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ANTIOXIDANT ACTIVITIES AND FREE AMINO ACID CONTENT OF RICE RESPONDED TO *XANTHOMONAS ORYZAE PV ORYZAE* INFECTION UNDER PHOSPHATE STARVATION CONDITION

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SUMMARY

Rice productivity strongly affects food security worldwide, especially in Asia, where rice is served as a staple food. However, rice productivity was threatened by numbers of environmental stresses, including *Xanthomonas oryzae pv oryzae* (*Xoo*) disease. *Xoo* is causing significant losses to rice production around the world. Furthermore, thanks to the significant function of phosphate (Pi) in nucleic acid and cell membrane structure, energy-transporting molecules, photosynthesis and respiration, a sufficient supply of Pi for rice plants is essential. Our previous studies had demonstrated the effect of low Pi in increasing *Xoo* tolerance in rice. In the present study, two-biochemical processes responsible for this tolerance were investigated by analyzing antioxidant enzyme activities and free amino acid profile produced by *Xoo* infected rice plants under Pi starvation and full Pi conditions. Two selected rice cultivars, G22 and G299, were used as plant materials. After two weeks of 6-week-old plants infected with *Xoo*, peroxidase and catalase activities in the plants under the low Pi condition were significant increased. Amino acids belonging to the glutamic, serine and pyruvate families, especially serine and proline accumulated the higher levels in plants under low Pi conditions compared to full Pi. In our two studied rice cultivars, the improvement in both antioxidant enzymes and free amino acid was found cultivar-dependence, the sensitive to low Pi G299 cultivar responsed stronger than the tolerant to low Pi G22 cultivar. The results from this study provide more detail about the possible mechanism how rice plants tolerant to *Xoo* under the Pi starvation condition and can suggest a strategy for green agriculture in dealing with *Xoo* infection by considering the interaction between Pi starvation and the *Xoo* response.

Keywords: amino acids, antioxidant enzymes, *Oryza sativa* L, phosphate starvation**,** *Xanthomonas Oryza sativa*

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population and grown in more than 100 countries, of which 90% are in Asia (Fukagawa, Ziska, 2019). Vietnam is among the leading rice-producing and exporting countries worldwide (FAOSTAT, 2020). However, rice productivity has been affected by many factors, including biotic and abiotic stresses (Rasheed *et al*., 2020), which can cause a big threat to the food security worldwide.

It was estimated that approximately 52% of the global productivity of rice grain yield is severely damaged by biotic factors (Park *et al*., 2007). Among that, the bacteria leaf blight disease caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*) is one of the most devastating diseases and might reduces rice production by

20%–50% (Fiyaz *et al*., 2022). Therefore, one of the main objectives of rice breeding programs is the resistance improvement of the rice plants to environmental stresses. Besides the biotic stress, the abiotic stress caused by low phosphate (Pi) which happened in approximately 70% of globally cultivated land (Kirkby and Johnston, 2008), is also a severe problem affecting rice productivity worldwide. Because Pi is the second-most essential element after nitrogen (Brady and Weil, 2008) for root development, tillering, early flowering, and ripening, the low Pi stress can lead to delayed phenological development and flowering (Dobermann, Fairhurst, 2001), which consequently strongly reduce the rice productivity.

Breeding for disease-resistant rice varieties was one of the best options to counter these disease outbreaks. On the other hand, rice plants also have acquired a range of developmental strategies to cope with these stresses during evolution. Several studies have reported the efficiency of low Pi on pathogen resistance in plants. Low Pi enhanced the defense of *Arabidopsis*, tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* (Khan *et al*., 2016), sorghum (Zhang *et al*., 2019), and of cotton (cv. YZ1) (Luo *et al*., 2021) against pathogens. However, excess Pi increased the sensitivity to *Magnaporthe oryzae* infection reported in rice (Campos-Soriano *et al*., 2020).

