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Research Article

The Effect of Tapak Liman (*Elephantopus scaber* L.) Extract on *Xa4* Gene Expression in Rice IR64 Infected by Bacterial Leaf Blight (*Xanthomonas oryzae*)

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Abstract: Bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), represents a significant threat to rice (*Oryza sativa*) production. Induce plant resistance has emerged as a promising control strategy. The extract of Tapak Liman (*Elephantopus scaber*) has been considered a promising agent due to its antimicrobial properties, with several of its compounds showing its potential as inducers of plant resistance. This study aimed at elucidating the impact of Tapak Liman extract on the expression of resistance *Xa4* gene in rice that plays a crucial role in the synthesis mechanism leading to cell wall thickening. To explore this effect, we analyzed *Xa4* gene expression using the reverse transcription-polymerase chain reaction (RT-PCR) technique, followed by a semi-quantitative descriptive analysis. Our results demonstrate that the application of Tapak Liman extracts at a concentration of 10 mg/ml significantly upregulates *Xa4* gene expression in the IR64 compared with other concentrations, 1 mg/ml or 5 mg/ml. Furthermore, the observed higher expression of the *Xa4* gene persists until 5 days after pathogen inoculation, which is also implicated with a less developed lesion on rice leaves by 76% compared with the control.

1. INTRODUCTION

Bacterial Leaf Blight (BLB) is caused by the pathogen *Xanthomonas oryz*ae pv. *oryzae* (*Xoo*), which attacks rice (*Oryza sativa* L) around the world, including in Indonesia (Jiang *et al*. 2020). *Xoo* enters plants through wounds on the leaves and then develops in plant tissues to cause symptoms such as spots/lesions parallel to the edges of the leaves and will continue to spread to the entire leaf resulting in the damage to the growing point and the death of the rice plant (Ke *et al*., 2017). Based on the impact caused by this disease, it is necessary to carry out appropriate control to suppress the development of the *Xoo* pathogen.

One of the strategies for controlling the BLB is through the induction of rice plant resistance (Liu & Wang, 2016; Fiyaz *et al*., 2022). A wide variety of biotic and abiotic agents (stimuli) can induce disease resistance in plants (Walter *et al*., 2013). Exogenous application of natural

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or synthetic chemicals to plants can activate their resistance and boost the innate defenses to disease (Nadhira *et al*., 2021; Liu & Wang, 2016; Krzyzaniak *et al*., 2018; Honig *et al*., 2023). Plant extracts are one type of plant-resistance-inducing substance that can be employed (Abo-Elyousr *et al*., 2020).

The Tapak Liman (*Elephantopus scaber*) is an ethnobotanical species in the Asteraceae family containing bioactive compounds that are thought to induce resistance in rice plants to BLB because it can inhibit the growth of Xoo (Rejeki *et al*., 2017). *E. scaber* has been reported to contain biochemical flavonoids, terpenoids, saponins, tannins, carbohydrates, and proteins (Kabiru & Por, 2013). In addition, phenylpropanoids and flavonoids play an important role in plant development by developing resistance to various biotic and abiotic stress (Ramaroson *et al*., 2020).

Induction of plant resistance is also considered to be able to activate genes related to plant resistance responses directly and indirectly, as well as priming cells that cause stronger elicitation of resistance (Walters *et al*., 2005). Rice was identified as having 46 types of natural resistance genes against Xoo (*Xa* or *xa* genes) (Arunakumari *et al*., 2016). Each of these genes has a specific resistance rule to the Xoo pathotype (Jiang *et al*. 2020). The *Xa* genes which have an important function in controlling the Xoo, especially in Asia, are the *Xa4*, *Xa3/Xa26* genes. The *Xa4* gene itself has the function of activating the expression of the *CesAs* gene which can thicken cell walls so that plants are more resistant to Xoo bacteria (Mazerai *et al*., 2018). However, the level of gene expression in each plant varies depending on the variety or cultivar of the plant in expressing the resistance gene. As seen in previous studies, IR64 variety rice is very susceptible to HDB disease strains III, IV, and VIII and has a high incidence rate of 61%, where IR64 expresses the *Xa4* gene before Xoo inoculation, but expresses the *Xa10* gene and *xa13* gene after Xoo inoculation (Nadhira *et al*., 2022).

