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# Combinations of seed soaking and foliar spraying of methanol *Kalanchoe pinnata* leaf extracts induce rice resistance against bacterial leaf blight

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**ABSTRACT**

*Bacterial leaf blight (Xanthomonas oryzae pv. oryzae) is an important rice disease. This study tested disease-reducing effects of methanol Kalanchoe pinnata leaf extracts prepared by the liquid-liquid extraction method and investigated the mechanism involved in the observed disease reduction. Three extract concentrations (1, 1.5, and 2%) were tested under net house conditions using three application methods [seed soaking combined with foliar spraying at (1) 14 days before inoculation (DBI), (2) 7 DBI and (3) both 14 and 7 DBI]. The disease was assessed through lesion lengths at 7, 14 and 21 days after inoculation. Seed soaking combined with foliar spraying at 14 DBI using 1.5% extract showed significant disease reduction similar to that of the chemical control using Starner 20WP. Induced resistance involved in the disease reduction through increased activities in rice tissues of the four defense-related enzymes, i.e., peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL). Activities of POX, CAT and PPO generally increased while those of PAL decreased after pathogen inoculation. By extract application, activities of POX, PPO and PAL increased earlier compared to those of CAT. Activities of these enzymes reached higher levels with the presence of both extract application and pathogen inoculation.*

## 1. INTRODUCTION

Bacterial leaf blight disease (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae (Xoo*) is one of the most devastating diseases in paddy fields and commonly occurs in major rice-growing countries of Asia (Teja et al., 2025). Chemical pesticides are commonly used to manage the disease; however, over-application may result in detrimental effects on human health and the ecosystem (Ahmad et al., 2024). Induced disease resistance has attracted increasing attention as an eco-friendly alternative method for plant disease control. Induced resistance is described as a method that uses abiotic or biotic elicitors (chemical compounds, microorganisms, and plant extracts) to activate host defence responses, leading to enhanced physical and chemical barriers against infection and the proliferation of phytopathogens (Kloepper et al., 1992; Walters et al., 2005). The defense mechanism is commonly associated with the activation of four key antioxidant or defense enzymes, including peroxidase (POX), catalase (CAT), phenylalanine ammonia lyase (PAL), and polyphenol oxidase (PPO). These enzymes are involved in defense-related signaling and metabolic regulation, contributing to reactive oxygen species (ROS)

regulation and the production of phenolic and antibacterial compounds (van Loon, 1997; van Loon et al., 1998, van Loon & Van Strien, 1999).

Among various elicitors, plant extracts act as natural elicitors that stimulate plant immune system and due to their diverse bioactive compounds and are considered safe for human health and the environment (Tucuch Pérez et al., 2024). Several plant extracts have been reported to induce resistance against BLB, including *Datura metel* (Kagale et al., 2004), *Chromolaena odorata* (Khoa, 2010; Xa et al., 2024), *Argemone mexicana*, *Eucalyptus globulus*, *Bassia scoparia*, *Mallotus philippensis*, and *Shorea robusta* (Mansoori et al., 2025). *Kalanchoe pinnata* is commonly found in Viet Nam. This plant has been used as a conventional treatment for wound healing, burn treatment, and inflammation treatment since its extract consists of rich phenolic compounds, which are considered antimicrobial substances (Quazi et al., 2011; Nguyen et al., 2025). These compounds are also associated with elicitor activity in plant defense responses (Saini et al., 2024).

Previous studies have demonstrated the potential of *crude Kalanchoe pinnata* extract to reduce BLB disease. Screening of aqueous plant extracts identified *K. pinnata* extract as the most effective (Xa, 2015), and subsequent studies demonstrated its effectiveness in reducing BLB (Khoa et al., 2017; Huong et al., 2018). In addition to the biological potential of plant extracts, their effectiveness is also influenced by extraction solvent. Using appropriate extraction solvents may improve the activity of plant extracts. Methanol (polarity index 0.762) is effective in extracting phenolics, flavonoids, alkaloids and other moderately polar secondary metabolites that are likely contributors to elicitation and antimicrobial activities (Sultana et al., 2009; Abubakar & Haque, 2020). A previous study showed that methanol extracts had higher activity against BLB compared to aqueous and dichloromethane extracts (Xa et al., 2023).

The disease-reducing effect of methanol *Kalanchoe pinnata* extract on BLB has been reported using both the seed soaking (Xa et al., 2023), and foliar spraying method (Duy et al., 2024). Although methanol extracts of *K. pinnata* have shown promising effects against the disease when applied as either seed soaking or foliar spraying, effects of combinations of both application methods should be investigated for further selection and optimization of the extract usage in practice.