High production of Reactive Oxygen Species (ROS) during environmental stresses can cause significant damage to the cells. Naturally, plants develop antioxidant defense system to detoxify ROS to protect plant cells and their organelles against the toxic effect of these species (Apel and Hirt, 2004) by using two components - enzymatic and non-enzymatic system. Peroxidase (POD) and catalase (CAT) enzymes are the two most important antioxidant enzymes, which can break down two H_2O_2 molecules into one molecule of oxygen, and two molecules of water (von Ossowski *et al*., 1993; Caverzan *et al*., 2012).

The Pi starvation increased ROS bursts and activated the antioxidant enzymes have been reported in a number of plants, including maize (Zhang *et al*., 2014), rice (Xu *et al*., 2007; Fu *et al*., 2014), tomato (Muneer and Jeong, 2015), sheepgrass (Li *et al*., 2019), *Arabidopsis* (Tyburski *et al*., 2009), and beans (*Phaseolus vulgaris)* (Juszczuk *et al*., 2001). This is a solution of plants to defend the plant against oxidative damage (Zhang *et al*., 2014).

Furthermore, the changes in amino acid metabolism have been reported as the solutions to respond to environmental stresses in number of plants. Extensive amino acid accumulation in response to drought stress has been reported in *Sporobolus stapfianus* (Martinelli *et al*., 2007), *Gossypium hirsutum* (L.) (Showler, 2002), *Arabidopsis* (Batista-Silva *et al*., 2019), and to Pi starvation in many plants including *Arabidopsis* (Aleksza *et al*., 2017), and tea (*Camellia sinensis*) (Santosh KC *et al*., 2018). In *Arabidopsis*, to deal with salt stress, the content of proline experienced a strong induction after salt treatment. Concentrations of the low abundant amino acids (branched-chain amino acids, lysine, methionine, histidine, and aromatic amino acids) also showed the strongest relative increase after polyethylene glycol treatment in drought stress (Batista-Silva *et al*., 2019).

In our previous study, low Pi can increase the tolerance of rice plants toward *Xoo* infection, and this response was cultivar dependence. In the present study, further investigation how the rice plants respond to *Xoo* under Pi starvation stress in terms of amino acid metabolism and antioxidant enzyme activities was conducted using the sensitive to low Pi cultivar - G299 and tolerant to low Pi cultivar - G22 as plant materials.

MATERIALS AND METHODS

Plant materials

Two cultivars, including *indica* rice G22 (Trung Trang Tuyen Quang, a low Pi-resistant cultivar) and G299 *japonica* rice (Blao sinh sai, a low Pi-sensitive cultivar), were selected for this study. Their seeds were supplied by the Plant Resources Center in Hanoi, Vietnam.

Infection of rice plants with *Xoo*

Rice cultivars were grown in sand supplemented with liquid Yoshida medium (To *et al*., 2020). One batch was watering with full-Pi-containing yoshida medium (320 µM Pi) as the control condition, and the other batch was watering with the low-Pi-containing Yoshida medium (10 µM Pi) as the treatment condition representing Pi deficiency (Mai *et al*., 2021). The medium was supplemented every week. The leaves of 6-week-old rice plants were artificially inoculated with *Xoo*, which was isolated from the leaves of rice blight disease plants in Yen Bai, using clipping method (Kauffman *et al*., 1973). Infection protocol was followed by one described by To *et al*., 2022. In brief, three newly expanded leaves of each plant after clipping were dipped in bacterial suspension (1x10-8 cfu/ml), which was previously grown in liquid PSA media (10 g/L peptone, 1 g/L glutamic acid, 10 g/L sucrose, and 20 g/L agar) for two days, or sterile distilled as the negative control. Following inoculation, leaves were covered with polyethylene bags in high humidity for 24 h. Plants were maintained at 27 °C and 70% relative humidity during the study. The aerial plant parts were harvested after two weeks of infection. The experiment was performed in triplicate.

