OPEN ACCESS Int. J. Phytopathol. 13 (02) 2024. 199-209 DOI: 10.33687/phytopath.013.02.4888

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Available Online at EScience Press
International Journal of Phytopathology
ISSN: 2312-9344 (Online), 2313-1241 (Print)
https://esciencepress.net/journals/phytopath

AQUEOUS *CHROMOLAENA ODORATA* LEAF EXTRACTS INDUCE RICE RESISTANCE AGAINST BACTERIAL LEAF BLIGHT

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ARTICLE INFO

Article History
Received: December 10, 2023
Revised: May 10, 2024
Accepted: June 12, 2024

Keywords
Chromolaena odorata
X. oryzae pv. *oryzae*
Bacterial leaf blight
Induced resistance
Oryza sativa

ABSTRACT

Aqueous *Chromolaena odorata* leaf extracts enhanced rice seed germination and seedling growth. Five extract concentrations (1, 2, 3, 4, and 5%, w/v) applied as seed soaking and foliar spraying were tested for their disease-reducing effects against rice bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae*. For foliar sparying, the extracts were applied using three different methods, *i.e.*, at 7 days before inoculation, at 14 days before inoculation and their combination. It was efficient to apply the 3% extract by seed soaking and foliar spraying at 14 days before inoculation to control BLB as it was the lowest concentration where the disease-reducing effects were observed until 21 DAI. The effects involved induced resistance. Indeed, activities of the four defense-related and antioxidant enzymes, *i.e.*, peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL), increased after extract application and reached higher levels with both extract application and pathogen inoculation. Activities of POX and CAT were induced earlier and stronger using seed soaking than foliar spraying while those of PPO and PAL increased earlier using foliar spraying than seed soaking. This suggests a combination of both application methods to obtain coordinate increases in activities of the four defense-related and antioxidant enzymes which could provide sufficiently protection to rice plants again the disease.

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INTRODUCTION

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most important diseases in rice fields, particularly in Vietnam (Mew *et al.*, 1993; Khoa, 2018). Various management methods for the disease have been investigated, *i.e.*, antibiotics (Khan *et al.*, 2012), antagonistic bacteria (Khoa *et al.*, 2016), genetic resistance (Kumar *et al.*, 2020), and induced resistance (Kagale *et al.*, 2004; Nisha *et al.*, 2012; Khoa *et al.*, 2017). Induced resistance is defined as the process of active resistance dependent on the host plant's physical and chemical barriers, activated by biotic or abiotic agents (Kloepper *et al.*, 1992; Walters *et al.*, 2005). Physically, induced resistance may involve in papilla formation, lignification, and callose accumulation which prevent the direct penetration of pathogen to plant tissues (Kang *et al.*, 2002; Latz *et al.*, 2018). Likewise, the productions of phenolic compounds (Santiago *et al.*, 2009; Fan *et al.*, 2017), phytoalexins (Cho and Lee, 2015; Singh and Chandrawat, 2017) and defense-related enzymes like peroxidases (POX), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) (van Loon *et al.*, 2006; Khoa *et al.*, 2017) may involve in induced resistance. POX possess numerous defensive functions in

plant resistance such as crosslinking of cell wall polysaccharides and regulation of the production of reactive oxygen species (ROS) (Hiraga *et al.*, 2001). Among the ROS, hydrogen peroxide (H_2O_2) plays an important role in plant-pathogen interactions (Shetty *et al.*, 2008) and its metabolism is tightly correlated with the antioxidant enzyme CAT which decomposes H_2O_2 at a rapid rate (Apel and Hirt, 2004). As a biotrophic pathogen, *Xoo* infection is inhibited by H_2O_2 (Yu *et al.*, 2016). PPO mediates phenolic oxidation to prevent plant disease development (Li and Steffens, 2002). PAL is a key enzyme in the phenylpropanoid pathway, it catalyzes the conversion of *L*-phenylalanine to trans-cinnamic acid, the initiate reaction in the biosynthesis of phenolic compounds, and generates lignin monomer which contributes to strengthening plant cell walls (Solekha *et al.*, 2020). Increases of PAL and PPO activities could assist rice plants to inhibit *Xoo* infection as they enhance the biosynthesis of secondary metabolites in rice tissues (Kagale *et al.*, 2004; Govindappa *et al.*, 2011; Nisha *et al.*, 2012; Khoa *et al.*, 2017; Hương *et al.*, 2018; Solekha *et al.*, 2020; Xạ *et al.*, 2023).

Several chemicals, bacteria and plant extracts can induce rice resistance against BLB. They include salicylic acid (Mohan Babu *et al.*, 2003), vitamin B_1 (Ahn *et al.*, 2005), *Bacillus* spp. (Udayashankar *et al.*, 2011), *Pseudomonas fluorescens* (Shivalingaiah and Umesha, 2013), methanol extract of *Datura metel* (Kagale et al., 2004), aqueous and methanol extract of *Adhatoda vasica* (Govindappa et al., 2011), aqueous extract of *Vitex negundo (Nisha et al., 2012)*, and aqueous extract of *Kalanchoe pinnata* (Khoa *et al.*, 2017; Hương *et al.*, 2018). Indeed, seed soaking using an aqueous *K. pinnata* extract exhibited disease-reducing effects until 21 days after inoculation (DAI) with the pathogen. Foliar spraying using this extract showed similar effects as activities of PAL and PPO increased at 2 and 4 DAI, respectively (Hương *et al.*, 2018; Xạ *et al.*, 2023).

