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RESEARCH PAPER

Induction of defense-related enzymes in tomato plants inoculated with selected rhizobacteria against bacterial wilt disease

Yulmira Yanti, Hasmiandy Hamid, Yaherwandi, & Reflin

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ABSTRACT

Bacterial wilt caused *Ralstonia syzygii* subsp. *indonesiensis* is one of the most destructive disease of tomato and results in severe yield losses. Plant growth-promoting rhizobacteria (PGPR) are known to enhance plant growth and health. In previous research, seven selected PGPR strains were identified for their ability to suppress *R. syzygii* subsp. *indonesiensis* and promote tomato growth. The present study aimed to evaluate the enzymatic defense responses of tomato plants inoculated with two selected rhizobacterial strains. The strains were assessed for their ability to induce the production of defense-related enzymes, including peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and lipoxygenase (LO). *R. syzigii* subsp. *indonesiensis* was inoculated onto tomato plants seven days after rhizobacterial treatment at the seedling stage. Enzyme activities were recorded from 0 to 30 days after pathogen inoculation at two-day intervals. The results showed a reduction in disease incidence and severity in tomato plants treated with the two rhizobacterial strains compared with the control. *E. oryzendophyticus* RZ.2.2.AG2 demonstrated greater effectiveness in reducing disease incidence (26%) and severity (2.5) than *B. thuringiensis* RZ.1.1.AG4. Enzyme activity assays further confirmed that both strains induced plant resistance by increasing PAL, PO, PPO, and LO activities, with significantly higher enzyme activity observed in roots than in leaves. Tomato plants treated with *E. oryzendophyticus* RZ.2.2.AG2 exhibited the highest enzyme activities in both roots and leaves, indicating that disease suppression was associated with induced systemic resistance (ISR) mediated through activation of defense-related enzymes.

Keywords: Induced systemic resistance, plant defense enzyme, plant growth-promoting rhizobacteria, *Ralstonia syzygii* subsp. *indonesiensis*, tomato

INTRODUCTION

Ralstonia syzygii subsp. *indonesiensis* (previously *R. solanacearum*) is the causal agent of bacterial wilt disease (Nurdika et al., 2022), one of the most destructive vascular diseases affecting tomato crops worldwide. This disease causes severe yield losses, estimated to range from 15% to 55% globally (Edy et al., 2022; El-Argawy & Adss, 2016). Effective management of bacterial wilt remains difficult due to the high genetic variability of the pathogen, its ability to survive under diverse environmental conditions, limited chemical control options, and its extremely wide host range (Balamurugan et al., 2021).

Biological control is a key component of integrated disease management, and considerable attention has been given to rhizosphere microorganisms that promote plant growth and enhance plant health, collectively known as Plant Growth-Promoting Rhizobacteria (PGPR). Among them, species belonging to the genera *Bacillus* and *Pseudomonas* are the most extensively studied. *Bacillus* spp. are naturally abundant in the rhizosphere and are well known for their PGPR activities through the production of various bioactive metabolites that improve plant nutrient availability (Salazar et al., 2023). Similarly, *Pseudomonas* spp., particularly fluorescent *Pseudomonas* (FLPs), are ubiquitous soil bacteria and exhibit multiple traits that make them highly effective PGPR.

PGPR can suppress plant diseases by stimulating inducible plant defense mechanisms, thereby enhancing host resistance to subsequent pathogen invasion. This phenomenon, known as Induced Systemic Resistance (ISR) (Seth et al., 2023), is long-lasting and does not promote resistance development in the target pathogen. Consequently, ISR provides a promising basis for novel plant disease management strategies applicable under both greenhouse and field conditions, particularly within integrated disease management frameworks (Yang et al., 2024). The induction of

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Corresponding author:
Yulmira Yanti (yy.anthie79@gmail.com)

Department of Plant Protection, Faculty of Agriculture, Andalas University, Padang, West Sumatera, Indonesia 25163

systemic resistance by PGPR against various plant pathogens has therefore been considered one of the most sustainable and desirable approaches in modern crop protection (Patel et al., 2021).

