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Effects of seed soaking and foliar spraying of *Kalanchoe pinnata* aqueous leaf extracts against rice bacterial leaf blight

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ABSTRACT

This study aims at evaluating the disease-reducing effects against rice bacterial leaf blight (Xanthomonas oryzae pv. oryzae). Under greenhouse conditions, the activities of the four enzymes [peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL)] after application of Kalanchoe pinnata aqueous leaf extracts using the combination of seed soaking and foliar spraying were studied. Overall, two extract concentrations [1 and 2% (w/v)] applied as seed soaking combined with the five extract concentrations [1, 2, 3, 4 and 5% (w/v)] applied as foliar spraying were tested. Three application methods were furthermore used for foliar spraying (7 days before pathogen inoculation (DBI), 14 DBI and their combination). Results showed the effects increased with the increase of extract concentrations and durations from application time points prior to pathogen inoculation. The combination of foliar spraying at 7 and 14 DBI provided stronger protection compared to single sprays. The effects involved induced resistance. Indeed, the activities of POX and CAT increased until 4 days after inoculation (DAI) and remained until 7 DAI, while those of PPO and PAL increased similarly then decreased until 7 DAI. Activities of these enzymes increased after pathogen inoculation and reached higher levels with extract applications.

1. INTRODUCTION

Bacterial leaf blight (BB) is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Swings et al., 1990). In Viet Nam, the disease is more destructive during rice harvest where losses can reach 65% of yields (Dinh et al., 2008; Son, 1993). In addition, the disease also causes serious damage to the quality of rice, which affects the export of high-quality rice (Dinh et al., 2008; Khoa, 2018; Son, 1993).

BB management has centered on methods that reduce the initial inoculum and subsequent development of the patroon rice plants. Induced resistance is a sustainable and environmentally friendly way to control the BB disease (Kloepper et al., 1992, Khoa et al., 2011, 2017; Lyon et al., 2007; Khoa, 2018; Pieterse et al., 2014; Walters et al., 2007). For this method, the resistance does not directly affect the pathogens but generates signals to stimulate the self-defense mechanism in the plants. Here there is an increased accumulation of phenolic compounds, phytoalexins and disease-related proteins (PR-proteins) such as peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) to prevent the infection and growth of pathogens (Van Loon et al., 1998; Vidhyasekaran et al., 1997).

As reported by Khoa et al. (2017), seeds soaking in fresh extracts observed disease-reducing effects up to 40%. Mechanism of induced resistance involved POX, CAT increased earlier and was stronger when pathogens inoculation. In addition, the study also recorded an increase in the PPO and PAL activities at the early times. According to Huong et al. (2018), to investigate disease-reducing effects and the mechanism of induced resistance involved PAL and PPO of *K. pinnata* extracts using foliar spraying, the extracts were investigated at five concentrations (1, 2, 3, 4, 5 and 10% (w/v)) at 14 and 7 days before inoculation (DBI). Those results showed a disease-reducing effect until 21 days after inoculation (DAI) with 1% extract at 14 DBI. When rice plants were sprayed with extracts and inoculated with *Xoo*, the two enzyme activities increased, in which PAL increased at 2 DAI and PPO increased at 4 DAI. In individual treatment, seeds soaking and spraying showed disease-reducing effects of BB disease. Thus, this study observed disease-reducing effects through the induced resistance mechanism of *K. pinnata* extracts using a combination of seed soaking and foliar spraying.

2. MATERIALS AND METHODS

2.1. Plant materials and pathogens

Healthy mature plants (one-year-old) grown in the greenhouse of the Vinh Long University of Technology Education (Vinh Long Province, Viet Nam) were harvested at 7:00 a.m. Uniform mature leaves (approx. 10 cm in length) were then collected under laboratory conditions to prepare extracts.

The rice cv. Jasmine 85 and *Xoo* were kindly provided by the Can Tho University (Can Tho City, Viet Nam).

2.2. Disease-reducing effects of plant extracts on BB under greenhouse conditions

The experiment was arranged in a completely randomized design with 3 replications and 2 factors, such as (1) concentration of plant extracts: seed soaking and foliar spraying; (2) time points: spraying at 14 and 7 DAI and their combination.

For seed soaking treatment, rice seeds were soaked in *K. pinnata* extracts for 24 hours. The seeds were then incubated at 28 ± 2ºC for 48 hours before sowing.

Starner 20WP (Sumitomo Chemical Co., Ltd, Osaka, Japan) was used as a chemical control and sprayed at three time points at 5, 10, 15 DAI (Khoa et al., 2017). Water was utilized as the untreated control.

Rice leaves were inoculated by the leaf-clipping method at 45 days after sowing (DAS). The sterilized scissors were submerged in 10^9 CFU/mL *Xoo* suspension and cut 5 fully expanded leaves per plant. The cut position was about 2-3cm from the leaf tip (Kauffman, 1973; Khoa et al., 2017).

