PO) activity assay

For peroxidase activity, the guaiacol oxidation was determined in the presence of hydrogen peroxide as described by Fecht-Christoffers *et al*. (2003). The reaction mixture consisted of 100 µL supernatant and 3 mL of mix-solution (1 mL of 0.25% guaicol (w/v), 1 mL of 0.1 M hydrogen peroxide, and 1 mL of 10 mm phosphate buffer). The changes in absorbance at 470 nm were measured at every 30 sec of interval for 2 min by using a UV spectrophotometer (Model UV-1800, Shimadzu, Japan). A mixture without supernatant was used as a blank. Peroxidase (PO) activity was expressed as a change in absorbance (unit)/min/g of protein.

Phenylalanine ammonia-lyase (PAL) activity assay

The PAL activity reaction mixture consisted of the supernatant (100 µL) 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 PAL activity was measured using a UV spectrophotometer (Model UV-1800, Shimadzu, Japan) at 290 nm as the amount of trans-cinnamic acid formed from L-phenylalanine. The PAL activity was expressed in nmol of trans-cinnamic acids produced/min/g of protein (Mozzetti *et al*., 1995).

Experimental design and statistical data analysis

The experiment in the glasshouse was arranged in a randomized completed block design (RCBD) with four different treatments and three replications. Post Hoc test; Least significant difference (LSD) was applied to reveal the significant differences ($p<0.05$). All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) program version 25.0. The disease severity in each treatment was further expressed as the area under the disease progress curve (AUDPC) using the Logit model described by Campbell and Madden (1990) in the Sigma Plot software.

RESULTS AND DISCUSSION

Rice blast disease evaluation

Silicon is an important nutrition element although Si is not considered as one of the essential elements in plants (Balakhnina & Borkowska *et al.*, 2012). Rice is a high active Si-uptake plant that can absorb 150-300 kg/ha during cltivatios (Sahebi *et al*., 2015). Numerous studies reported that Si can influence positively plant growths and yields, particularly under unfavorable and stressed conditions. In plants, Si is reported to be essential for the improvement of a nutrient imbalance, reduction of mineral toxicities, improvement of mechanical properties of plant tissues, and enhancement of resistance to other various biotic and abiotic (Hattori *et al*., 2005). In the current study, both of the rice cultivars foliar-sprayed with 9 mg/L of calcium silicate significantly reduced rice blast disease severity. For instance, the DSI at 8 DAI were 5.4% in T1 and 10.42% in T3 (MR219-4) and 1.33% in T1 and 4.28% in T3 (MARDI Aerob 1) (Figure 1). The disease severity index for treatment T1 and T3 increased gradually from Day 2 until Day 8. Rice seedling without foliar Si treatment exhibited significantly high rice blast disease severity compared with treatments. There is no rice blast disease symptom observed on rice seedlings without *P. oryzae* inoculation for both rice cultivars tested.

The DSI was transformed to Area under progress curve (AUDPC) for both rice cultivars tested. Generally, the susceptible rice cultivar MR219-4 formed diamond-shaped lesions with pointed end similar as described by Webster (2000) with AUDPC of 148.57 unit^2 while on the resistant rice cultivar (MARDI Aerob 1), the disease severity is much lower with small brown pin-points size lesions formed on the infected rice leaves with AUDPC of 53.71 unit^2 (Table 3). Interestingly, the foliar application of calcium silicate (T1) effectively reduced the AUDPC of MR219-4 to 96.57 unit^2 and MARDI Aerob 1 to 21.90 unit^2. A similar finding was also reported by Datnoff *et al*. (1997) that the effectiveness of calcium silicate through root

Table 3. The AUDPC of rice cultivars MARDI Aerob 1 and MR219-4

Treatments	Area under progress curve (AUDPC)	
	MARDI Aerob 1	MR219-4
T1	21.90	96.57
T2	0.00	0.00
T3	53.71	148.57
T4	0.00	0.00