Many defense mechanisms involved in ISR have only recently been elucidated. These include reinforcement of cell wall structures through lignification or callose deposition (Zhu et al., 2022; Tahsili et al., 2014), accumulation of antimicrobial phytoalexins (Hamid et al., 2021), and induction of defense-related proteins with antifungal properties (Seth et al., 2023). Defense-related enzymes constitute an essential protective system in plants against pathogen invasion. Several enzymes, including superoxide dismutase (SOD), peroxidase (PO), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL), have been identified as vital components of plant defense and are therefore widely studied in plant–pathogen interaction research (Resti et al., 2016; Han et al., 2016). SOD, PO, and PPO are representative antioxidant enzymes that play crucial roles in protecting plant cells against membrane lipid peroxidation and oxidative stress during pathogen infection (Neshat et al., 2022). Activation of systemic resistance by nonpathogenic rhizobacteria has also been associated with the induction of lipoxygenase (LOX) activity in bean and tomato plants (Gogoi et al., 2024).

Peroxidase (EC.1.11.1.7) are a family of enzymes capable to oxidizing a wide range of substrates using hydrogen peroxide H_2O_2. In plant's, peroxidases belong to class III and involved in numerous physiological processes, including pathogen defense, wound healing (Pandey et al., 2017), and cell development (Devi et al., 2023). These oxidoreductive enzymes participate in cell wall polysaccharide modification processes such as phenol oxidation, suberization, and lignification during plant defense responses against pathogenic agents (Khan et al., 2023). PPO is involved in the oxidation of polyphenols into quinones and contributes to lignification of plant tissues during microbial invasion (Mohammadi & Kazemi, 2002). PAL is the key enzyme in the phenylpropanoid pathway and plays a critical role in the biosynthesis of several defense-related secondary metabolites, including phenolic compounds and lignin (Tahsili et al., 2014). The activation of systemic resistance by nonpathogenic rhizobacteria has also been associated with enhanced LOX activity in bean and tomato plants (Sailaja et al., 1998; Silva et al., 2004; Ongena et al., 2004; Ongena et al., 2007). Plant LOX (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is involved not only in growth and developmental regulation through the biosynthesis of signaling molecules and volatile compounds related to insect attraction, but also in defense responses to pathogens, wounding, and environmental stress (Feussner & Wasternack, 2002; La Camera et al., 2004; Baysal & Demirdoven, 2007; Shah, 2005).

Several PGPR antagonists have been evaluated for the control of *R. solanacearum* varying degrees of success, including *P. fluorescens* (Seleim et al., 2011), and *Stenotrophomonas maltophilia* (Messiha et al., 2007) in potato crops. *Bacillus*-based biocontrol agents have also shown significant potential for controlling bacterial wilt in mulberry (Ji et al., 2008) and tobacco crop (Maketon et al., 2008). Nguyen & Ranamukhaarachchi (2010) reported that two soil-derived bacterial antagonists, *B. megaterium* and *Enterobacter cloacae*, exhibited high potential for suppressing bacterial wilt in chili crops while also increasing fruit weight, biomass, and plant height.

Our previous in planta study screened 13 indigenous PGPR strains and demonstrated their ability to suppress *R. syzygii* subsp. *indonesiensis* while promoting growth in chili (Yanti et al., 2017). However, subsequent in vitro characterization revealed that only one strain exhibited consistent antagonistic traits, indicating that effective disease suppression may not solely depend on direct antagonism (Habazar et al., 2018). Despite extensive reports on the use of PGPR to control bacterial wilt caused by *Ralstonia* spp., most studies emphasize direct antagonistic activity rather than plant-mediated defense responses. Consequently, the role of induced systemic resistance (ISR), particularly through the activation of defense-related enzymes in tomato plants, remains insufficiently understood. Therefore, this study aimed to evaluate ISR-mediated defense responses in tomato plants treated with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 by quantifying the activities of key defense-related enzymes—superoxide dismutase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, and lipoxygenase—following inoculation with *R. syzygii* subsp. *indonesiensis*. The results are expected to clarify the mechanistic basis of PGPR-mediated resistance and support the development of sustainable biological control strategies for bacterial wilt in tomato.

MATERIALS AND METHODS

Research Site. This study was conducted through laboratory and greenhouse experiments at the Laboratory of Microbiology, Department of Plant

Protection, Faculty of Agriculture, Andalas University, Padang, Indonesia.

Preparation of Bacterial Inocula. The pathogenic bacterium *R. syzygii* subsp. *indonesiensis* and two selected plant growth-promoting rhizobacteria (PGPR), *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2, were obtained from our culture collection (Yanti et al., 2017). All bacterial strains were initially grown on nutrient agar (NA) to confirm purity.