The lesion length was assessed by measuring at three time points 7,14 and 21 DAI (Huong et al., 2018; Khoa et al., 2017).

2.3. Enzyme assays

The experiment was arranged in a completely randomized design with 3 replications, including 4 treatments: (1) seeds soaked in sterile distilled water, rice plants were not inoculated; (2) seeds soaked in *K. pinnata* extracts, rice plants were not inoculated; (3) seeds soaked in sterile distilled water, rice plants were inoculated; (4) seeds soaked in *K. pinnata* extracts, rice plants were inoculated. Whereas, seeds were soaked in 2% of *K. pinnata* extracts; sprayed 1% of *K. pinnata* extracts at 14 days before pathogen inoculation (DBI) (minimize concentrations which disease-reducing effects after 21 DAI) and applied the combination of soaking at 2% and sprayed 5% of *K. pinnata* extracts at 14 DBI.

2.3.1. Rice leaf collection and enzyme extraction

Rice leaves after pathogen inoculation at 45 DAS were collected and immediately frozen in liquid nitrogen, continuously collected at 24h intervals during 7 DAI.

For extraction of enzymes: 10 g rice leaf was ground in liquid nitrogen using retsch mixer mill (MM200, Retsch Co., Germany) and then (for each assay) took 0.1 g per time point subsequently homogenized in 1.5 mL of buffer solution (specific buffer for each enzyme). The homogenate was then centrifuged at 10.000 rpm at 4°C for 30 min. Then, 1 mL of the supernatant was used as the crude enzyme extract for assaying enzymatic activity. All of the samples were kept in the ice during assay.

2.3.2. Peroxidase assay

POX activity was expressed as changes in absorbance at 470 nm at 30s intervals during 2 minutes since the reaction occurred as the rate of conversion of tetraguaiacol and guaiacol using the method described by Hammerschmidt et al. (1982) and Khoa et al. (2017). The mixture of 1.6 mL of

0.05 M hydrogen peroxide (H_2O_2) solution, 0.15 mL of 0.15 M guaiacol solution, 0.15 mL of 0.1M sodium phosphate buffer solution and pH 6.5 was used as blank. The reaction mixture comprised 1.6 mL of 0.05 M H_2O_2 solution, 0.15 mL of 0.15 M guaiacol solution and 0.15 mL of the enzyme extract diluted two-fold with the extraction buffer. The experiments were designed with three replications.

2.3.3. Catalase assay

CAT activity was recorded at 240 nm at 30s intervals for 2 minutes because of the breakdown of H_2O_2 by the enzyme followed the method described by Beers and Sizer (1952) and Khoa et al. (2017). The experiments were designed with three replications. Blank sample was valued as 0, which comprised 1.75 mL of 0.1 M H_2O_2 solution and 0.15 mL of 0.1 M sodium potassium phosphate buffer, pH 7.0. The reaction mixture comprised 1.75 mL of 0.1 M H_2O_2 solution and 0.15 mL of the enzyme extract diluted five-fold with the extraction buffer.

2.3.4. Polyphenol oxidase assay

PPO activity was determined as the rate of conversion of colorless catechol to brown benzoquinone at 490 nm at 30s intervals for 2 minutes followed the method described by Mayer et al. (2006), Nisha et al. (2012) and Khoa et al. (2017). The experiments were designed with three replications. The blank sample comprised 1.75 mL of 0.2 M catechol solution and 0.15 mL of 0.1 M sodium phosphate buffer, pH 6.5 was set as 0. The reaction mixture contained 1.75 mL of 0.2 M catechol solution and 0.15 mL of the enzyme extract diluted two-fold with the extraction buffer.

2.3.5. Phenylalanine ammonialyase assay

PAL activity was measured as the rate of deamination of *L*-phenylalanine to produce *trans*-cinnamic acid and absorbance of the product was read at 290 nm following the method described by Dickerson et al. (1984), Kagale et al. (2004) and Khoa et al. (2017). The experiments were designed with three replications. The blank sample was made by 0.7 mL of 0.1 M sodium borate buffer (pH 8.7), 1 mL of 0.1 M *L*-phenylalanine solution, and 0.15 mL of distilled water. The reaction mixture comprised 0.5 mL of 0.1 M sodium borate buffer (pH 8.7), 1 mL of 0.1 M *L*-phenylalanine solution, 0.15 mL of distilled water and 0.2 mL of the enzyme extract. The reaction occurred in a test tube at 32 ± 1°C for 40 min. Then, 0.2 mL of 5.0 N HCl solution was used to stop the reaction.