The study aimed to assess the effectiveness of *Kalanchoe pinnata* extract by methanol when using seed soaking combined with foliar spraying against BLB under net house conditions. Furthermore, the changes of four defense-related enzymes (POX, CAT, PPO, and PAL) in response to the application of the extract were investigated.

## 2. MATERIALS AND METHOD

### 2.1. Sources of *Kalanchoe pinnata* and *Xanthomonas oryzae* pv. *oryzae*

Leaves of *Kalanchoe pinnata* used in this study were obtained from plants cultivated under net house conditions at Vinh Long University of Technology Education.

The rice cultivar Jasmine 85, known to be susceptible to BLB and the highly virulent *Xanthomonas oryzae* pv. *oryzae* XCT13 were supplied by the Plant Pathology research group, Laboratory of Molecular Biology, Institute of Food and Biotechnology. Before sowing, rice seeds were treated in hot water at 55ºC for 15 minutes to enhance the germination capacity of rice seed.

### 2.2. Preparation of *Kalanchoe pinnata* extract

Methanol *Kalanchoe pinnata* extract preparation protocol followed by Kagale et al. (2004) and modified by Xa et al. (2023). Three hundred grams of leaves were harvested in the morning and washed with distilled water. Then, the leaves were grounded with 1,500 mL methanol 100% for 5 minutes. To obtain the concentrated leaf extract of methanol, the mixture was then placed in an ultrasonic bath at 120 W for 60 minutes, followed by filtering through $Na_2SO_4$ and filter paper. Filtrates were completely evaporated by using a rotary evaporator at 50 °C and 5,000 rpm. The residue was dissolved in 100 mL of sterile distilled water to obtain methanol *Kalanchoe pinnata* extract (1 g/mL). Then the extracts were stored at 4ºC.

### 2.3. Testing for the disease-reducing effects of methanol *Kalanchoe pinnata* leaf extracts on bacterial leaf blight disease under net house conditions

The experiment was conducted using a completely randomized design comprising three replicates for each treatment. Each replicate consisted of one pot containing three rice plants. *K. pinnata* leaf extract at three concentrations including 1, 1.5, and 2% (w/v) was investigated. Combined with seed soaking, the extracts were sprayed on rice plants using three different methods, i.e., (1) spraying at 14

days before inoculation (DBI), (2) at 7 DBI and (3) at both 14 and 7 DBI. Water was utilized as the untreated control, while the bactericide, oxolinic acid (Starner, 20 WP, Sumitomo Chemicals Co., Osaka, Japan) was used as chemical control. Five milliliters of the bactericide at 1 mg/ml were applied per rice plant and sprayed at 5, 10, and 15 DAI. The bactericide (1mg/mL) was applied by spraying 5 mL per rice plant at 5, 10, and 15 DAI (Khoa et al., 2017; Huong et al., 2018).

#### *2.3.1. Inoculum Preparation*

The bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* was grown on Petri plates that contained modified Wakimoto's medium and incubated at 28 ± 2°C for 72 hours. To prepare the inoculum, 2-day-old bacterial cells were collected using a 2-mm inoculation loop and added into 10 mL of sterile distilled water. The bacterial inoculum was adjusted to a cell density of approximately $10^9$ CFU/mL (Khoa, 2005; Khoa et al., 2017).

#### *2.3.2. Pathogen Inoculation and Disease Assessment*

At 45 days after sowing, rice plants were inoculated with *Xoo* using leaf-clipping method described by Kauffman et al. (1973). Sterile scissors were treated with 70% (v/v) ethanol and dipped in the inoculum and used to clip the tips (2-3 cm) of 5 fully expanded leaves per plant (Khoa et al., 2017). Lesion lengths (mm) were measured at 7, 14, and 21 DAI. The mean lesion length of each replicate (pot) was then calculated as the average of the three plants within the pot.

### 2.4. Investigating the effect of methanol *Kalanchoe pinnata* extracts to induce resistance through defense-related and antioxidant enzymes

There are four treatments performed in triplicate. (1) seed soaking combined with foliar spraying using *K. pinnata* extract followed by pathogen inoculation (extract + pathogen); (2) seed soaking combined with foliar spraying by *K. pinnata* extract, without pathogen inoculation (extract + no pathogen); (3) seeds soaking combined with foliar spraying by sterile distilled water, followed by pathogen inoculation (water + pathogen); and (4) seeds soaked in sterile distilled water, without pathogen inoculation (water + no pathogen).