For inoculum preparation, a loopful of a 2-day-old culture was transferred into 100 mL of nutrient broth (NB) in a 250 mL Erlenmeyer flask and incubated at 28 ± 2 ºC on an orbital shaker at 150 rpm for 48 hours (preculture). The main culture was prepared by transferring 1 mL of the preculture into 50 mL of sterile coconut water in a 250 mL flask and incubating under the same conditions. After 48 hours, the bacterial suspension reached a density of approximately 9×10^8 CFU mL^{-1}, as determined using McFarland standard No. 8 ($\approx 10^8$ cells mL^{-1}).

The *R. syzygii* subsp. *indonesiensis* isolate was cultured on tetrazolium triphenyl tetrachloride (TZC) selective medium and incubated at room temperature for 48 hours to confirm colony morphology.

Inoculation of Selected Rhizobacterial Strains on Tomato Plants. Tomato plants of the *Warani* variety were used in this study. The selected rhizobacterial strains were applied to tomato plants using a two-step inoculation method: 1) Seed treatment: Tomato seeds were surface-sterilized with 1% NaCl, rinsed twice with sterile distilled water, and then dipped in bacterial suspension for 15 min. The treated seeds were drained and air-dried under shade for 30 min before sowing (Habazar et al., 2014). 2) Seedlings treatment: Three-week-old tomato seedlings were inoculated with rhizobacterial isolates using the root-dipping technique prior to transplanting. Plants were grown under greenhouse conditions, with temperature and humidity dependent on natural environmental conditions.

Inoculation of *R. syzygii* subsp. *indonesiensis* on Tomato Plants. A suspension of *R. syzygii* subsp. *indonesiensis* (10^8 CFU mL^{-1}) were inoculated onto two- week-old tomato plants using the soil-drenching method. Plants treated with sterile distilled water served as controls. Standard agronomic practices, including watering and weeding, were applied throughout the experiment.

Incidence and Severity of Bacterial Wilt Disease. A total of 50 tomato plants treated with two PGPR strains and one control were used for the assessment of bacterial wilt incidence and severity following inoculation with *R. syzygii* subsp. *indonesiensis.* Disease severity was evaluated using the scale described by Horita & Tsuchiya (2001): 1 = No symptoms; 2 = Wilting of top young leaves; 3 = Wilting of two leaves; 4 = Wilting of four or more leaves; 5 = Plant death.

Enzyme Extraction. Plants were carefully removed from pots at 0, 1, 3, 5, 7, 9, 12, 15, 17, 19, 21, 23, 25, 27 and 30 days after inoculation with *R. syzygii* subsp. *indonesiensis*. Roots were washed thoroughly with sterile distilled water and separated from aerial parts. Root and leaf tissues (1 g) from three biological replicates were homogenized separately in liquid nitrogen using a pre-chilled mortar and pestle. The homogenized samples were stored at −70 ºC until enzyme analysis.

Peroxidase (POD) Assay. Peroxidase activity was assayed according to Bateman (1967), with modifications by Yanti (2015). One gram of root or or leaf tissue was homogenized in 2 mL of 0.1 M phosphate buffer (pH 7.0) at 4 ºC and centrifuged at 16,000 × g for 15 min at 4 ºC. The supernatant was used as the enzyme extract.

The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract, and 0.5 mL of 1% H_2O_2. Changes in optical density were recorded at 30 s intervals for 3 min at 420 nm. Enzyme activity was expressed as the change in absorbance min^{-1} g^{-1} protein (Hammerschmidt et al., 1982).

Phenylalanine Ammonia-Lyase (PAL) Assay. Root and leaf tissues (1 g) were homogenized separately in 3 mL of ice-cold 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM 2-mercaptoethanol and 0.1 g insoluble polyvinylpyrrolidone (PVP). The homogenate was filtered through cheesecloth and centrifuged at 16,000 × g for 15 min at 4 ºC.

A reaction mixture containing 0.4 mL enzyme extract, 0.5 mL of 0.1 M borate buffer (pH 8.8), and 0.5 mL of 12 mM L-phenylalanine was incubated at 30 ºC for 30 min. Absorbance was recorded at 290 nm. PAL activity was calculated as trans-cinnamic acid production according to Dickerson et al. (1984) and expressed as µmol trans-cinnamic acid min^{-1} g^{-1} protein.