Chromolaena odorata is a shrub, belonging to the family Asteraceae (Muniappan *et al.*, 2005). It is considered an invasive weed (Van Chi and Hop, 1999). Aqueous fresh leaf extract of this plant is styptic and is a folk remedy that is utilized as a treatment for human skin wounds (Olawale *et al.*, 2022; Van Chi and Hop, 1999). *C. odorata* extracts were found to protect rice plants against sheath blight (*Rhizoctonia solani*) (Khoa *et al.*, 2011; Rodríguez-Algaba *et al.*, 2015). Indeed, foliar spraying and seed soaking application of extracts of either fresh or dried leaves of *C. odorata* gave up to 68% reduction in sheath blight lesion lengths under controlled and semi-field conditions (Khoa *et al.*, 2011). Activity-guided separation of the *C. odorata* extracts indicated that the bioactive compounds are hydrophilic, low molecular weight compounds. Further purification yielded fractions with disease reducing effects of up to 72 % at 15 DAI and the effects involved induced resistance as activities of *β*-1,3-glucanase increased after extract application (Rodríguez-Algaba *et al.*, 2015).

This paper presents the disease-reducing effects under greenhouse conditions of aqueous *C. odorata* leaf extracts using seed soaking and foliar spraying to control rice BLB and investigating the involvement of induced resistance behind the observed disease reduction, focusing on activities of the four defense-related and antioxidant enzymes, *i.e.*, POX, CAT, PPO, and PAL.

MATERIALS AND METHODS

Materials

Healthy mature plants (one-year old) grown in the greenhouse of the Vinh Long University of Technology Education (Vinh Long Province, Vietnam) were harvested at 7:00 a.m. to ascertain their physiologically comparable tissues. Uniform mature leaves (aprox. 5 cm in length) were then collected under laboratory conditions to prepare aqueous extracts.

The susceptible rice cv. Jasmine 85 and the highly virulent isolate *Xoo* XCT-13 were kindly provided by the Plant Pathology Research Group, Molecular Biology Laboratory, Institute of Food and Biotechnology, Can Tho University (Can Tho City, Vietnam).

Preparation of Plant Extracts and Seed Treatment

Aqueous *C. odorata* leaf extracts were prepared as described by Khoa *et al.* (2017). Initially, the collected leaves were rinsed with sterile distilled water. They were then cut into small pieces and thoroughly mashed in sterile distilled water with a pestle and mortar. After 30 minutes of soaking, the mixes were filtered through cheesecloth to eliminate debris. All plant extracts collected were used immediately. Rice seeds were soaked in sterile distilled water at 50 - 55^{o}C for 30 minutes before being treated with plant extracts for 24 hours. The seeds were then incubated for 48 hours at 28 ± 2^{o}C before sowing.

Effects of *C. odorata* Leaf Extracts on Rice Seed Germination and Seedling Growth

The aim of this test is to desmonstrate that aqueous *C. odorata* leaf extracts have no adverse effects on rice seed

germination and seedling growth, using the methods described by Singh and Rao (1977) and Khoa *et al.* (2017). Five extract concentrations were tested [1, 2, 3, 4 and 5% (w/v)], sterile distilled water served as a control.A hundred seeds were evenly placed in ten rows (ten seeds/row) between two moist sheets of filter papers. These sheets were rolled and stored vertically in a plastic bag at 28 ± 2°C. After 3, 5 and 7 days, germination rates (% germinated seeds over total number of seeds) and the shoot and root lengths of seedlings were recorded.

Disease-Reducing Effects of Aqueous *C. odorata* Leaf Extracts on BLB Lesion Lengths under Greenhouse Conditions

The experiment was arranged in a completely randomized design with three replications. Three concentrations of aqueous *C. odorata* leaf extracts including 1, 2, 3, 4 and 5% (w/v) were investigated. For seed soaking treatment, rice seeds were soaked in *C. odorata* extracts for 24h. For foliar spraying treatment, the extracts were sprayed on rice leaves using three application methods, *i.e.*, at 14 days before inoculation (DBI), 7 DBI, and a combination of 14 and 7 DBI. The bactericide oxolinic acid (Starner 20 WP, Sumitomo Chemicals Co., Osaka, Japan) was used as a chemical control and water was used as an untreated control. Five milliliters of the bactericide (1 mg/mL) was applied per rice plant and sprayed three times at 5, 10, and 15 days after pathogen inoculation (Khoa *et al.*, 2017; Hương *et al.*, 2018).

Inoculum Preparation

Xoo was cultured on modified Wakimoto's medium at 28 ± 2 °C (Karganilla *et al.*, 1973) for 72 hours. A loopful (2-mm diameter) of bacterial cells was transferred into 10mL sterile distilled water. Cell density of the bacterial suspension was adjusted to 10^9 colony-forming units (CFU/mL) (Khoa, 2005; Khoa *et al.*, 2017).

Pathogen Inoculation and Disease Assessment

The inoculation of rice plants was conducted at 45 days after sowing (DAS) by leaf-clipping method (Kauffman et al., 1993). A pair of scissors was sterilized with 70% (v/v) ethanol and submerged in the inoculum that was utilized to clip 5 fully expanded uppermost leaves (approximately 2-3 cm from leaf tips) (Khoa *et al.*, 2017). The disease was assessed by measuring lesion lengths (mm) at 7, 14 and 21 DAI. The experiment was repeated thrice with five replications (three plants with 5 leaves per plant).