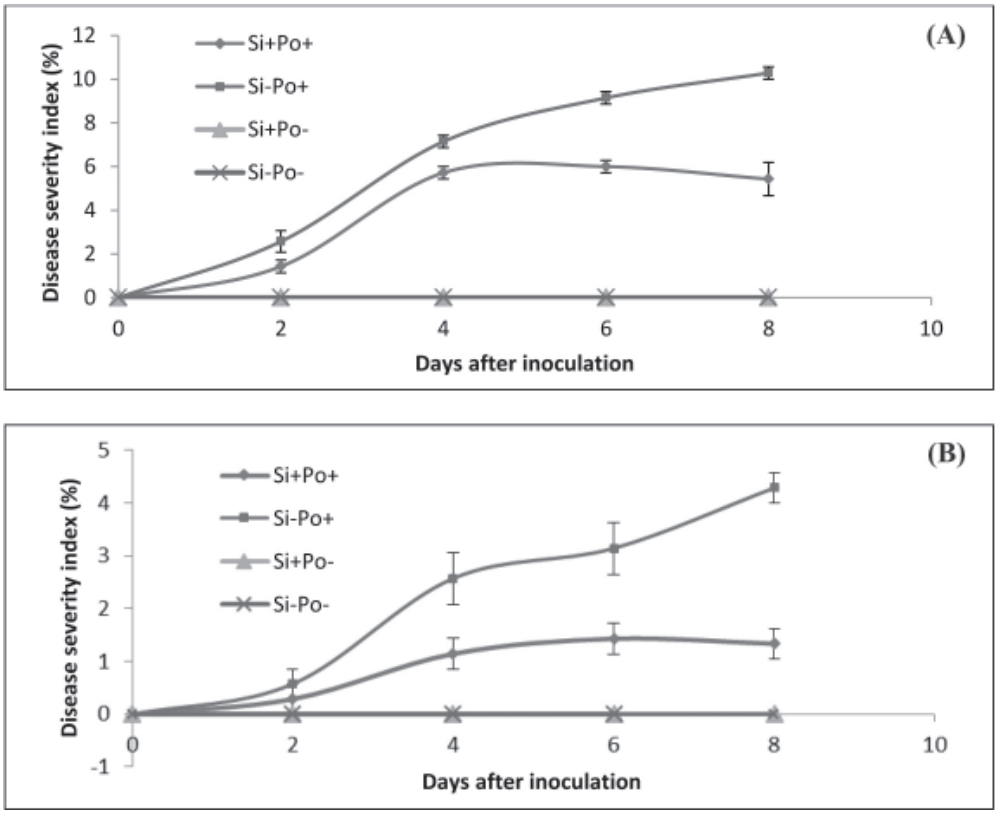

Fig. 1. The rice blast disease severity index at 8 days after inoculation for rice cultivar (A) MR219-4 and (B) MARDI Aerob 1. Values are the mean of three replications and bars ± represent standard error.

application reduced neck blast disease incidence to 32.4% over the control when applied at 15 mg/ha.

Most studies reported the disease control of the plant by Si involved root absorption, either from soils or from soil-less media while little information is available on foliar application of Si especially in rice. In cucumber, muskmelon and zucchini squash (Menzies *et al*., 1992) and grape (Bowen *et al*., 1992), foliar application of Si were reported effectively in controlling powdery mildew and was suggested as an important method in reducing the infection of several pathogens (Laane *et al.*, 2018). The physical barrier of Si deposited on leaf surfaces or an osmotic effect of the foliar silicate applied through the formation of a coating on the leaves (Menzies *et al*., 1992) and the formation of cuticle-double layers (Ng *et al*., 2018) was explained to strengthen the cuticle activity as a mechanical barrier to hinder pathogen penetrations.

Defense-related enzymatic activity (PPO, PO, and PAL)

Secondary metabolic compounds are playing important role in plant resistance to biotic stress (Gomes *et al*., 2005; Ye *et al*., 2013). While, PPO, PO, and PAL are the enzymes that contribute to the biosynthesis of those secondary metabolic compounds such as phytoalexins, phenol, and lignins (Liu *et al*., 1997).