Polyphenol Oxidase (PPO) Assay. Polyphenol oxidase activity was determined following Mayer et al. (1965). Freeze-dried root and leaf tissues (1 g) were homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 × g for 15 min at 4 ºC. The supernatant served as the enzyme source.

The assay mixture consisted of 0.2 mL enzyme extract, 1.5 mL sodium phosphate buffer, and 0.2 mL of 0.01 M catechol. Absorbance was recorded at 420 nm at 30 s intervals for 3 min. Enzyme activity was expressed as the change in absorbance min^{-1} g^{-1} fresh weight.

Lypoxigenase (LOX) Assay. Leaf extracts were prepared according to Ohta et al. (1986) with modifications, using 0.05 M sodium phosphate buffer (pH 6.5) without Triton X-100. Protein concentration was determined using the bicinchoninic acid assay (Smith et al., 1985).

LOX activity was measured using linolenic acid as substrate following Axelrod et al. (1981), based on the increase in absorbance at 234 nm due to the formation of conjugated double bonds in linoleic acid hydroperoxides. Optimal pH was determined using buffer systems (50 mM) covering a pH range of 2–10. Optimal temperature was assessed in 50 mM sodium phosphate buffer (pH 6.0) across a temperature range of 20–50 ºC. All assays were conducted in triplicate.

RESULTS AND DISCUSSION

The selected rhizobacteria isolated from the tomato rhizosphere, *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2, were effective in suppressing *R. syzygii* subsp. *indonesiensis* and controlling bacterial wilt in pot-grown tomato plants. Both strains were previously reported to induce systemic resistance in tomato against *R. syzygii* subsp. *indonesiensis* (Habazar et al., 2018). In the present study, seed and seedling treatments with these bacterial strains significantly increased the activities of several defense-related enzymes, including peroxidase (PO), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and lipoxygenase (LO), following pathogen inoculation.

Incidence and Severity of Bacterial Wilt. Both rhizobacterial strains significantly reduced the incidence and severity of bacterial wilt caused by *R. syzygii* subsp. *indonesiensis* compared with the untreated control (Table 1). Among the two strains, *E. oryzendophyticus* RZ.2.2.AG2 showed superior efficacy, reducing disease incidence to 26% and disease severity to a score of 2.5, compared with *B. thuringiensis* RZ.1.1.AG4.

Peroxidase (PO) Activity. An increase in peroxidase (PO) activity was observed in tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 following challenge inoculation with *R. syzygii* subsp. *indonesiensis* (Figure 1). PO activity reached its maximum at 15 days after pathogen inoculation and gradually declined thereafter. In the roots of tomato plants treated with *B. thuringiensis* RZ.1.1.AG4, PO activity was 16.10–43.55% higher than in the control, whereas in plants treated with *E. oryzendophyticus* RZ.2.2.AG2, PO activity increased by 14.41–38.71% relative to the control (Figure 2). Similarly, PO activity in the leaves increased by 16.81–45.61% in *B. thuringiensis*-treated plants and by 7.96–35.09% in *E. oryzendophyticus*-treated plants compared with the control. Overall, PO activity was consistently higher in plants treated with *B. thuringiensis* RZ.1.1.AG4 than in those treated with *E. oryzendophyticus* RZ.2.2.AG2.

Peroxidase is a key enzyme involved in multiple physiological processes, including lignification, wound healing, phenol oxidation, and defense against pathogens (Passardi et al., 2004). In addition to scavenging reactive oxygen species (ROS), PO can also generate ROS such as H_2O_2, O_2-, and OH^-, which are toxic to pathogens and contribute to cell wall strengthening and defense signaling (Kawano, 2003). Enhanced PO activity has frequently been associated with increased resistance and lignin accumulation in plants (Reuveni et al., 1992). Higher PO activity was observed in tomato roots treated with *Phytium corrugata* and challenged with *Phytium aphanidermatum* (Chen et al., 2000).

In the present study, seed and seedling

Table 1. Incidence and severity of tomato plants treated with PGPR strains against *R. syzygii* subsp. *indonesiensis*

Strains	Incidence (%)	Severity
B. thuringiensis RZ.1.1.AG4	40.0	3.0
E. oryzendophyticus RZ.2.2.AG2	26.0	2.5
Control	100	5.0

treatments with *B. thuringiensis* RZ1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 resulted in higher PO activity at 15 days after pathogen inoculation in both roots and leaves, with slightly higher activity in the roots (Figure 2). The induction or accumulation of PO in response to pathogen inoculation has been demonstrated in several pathosystems, including tomato–*P. aphanidermatum* (Nakkeeran et al., 2006), rice–*Pyricularia oryzae* and *Bipolaris sorokiniana*, pumpkin–*Colletotrichum lagenarium*, cassava–*Xanthomonas axonopodis* pv. *manihotis* (Pereira et al., 2000), and *Capsicum baccatum* var. *pendulum*–Pepper yellow mosaic virus (PYMV) (Gonçalves et al., 2013).