#### *2.4.1. Tissue collection and enzyme extraction*

Rice plants were challenged to inoculate with *Xoo* at 45 days after inoculation (DAI) under net house conditions. Rice leaves were harvested from 0 to 7 DAI (once a day) (1 leaf per plant), and leaf samples were immediately stored in liquid nitrogen. For enzyme extraction, 0.1 g of rice leaf samples were finely chopped and transferred to a 2 mL Eppendorf tube containing a pasteurized bearing ball, then soaked in liquid nitrogen for 20 minutes and ground to a fine powder. Then, they were homogenized in 1.5 mL of buffer solution corresponding to each enzyme, including 0.1 M sodium phosphate buffer (pH 6.5) for POX and PPO, 0.1 M sodium-potassium phosphate buffer (pH 7) for CAT, and 0.1 M sodium borate buffer (pH 8.7). Then, the mixture was centrifuged at 10,000 rpm for 30 min at 4°C. After centrifugation, supernatant was transferred to a new tube and used as the source of the crude enzyme extract. Enzyme extracts were stored at 4°C.

#### *2.4.2. Peroxidase assay*

The activities of POX were determined according to the method of Hammerschmidt et al. (1982) by monitoring the formation of tetraguaiacol from guaiacol at 470 nm. Measurements were conducted in triplicate at 90-second intervals. The reaction mixture contained 1.6 mL of 0.05 M $H_2O_2$, 0.15 mL of 0.15 M guaiacol, and 0.15 mL of enzyme extract diluted two-fold with 0.1 M sodium phosphate buffer (pH 6.5). A blank containing all reagents except the enzyme extract to calibrate the spectrophotometer to 0. Enzyme activities were expressed as changes in absorbance. POX activities were expressed as the increase in absorbance at 470 nm per min per gram of fresh leaf tissue.

#### *2.4.3. Catalase assay*

The activities of CAT were determined according to Beers and Sizer (1952) by monitoring the decomposition of $H_2O_2$ at 240 nm. Measurements were conducted in triplicate, and absorbance values were recorded at 20-second intervals for 2 minutes after reaction initiation. The reaction mixture contained 1.75 mL of 0.1 M $H_2O_2$, and 0.15 mL of enzyme extract prepared in 0.1 M potassium phosphate buffer (pH 7.0). A blank containing 2 mL of the extraction buffer to calibrate the spectrophotometer to 0. CAT activities were expressed as the change in absorbance at 240 nm per min per gram of fresh leaf tissue.

#### *2.4.4. Polyphenol oxidase assay*

The activities of PPO were determined according to Mayer et al. (1966) and Khoa et al. (2017) by monitoring the oxidation of catechol into brown-

colored benzoquinone at 490 nm. Measurements were conducted in triplicate, and absorbance values were recorded at 30-second intervals for 2 minutes. The reaction mixture contained 1.75 mL of 0.2 M catechol, and 0.15 mL of enzyme extract diluted two-fold using sodium phosphate buffer (pH 6.5). A blank containing all reagents except the enzyme extract to calibrate the spectrophotometer to 0. PPO activities were expressed as changes in absorbance at 490 nm per min per gram of fresh leaf tissue.

*2.4.5. Phenylalanine ammonia lyase assay*

The activities of PAL were determined according to Dickerson et al. (1984) by quantifying the conversion of L-phenylalanine into trans-cinnamic acid. The formation of trans-cinnamic was monitored at 290 nm. Measurements were conducted in triplicate. The reaction mixture contained 0.5 mL of 0.1 M sodium borate buffer (pH 8.7), 1.0 mL of 0.1 M L-phenylalanine, 0.15 mL of distilled water, and 0.2 mL of enzyme extract. After incubation at 32 ± 1°C for 40 minutes, the reaction was terminated by adding 0.2 mL of 5 N HCl. A blank containing all reagents except the enzyme extract to calibrate the spectrophotometer to 0. PAL activities were expressed as changes in absorbance at 290 nm per min per g of fresh leaf tissue.

### 2.5. Data analyses

All experiments were conducted using three triplicates. Lesion length data and the absorbance value in enzymatic assays were represented in a chart using Excel 2019. Statistical analyses were performed with IBM SPSS Statistics 20. Differences among treatments were evaluated by one-way analysis of variance (ANOVA) and significant differences among means were determined using Duncan test (Multiple Range Test 5%).