Determination of free amino acid (FAA) content

Leaf samples (100 mg dry weight) were ground into powder in liquid nitrogen using pestle and mortar and extracted with 1.5 mL of boiling Mili Q water. The extraction protocol was followed Jia *et al*., (2018). Briefly, the mixture was first placed in ultrasonicator bath for 15 min and then incubated at 100 °C for 45 min with gentle agitation on a shaker (Eppendorf, Germany). After that, the sample was filtered through a 0.2 µm pore membrane (Sartorius AG, Germany) to remove the purities. Finally, based on ion-exchange chromatography, the amino acid profile was determined using an amino acid analyzer (Biochrom 30 +, Biochrom, Cambridge, UK). Colorimetric detection was performed at 570 nm for most of the amino acid and at 440 nm for proline and N-leucine after post-column derivatization with the ninhydrin reagent. Standard amino acids, which were purchased from Thermo Scientific (Waltham, MA, USA), were used to determine the concentration of individual amino acids in leaf samples. The total free amino acid content was calculated as the sum of each free amino acid content.

Antioxidant enzyme activities

Crude protein extraction

The crude protein extraction was carried out according to the method described by Chen and Zhang (2016). Briefly, the aerial plant parts were stored in liquid nitrogen after harvesting. Two hundred milligrams of them were grind using a mortar and pestle in liquid nitrogen into a fine powder and homogenized with 3 ml of 100 mM PBS buffer (pH 7.8) (sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic). The homogenate was transferred to 1.5 ml centrifuge tubes and centrifuged for 20 min at 15000 g at 4 °C. The supernatant was collected and assayed for enzyme activity. The concentration of crude protein (mg/ml) in the supernatant was measured using a nanodrop 2000/2000c spectrophotometer machine (Thermo Scientific, Waltham, MA, USA) at the wavelength of 280 nm.

Peroxidase (EC 1.11.1.7) activities

Peroxidase assay was carried out according to the method described by Chen and Zhang (2016). The reaction buffer contained 0.2% guaiacol, 100 mM PBS (pH 7.0) and 30% H2O2. The reference contained 50 µl 100 mM PBS (pH 7.8) and 1 ml of the reaction buffer. The reaction solution contained 50 µl crude enzyme solution and 1 ml of the reaction buffer. This mixture was put into the cuvette and immediately recorded the dynamic absorbance at 470 nm every 15 sec for 1 min by using a UV-1800 UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan).

The peroxidase activity was calculated based on the formula:

$$\text{POD activity (unit: u/mg protein)} = \frac{\triangle \text{A470 x (V/Vt)}}{(0.1 \text{ x t})/\text{Cp}}$$

Where: △A470: the change of absorbance at 470 nm every 15 sec

V: total volume of crude enzyme solution

Vt: volume of crude enzyme used in the testing tube

t: reaction time (min)

Cp: crude protein concentration (mg/ml)

0.1: One unit of POD is defined as the amount of enzyme that increases 0.01 of absorbance at 470 nm per minute.

Catalase (EC 1.11.1.6) activities

Catalase assay was also carried out according to the method described by Chen and Zhang (2016). The reaction buffer contained 30% H_2O_2 in 100 mM PBS (pH 7.0). The reference contained 50 µl 100 mM PBS (pH 7.8) and 1 ml of the reaction buffer. The reaction solution contained 50 µl crude enzyme solution and 1 ml of the reaction buffer. This mixture was put into the cuvette and immediately recorded the dynamic absorbance at 240 nm every 15 sec for 1 min by using a UV-1800 UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan).

The catalase activity was calculated based on the formula:

$$\text{CAT activity (unit: u/mg protein)} = \frac{\triangle \text{A240 x (V/Vt)}}{(0.1 \text{ x t})/\text{Cp}}$$

Where: △A240: the change of absorbance at 240 nm every 15 sec

V: total volume of crude enzyme solution

Vt: volume of crude enzyme used in the testing tube

t: reaction time (min)

Cp: crude protein concentration (mg/ml)

0.1: One unit of CAT is defined as the amount of enzyme that decreases 0.1 of absorbance at 240 nm per minute.