Phenylalanine Ammonia-Lyase (PAL) Activity. Increased PAL activity was recorded in the leaves and roots of rhizobacteria-pretreated tomato seeds and seedlings challenged with *R. syzigii* subsp. *indonesiensis*. In plants treated with *E. oryzendophyticus* RZ.2.2.AG2, PAL activity reached a maximum at 15 day post inoculation (dpi), whereas in *B. thuringiensis* RZ.1.1.AG4-pretreated plants, the maximum was observed at 17 dpi (Figure 3). Thereafter, PAL activity gradually declined. In control plants without rhizobacterial treatment, PAL activity increased slightly and declined slowly.

PAL activity in the roots of *B. thuringiensis* RZ.1.1.AG4-pretreated tomato plants was 3.23-90.48% higher than in the control (Figure 3), whereas in the roots of *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants, PAL activity was 19.36–78.57% higher than in the control. In the leaves, PAL activity in *B. thuringiensis* RZ.1.1.AG4-pretreated plants was 3.23–87.50% higher than in the control, while in *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants it was 19.36–67.65% higher than in the control. Similar to the trend observed for PO activity, PAL activity in rhizobacteria-inoculated tomato plants was generally higher in the roots than in the leaves (6.25–30.61%). Overall, PAL activity was greater in plants treated with

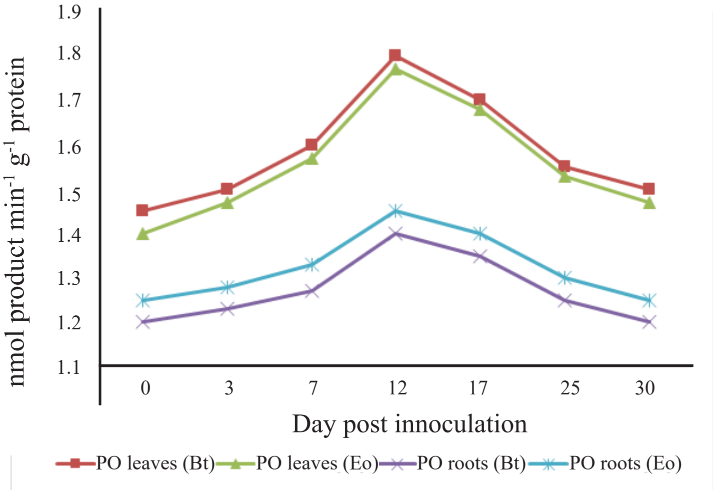

Figure 1. Peroxidase activity in the roots and leaves of tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4- or *E. oryzendophyticus* RZ.2.2.AG2 and inoculated with *R. syzygii* subsp. *indonesiensis*. PO leaves (Bt) = 1.77; PO leaves (Eo) = 1.80; PO roots (Bt) = 1.40; PO roots (Eo) = 1.45.

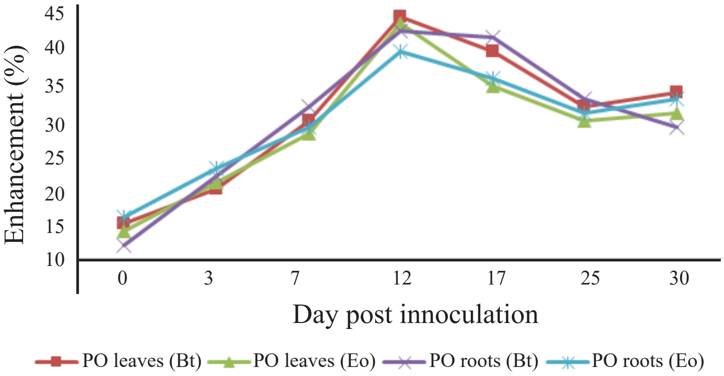

Figure 2. Polyphenol oxidase activity in the roots and leaves of tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 or *E. oryzendophyticus* RZ.2.2.AG2 following inoculation with *R. syzygii* subsp. *indonesiensis*. PO leaves (Bt) = 4.5; PO leaves (Eo) = 4.3; PO roots (Bt) = 4.2; PO roots (Eo) = 4.0.