Assays of Enzyme Activities

For seed soaking, four treatments were included in these assays (1) seed soaking with aqueous *C. odorata* leaf extract and inoculation with *Xoo* (extract + inoculated); (2) seed soaking with aqueous *C. odorata* leaf extract and no inoculation with *Xoo* (extract + unnoculated); (3) seed soaking with distilled water and inoculation with *Xoo* (water + inoculated); (4) seed soaking with distilled water and no inoculation with *Xoo* (water + uninoculated).

For foliar spraying, four treatments were included in these assays (1) foliar spraying with aqueous *C. odorata* leaf extract and inoculation with *Xoo* (extract + inoculated); (2) foliar spraying with aqueous *C. odorata* leaf extract and no inoculation with *Xoo* (extract + uninoculated); (3) foliar spraying with distilled water and inoculation with *Xoo* (water + inoculated); (4) foliar spraying with distilled water and no inoculation with *Xoo* (water + uninoculated).

Sample Collection and Enzyme Extraction

Rice leaves were collected from 0 to 7 DAI (once a day) which were rapidly frozen in liquid nitrogen and stored at -80°C. For enzyme extraction, 1 g of rice leaf sample was crushed in liquid nitrogen using a Retsch mixer mill (MM200, Retsch Co., Germany). An amount of 0.1 g sample was taken at each time point and homogenized in 1.5 mL of buffer solution (a specific buffer was used for each enzyme in the experiment). The homogenate was centrifuged at 10,000 rpm and 4 °C for 30 minutes. The supernatant containing enzymes were collected and kept on ice during the investigation. Each experiment was performed in triplicate.

Peroxidase Assay

POX activity was recorded at 470 nm (Labomed 2602, UV/Vis spectrometer, PerkinElmer) by measuring the rate at which brown tetraguaiacol was converted from guaiacol following the method described by Hammerschmidt *et al.* (1982) and Khoa *et al.* (2017). The experiments were done with three replications. POX was extracted using 0.1 M sodium potassium phosphate buffer, pH 6.5. The reaction mixture consisted of 1.6 mL 0.05 M H_2O_2 solution, 0.15 mL 0.15 M guaiacol solution and 0.15 mL enzyme-containing extract. POX activity was expressed as changes in absorbance at 470 per min per g fresh leaf tissue. The blank sample was valued as 0 at 470 nm, consisting of 1.6 mL 0.05 M H_2O_2 solution, 0.15 mL 0.15 M guaiacol solution, 0.15 mL 0.1M sodium phosphate buffer solution, pH 6.5.

Catalase Assay

CAT activity was expressed as changes in absorbance at

240 nm of the decreased concentration of H_2O_2 followed the method described by Beers and Sizer (1952) and Khoa *et al.* (2017). The experiments were done with three replications. CAT was extracted using 0.1 M sodium potassium phosphate buffer, pH 7.0. The reaction mixture consisted of 1.75 mL 0.1 M H_2O_2 solution and 0.15 mL enzyme-containing extract. The absorbance of all samples was recorded at 240 nm per min per g fresh leaf tissue. The blank sample was valued as 0 at 240 nm, consisting of 1.9 mL extract buffer solution.

Polyphenol Oxidase Assay

PPO activity was evaluated by the rate of conversion of colorless catechol to brown benzoquinone at 490 nm following the method described by Mayer (2006) and Khoa *et al.* (2017). The experiments were done with three replications. PPO was extracted using 0.1 M sodium phosphate buffer, pH 6.5. The reaction mixture consisted of 1.75 mL 0.2 M catechol solution and 0.15 mL enzyme-containing extract. The absorbance of all samples was recorded at 490 nm per min per g fresh leaf tissue. The blank sample consisted of 1.75 mL 0.2 M catechol solution and 0.15 mL 0.1 M sodium phosphate buffer, pH 6.5.

Phenylalanine Ammonia Lyase Assay

PAL activity was assessed by the rate of deamination of *L*-phenylalanine to produce trans-cinnamic acid and absorbance of the product was recorded at 290 nm following the method described by Dickerson *et al.* (1984) and Hương *et al.* (2018). The experiments were done with three replications. PAL was extracted using 0.1 M sodium borate buffer, pH 8.7. The reaction mixture consisted of 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 distilled water and 0.2 mL of the enzyme extract. After allowing the reaction to occur in a test tube at 32 ± 1°C for 40 min, 0.2 mL of 5.0 N HCl solutions was used to terminate the reaction. The absorbance of all samples was recorded at 290 nm per min per g fresh leaf tissue. The blank sample was made by 0.7 mL of 0.1 M sodium borate buffer, pH 8.7, 1 mL 0.1 M *L*-phenylalanine solution and 0.15 mL distilled water.

Data Analyses

All experiments were done in a completely randomized design and each treatment had three replications (five plants/replication). Three independent experiments were carried out. For greenhouse experiments, lesion lengths were presented using means of those from five inoculated leaves/plant. Data were analyzed by analysis of variance without transformation using PC-SAS® version 9.1 (SAS Institute Inc., Cary, NC, USA). All hypotheses were rejected at $P \leq 0.05$.

For enzyme activity assays, mean changes in absorbance per min per mg protein of fresh leaf tissue of each treatment were calculated and line charts representing changes in enzyme activity of four treatments for 7 days were made using Microsoft Excel 2016.