2.4. Data analyses

A completely randomized design was used for all experiments, with three replications for each treatment. In the greenhouse experiments, lesion lengths were measured using the mean of five inoculated leaves per rice plant replication. Normality showed that data of all dependent variables represent continuous variables with normal distribution. Thus, they were analyzed by analysis of variance (ANOVA) without transformation of data. All data were analyzed by PC-SAS® version 9.1 (SAS Institute Inc., Cary, NC, USA) and all hypotheses were rejected at $P \leq 0.05$.

Enzymatic activity assays were independently repeated three times. Mean changes in absorbance per minute per gram of fresh leaf tissue were calculated for each treatment and line charts were constructed using Microsoft Excel 2016 to represent changes in enzymatic activity during 7 DAI.

3. RESULTS AND DISCUSSION

3.1. Effects of *K. pinnata* extracts on BB under greenhouse conditions using seeds soaking and foliar spraying

For experiment 1, the treatments applied as seeds soaking in *K. pinnata* extracts at 2% combined with spraying at five concentrations (1, 2, 3, 4, 5%) showed short lesion length compared to negative control at 7 days before inoculation (DBI) (Figure 1B). While the lesion length of treatments was applied seeds soaking at 1% and spraying at 4, 5% were recorded significantly difference to the water control at 14 and 21 DAI (Figure 1A).

Described as by Khoa et al. (2017), seeds soaking within *K. pinnata* extracts at 1% did not give any observable effects on reducing the lesion length, whereas at 2% gave a significant difference compared to water control until 21 DAI. Reductions in lesion length were recorded no significant difference in spraying an aqueous extract at five concentrations (1, 2, 3, 4 and 5%) compared to water control at 7 DBI (Huong et al., 2018). Those data showed treatments applied seeds soaking and spraying gave higher disease-reducing effects than individual methods.

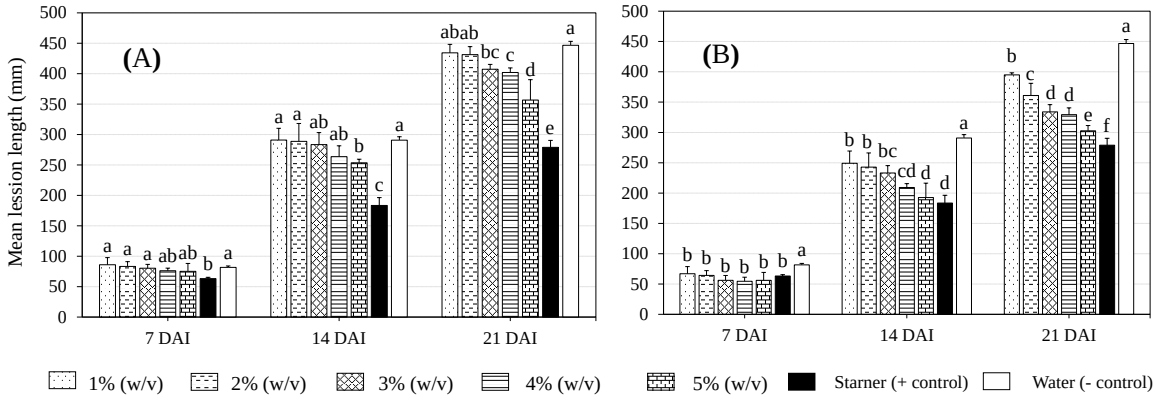

Figure 1. Mean lesion lengths (mm) of bacterial leaf blight on 45 DAS inoculated leaves of rice cv. Jasmine 85 using seed soaking with aqueous extract of *K. pinnata* at concentrations of 1% (A) and 2% (B) combined with foliar spraying at 1, 2, 3, 4 and 5% (w/v) at 7 DBI

Starner (+ control) was used as Starner 20WP (oxolinic acid 20%, 1 mg/mL). Bars with the same letters at the same time are not significantly different at P ≤ 0.05 by Duncan's multiple range test. Comparison is possible between treatments of two concentrations. DAI: days after inoculation. DBI: days before inoculation.

Experiment 2 was performed similarly to the experiment above, but the time of spraying the extract was applied at time 14 DBI. Disease-reducing effects were observed the same as positive control and maintained until 21 DBI, respectively, the treatments of seed soaking at 1% combined with foliar spraying at 5% (Figure 2A); and three treatments of seed soaking at 2% in combination with spraying at 3, 4 and 5% (Figure 2B).

According to Huong et al. (2018), when applied spraying with an aqueous extract 1% at 14 DBI, disease-reducing effects were always higher than that at 7 DBI. Similarly, the results recorded that the treatments of soaking seeds with 1% combined with foliar spraying at 14 DBI were more effective than those at 7 DBI. This result was also recorded similar to the treatments of soaking seeds with 2% extract combined with foliar spraying at the same time. Therefore, the farther (14 DBI) the *K. pinnata* extracts were sprayed, the more effective it was to reduce the disease.