In this study, *P. oryzae* infection significantly increased PPO activity in rice leaves for both rice cultivars tested regardless of Si treatment (Figure 2). Rice seedlings foliar sprayed with Si alone did not change the PPO activity for both rice cultivars tested. However, rice seedlings inoculated with *P. oryzae* after foliar Si application increased gradually on PPO activity from 0 DAI and reached the highest level at 4th DAI in both rice cultivars. Foliar Si application significantly increased the PPO activity

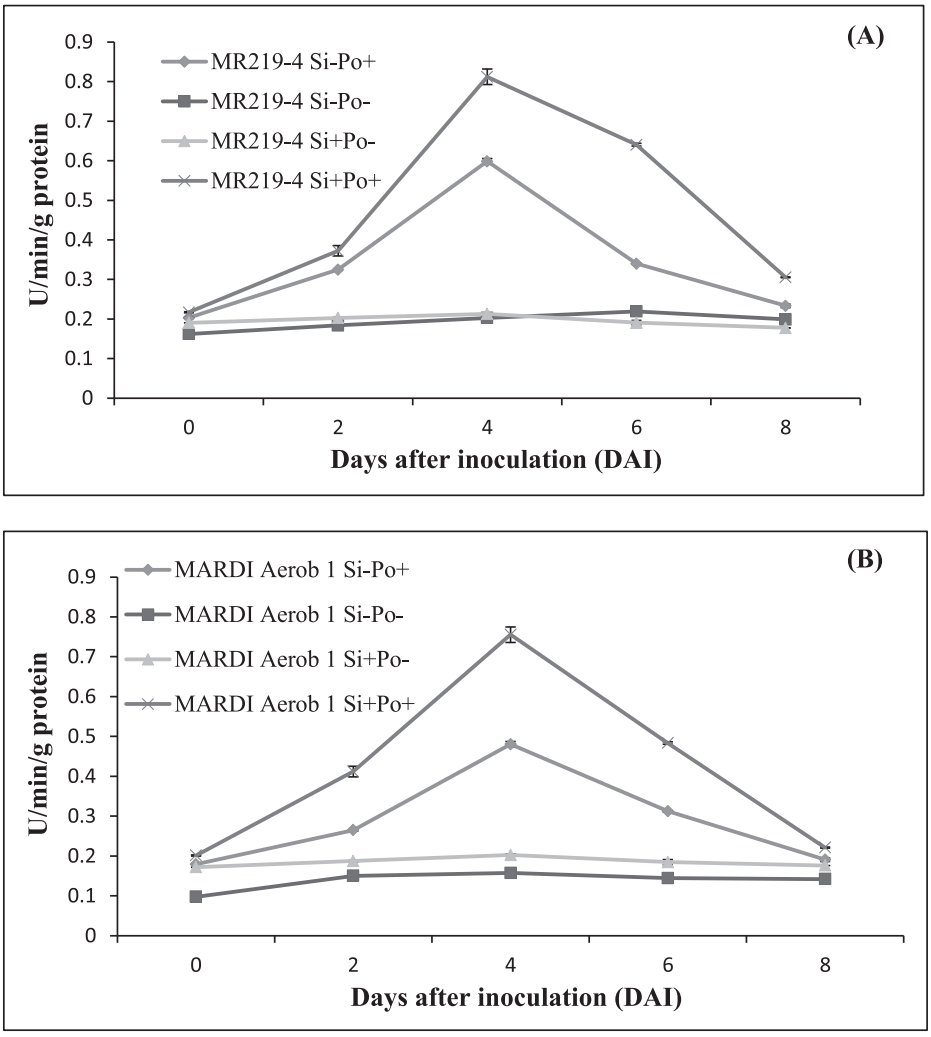

Fig. 2. The activity of polyphenol oxidase (PPO) after *P. oryzae* inoculation for rice cultivar (A) MR219-4 and (B) MARDI Aerob 1. Values are the mean of three replications and bars ± represent standard error.

from 0.59 to 0.81 u/min/g protein (37%) in rice cultivar MR219-4 and 0.48 to 0.75 u/min/g protein (56%) in rice cultivar MARDI Aerob 1.