RESULTS

Effects of Aqueous *C. odorata* Leaf Extracts on Rice Seed Germination and Seedling Growth

Seed germination and seedling growth were generally enhanced after extract application (Table 1). Using the 4% and 5% extracts, root lengths and shoot lengths were significantly increased compared to those of the water control. After 7 days, application of the extract did not show any adverse effects on rice seed germination. Indeed, the germination rates of rice seeds treated with the extracts were not significantly different compared to those of the water control. The aqueous *C. odorata* leaf extracts could therefore be tested for their disease-reducing effects against rice BLB using seed soaking and foliar spraying under greenhouse conditions.

Effects of Aqueous *C. odorata* Leaf Extracts on BLB Lesion Lengths under Greenhouse Conditions

Using seed soaking application, the 3%, 4%, and 5% extracts significantly reduced lesion lengths on rice leaves at 21 dai (336.3 ± 15.7 mm, 317.4 ± 28.9, and 319.3 ± 9.4, respectively). Significant reduction in lesion lengths was shown between the extract treatments and the untreated control (387.6 ± 39.5 mm) while no significant difference was observed among the mean lesion lengths of the extract treatments and the chemical control (305.1 ± 7.4 mm) (Figure 1A).

Using foliar spraying at 7 DBI, the 3%, 4%, and 5% extracts showed significant differences in mean lesion lengths (345.4 ± 11.1 mm, 333.8 ± 29.5 mm, and 319.4 ± 11.4 mm, respectively) compared to that of the untreated control (387.6 ± 39.5 mm) (Figure 1B). The 3%, 4%, and 5% extracts sprayed at 14 DBI also reduced mean lesion lengths significantly (344.5 ± 11.0 mm, 314.1 ± 22.2 mm, and 312.5 ± 6.1 mm, respectively) (Figure 1C). Using a combination of foliar spraying at 14 and 7 DBI, all extracts tested provided better protection compared to the untreated control did (Figure 1D).

Table 1. Effects of aqueous *Chromolaena odorata* leaf extracts on rice seed germination and seedling growth 3, 5 and 7 days after soaking.

Treatments	3 days		5 days		7 days		Germination rates (%)
	SL	*RL*	*SL*	*RL*	*SL*	*RL*	
1% (w/v)	5.6±1.3 a	21.6±1.0 cd	33.0±2.6 a	58.6±6.6 ab	57.0±2.5 bc	90.3±3.3 b	83±2.9 a
2% (w/v)	6.4±2.6 a	13.2±1.3 ab	32.7±4.8 a	64.4±7.3 b	52.6±3.2 ab	90.7±9.2 b	88±5.8 a
3% (w/v)	4.9±0.6 a	14.8±2.7 ab	32.1±2.7 a	68.5±10.1 b	53.3±2.5 ab	93.9±8.7 b	88±7.6 a
4% (w/v)	10.9±2.4 b	18.7±6.6 bc	30.5±3.4 a	60.0±4.7 ab	61.3±4.5 cd	114.8±8.2 c	83±7.6 a
5% (w/v)	10.8±0.4 b	27.2±2.0 d	33.6±2.3 a	69.3±6.7 b	64.9±4.1 d	111.5±12.1 c	90±10.0 a
Control	5.2±1.1 a	11.5±2.4 a	28.7±1.6 a	46.2±8.2 a	47.5±4.2 a	68.0±10.6 a	83±2.9 a

SL: shoot lengths (mm); RL: root lengths (mm). At the same time point, means followed by same letters within a column are not significantly different at $P \leq 0.05$ by Duncan's multiple range test. Each value is the mean ± standard deviation of three replications.