## 3. RESULTS AND DISCUSSION

### 3.1. Disease-reducing effects of methanol *Kalanchoe pinnata* extract on bacterial leaf blight under net house conditions

At 7 DAI, all treatments reduced mean lesion lengths as the chemical control did (1.22±0.19), except the combination treatments of seed soaking (1% extract) and foliar spraying (2%) at either 7 DBI or 14 DBI, which did not show any differences as compared to the untreated control (Figure 1A).

At 14 DAI, all treatments showed significant disease reductions in BLB lesion lengths as compared to those of the untreated control, except seed soaking (1% and 1.5% extracts) combined with spraying (1% extract) at 7 DBI (18.56±3.56 and 16.44±0.51, respectively) (Figure 1B). Applications of 1% extracts could not have similar effects as those of the chemical control, while applications of 1.5% and 2% extracts could.

At 21 DAI, treatments using either higher extract concentrations or foliar spraying at both 14 and 7 DBI provided similar protection as the chemical control did (Figure 1C). Seed soaking combined with foliar spraying using 1% extract could not show any effects at this assessment time point. These results indicate that extract concentrations and application methods, particularly spraying time points, have possible impacts on the disease-reducing effects *K. pinnata* extracts.

Seed soaking combined with foliar spraying 14 DBI using 1.5% extract was the most efficient method among others as it could provide similar effects compared to that of the chemical treatment while requiring lower amount of the extracts (vs. 2% extract) and less labor for application (spraying only one time). Spraying at 14 DBI provides enough time for the defense responses to be activated in rice plants. In addition, spraying one time instead of two times reduces extract and labor consumption, thus application costs. Spraying at 14 DBI has also been recommended in a number of earlier studies where significant disease reductions were observed (Khoa et al., 2017; Huong et al., 2018; Duy et al., 2024). The combination of seed soaking and foliar spraying at 14 DBI of 1.5% *K. pinnata* leaf extract is therefore selected for further investigations.

This study presented the effects of combinations of seed soaking and foliar spraying of *K. pinnata* leaf extracts. Further studies could be performed to compare the effects of lone applications (either seed soaking or foliar spraying) with those of their combinations.