The effect of Si foliar-sprayed with *P. oryzae* inoculation on the peroxidase (PO) activity for both rice cultivars MR219-4 and MARDI Aerob 1 was similar to those PPO activities (Figure 3). Without *P. oryzae* inoculation, Si foliar-sprayed alone did not significantly increase the PO activity in both rice cultivars tested. Rice seedlings foliar-sprayed with calcium silicate (Si+, Po+) significantly increased PO activity at 4th DAI for the moderate resistant (MR219-4) and resistant cultivars (MARDI Aerob 1) compared with *P. oryzae* inoculation alone (Si-, Po+). The activity of PO at 4th DAI induced by foliar Si application was increased 8.40% (0.90 in Si+ Po+ compared with 0.83 in Si-, Po+) for MR219-4 and 13.13% (1.12 in Si+ Po+ compared with 0.99 in Si-, Po+) for MARDI Aerob 1.

Our results indicate that the low rice blast disease severity in the foliar Si-sprayed plant was associated with the high activity of PPO, PO, and PAL in both rice cultivars tested. This finding was in agreement with the previous report that ground application of Si improves the activity of PPO, PO, and PAL in *Pythium* infection in cucumber root (Liang *et al*., 2005) and *P. oryzae* in rice blast disease (Cai *et al*., 2008). The soluble Si in plant tissue may be associated with an increase in rice resistance to blast through the production of phenolic-like compounds, diterpenoid phytoalexins, and the activation of some PR-genes (Rodrigues *et al*., 2003, 2004, 2005).

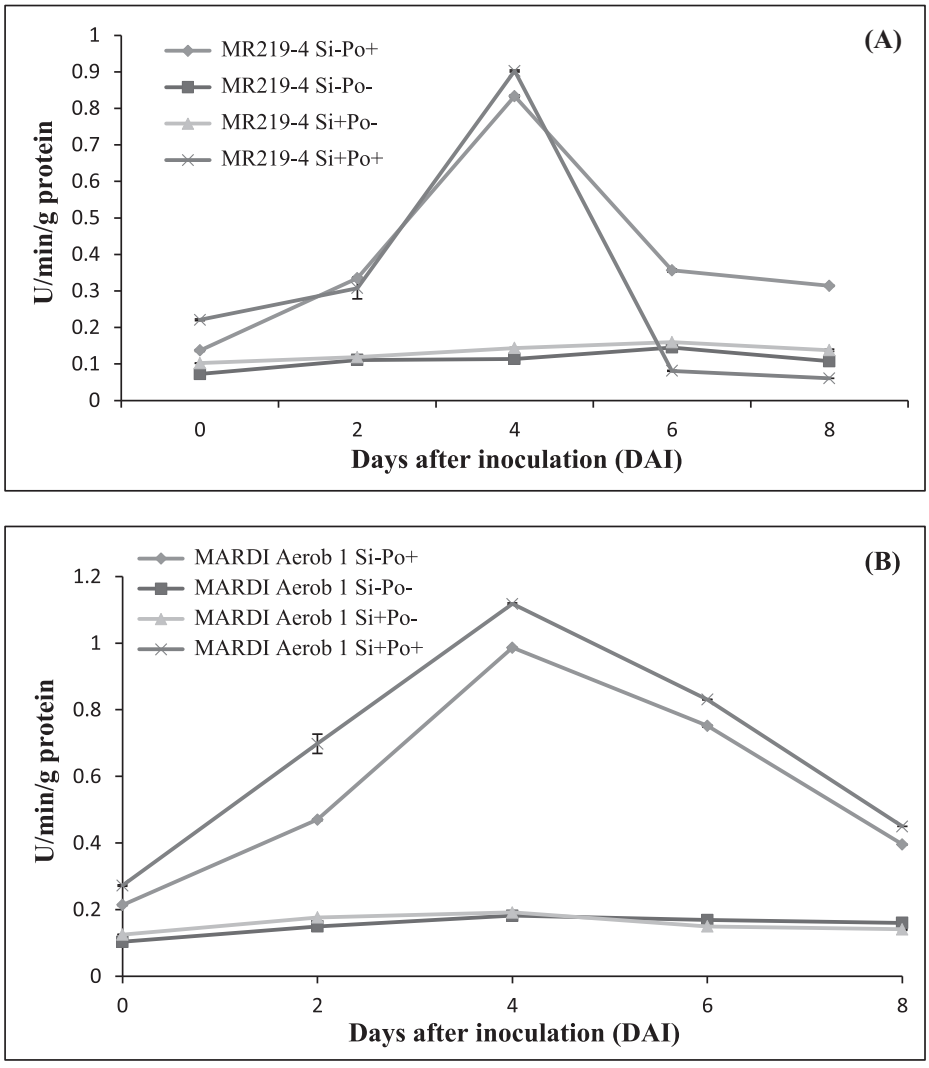

Fig. 3. The activity of peroxidase (PO) after *P. oryzae* inoculation for rice cultivar (A) MR219-4 and (B) MARDI Aerob 1. Values are the mean of three replications and bars ± represent standard error.