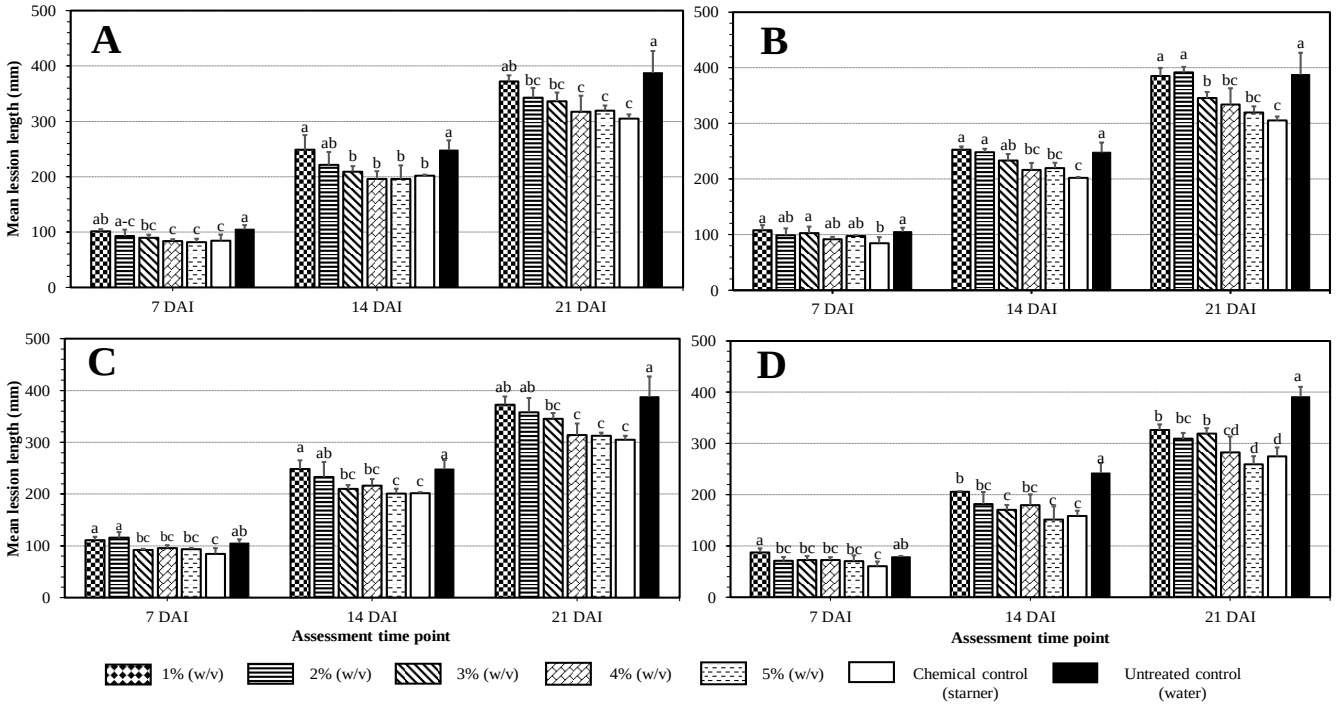

Figure 1. Mean lesion lengths (mm) of bacterial leaf blight on 45-day-after-sowing inoculated leaves of rice cv. Jasmine 85. Five concentrations of the extract were tested, *i.e.,* 1, 2, 3, 4 and 5% (w/v). These extracts were applied as seed soaking for 24 h in aqueous *Chromolaena odorata* leaf extracts (A); these extracts were sprayed at 7 days before inoculation (B); 14 days before inoculation (C) combination of 14 and 7 days before inoculation (D). At the same time point, bars with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test. Each value is the mean ± standard deviation of three replications. DAI: days after inoculation.

The 3% extract was selected to investigate the involvement of induced resistance in the observed disease reduction, focusing on activities of the four enzymes, *i.e.*, POX, CAT, PPO, and PAL., as it provided strong protection against BLB until 21 DAI using seed soaking and foliar spraying at 14 DBI.

Enzyme Activities

Peroxidase activities

POX activities generally increased from 1 to 4 DAI in the two treatments with pathogen inoculation (Figure 2). The activities increased dramatically with pathogen inoculation 0 - 4 DAI, from 22.79 ± 1.27 to 64.83 ± 1.86 in

the extract + inoculated treatment and from 20.97 ± 1.42 to 32.82 ± 1.09 in the water + inoculated treatment. Extract application induced POX activities slightly 0-6 DAI (from 22.84 ± 1.54 to 28.44 ± 0.99 in the extract + uninoculated treatment) but the activities reached higher levels 2-6 DAI with the presence of both pathogen inoculation and extract application(from 37.91 ± 3.04 to 85.32 ± 1.66).

When rice seeds were soaked with 3% extract and rice plants were inoculated with the pathogen, POX activities increased 4.1 times 1 to 4 DAI and reached the highest level at 4 DAI (83.79 ± 3.12) (Figure 2A). Meanwhile, foliar spraying using 3% extracts, POX activities increased 2.9 times 1 to 4 DAI and reached the highest level at 6 DAI (85.32 ± 1.66) (Figure 2B). POX activities increased with the presence of both extract application and pathogen inoculation. Seed soaking was found to increase POX activity earlier and stronger as compared to foliar spraying.

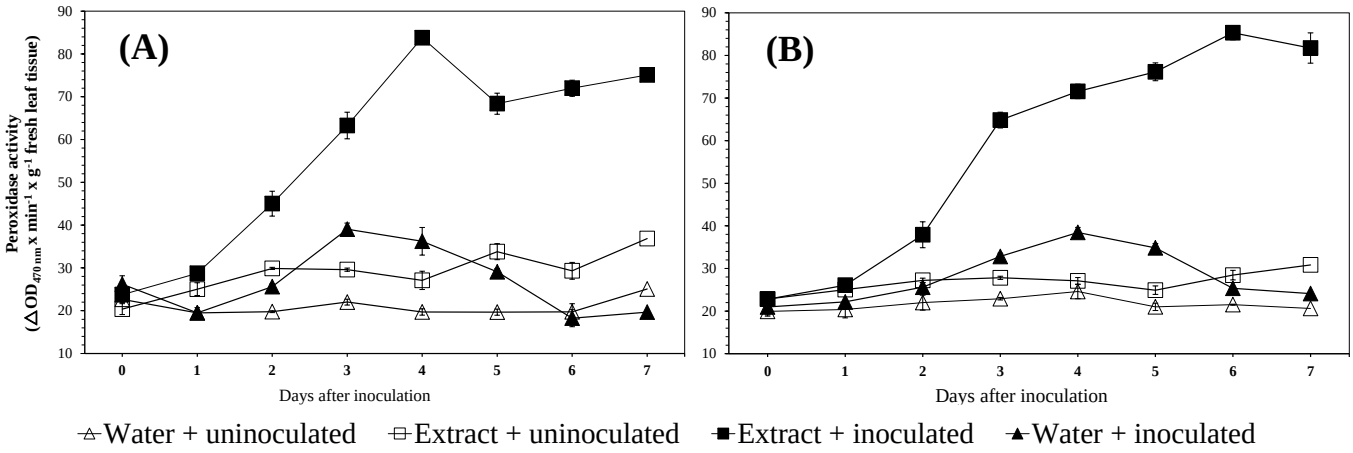

Figure 2. Peroxidase (POX) activities in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xanthomonas oryzae* pv. *oryzae* at 45 days after sowing. Seed soaking using 3% of aqueous *Chromolaena odorata* leaf extracts (A). Foliar spraying using 3% of aqueous *C. odorata* leaf extracts at 14 days before inoculation (B). Each value is the mean ± standard deviation of three replications.