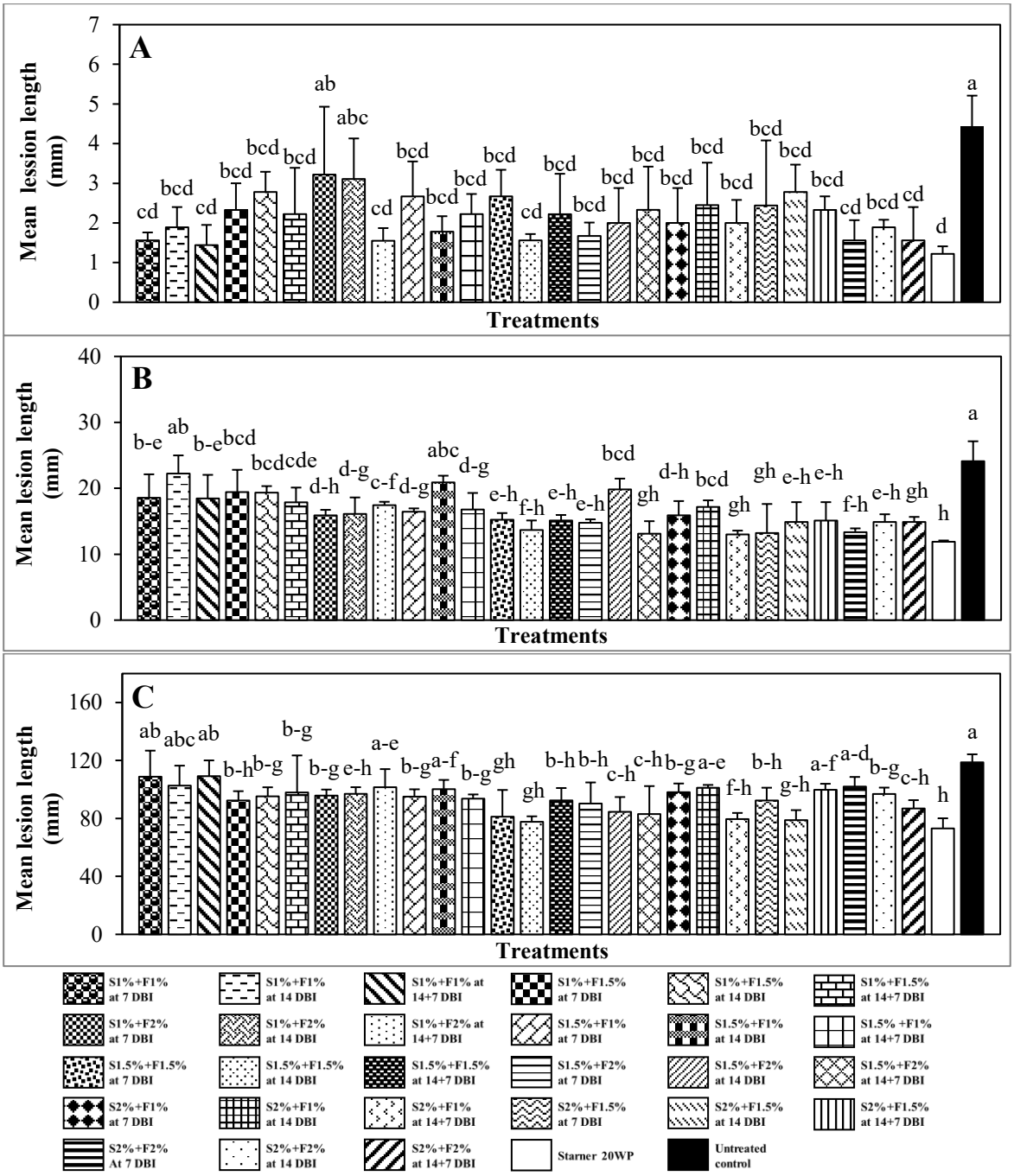


**Figure 1. Mean lesion lengths (mm) of bacterial leaf blight on Jasmine 85 rice plants inoculated at 45 days after sowing and assessed at 7 days after inoculation (DAI) (A), 14 DAI (B), and 21 DAI (C). Rice plants were treated with *Kalanchoe pinnata* at 1, 1.5, and 2% (w/v) by using seed soaking combined with foliar spraying at 14 days before inoculation (DBI), 7 DBI, and combination of 14 and 7 DBI. Starner 20WP (1mg/mL) was used as a chemical, and distilled water was used as an untreated control**

*Bars sharing the same letter are not significantly different at P≤0.05 by Duncan's Multiple Range Test. S, seed soaking; F, foliar spraying.*

### 3.2. Enzyme activities

The changes in POX activities in four treatments are illustrated in Figure 2A. The POX activities tended to increase in water + no pathogen from 0 to 7 DAI (statistically significant difference). The enzyme activity tended to peak at 1, which may be partly attributable to mechanical stress during handling (Wang et al., 2006). Then, the activities decreased moderately from 1 to 3 DAI and remained at that level thereafter. These results indicate that in the absence of both the extract and the pathogen, POX activities were maintained at low levels. and significantly lower than other treatments from 1 to 5 DAI. The enzyme activities had an increase from 0 to 7 DAI in the inoculated rice plant treated with water (water + pathogen). Enzyme activities reached a maximum at 1 DAI. Then, the activities declined moderately at 3 DAI. Thus, the results showed that POX activity generally increased from 0 to 7 DAI in both water treatments. However, the activities in water + pathogen treatment increased further and remained significantly higher than water + no pathogen treatment. The presence of *Xoo* may have stimulated the plant's defense response, resulting in an increase in enzyme activities.