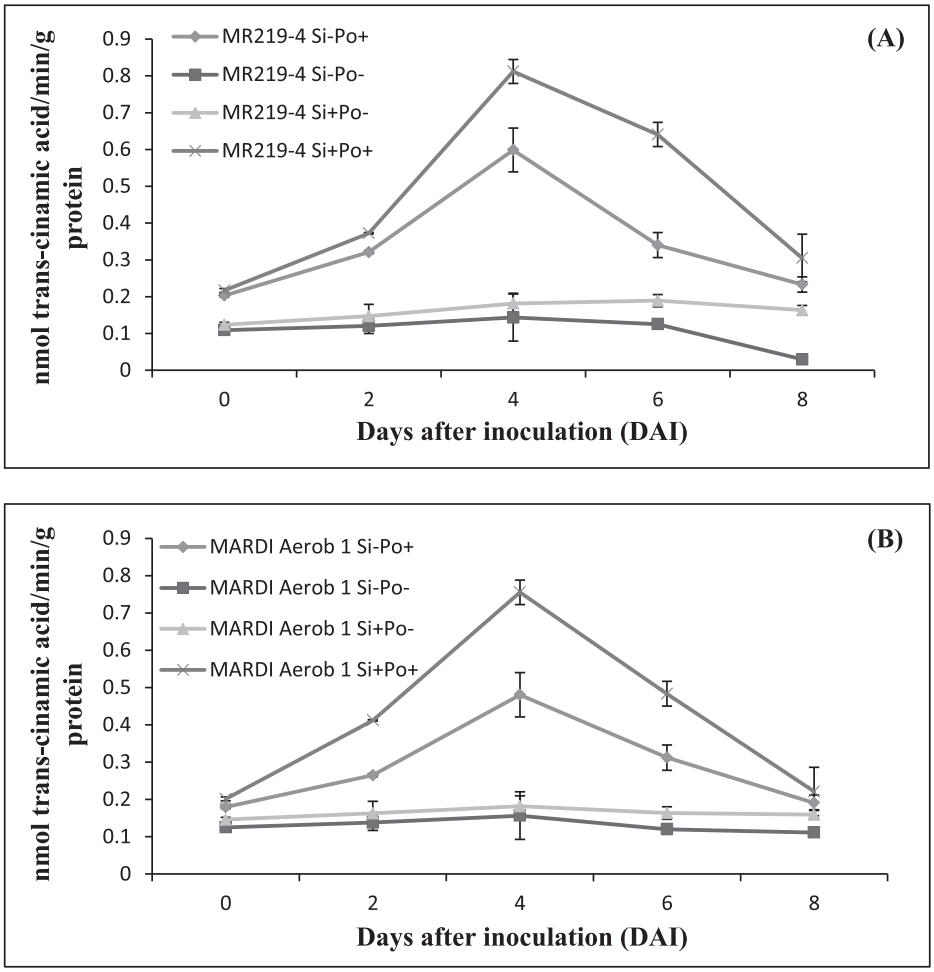

Fig. 4. The activity of phenylalanine ammonia lyase (PAL) after *P. oryzae* inoculation for rice cultivar (A) MR219-4 and (B) MARDI Aerob 1. Values are the mean of three replications and bars ± represent standard error.

CONCLUSION

This study indicated that foliar Si application significantly increased rice blast disease resistance for both partially resistant (MR219-4) and resistant rice (MARDI Aerob 1) through the induction of PPO, PO, and PAL production in rice leaves. The increased activity of these defense-related enzymes might contribute to the activation and biosynthesis of the secondary metabolic compounds associated with the low rice blast disease severity. Plant pathogen interactions with Foliar Si application involve a complex and dynamic network. Therefore, the molecular prospective in gene regulation and expression after foliar Si application is important to be explored.

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