Catalase activities

CAT activities generally increased from 0 to 5 DAI in the treatments with pathogen inoculation and extract application (Figure 3). When rice seeds were soaked with 3% extract and rice plants were inoculated with the pathogen, CAT activities increased 3.0 times at 1 DAI (6.00 ± 0.53) and reached the highest level at 5 DAI (18.13 ± 0.22)(Figure 3A). Meanwhile, using foliar spraying, CAT activities increased 2.7 times at 1 DAI (6.80 ± 0.35) and reached the highest level at 6 DAI (18.48 ± 0.45) (Figure 3B). Similar to POX, CAT activities significantly increased with the presence of both pathogen inoculation and extract applicatio. Foliar spraying was found to increase CAT activities earlier and stronger as compared to seed soaking.

Polyphenol oxidase activities

PPO activities generally increased from 0 to 7 DAI in both water + uninoculated and extract + uninoculated treatments (Figure 4). PPO activities increase earlier, 0-2 DAI from 1.98 ± 0.24 to 3.44 ± 0.23 in the water + inoculated treatment and from 1.63 ± 0.16 to 3.60 ± 0.12 in the extract + inoculated treatment. The presence of both extract application and pathogen inoculation induced PPO activities significantly 0-4 DAI (from 1.98 ± 0.24 to 4.19 ± 0.04) using seed soaking (Figure 4A) and 0-3 DAI (from 1.83 ± 0.07 to 4.72 ± 0.07) using foliar spraying (Figure 4B). PPO activities increased significantly with the presence of both pathogen inoculation and extract application. Foliar spraying was found to increase PPO activities stronger as compared to seed soaking.

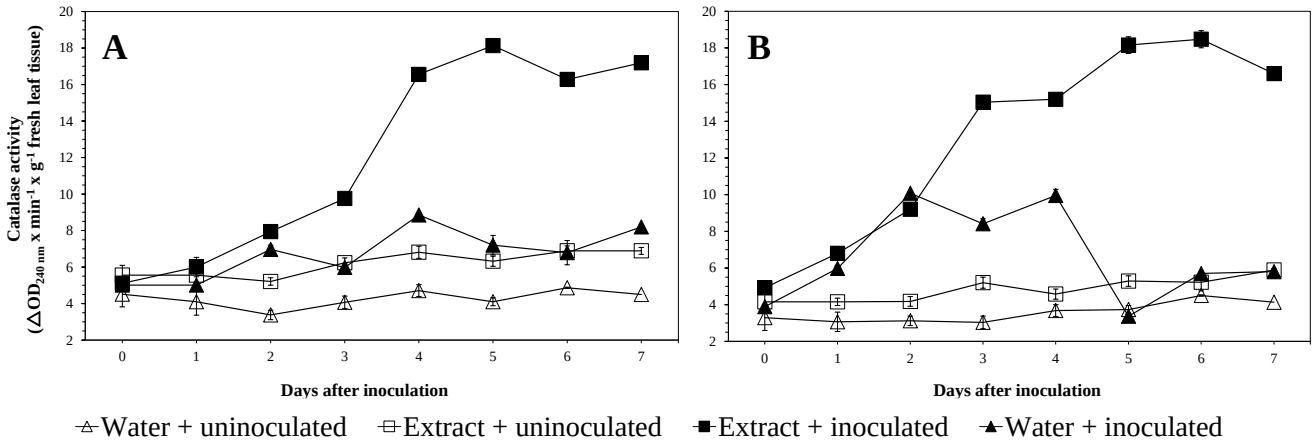

Figure 3. Catalase (CAT) activities in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xanthomonas oryzae* pv. *oryzae* at 45 days after sowing. Seed soaking using 3% of aqueous *Chromolaena odorata* leaf extracts (A). Foliar spraying using 3% of aqueous *C. odorata* leaf extracts at 14 days before inoculation (B). Each value is the mean ± standard deviation of three replications.