B. thuringiensis RZ.1.1.AG4 than in those treated with *E. oryzendophyticus* RZ.2.2.AG2.

Early induction of PAL plays a critical role in activating plant defense, as lignin biosynthesis is initiated from L-phenylalanine. During the conversion of L-phenylalanine to trans-cinnamic acid, PAL acts as the first enzyme in the phenylpropanoid pathway, catalyzing the trans-elimination of ammonia from L-phenylalanine (Duan et al., 2014). PAL is the rate-limiting enzyme in the production of secondary metabolites and is responsible for the biosynthesis of phenolic compounds and phytoalexins.

In the present study, seed and seedling treatments with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2, followed by inoculation with *R. syzygii* subsp. *indonesiensis*, resulted in higher PAL activity up to 17 days after pathogen inoculation in both roots and leaves, with slightly higher activity in the roots (Figure 3).

Jha (2019) reported that induction of PAL in maize plants inoculated with *P. aeruginosa* and *B. megaterium* and infected with *Aspergillus niger* showed the highest activity in plants inoculated with *B. megaterium* at 45 days after infection, representing a threefold increase compared with the control. PAL activity was more moderate in plants inoculated with *P. aeruginosa* (approximately 1.5-fold) than in plants treated with a combination of both isolates (approximately 2-fold) compared with the control.

De Meyer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots and increased salicylic acid levels in leaves. Similarly, seed bacterization with *B. subtilis* AF1 resulted in a distinct increase in PAL activity in pigeon pea within 24 hours (Podile & Laxmi, 1998). Nakkeeran et al. (2006) also reported increased PAL activity in *P. chlororaphis* strain PA23-treated tomato seedlings challenged with *P. aphanidermatum*, with maximum activity observed at 12 days, followed by a decline. In plants inoculated with the pathogen alone, PAL activity declined rapidly.

Polyphenol Oxidase (PPO) Activity. Increased polyphenol oxidase (PPO) activity was observed in tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 following inoculation with *R. syzygii* subsp. *indonesiensis* (Figure 4). PPO activity reached a maximum at 15 days after pathogen inoculation and declined thereafter.

In the roots of *B. thuringiensis* RZ.1.1.AG4-pretreated tomato plants, PPO activity was 17.36–35.94% higher than in the control (Figure 4), whereas in the roots of *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants, PPO activity was 15.70–34.38% higher than in the control. In the leaves, PPO activity in *B. thuringiensis* RZ.1.1.AG4-pretreated plants was 17.09–40.98% higher than in the control, while in *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants it was 7.69–37.71% higher than in the control (Figure 4). Overall, PPO activity was higher in the roots than in the leaves (1.16–21.00%). Furthermore, PPO activity was greater in plants treated with *B. thuringiensis* RZ.1.1.AG4 than in those treated with *E.*

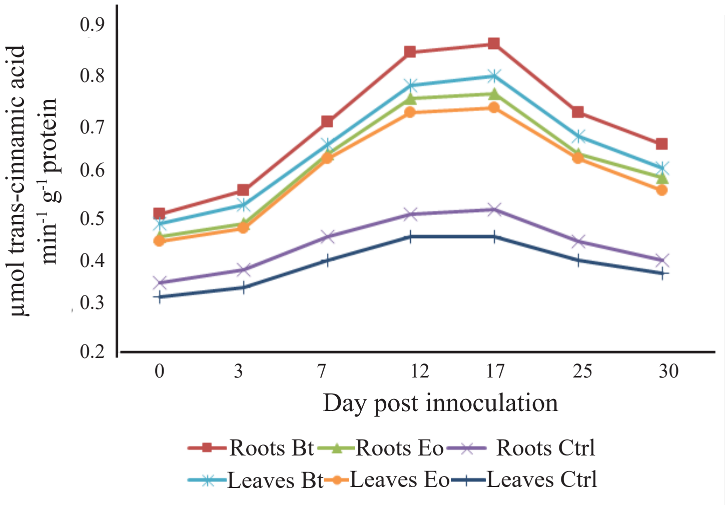

Figure 3. Phenylalanine ammonia-lyase (PAL) activity in the roots and leaves of tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 or *E. oryzendophyticus* RZ.2.2.AG2 and inoculated with *R. syzygii* subsp. *indonesiensis*. Roots Bt = 0.87; Roots (Eo) = 0.76; Leaves Bt = 0.79; Leaves Eo = 0.81; Roots Ctrl = 0.46; Leaves Ctrl = 0.45.