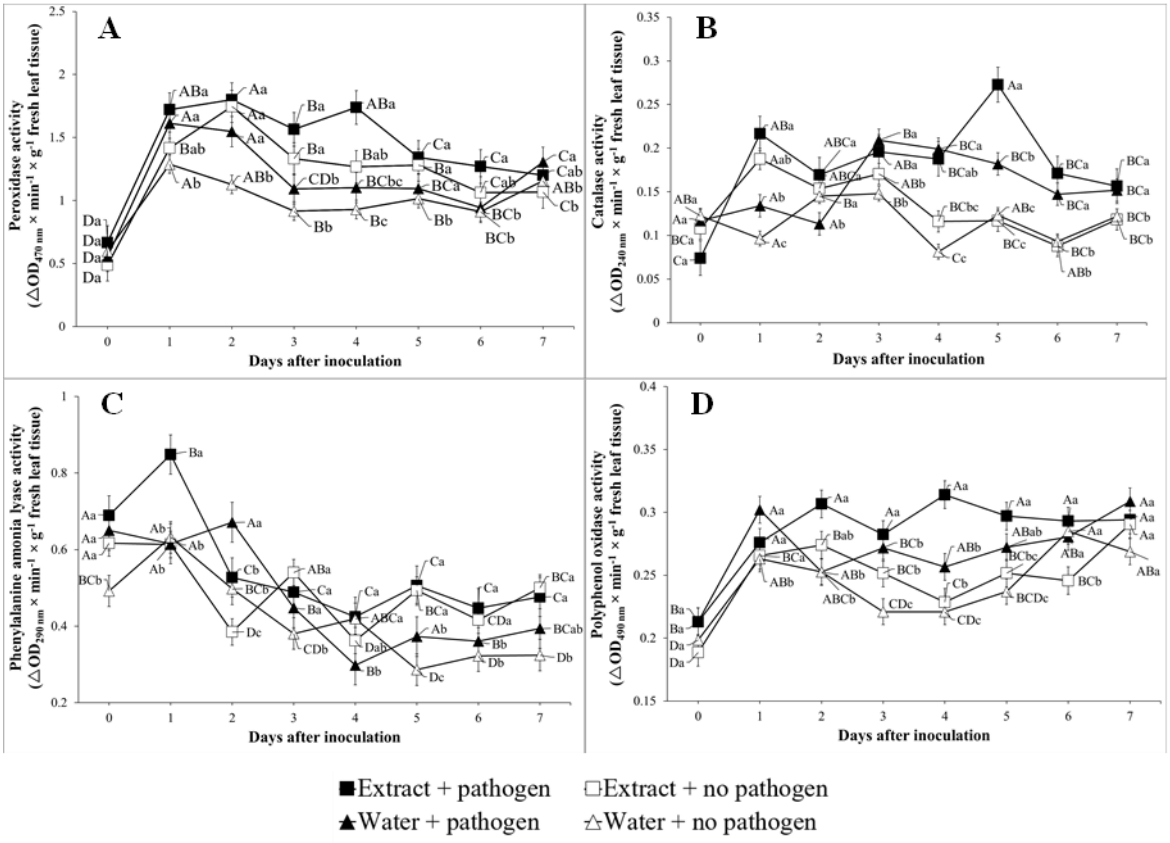


**Figure 2. The changes in activities of peroxidase (A), catalase (B), phenylalanine ammonia lyase (C), polyphenol oxidase (D) through time points (0-7 days after inoculation)**

*Uppercase letters indicate differences among assessment time points within each treatment (0-7 days after inoculation). Lower case letters indicate differences among treatments within each assessment time point. Values associated with a similar letter are not significantly different at p≤0.05 by Duncan's Multiple Range Test.*

An increase in POX activities was observed in treatment with methanol *K. pinnata* extract and without pathogen inoculation (extract + no pathogen) from 0 to 7 DAI (Figure 2A). The enzyme activities increased dramatically from 0 to 2 DAI, peaked at 2 DAI, remained stable until 4 DAI, and then declined thereafter. An early and significant increase in POX activities was observed in the presence of both extract and pathogen (extract + pathogen) from 1 to 7 DAI. Enzyme activities dramatically increased from 0 to 1 DAI and reached a maximum at 2 DAI. The combination of extract

application and pathogen challenge resulted in hihger POX activities than either treatment applied individually. This enzyme participates in several physiological processes, particularly hormone production and cell wall fortification through accumulation of lignin, suberin, and other structural polymers. POX is also involved in $H_2O_2$ metabolism (Bestwick et al., 1998). Thus, the presence of both methanol *K. pinnata* extract and pathogen is sufficient to trigger an early and significant increase in $H_2O_2$ production in rice plants, thereby preventing the penetration and rapid proliferation of *Xoo*. This is concordant with previous studies. For instance, Khoa et al. (2017) reported an increase in POX activities from 1 to 6 DAI when using seed soaking with aqueous crude extract of *K. pinnata*. Kagale et al. (2004) reported that POX activities increased from 24 to 96 hours after inoculation (HAI) using leaf extract of *Datura metel*. In addition, Nisha et al. (2012) applied the *Vitex negundo* extract to induce the resistance in rice plants against BLB; the results indicated an increase in POX activities from 20 to 100 HAI.