Therefore, based on the case study above, the expression of the *Xa4* resistance gene needs to be quantified to identify that the *E. scaber* extract can induce the resistance gene of the IR64 variety Rice plant infected with the BLB.

2. MATERIAL and METHODS

2.1. Planting and Seedling Material

Rice IR64 seeds were disinfected using 10% sodium hypochlorite and rinsed with distilled water and soaked for 24 hours. Seeding was carried out on containers filled with paddy soil for 14 days. Rice seeds were then transferred to the planting medium of paddy soil in polybags 15 × 15 × 7 cm to the age of 21 days after planting where each polybag contains 10 plants.

2.2. Preparation of *E. scaber* Extract

A total of 50 grams of simplicial powder of *E. scaber* leaves was mixed with 150 mL of methanol solvent and shaken for 48 hours. The suspension of the *E. scaber* in methanol was then passed through a Whatman paper to obtain a debris-free solution. The solution was evaporated using an evaporator rotary device (Hahnvapor HN 2005 V-N, Korea). The remaining solvent in the extract was evaporated using an oven with a temperature of 45°C until a paste extract was obtained.

2.3. Application of *E. scaber* Extract

The application of *E. scaber* extract was carried out on rice plants aged 14 days after sowing. The doses of *E. scaber* extract given were 1 mg/ml, 5 mg/ml, and 10 mg/ml with an application volume of 10 mL per polybag. The application of salicylic acid (SA) 10 mM was used as a positive control and distilled water as a negative control.

2.4. Preparation of the Inoculum and Inoculation of Pathogens

Xoo strain H01 was obtained from the collection of the Plant-Microbe Interaction Laboratory,

CDAST, University of Jember. Xoo isolates were grown on liquid media yeast extract dextrose at a temperature of 28 °C for 24 hours (Rejeki *et al*., 2021). The inoculum obtained is then diluted with distilled water until it reaches an optical density (OD600) equal a 10^8 CFU per milliliter. Inoculation of the pathogen was carried out on rice aged 7 days after planting (DAP) using the leaf clipping method by dipping sterile scissors in the inoculum suspension which was then used to cut 2-3 cm of the tip of the rice leaf (Nadhira *et al*., 2022).

2.5. RNA Total Isolation

Rice leaves were harvested on day 0 (before Xoo inoculation), day 3rd, and day 5th (after Xoo inoculation). A total of 50-100 mg of rice leaf samples was ground to a fine powder with liquid nitrogen. Total RNA was isolated using Total RNA Mini-kit (Plant) (Geneaid, Taiwan) and pure RNA samples were visualized by electrophoresis on a 1% agarose gel.

2.6. The cDNA (Complementary DNA) Synthesis

The pure total RNAs were reverse-transcribed into complementary DNA (cDNA) using the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The reverse transcription process was carried out by the one-step protocol including gDNA removal according to the manual guideline. Before cDNA synthesis, the 4× DN Master Mix (containing 50:1 gDNA remover) was mixed with a 2 µL RNA template (0.5 µg) and adjusted with the nuclease-free water to a total of 8 µL. About 5 minutes after incubation at 37 °C, about 8 µl of pure RNA was added with 2 µl 5× RT Master mix. Furthermore, incubation was carried out with a program at a temperature of 37 °C for 30 minutes, and then inactivated (reverse transcriptase inactivation) at a temperature of 98°C for 5 minutes.