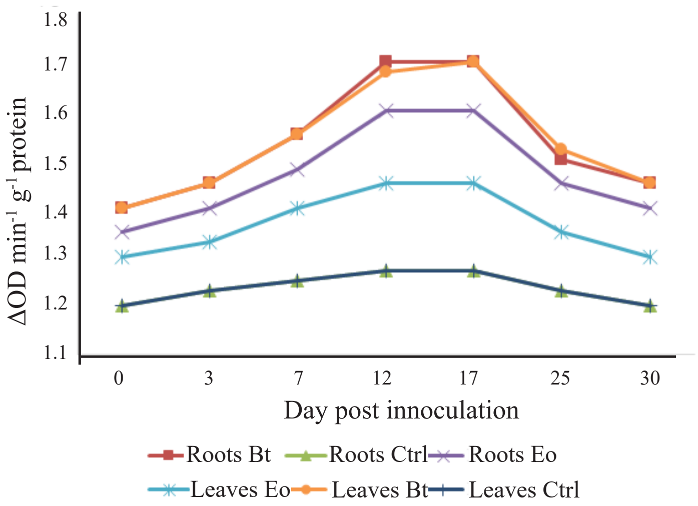

Figure 4. Polyphenol oxidase (PPO) activity in the roots and leaves of tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 or *E. oryzendophyticus* RZ.2.2.AG2 and inoculated with *R. syzygii* subsp. *indonesiensis.* Roots Bt = 1.70; Roots (Eo) = 1.70; Leaves Bt = 1.60; Leaves Eo = 1.45; Roots Ctrl = 1.28; Leaves Ctrl = 1.27.

oryzendophyticus RZ.2.2.AG2.

Polyphenol oxidase (catechol oxidase; EC 1.10.3.2) has been purified and characterized from a wide range of plant species and tissues (Constabel et al., 1996; Mayer, 2006). PPOs are copper-containing enzymes that catalyze the oxidation of hydroxyphenols to quinone derivatives, which possess antimicrobial activity (Shi et al., 2001). Due to its reaction products and wound inducibility, PPO plays an important role in plant defense against pathogens (Mayer & Harel, 1979). Upon pathogen attack, plants often exhibit a rapid increase in PPO activity, indicating enhanced synthesis of antimicrobial compounds.

Li & Steffens (2002) proposed several mechanisms underlying PPO-mediated defense, including the general toxicity of PPO-generated quinones to pathogens and plant cells, acceleration of cell death, alkylation and reduced bioavailability of cellular proteins to pathogens, cross-linking of quinones with proteins or other phenolics to form physical barriers in the cell wall, and quinone redox cycling leading to the production of H_2O_2 and other reactive oxygen species (Jiang & Miles, 1993). Reactive oxygen species are well known to play critical roles in plant–pathogen interactions and defense signaling. PPO has also been implicated in the formation of melanin-like polymers in potato black spot lesions (Stevens et al., 1998).

In the present study, seed and seedling treatments with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 resulted in elevated PPO activity at 15 days after inoculation with *R. syzygii* subsp. *indonesiensis,* both in roots and leaves, with slightly higher activity in the roots. The correlation between the protective effects of rhizosphere bacteria and the induction of defense enzymes, including PPO, has been reported by Chen et al. (2000) and Ramamoorthy et al. (2002). Three- to four-fold increases in peroxidase and PPO activities were observed in *B. subtilis* strain BSCBE4- and *P. chlororaphis* strain PA23-pretreated tomato seedlings inoculated with *P. aphanidermatum* (Nakkeeran et al., 2006). Increased PPO activity was also reported in banana roots treated with a *Fusarium oxysporum* -derived elicitor (Thakker et al., 2013). A marked increase in PPO activity was observed in banana roots treated with *Pseudomonas fluorescens* against Fusarium wilt (Sarvanan et al., 2004). Similarly, studies demonstrating correlations between high PPO levels and enhanced pathogen resistance continue to support the role of PPO in plant defense (Raj et al., 2006).