CAT activities tended to remain stable in water + no pathogen treatment from 0 to 7 DAI (statistically non-significantly different) (Figure 2B). The enzyme activities tended to reach a peak at 3 DAI and remained stable thereafter. The results indicated that without the presence of both extract and pathogen, enzyme activities were significantly lower than in other treatments. CAT activities generally increased after inoculation (water + pathogen) from 1 to 7 DAI. The activities increased significantly from 2 to 3 DAI, reached a maximum at 3 DAI, and remained stable thereafter. The activities in water + pathogen treatment increased further and remained significantly higher than water + no pathogen treatment. *Xoo* might have stimulated the CAT production to scavenge $H_2O_2$, which is toxic to the pathogen. However, the accumulation of $H_2O_2$ is potentially harmful to the rice plant. Thus, the increase in CAT activities from 2 to 3 DAI rapidly catalyzes the decomposition of H2O2, thereby detoxifying rice plants. The increase in the activities of CAT was observed in the treatment with methanol extract of *K. pinnata* and without pathogen inoculation (extract + no pathogen). The enzyme activities began to moderately increase from 0 to 1 DAI and slightly reduced from 2 to 4 DAI, then remained stable thereafter. CAT activities in the extract + no pathogen treatment increased compared to those in the water + no pathogen treatment. The result indicated that the extract application could also trigger CAT accumulation in rice plants, but this effect did not persist due to the presence of *Xoo*. CAT activities in rice plants were treated with methanol *K. pinnata* extract followed by pathogen inoculation (extract + pathogen) increased from 0 to 7 DAI. The enzyme activities dramatically increased from 1 to 5 DAI higher than in other treatments, then declined drastically at 6 DAI. Besides, CAT activity in the extract + pathogen treatment was significantly greater than that in the extract + no pathogen treatment. The combination of methanolic *K. pinnata* extract and pathogen inoculation induced a stronger CAT response than either treatment applied individually. This observation suggests that the simultaneous presence of the extract and pathogen was sufficient to stimulate catalase activity, thereby enhancing detoxification processes in rice plants. The result supports those by Khoa et al. (2017) where the CAT activities increased from 1 to 6 DAI using seed soaking with aqueous *K. pinnata* crude extract.

Activities of PAL decreased from 0 to 7 DAI in the water + no pathogen, except for a peak at 1 DAI (Figure 2C). The activities decreased dramatically from 1 to 3 DAI and remained stable from 4 to 5 DAI. The enzyme activities decreased from 0 to 7 DAI in inoculated rice plants treated with water (water + pathogen). Although the activities decreased further, they were significantly higher at 2 DAI than in the water + no pathogen treatment. This can be explained by the presence of pathogen in the water + pathogen treatment triggers the production of PAL in plants. A decrease in activities of PAL was observed in the treatment of methanol *K. pinnata* extract, and without pathogen inoculation (extract + no pathogen), the enzyme reached a high level at 0-1 DAI, then declined from 1 to 7 DAI. The results indicated that the extract triggered PAL at the initial time point for the secondary metabolites biosynthesis and played an essential role in salicylic acid biosynthesis, an important plant defense signal (Appu et al., 2021). On the other hand, in the absence of the pathogen, enzyme activities did not increase steadily in rice plants to save plant energy. In the presence of both extract application and *Xoo* inoculation (extract + pathogen), PAL generally decreased from 1-7 DAI. Enzyme activities increased significantly from 0 to 1 DAI, then decreased dramatically at 2 DAI and remained low thereafter. Besides, the enzyme activities in extract + pathogen treatment showed no significant difference compared to extract + no pathogen treatment. A significant increase in

enzyme activities was observed at 1 DAI in the treatment with both extract application and pathogen inoculation, compared with either the extract or the pathogen alone. The induction of PAL observed in rice plants may have resulted from the combined effects of methanolic *K. pinnata* extract and pathogen inoculation, leading to enhanced resistance against *Xoo* colonization and multiplication. The increase in PAL activities in extract + pathogen treatment from 1-2 DAI due to the penetration and rapid proliferation of *Xoo*, which initially damages rice plants at the first stage, leading to the triggering of the induced systemic resistance signals in rice plants, the decrease in PAL activities while other enzymes increased can be explained by their distinct roles and temporal regulation during rice and *Xoo* interaction. PAL functions as an upstream enzyme in the phenylpropanoid pathway and is typically associated with early defense responses, showing transient induction after infection (Kim et al., 2020). In contrast, downstream and oxidative enzymes such as PPO are involved in the utilization of phenolic compounds and are often sustained or further induced during later stages of infection (Yang et al., 2024). Previous studies on rice–*Xoo* interactions have demonstrated that defense-related enzymes are differentially regulated over time rather than synchronously expressed (Shasmita et al., 2021). Therefore, the observed decline in PAL activities alongside increased activities of other enzymes likely reflects a shift from phenolic biosynthesis to downstream defense mechanisms such as oxidation and cell wall reinforcement. This result is supported by previous studies. For instance, Kagale et al. (2004) showed an increase in PAL activities from 24 to 72 HAI. In another study, Khoa et al. (2017) reported an increase in PAL activities from 1 to 3 DAI. Huong et al. (2018) reported that PAL activities increased from 0 to 1 DAI.