2.7. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The cDNA sample was subsequently confirmed using polymerase chain reaction (PCR). About 2 µl of cDNA were added with 25 µl of MyTaq HS Red Mix master mix (Bioline, UK) and 2 µl of Forward and Reverse primers of 2 µl each, distilled water added by 19 µl with a total volume of 50 µl. Primers used were Xa4 Forward (5'-ATCGATCGATCGATCTCACGAGG-3') and Reverse (5'-TGCTATAAAAGGCATTCGG-3') responsible for amplicon with the size of 150 bp (Rasmiyana *et al*., 2019). *β-actin* is used as an internal control gene or housekeeping gene to normalize the gene expression with Forward primer 5'-TGTATGCCAGTGGTCGTACCA-3' and Reverse primer of 5'-CCAGCAAGGTCGAGACGAA-3' responsible for amplicon with the size of 121 bp (Nadhira et al., 2022). DNA amplification was carried out in 25 cycles with pre-denaturation conditions of 95 °C for 3 minutes, denaturation of 95°C for 30 seconds, annealing of 53 °C (for *Xa4*) or 56 °C (for *β-actin*) for 30 seconds, temperature elongation of 72 °C for 1 minute, and final elongation of 72 °C for 5 minutes. RT-PCR results are subsequently electrophoresis on agarose gel 1%. The results of the RT-PCR band visualization were observed in 1% of agarose gel and quantified using ImageJ software (Hazman, 2022) by comparing the ratio of the target genes to the housekeeping gene (β-actin gene).

2.8. Evaluation of the Severity of the Disease

The BLB severity of rice plant disease was observed at 14 days after inoculation of the pathogen using a lesion length scale, namely resistant ≤ 3.0 cm; medium resistance 3.0 – 6.0 cm; moderately vulnerable 6.0 – 9.0 cm; and vulnerable to >9.0 cm (Nadhira *et al*., 2022).

2.9. Data Analysis

The data obtained were then carried out analysis using semi-quantitative descriptive analysis. Disease severity data was further statistically analysis using Duncan Multiple Range Test (DMRT) for the significance (p<0.05) using Microsoft Excel 2019.

3. RESULTS

3.1. The *Xa4* Gene Expression

The RT-PCR results showed that the *Xa4* gene indicated different levels of expression according to the *E. scaber* dose treatment and the harvest period both before Xoo inoculation and after Xoo inoculation. It is characterized by the difference in the brightness level of the *Xa4* gene DNA band when compared to *β-actin* as a housekeeping gene (Figure 1A). Gene expression analysis using ImageJ software showed that *Xa4* values tend to fluctuate. The application of *E. scaber* extract at a 10 mg/ml both after and before inoculation of the Xoo pathogen showed the highest *Xa4* gene expression value compared to other treatments, especially when compared to salicylic acid (SA) and dH_2O treatment (Figure 1B). In contrast, the treatment of 1 mg/ml showed the lowest expression of the *Xa4* gene, especially on the fifth day after inoculation (Figure 1B). Gene expression values tended to decrease on the fifth day after pathogen inoculation at the 10 mM salicylic acid (SA) treatment, dH_2O, and 1mg/ml of *E. scaber* dose but increased at the 5mg/ml and 10 mg/ml *E. scaber* dose treatments. In addition, *Xa4* gene expression at the SA of 10 mM treatment also showed the lowest *Xa4* gene expression value at the overall harvest time in all treatments, especially in harvesting before Xoo inoculation and the third day after Xoo inoculation.

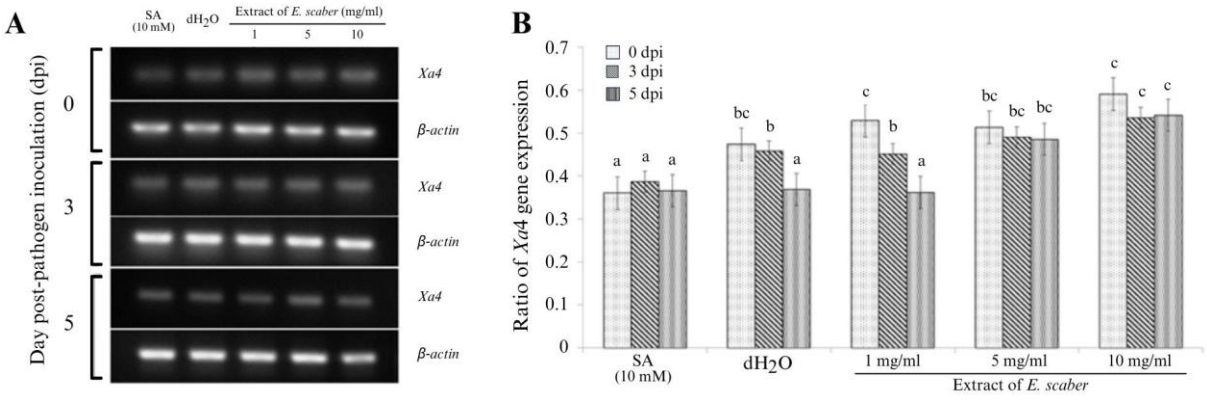

Figure 1. The *Xa4* expression ratio value (A) RT-PCR visualization results of *Xa4* expression compared to *β-actin* gene (B) *Xa4* gene value using ImageJ software; SA is salicylic acid. Different notations above the bars varied significantly by Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