Statistical analysis

The differences between investigated parameters were statistically analyzed using the ANOVA one-way test in the SPSS.

RESULTS

Antioxidant enzyme activities

Our previous study has shown that low Pi increased the tolerance of rice plants to *Xoo* by reducing the length of lesions and bacterial amount in the leaves of infected plants (unpublished data). In the present study, the antioxidant enzymes CAT activity in the leaves of *Xoo* infected rice was generally higher in low Pi conditions than in normal conditions (Fig. 1A). However, the level of CAT activity was different between the two selected cultivars. In the low Pi condition, CAT activities were 61.6 % and 153.22 % higher in G22 and G299, respectively, than in the full Pi condition. The highest activity of CAT was observed in the G299, a low Pi-sensitive cultivar. It reached approximately 1.808 ± 0.086 u/mg protein. In the normal condition, there was no significant difference in the CAT activity between G22 and G299 rice cultivars ($p > 0.05$).

The POD enzyme also experiences the same trend as the CAT enzyme, where the highest activity of POD was obtained when rice plants were grown in the low Pi medium (Fig. 1B). In the low Pi medium, POD exhibited 40.86 % and 231.16 % higher in the leaves of G22 and G299, respectively than in full Pi medium. The highest POD activity was 5.71 ± 0.2 u/mg protein in G299 grown in a low Pi medium. It was lower

in the G22 plant grown in the same medium, where it reached 4.39 ± 0.10 u/mg protein. Furthermore, we always obtained much higher activity of POD than CAT in any conditions.