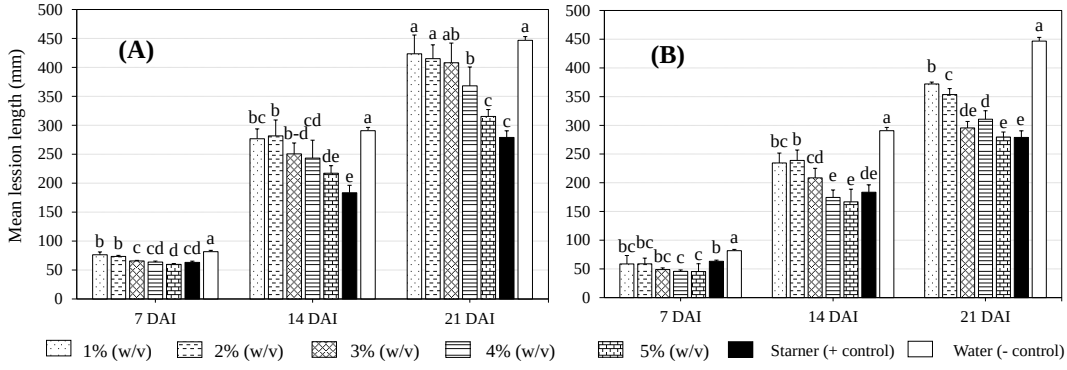

Figure 2. Mean lesion lengths (mm) of bacterial leaf blight on 45 DAS inoculated leaves of rice cv. Jasmine 85 using seed soaking with aqueous extract of *K. pinnata* at concentrations of 1% (A) and 2% (B) combined with foliar spraying at 1, 2, 3, 4 and 5% (w/v) at 14 DBI

Starner (+ control) was used as Starner 20WP (oxolinic acid 20%, 1 mg/mL). Bars with the same letters at the same time are not significantly different at P ≤ 0.05 by Duncan's multiple range test. Comparison is possible between treatments of two concentrations. DAI: days after inoculation. DBI: days before inoculation.

In addition, experiment 2 determined that treatments applied seed soaking at 2% combined with foliar spraying at 1% to reduce the disease and maintained until 21 DAI. The treatments which gave disease-

reducing effects as positive control and maintained until 21 DAI was applied seed soaking at 2% combined with foliar spraying at 5%. Those two treatments were used to show the induced resistance involved enzymes activities in the next experiments.

Experiment 3 was performed similar to the two experiments above, but the *K. pinnata* extracts were sprayed in a combination of 14 and 7 DBI. The experimental results showed that lesion length was the same as the positive control and maintained until 21 DAI, which were two treatments of 1% seed soaking combined with 4, 5% foliar spraying, respectively (Figure 3A); and three treatments of 2% seed soaking in combination with 3, 4 and 5% spraying (Figure 3B). When comparing the disease-reducing effects in the treatments applied seeds soaking at 1% combined with foliar spraying, the time of foliar spraying gave high disease-reducing effects was 7, 14 DBI and combinations of that two times, respectively. The results were similar to the treatment with applied seed soaking at 2% combined with foliar spraying.

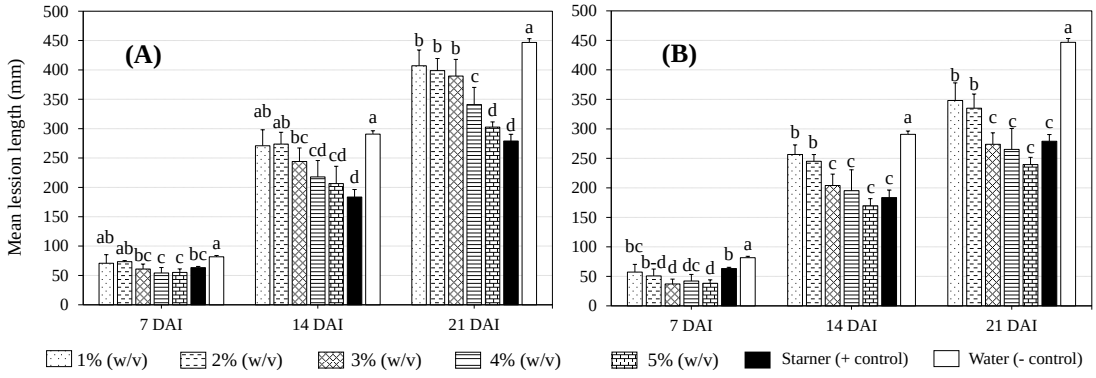

Figure 3. Mean lesion lengths (mm) of bacterial leaf blight on 45 DAS inoculated leaves of rice cv. Jasmine 85 using seed soaking with aqueous extract of different concentrations at 1% (A) and 2% (B) combined with foliar spraying at 1, 2, 3, 4 and 5% (w/v) at 14 and 7 DBI

Starner (+ control) was used as Starner 20WP (oxolinic acid 20%, 1 mg/mL). Bars with the same letters at the same time are not significantly different at P ≤ 0.05 by Duncan's multiple range test. Comparison is possible between treatments of two concentrations. DAI: days after inoculation. DBI: days before inoculation.