In the water + no pathogen treatment, activities of PPO increased from 0 to 7 DAI (Figure 2D). The activities increased from 0 to 2 DAI and tended to decrease from 2 to 6 DAI. The enzyme increased at all time points in inoculated rice plants treated with water (water + pathogen), reaching a maximum at 1 DAI and declining thereafter. However, enzyme activities increased from 4 to 7 DAI. PPO activities remained significantly higher compared to those in the water + no pathogen. An increase in the PPO activities was observed in extract application (extract + no pathogen) 0-7 DAI. The enzyme activities increased moderately from 0 to 2 DAI, decreased slightly until 4 DAI, and then increased thereafter. The treatments remained significantly higher than the water + no pathogen treatment. PPO activities increased in extract + pathogen treatment from 1 to 7 DAI. Enzyme activities increased dramatically during 0-1 DAI and then remained stable thereafter. Besides, the enzyme activities in extract + pathogen treatment were significantly higher than the extract + no pathogen treatment. The presence of both extract and pathogen was a sufficient condition to trigger the production of PPO in rice plants helping to prevent infection and multiplication of *Xoo*. PAL acts earlier in the phenylpropanoid pathway (increased at 1 DAI), whereas PPO functions later by oxidizing phenolic substrates (increased at 2 and 3 DAI). PPO utilizes phenolic compounds synthesized through the phenylpropanoid pathway as substrates, leading to the formation of ortho-quinones that exhibit toxic effects against *Xoo* (Bashan et al., 1987; Fuerst et al., 2014). This is concordant with the results of Xa et al. (2023), which indicated that the increase of PPO from 2-5 DAI.

In general, the findings indicated that the reduction in lesion length under net house conditions was associated with an increase in enzyme activities. Specifically, methanol *K. pinnata* leaves extract at 1.5% applied as seed soaking combined with foliar spraying showed similar disease reduction compared to that of the chemical control at all assessment time points (Figure 1). Additionally, the activities of POX, CAT, PAL, and PPO in treatments treated with the extract and challenged with *Xoo* increased early and remained higher than those in other treatments (Figure 2). By contrast, enzyme activities in the negative control remained low, which was associated with significantly longer lesion lengths. Although each enzyme has a different role, they function in a coordinated manner and collectively contribute to the defense response of rice plants against the pathogen. PAL was activated early from 0 to 1 DAI and was involved in the biosynthesis of salicylic acid, which participates in signaling pathways that activate defense mechanisms. PPO activity increased from 0 to 2 DAI and contributed to the production of *ortho*-quinones that inhibit pathogen growth. POX regulated ROS, particularly hydrogen peroxide with antimicrobial activity, whereas CAT reached its highest activity from 1 to 5 DAI, catalyzing hydrogen peroxide decomposition to prevent oxidative damage.

## 4. CONCLUSIONS

The results obtained under net house conditions indicate that the combination of seed soaking and foliar spraying effectively induced disease resistance in rice. Notably, seed soaking combined with foliar spraying at 14 DBI using 1.5% methanol extract of *K. pinnata* resulted in a disease-reducing effect comparable to the chemical control (Starner 20WP). This induced resistance was associated with changes in defense-related enzymes (POX, CAT, PAL, and PPO), where the activities of POX, CAT, and PPO generally increased, while PAL showed a decreasing trend in the presence of *Xoo*. Enzyme activities were further enhanced when the extract application was combined with pathogen inoculation. These findings highlight the potential of a combination of seed soaking and foliar spraying as an application strategy for controlling bacterial leaf blight; however, future studies should validate these results under field conditions and assess economic feasibility. In addition, detection of bioactive compounds in the extracts responsible for the observed resistance induction should also be investigated to understand the effects in detail. The disease reduction is associated with induced resistance through enhanced activities of the defense-related enzymes, but may also be contributed to by other mechanisms like direct antibacterial effects of the extracts, which could be an interesting topic for additional studies.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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