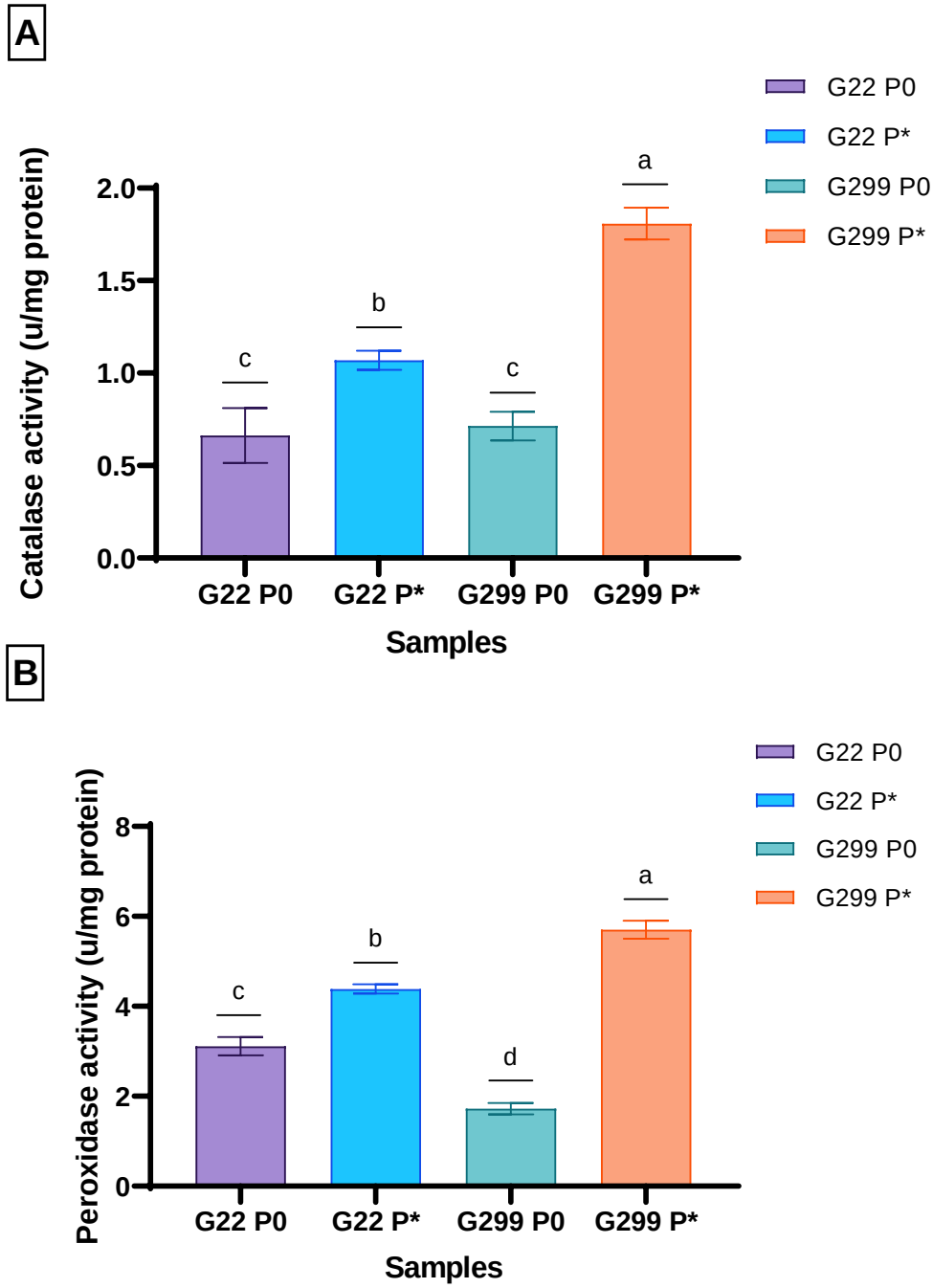

Figure 1. The activity of antioxidant enzymes in G22 and G299 rice cultivars grown in different phosphate culture conditions infected with *Xanthomonas oryzae pv oryzae*. P0 (full Pi), P* (low Pi). Different letters indicate significant differences between treatments ($p < 0.05$) according to the Tukey posthoc test.