Thus, seeds soaking at two concentrations (1 and 2%) and foliar spraying at five concentrations (1, 2, 3, 4 and 5%) at three spray times (7, 14 DBI and both of two) showed disease-reducing effects. At the higher the concentration, the greater the disease-reducing effects were shown (seeds soaking at 2% gave high disease-reducing effects than 1%). The bioactive substances in aqueous extracts are more sensitive when respraying, thereby increasing resistance against *Xoo*. However, this issue needs to be studied in more details.

Those results were completely consistent with the studies using seeds soaking in *Justicia adhatoda* L. extracts to induce resistance to BB disease (Govindappa et al., 2011); in methanol extracts of *Viticis negundo* (Nisha et al., 2012) and foliar spraying methanol extracts of *Datura metel* (Kagale et al., 2004).

An aqueous plant extract for seeds soaking and foliar spraying was applied earlier than the pathogens inoculation, but disease-reducing effects were still recorded in that study. Although only reducing the expression of BB disease but not completely controlling, those results were acceptable. Reducing lesion length would help maintain the leaf area required for photosynthesis and CO_2 fixation in rice, thereby reducing yield loss (Kumar et al., 2013).

3.2. Mechanisms of induced resistance against BB disease of *K. pinnata* extracts using seed soaking and foliar spraying

3.2.1. Peroxidase activities

The activity of POX increased from 0 to 7 DAI in all treatments (Figure 4). There was a slight increase from 19.93 ± 0.07 (0 DAI) to 30.83 ± 1.13 (7 DAI) in water - uninoculated treatment and extracts-uninoculated treatment. Meanwhile, in the extract - inoculated treatment, there was recorded a strong increase in PO activity and maintained at a higher level than all other treatments.

When seeds were soaked at 2% extracts combined with foliar spraying with 1% extracts, POX activity increased continuously from 0 to 4 DAI and remained stable at 7 DAI (Figure 4A). Meanwhile, PO activity strongly increased in the treatments using seeds soaking combined with 5% foliar spraying at 2-3 DAI and kept steady at a high level (Figure 4B).

The study observed that treatments used foliar sprayed at 5% had higher PO activity than the 1%.

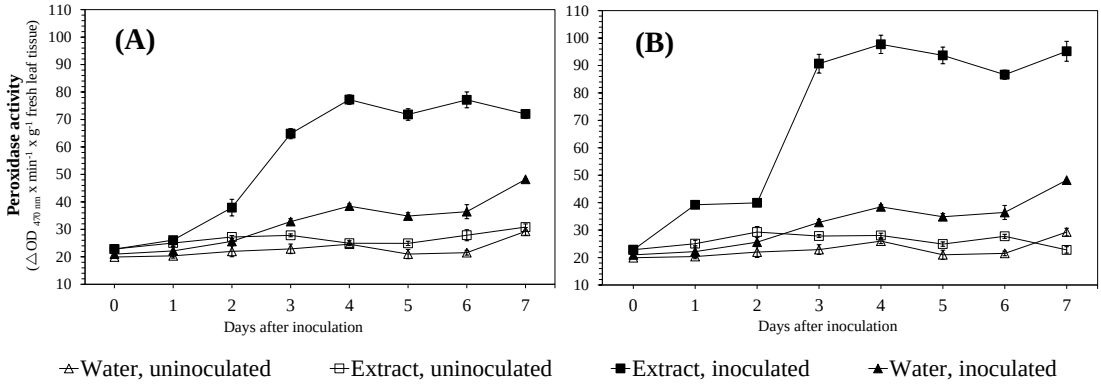

Figure 4. Changes in total activities of peroxidase (POX) in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xoo* at 45 DAS

(A): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 1% of extracts at 14 DBI. (B): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 5% of extracts at 14 DBI. Each value is the mean ± standard deviation of three replications. DAS: days after sowing.

The results were similar to those of Kagale et al. (2004) when using foliar spraying methanol extract of *Datura metel* and Govindappa et al. (2011) when using seeds soaking in *Justicia adhatoda* L. extracts. Both two studies showed that POX activity continuously increased from 0 to 96 hours after inoculation. According to Nisha et al. (2012), POX activity continuously increased from 0 to 100 hours after inoculation (methanol extract) and from 0 to 120 hours after inoculation (aqueous extract). According to Khoa et al. (2017), using seeds soaking with *K. pinnata* extracts, the activity of POX increased from 0 to 6 DAI (Khoa et al., 2017).