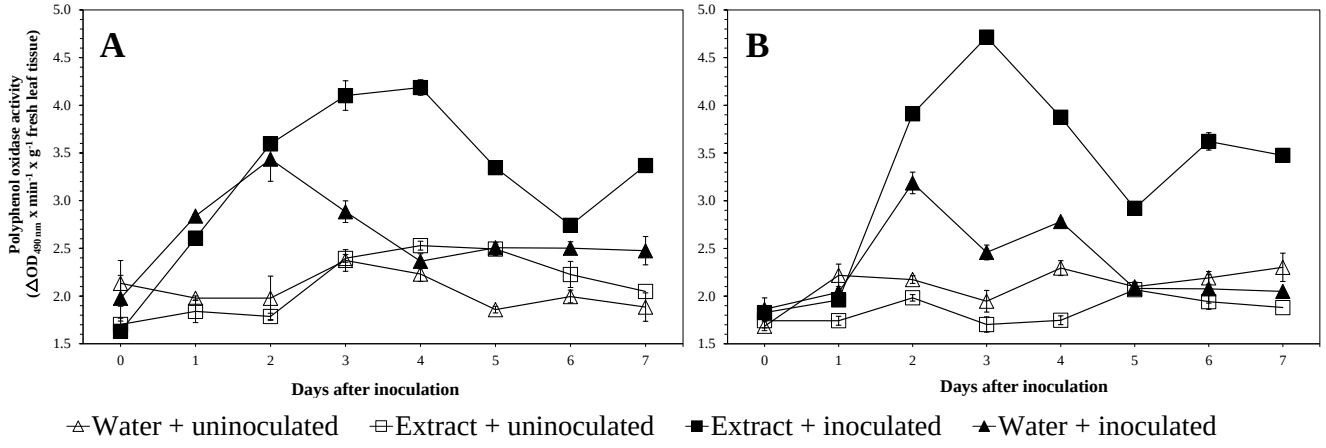

Figure 4. Polyphenol oxidase (PPO) activities in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xanthomonas oryzae* pv. *oryzae* at 45 days after sowing. Seed soaking using 3% of aqueous *Chromolaena odorata* leaf extracts (A). Foliar spraying using 3% of aqueous *C. odorata* leaf extracts at 14 days before inoculation (B). Each value is the mean ± standard deviation of three replications.

Phenylalanine ammonia lyase activities

PAL activities generally increased 0-2 DAI with pathogen inoculation, from 0.15 ± 0.01 to 0.22 ± 0.01 in the water + inoculated treatment and from 0.13 ± 0.01 to 0.26 ± 0.01 in the extract + inoculated treatment (Figure 5). The presence of both extract application and pathogen inoculation induced PAL activities significantly 0-4 DAI, from 0.13 ± 0.01 to 0.36 ± 0.01 using seed soaking (Figure 5A) and 0-3 DAI, from 0.16 ± 0.01 to 0.33 ± 0.01 using foliar spraying (Figure 5B). Similar to POX, PAL activities increased strongly 0-3 DAI in foliar spraying treatment.

DISCUSSION

Seed soaking and foliar spraying using aqueous extracts of *C. odorata* reduced BLB lesion lengths (Figure 1). The disease-reducing effects help maintain leaf areas required for photosynthesis and CO_2 fixation in rice plants, thereby reducing yield loss (Kumar *et al.*, 2013). These results were consistent with the studies using seeds soaking with *J. adhatoda* L. extracts (Govindappa *et al.*, 2011), seeds soaking with *V. negundo* extracts (Nisha *et al.,* 2012), foliar spraying with *D. metel* extracts (Kagale *et al.*, 2004), seeds soaking with *K. pinnata* extracts (Khoa *et al.*, 2017; Xạ *et*

al., 2023) and foliar spraying with *K. pinnata* extracts (Hương *et al.*, 2018; Xạ *et al.*, 2023). One of the common methods to study the involvement of induced resistance behind the reduction of plant diseases is to quantify changes in levels of PR-proteins and other defense-related enzymes in plant tissues. The enzyme assays have been applied to study a number of plant-microbe systems including rice-*Xoo* (Kagale *et al.*, 2004; Nisha *et al.*, 2012; Khoa *et al.*, 2017; Xạ *et al.*, 2023).

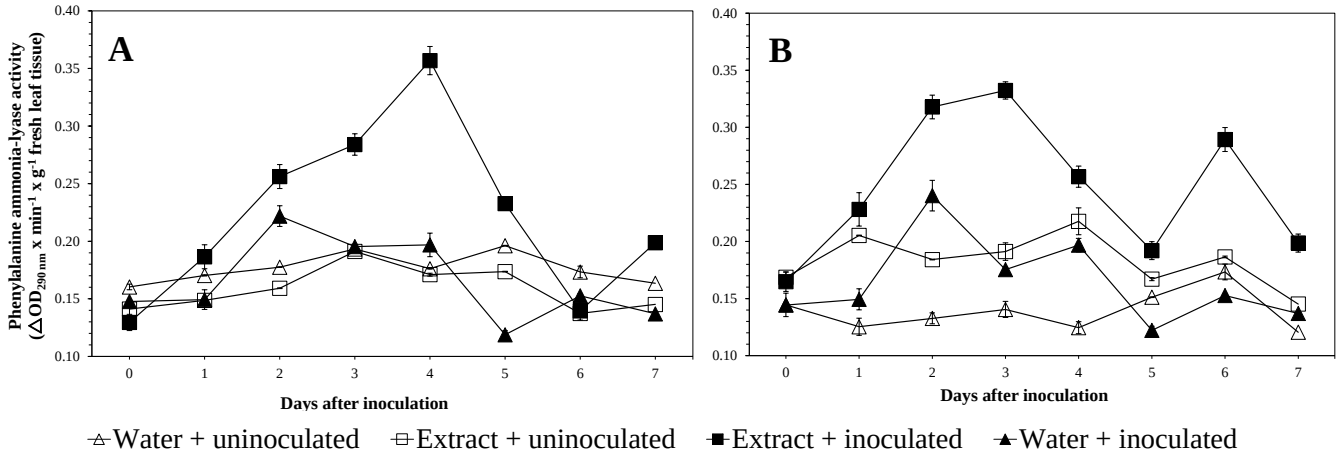

Figure 5. Phenylalanine ammonia lyase (PAL) activities in leaf tissues of rice cv. Jasmine 85 with and without challenge inoculation of *Xanthomonas oryzae* pv. *oryzae* at 45 days after sowing. Seed soaking using 3% of aqueous *Chromolaena odorata* leaf extracts (A). Foliar spraying using 3% of aqueous *C. odorata* leaf extracts at 14 days before inoculation (B). Each value is the mean ± standard deviation of three replications.