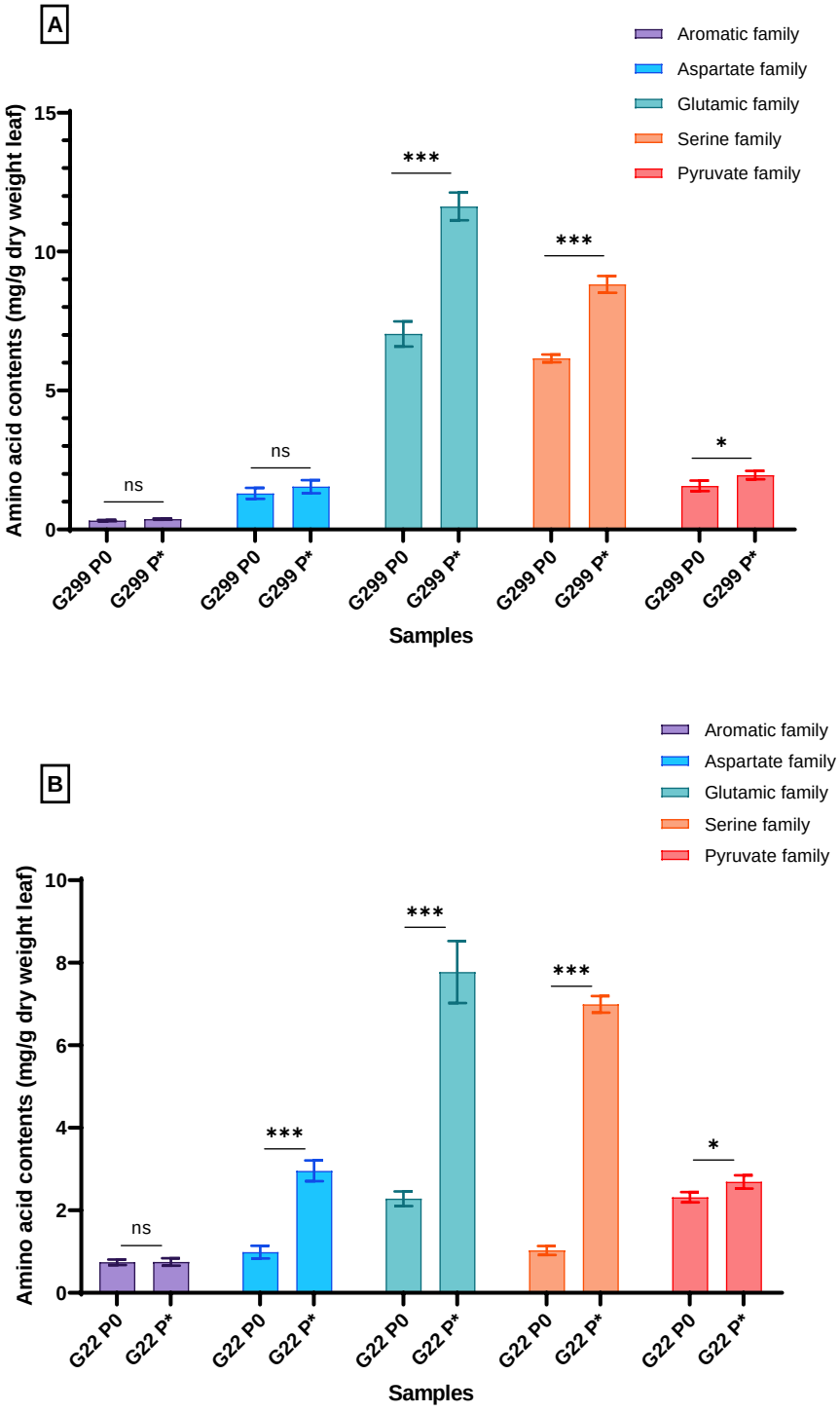

Figure 2. The amino acid content in G22 and G299 rice cultivars grown in different phosphate culture conditions infected with *Xanthomonas oryzae pv oryzae*. P0 (full Pi), P* (low Pi). (ns), (*) and (***) indicate no significant, significant difference with $p < 0.05$ and $p < 0.01$, respectively.

Variation of amino acid content

In the low Pi sensitive G299 rice cultivar infected with *Xoo*, the significantly higher amino acid contents of glutamic, serine and pyruvate families were obtained in plants grown in low Pi condition than in full Pi condition ($p < 0.05$) (fig. 2A). Especially, the content of glutamic and serine families were 66.48 % and 43.74 higher. The highest content of amino acid was found in the glutamic family including proline, arginine, histidine and glutamic. However, there was no significant difference in aromatic and aspartate families between plants grown in low Pi and full Pi conditions ($p > 0.05$).

In the low Pi tolerant G22 cultivar infected with *Xoo*, the situation was similar to the G299 cultivar. In most the case, we obtained higher amino acid content in the G22 plants grown in the low Pi medium than in the full Pi medium ($p < 0.05$) (fig. 2B). The highest content was also obtained in the glutamic family following by the serine family. The content of glutamic and serine families exhibited approximately 3.0 and 5.8 folds, respectively, higher in the low Pi medium than in the full Pi medium. There was no significant difference in the content of the aromatic family in plants grown in full and low Pi medium ($p > 0.05$).

The amino acid analysis of two rice cultivars shown in Table 1 reveals that there was a significant variation in total free amino acid (FAA) as well as the individual amino acid content between plants grown in two different conditions. The FAAs of G299 and G22 cultivars were increased in the low Pi condition, and reached 24.43 ± 0.338 and 18.68 ± 0.36 mg/g dry weight leaves, respectively, which were approximately 1.5 and 2.6 fold higher than rice cultivars grown in the normal Pi condition.

Apart from total FAA, most single amino acid contents of plants grown in low Pi medium also increased significantly, especially serine, proline, arginine and glutamine. However, lysine was found only in G22 but not in G299, and no tryptophan was detected in both the two cultivars and tested growth conditions (Table 1).