3.2. BLB Disease Severity

Analysis of disease severity in all treatments was shown by the presence of lesions on the tips of the leaves as a symptom of BLB disease (Figure 2A). Data on the severity of the disease showed a significant difference between the treatment with dH_2O with the treatment with *E. scaber* at doses of 5 mg/ml and 10 mg/ml. Leave pre-treated with dH_2O showed the longest lesions with an average of 0.33 cm compare with the *E. scaber* extracts of 5 mg/ml and 10 mg/ml which exhibited the shorter lesions, 0.12 cm and 0.08 cm, respectively (Figure 2B). In addition, the leave pre-treated with 10 mM salicylic acid showed insignificant differences when compared with the leave pre-treated with *E. scaber* extract at the dose of 5 mg/ml and 10 mg/ml (Figure 2B).

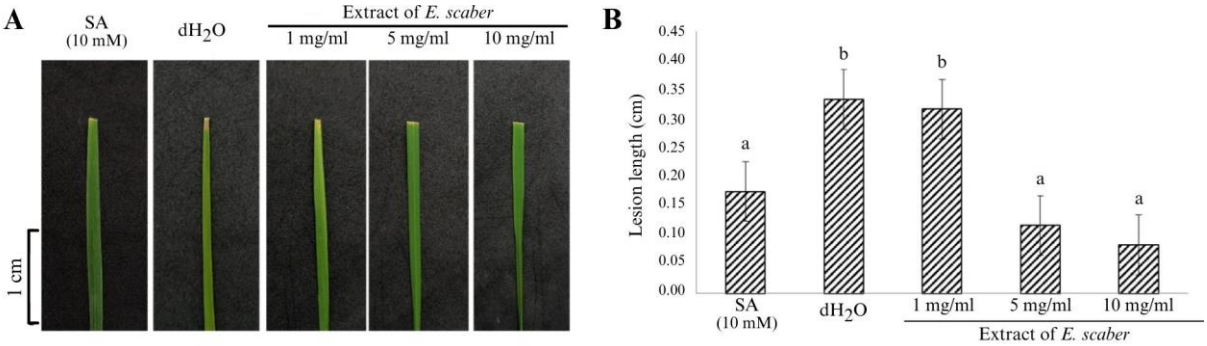

Figure 2. Symptoms of BLB characterized by the appearance of lesions (a) The size of the lesions seen on the tips of the leaves of plants affected by Xoo (b) The average value of the length of the lesions in each treatment; ES is an *Elephantophus scaber*. Different notations above the bars varied significantly by Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

4. DISCUSSION and CONCLUSION

Rice IR64 that are inoculated with the Xoo have symptoms of bacterial leaf blight (BLB) characterized by spots on the shoots or leaf edges (lesions) which then propagate to form pale yellow blight (Ke *et al*., 2017). The lesions are the result of mechanisms of cell death programmed to suppress the pathogen's growth and development in plant cells (Coll *et al*., 2011). In addition, the lesions on the leaves caused by the attack of pathogens are the result of a mechanism of oxidative damage. Oxidative damage will stimulate the occurrence of apoptosis (Demidchik, 2015). The development of lesions as an indication of disease severity shows that there is a difference in the high and low value of disease severity depending on the treatment. The lower severity of the disease under the treatment of *E. scaber* extract indicates that an *E. scaber* extract with a certain concentration can provide a more optimal resistance mechanism in rice against Xoo. This is related to previous studies by Abo-Elyousry *et al*. (2020) and Ashfaq *et al*. (2014) that pre-treating plant extracts such as *Nerium oleander*, *Eucalyptus chamadulonsis* and *Citrullus colocynthis* with certain doses and concentrations reduce the severity of disease in tomato (*Solanum lycopersicum* L.) and chili (*Capsicum annum* L.). In contrast, the higher severity of the disease is caused by the lack of resistance mechanisms of rice plants in resisting the Xoo pathogen, where plants are unable to provide early resistance signaling to pathogen attacks (Herrera-Vásquez *et al*., 2015). The mechanism of resistance to Xoo can be influenced by the expression of resistance genes found in Rice (Zafar *et al*., 2020). The Xa4 gene is one of the BLB resistance genes detected in IR64 rice, whereas in previous studies this gene had a lower gene expression value after inoculation from the Xoo pathogen, which caused plants to become more susceptible to BLB disease (Nadhira *et al*., 2022). Although all disease severity on the treated leaves is in the same resistant category (Figure 1A), however, it shows the differences depending on the treatment representing the resistance, statistically. This indicated that lower severity, a higher resistance response to the pathogen.