3.2.2. Catalase activities

Similar to the activity of POX, CAT activity also increased from 0 to 7 DAI (Figure 5). In which, CAT activity strongly increased and remained higher than all other treatments in the extract-inoculated treatment. When the pathogens inoculation, CAT activity is higher than that of the uninoculated treatment. In which, the extract treatment maintained a higher increase than that without extract treatment at all the assessment time points. Specifically, CAT activity continuously increased from 0 to 7 DAI when seeds soaked at 2% of extracts combined with foliar spraying at 1% (Figure 5A). Meanwhile, in the foliar spraying at 5% treatments, CAT activity increased from 0 to 5 DAI and slightly decreased at 7 DAI (Figure 5B).

CAT activity increased from 0 to 6 DAI, then decreased after 7 days which was similar to Khoa et al. using seeds soaking *K. pinnata* extracts (Khoa et al., 2017). According to Pal et al., CAT activity continuously increased from 0 to 96 hours after inoculation when seeds were soaked in *Ocimum sanctum* and *Cymbopogan citrus* extracts (Pal et al., 2011).

For parasitic pathogens in tissues, the mechanism of induced resistance related to signal pathways of the salicylic acid (SA) and the accumulation of high oxidative activity groups (H_2O_2, O^{2-}, OH^-) protecting plants against pathogens (Shetty et al., 2008, 2007; Singh and Rao, 1997). H_2O_2 participates in part of lignin, suberin, and others, stimulates genes involved in the protection and stimulates the synthesis of phytoalexins that are toxic to pathogens (Shetty et al., 2008; Van Loon et al., 2006, 1998). However, this group produce high oxidative activity, it can be toxic to cells. The two CAT and POX enzymes participate actively in the analysis H_2O_2 process to maintain equilibrium in plant cells (Govindappa et al., 2011; Kagale et al., 2004; Khoa et al., 2017; Nisha et al., 2012).

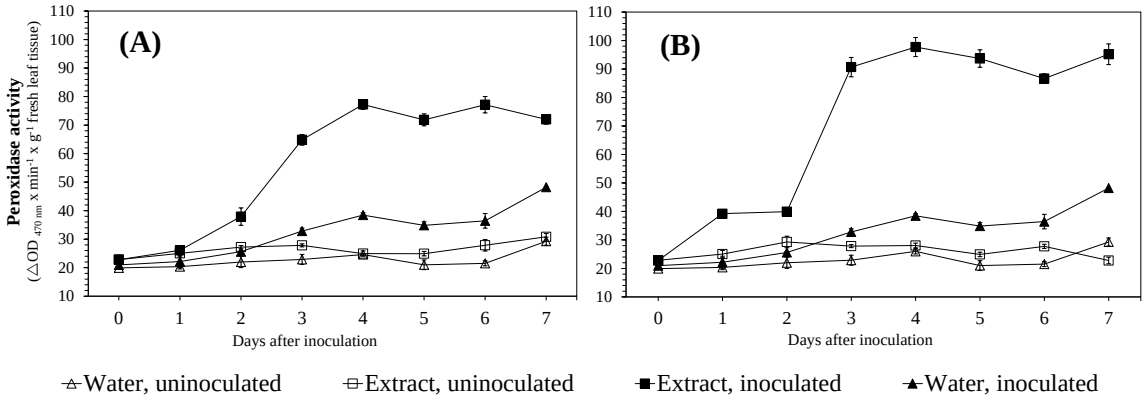

Figure 5. Changes in total activity of catalase (CAT) in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xoo* at 45 DAS

(A): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 1% of extracts at 14 DBI. (B): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 5% of extracts at 14 DBI. Each value is the mean ± standard deviation of three replications. DAS: days after sowing.

In addition, the study also showed that *Xoo* could induce resistance against disease after being artificially inoculated. The ability to induce resistance through a strong increase of POX and CAT activities in inoculation treatments. However, the availability of disease resistance in rice plants is not enough to prevent the growth of pathogens. It is necessary to have a method to activate the induced resistance of rice plants at the right time and strongly enough to prevent the infection and growth of pathogens.

3.2.3. Polyphenol oxidase activities

In both of water - uninoculated and –extract-uninoculated treatments, PPO activity was recorded at a stable level from 0 to 7 DAI (2.22 ± 0.04 to 2.30 ± 0.09, respectively) (Figure 6).

PPO activity started to increase earlier when pathogens inoculation. In which, PPO activity in the extract-inoculated treatment increased and was stronger than water-inoculated treatment. Specifically, the treatments using seeds soaking at 2% combined with foliar spraying at 1% had PPO activity sharply increased from 0 to 4 DAI; then decreased at the next assessment time points (Figure 6A). The treatments using seeds soaking at 2% combined with foliar spraying at 5% showed an increasing trend, but stronger at 2 to 4 DAI (Figure 6B).