Table 1. A variation of amino acid content (mg/g dry weight leaf) from leaves of G22 and G299 plants grown in different phosphate culture conditions infected with *Xanthomonas oryzae pv oryzae*.

Amino acids	G299 P*	G299 P0	G22 P*	G22 P0	Family
tryptophane	0	0	0	0	aromatic family
tyrosine	0.22 ± 0.003^{a}	0.05 ± 0.002^{c}	0.15 ± 0.02^{b}	0.18 ± 0.09^{b}	
phenylalanine	0.56 ± 0.003^{a}	0.29 ± 0.007^{b}	0.23 ± 0.004^{b}	0.15 ± 0.01^{c}	
iso-leucine	0.82 ± 0.02^{c}	0.39 ± 0.002^{b}	0.4 ± 0.005^{b}	0.38 ± 0.01^{b}	aspartate family
threonine	0.52 ± 0.002^{a}	0.16 ± 0.01^{c}	0.26 ± 0.04^{b}	0.13 ± 0.002^{c}	
methionine, and derivartives	1.64 ± 0.005^{a}	0.41 ± 0.035^{b}	0.31 ± 0.01^{b}	0.26 ± 0.032^{b}	
lysine	0	0	0.6 ± 0.002^{a}	0.53 ± 0.03^{a}	
proline	4.25 ± 0.03^{a}	0.7 ± 0.02^{d}	2.86 ± 0.011^{b}	1.02 ± 0.05^{c}	glutamic family

arginine	3.2 ± 0.02[a]	0.37 ± 0.025[d]	2.49 ± 0.01[b]	3.9 ± 0.012[c]	
histidine	0.56 ± 0.004[a]	0.09 ± 0.001[c]	0.25 ± 0.005[b]	0.15 ± 0.02[c]	
glutamine	1.96 ± 0.07[b]	1.14 ± 0.02[c]	2.17 ± 0.005[a]	1.96 ± 0.01[b]	
serine	6.75 ± 0.05[a]	0.60 ± 0.002[d]	6.02 ± 0.11[b]	5.48 ± 0.08[c]	
glycine	0.54 ± 0.03[a]	0.1 ± 0.002[d]	0.39 ± 0.04[b]	0.23 ± 0.01[c]	serine family
cysteine	0.18 ± 0.02[b]	0.04 ± 0.002[c]	0.38 ± 0.03[a]	0.25 ± 0.015[b]	
taurine	3.35± 0.026[a]	0.28 ± 0.002[b]	0.2 ± 0.003[b]	0.2 ± 0.01[b]	
alanine	0.51± 0.01[c]	1.33 ± 0.02[a]	1.38 ± 0.004[a]	0.84 ± 0.007[b]	
n-leucine	1.11 ± 0.015[a]	0.92 ± 0.005[b]	0.51 ± 0.011[c]	0.63 ± 0.002[c]	pyruvate family
valine	0.14 ± 0.03[a]	0.07 ± 0.01[b]	0.08 ± 0.01[b]	0.07 ± 0.01[b]	
Total	**24.43 ± 0.338**	**16.33 ± 0.165**	**18.68 ± 0.36**	**7.3 ± 0.4**	

Note: P0: full Pi, P*: low Pi. Different letters indicate significant differences between treatments ($p < 0.05$) according to the Tukey posthoc test.

DISCUSSION

In the present study, in order to open a larger view of understanding how low Pi can increase the tolerance to *Xoo* infection, antioxidant enzymes activities and free amino acid profile of rice cultivars under *Xoo* infected in case low Pi condition were investigated.