Using a similar approach, efficient induction of resistance was shown using aqueous *C. odorata* leaf extracts to protect rice plants against BLB. Increased activities of the four defense-related and antioxidant enzymes POX, CAT, PPO and PAL were shown in Figures 2 to 5. Data from the assays suggest that enzyme-mediated defense mechanisms were activated 1-5 DAI, which complies with previous studies on the infection process and growth kinetics of *Xoo* in rice leaves. Indeed, bacterial multiplication commences at 2 DAI and the populations increase steadily until 4 DAI, the pathogen after sufficient growth could invade rice vascular systems and continue to multiply until 14 DAI (Swings *et al.*, 1990; Niño-Liu *et al.*, 2006). Increased activities of defense-related and antioxidant enzymes 1-5 DAI could therefore reduce the *Xoo* inoculum level at the infection stage and subsequent development of the pathogen in rice plants thus reduce BLB lesion lengths (Figure 1).

In general, there were coordinated increases in activities of the defense-related and antioxidant enzymes POX, CAT, PPO, and PAL in rice tissues inoculated with *Xoo* after application of aqueous *C. odorata* leaf extracts. Similar activity increases of the enzymes were shown in different studies on induced resistance against BLB (Kagale *et al.*, 2004; Govindappa *et al.*, 2011; Nisha *et al.*, 2012; Chanprapai and Chavasiri, 2017). The observed disease reduction therefore involves induced resistance.

Data obtained from the POX assay suggest the active role of this enzyme in induced resistance against BLB. Early and significant increases in POX activities were observed with the presence of both extract application and pathogen inoculation (Figure 2). In this study, pathogen inoculation did not give any clear effects on CAT activities while the presence of both extract application and pathogen inoculation induced the activity of this enzyme significantly 1-5 DAI (Figure 3). Through this ability, *Xoo* might have triggered the production of the host CAT to scavenge the toxic H_2O_2 (Yu *et al.*, 2016). This agrees with the results of previous studies. For instance, Kagale *et al.* (2004) and Govindappa *et al.* (2011) showed a continuous increase in POX activities from 24 to 96 hours after inoculation (HAI) using foliar spraying with methanol *D. metel* leaf extract and seed soaking with *A. vasica* extracts. Nisha *et al.* (2012) reported a continuous increase of POX activities from 24 to 120 HAI using seed soaking with water extract and from 24 to 100 HAI using

methanol extract of *V. negundo*. Khoa *et al.* (2017) also observed an increase in POX activity 1-6 DAI and CAT enzyme activities 1-6 days using seed soaking with aqueous *K. pinnata* leaf extracts. Pal *et al.* (2011) also observed continuous increases of CAT activities 0-96 HAI when rice seeds were soaked with extracts of *Ocimum sanctum* and *Cymbopogan citrus*.

The key enzyme in the phenylpropanoid pathway like PAL could catalyse the first reaction in the biosynthesis of phenolic compounds and generate lignin monomer contributing to strengthening plant cell walls (Solekha *et al.*, 2020). Phenolic compounds generated from the phenylpropanoid pathway are PPO substrates (Araji *et al.*, 2014). PPO is a copper-containing bifunctional enzyme that hydroxylates and oxidizes phenolic compounds to highly reactive *ortho*-quinones against biotrophic pathogens like *Xoo* (Constabel and Barbehenn, 2008). With the presence of both extract application and *Xoo* inoculation, activities of both PPO and PAL were higher compared to those of the untreated control (Figure 4 and 5). PPO was induced stronger (1-4 DAI) than PAL (0-4 DAI). These results support the findings of previous studies. For example, Kagale *et al.* (2004) and Govindappa *et al.* (2011) showed continuous increases in PAL activities 24-72 HAI. Khoa *et al.* (2017) reported a similar increase of 1-3 DAI when rice seeds were soaked with aqueous *C. odorata* leaf extracts.

CONCLUSION

The disease-reducing effects of aqueous *C. odorata* leaf extracts against rice bacterial leaf blight using seed soaking and foliar spraying were shown under greenhouse conditions. The effects of seed soaking and foliar spraying 14 DBI using the extracts were significantly different from that of the untreated control while the former provided the strongest protection at all disease assessment timepoints (7, 14 and 21 DAI). It was shown that 3% was the lowest concentration of *C. odorata* extracts where the disease-reducing effects were observed until 21 DAI. The disease reduction involves induced resistance as activities of the four defense-related and antioxidant enzymes, *i.e.*, POX, CAT, PPO and PAL, increased after extract application and reached high levels with the presence of both extract application and pathogen inoculation. Activities of POX and CAT were induced earlier and stronger using seed soaking than foliar spraying while those of PPO and PAL increased earlier using foliar spraying than seed soaking. These will serve as a basis to select appropriate extracts or bioactive compounds in the extracts to effectively control rice bacterial leaf blight thus reduce the current use of chemicals to protect human health and the ecosystem.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.

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