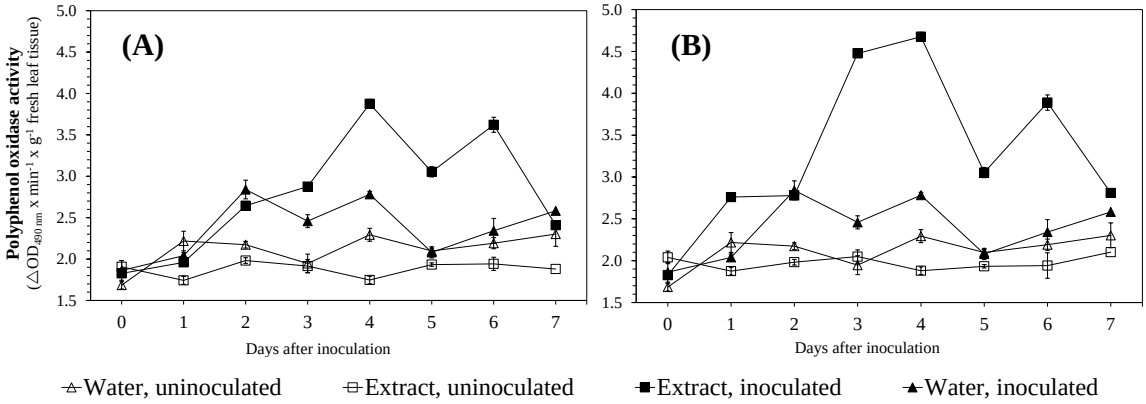

Figure 6. Changes in total activities of polyphenol oxidase (PPO) in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xoo* at 45 DAS

(A): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 1% of extracts at 14 DBI. (B): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 5% of extracts at 14 DBI. Each value is the mean ± standard deviation of three replications. DAS: days after sowing.

Thus, the study observed that when innocultating pathogen and applying the extracts, the PPO activity sharply increased from 0 to 4 DAI. When spraying the extract at 5%, the PPO activity increased stronger than that of 1%. According to Khoa et al. using seeds soaking in *K. pinnata* extracts, the PPO activity increased from 1 to 4 DAI (Khoa et al., 2017). Similarly, Huong et al. (2018) reported that the activity of PPO increased when sprayed the *K. pinnata* extracts. In that study using seeds soaking combined with foliar spraying, the PPO activity was recorded as stronger than the individual method.

In addition, the results were similar to those reported that using methanol extract of *Datura metel*, the PPO activity continuously increased from 0 to 164 hours after inoculation (Kagale et al., 2004). Using seeds soaking in *Justicia adhatoda* L. extracts, the activity of PPO increased from 0 to 72 hours after inoculation (Govindappa et al., 2011). When seeds were soaked in methanol extracts of *Folium Viticis negundo*, PPO activity increased from 0 to 100 hours after inoculation (Nisha et al., 2012).

3.2.4. Phenylalanine ammonialyase activities

In the extract-uninoculated treatment, PAL activity was higher than water-uninoculated treatments during the assessment time points at both methods (Figure 7).

The study recorded that PAL activity increased when the pathogen appeared (in inoculated treatment), even strongly increased when treated with the *K. pinnata* extracts. Specifically, activity of PAL increased at 1-2 DAI and 3-5 DAI; and decreased sharply at 7 DAI. Meanwhile, in the extract-inoculated treatment, results of PAL activity strongly increased at the time of 2-3 DAI and decreased slightly but still maintained at a higher level than all other treatments.

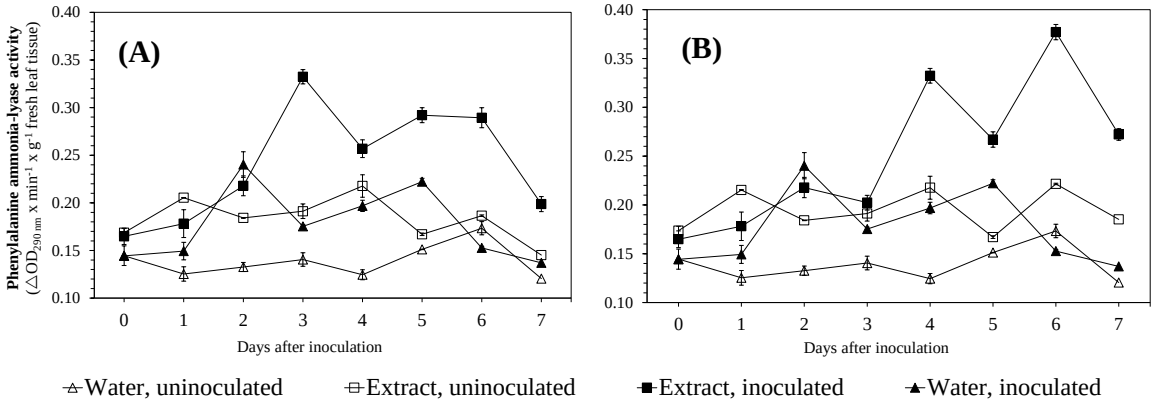

Figure 7. Changes in total activities of phenylalanine ammonia-lyase (PAL) in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xoo* at 45 DAS