Increased activities of antioxidant enzymes in dealing with stress have been reported in many studies. Low Pi can be considered abiotic stress for plant growth and development (López-Arredondo *et al*., 2014). However, in our study, activities of the antioxidant enzymes, peroxidase and catalase were found to be higher in the plants pre-treated with low Pi than in the plants grown in full Pi medium. These results could be suggested that the low Pi condition supported the higher tolerance to *Xoo* infection. The increased tolerant to pathogens in plants pre-treated with low Pi has been reported in some studies as the result of increased synthesis of jasmonic acid (Zhang *et al*., 2019; Luo *et al*., 2021); however, the increases of antioxidant enzymes and free amino acid accumulation have not been mentioned elsewhere. Although, the activities of CAT and POD were up in both treated cultivars, but different between them. In the study of Zhang *et al.*, changing enzyme activities during stresses was also the cultivar dependence (Zhang *et al*., 2011).

Besides serving as the materials for protein synthesis, some amino acids can act as precursors for synthesizing secondary metabolites and signaling molecules (Trovato *et al*., 2021). Polyamines, which play essential roles in plant growth and developmental and stress tolerance, are derived from arginine amino acids (Alcázar *et al*., 2006). N-hydroxy-pipecolic acid metabolites converted from lysine were the defense signals of bacterial infection (Chen *et al*., 2018). The hormone ethylene level, which was

controlled by the first metabolite of methionine, S-adenosylmethionine (Amir, 2010), also triggered the defense and survival mechanisms of plants in response to salt, flooding and heavy metal stresses (Chen *et al*., 2022). Furthermore, many studies have shown the involvement of amino acids in dealing with environmental stresses. The accumulation of FAA, especially proline, has been reported to respond to abiotic stress, including Pi starvation in *Arabidopsis* (Aleksza *et al*., 2017) or drought stress in tomatoes (Ferreira Júnior *et al*., 2018). Arginine and glycine were also alleviated the adverse effects of temperature in maize (*Zea mays*) (Matysiak *et al*., 2020) or drought stress in tomatoes (*Lycopersicon esculentum*) (Hamid and Idan, 2019). In *Arabidopsis,* the *c*oncentration of serine was increased in low temperatures and elevated salinity (Ho and Saito, 2001; Rosa-Téllez *et al*., 2020).

In our study, the concentration of total FAA was significantly increased in plants grown in the low Pi condition and infected with *Xoo*, especially serine, arginine, proline and glutamine. These results were in agreement with previous studies when they reported the involvement of proline, arginine, and serine in diverse stress responses. We also can partly explain that the better *Xoo* tolerance in rice plants pre-treatment with the low Pi condition than in full Pi condition was because of the increase in the total FAA concentration and some specific amino acids. The increase of total FAA in response to stress was reported in some plants, such as sunflower and sorghum, because of osmotic adjustment to alleviate detrimental effects (Manivannan *et al*., 2007). In contrast, stress decreased the total FAA content in wheat (El-bassiouny, Bekheta, 2005), Chinese cabbage (Shawon *et al*., 2018) or *Sporobolus stapfianus* (Martinelli *et al*., 2007). Similar to increases in antioxidant enzymes, the rise in FAA upon low Pi and *Xoo* infection in our two studied rice cultivars was cultivar-dependent, where they were higher in G99 than G22 cultivars. Furthermore, the G22 cultivar was identified as a low Pi tolerant cultivar; therefore, the FAA level was lower than the other - a low Pi sensitive one.

CONCLUSIONS

This study investigated the activities of antioxidant enzymes and amino acid content in rice plants, which were pre-treated with low Pi in response to *Xanthomonas oryzae pv oryzae*. We obtained the higher activities of these enzymes in the plants infected with *Xoo* and pre-treated with the low Pi condition than those grown in the full Pi condition. The pre-treated with low Pi condition also resulted in higher content of amino acids belonging to the glutamic, serine and pyruvate families. Finally, the response to *Xanthomonas oryzae pv oryzae* infection was cultivar – dependent in our two studied rice cultivars. The obtained results are expected to provide a further understanding of the *Xoo* tolerance in rice plants grown under low Pi condition and its potential application in sustainable agriculture development with an understanding of the role of Pi homeostasis.

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