The induction of resistance genes through the extraction of the *E. scaber* is intended to increase the expression of the *Xa4* gene, with the aim that the plant is more resistant to Xoo. This can be proven by the pattern of increasing Xa4 gene expression in the treatment of tread dose administration, even before the inoculation of the Xoo pathogen (Figure 1B). The increase in expression of the *Xa4* gene occurs because the *E. scaber* extract contains carotenoid compounds, alkaloids, and flavonoids that have anti-microbial properties (Rejeki *et al*. 2017). This antimicrobial compound is thought to induce the expression of the *Xa4* gene, a type of Wall-Associated Kinase (WAK) gene that works in the thickening of cell walls. The *Xa4* gene can also increase the *Cellulose Synthase* (*CesAs*) gene and inhibit the α-expansin (EXPA) gene resulting in increased mechanical strength and the cell wall of rice plants (Mazerai *et al*., 2018). However, in addition to the effect of increasing *Xa4* expression, there is also the phenomenon

of gene expression value decreasing and then increasing (fluctuating) allegedly due to the growth and development activity of the pathogen so that it affects the activity of the *Xa4* gene. According to Ishihara *et al*. (2019), the decrease in resistance gene expression occurred in the treatment of hot water extract from the *Hypsizygus marmoreus* fungus substrates which influenced the expression of *PR1b* and *PBZ1* genes in rice plants infected with *Pyricularia oryzae*. The *PR1b* and *PBZ1* genes at the beginning of inoculation experienced an increase in gene expression but showed a decrease in the expression value of the *PR1b* and *PBZ1* genes at 2 days after inoculation indicating that a pathogen spread in plants.

The application of *E. scaber* extract can also stimulate the expression of resistance genes through signaling complexes such as reactive oxygen species (ROS) signals and systemic acquired resistant (SAR) transduction signals mediated by SA (salicylic acid) (Chan, 2013; Khataee *et al*., 2019). In addition, the *Xa4* gene stimulates the formation of phytoalexins, molilactone A, and sakruranetin so that plants are more resistant to pathogen infection (Ji *et al*., 2018). On the other hand, the expression value of the *Xa4* gene in SA treatment is smaller than that of dH_2O. It is suspected that the low expression of the *Xa4* gene is due to oxidative stress. The previous studies by Horvath *et al*. (2007) reported that rice plants have a higher basal (endogenous) SA compared to other plants. Accordingly, the application of an exogenous SA on the rice plants experiences faster oxidative stress. Moreover, salicylic acid also stimulates the formation of *NPR1* (*Nonexpressor of Pathogenesis-Related Genes 1*) to transcribe resistance genes as well as the elicitation of resistance due to pathogen infection (Chan, 2013).

In conclusion, this study demonstrated that the *E. scaber* extract at a concentration of 10 mg/ml elicits the most pronounced induction of resistance by increasing the *Xa4* gene expression and consequently slower the BLB symptom development in rice.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hardian Susilo Addy: Design, analysis, interpretation, and supervision. **Ali Wafa**: Supervision and interpretation. **Nur Habibullah**: Performing the experiment and data analysis. **Hardian Susilo Addy** and **Wulan Arum Hardiyani**: writing the manuscript. All author agrees with the manuscript.

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