(A): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 1% of extracts at 14 DBI. (B): Soaking rice seed Jasmine 85 in 2% of K. pinnata extracts combined with foliar spraying at 5% of extracts at 14 DBI. Each value is the mean ± standard deviation of three replications. DAS: days after sowing.

Thus, PAL activity increased in the initial days when the pathogen inoculation, then decreased in the next 2 days in extract treatments. This result was also recorded when using seeds soaking in live leaf extracts (Khoa et al., 2017). Moreover, that tendency is similar to the published work by Kagale et al. (2004) when foliar spraying methanol extracts of *Datura metel* and Govindappa et al. (2011) when using seeds soaking in *Justicia adhatoda* L. extracts. Both of those studies reported a continuously increased in PAL activity from 0 to 72 hours after inoculation (Govindappa et al., 2011; Kagale et al., 2004). Khoa et al. (2017) and Huong et al. (2018) recorded a deep decrease in PAL activity after a sharp increase in the early days when the pathogen inoculation; while Kagale et al. (2004) and Govindappa et al. (2011) observed slightly decreased but always remained at high level (Govindappa et al., 2011; (Huong et al., 2018; Kagale et al., 2004; Khoa et al., 2017). Therefore, the results showed that the ability to induce resistance helps to increase the enzyme activity, which contributes to the control BB diseases.

PPO enzyme catalyzes the hydroxylation and oxidation of phenolic compounds to ortho-quinone, which has strong antimicrobial effects (Constabel & Barbehenn, 2008; Mayer, 2006; Yoruk & Marshall,

2003). In addition, it is also capable of oxidative polymerization or alkylation of proteins, forming polymers that are toxic to pathogens or melanin pigments. Those act as physical barriers to prevent the pathogens infected into healthy tissues, especially saprophytes fungi (Constabel & Barbehenn, 2008; Leach et al., 1989; Li & Steffens, 2002; Mayer, 2006)

PAL enzyme plays an important role in the phenylpropanoid cycle for the biosynthesis of phenolic compounds such as flavonoids, isoflavonoids, phytoalexins, and lignin monomers (Garcion et al., 2014; Potato et al., 1989). Flavonoids, isoflavonoids, and other phenolic compounds are generally secondary metabolites in plants with a wide variety of chemical structures. Thus they have many functions such as antioxidant and plant protection against pathogens by activating a hypersensitive response or inhibiting important enzymes of the pathogens (Hammerschmidt et al., 1982; Mierziak et al., 2014; Nicholson and Hammerschmidt, 1992; Van Loon et al., 2006, 1998). Therefore, the activity of PAL rapidly increases at 2 and 3 DAI (Figure 7) would promote the biosynthesis of the metabolic compounds; thereby helping to prevent the infection of *Xoo* into rice leaf tissue.

When *Xoo* infects the libe circuit and uses nutrient sources to growth after 2-3 days; then, causes disease (Leach et al., 1989; Niño-Liu et al., 2006; Swings et al., 1990). The activities of two enzymes PAL and PPO increased from 1 to 4 DAI which protected rice plants from pathogens.

Results were consistent with the studies using *Justicia adhatoda* L. extracts, methanol extract of *Folium Viticis negundo* and methanol extract of *Datura metel* to reduce BB disease (Govindappa et al., 2011; Kagale et al., 2004; Khoa et al., 2017; Nisha et al., 2012; Shivalingaiah et al., 2013). Those studies recorded the highest increase in PAL and PPO enzyme activities at 3 and 4 DAI, respectively, showing that these two enzymes play an important role in control BB disease.

4. CONCLUSIONS

This study investigated the disease-reducing effects of aqueous leaf extracts of *K. pinnata* against rice bacterial leaf blight using seed soaking combined with foliar spraying at different assessment time points. The results showed that disease reduction was higher with a higher concentration of the extract and when the application was far from the time of inoculation.

Activities of the enzymes peroxidase (POX) and catalase (CAT) sharply increased earlier after pathogen inoculation and more strongly when the extracts were applied. The combination of seed soaking at 2% with foliar spraying at 5% showed stronger enzyme activities than seed soaking at 2% combined with spraying at 1%. Similar increasing trends were observed for the enzymes polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL), but they sharply increased at 2-3 days after inoculation (DAI).

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