Lipoxygenase (LO) Activity. Increased lipoxygenase (LO) activity was observed in tomato plants pretreated with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 following inoculation with *R. syzygii* subsp. *indonesiensis* (Figure 5). LO activity reached a maximum at 12 days after pathogen inoculation and declined thereafter.

In the roots of *B. thuringiensis* RZ.1.1.AG4-pretreated tomato plants, LO activity was 7.14–53.33% higher than in the control (Figure 5), whereas in the roots of *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants, LO activity was 6.67–60.00%

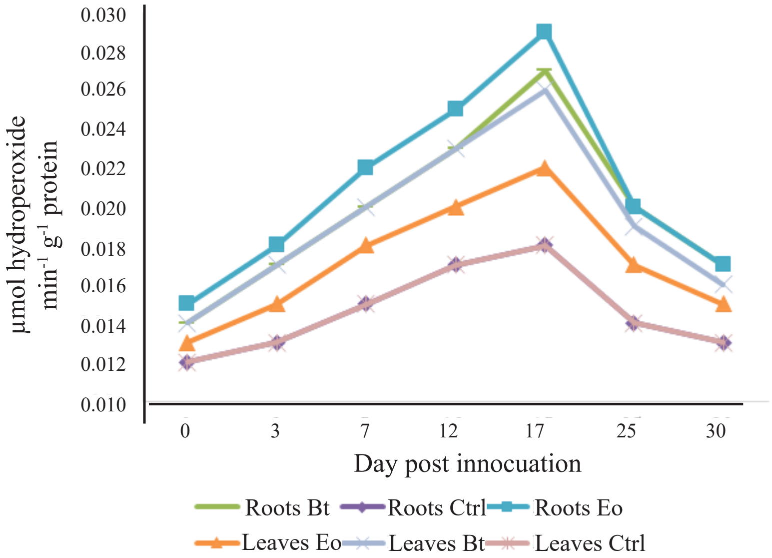

Figure 5. Lipoxygenase (LO) activity in the roots and in the leaves of with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 pretreated tomato plants inoculated with *R. syzygii* subsp. *indonesiensis.*

higher than in the control. In the leaves, LO activity in *B. thuringiensis* RZ.1.1.AG4-pretreated plants was 9.09–63.64% higher than in the control, while in *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants it was 20.00–127.27% higher than in the control (Figure 5). Overall, LO activity was higher in the roots than in the leaves (4.35–81.82%).

Unlike the other defense enzymes examined, LO activity was greater in *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants inoculated with *R. syzygii* subsp. *indonesiensis* than in *B. thuringiensis* RZ.1.1.AG4-pretreated plants.

Lipoxygenases (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) are dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids containing a cis,cis-1,4-pentadiene structure. Linolenic acid (C18:3) contains this structure and is one of the most abundant fatty acids in plant tissues (Hildebrand et al.,1988). LOX enzymes are associated with plant growth and development, senescence, defense against pathogens and insects, and the biosynthesis of regulatory molecules (Vieira et al., 2001).

Activation of systemic resistance by nonpathogenic rhizobacteria has also been associated with the induction of LOX activity in bean and tomato (Ongena et al., 2004; Ongena et al., 2007; Sailaja et al., 1998; Silva et al., 2004). In the present study, seed and seedling treatments with *B. thuringiensis* RZ.1.1.AG4 and *E. oryzendophyticus* RZ.2.2.AG2 resulted in higher LO activity following pathogen inoculation in both roots and leaves of tomato plants, with slightly higher activity in the roots.

Akram et al. (2008) reported significantly enhanced LOX activity in *Pseudomonas putida* BTP1-elicited tomato plants compared with controls, with elevated levels of hydroperoxide-degrading enzymes maintained for up to four days after pathogen challenge. In our study, the enhancement of LO activity was particularly pronounced in the leaves of *E. oryzendophyticus* RZ.2.2.AG2-pretreated plants inoculated with *R. syzygii* subsp. *indonesiensis* (Figure 5). In contrast, in the roots, enhancement of defense enzyme activity—particularly PAL—was more pronounced in *B. thuringiensis* RZ.1.1.AG4-pretreated plants (Figure 4).

Based on our previous study, these strains effectively controlled bacterial wilt caused by *R. syzygii* subsp. *indonesiensis* in tomato plants. We therefore propose that the biocontrol mechanism of these strains involves induced systemic resistance (ISR) (Habazar et al., 2018). In the present study, both selected rhizobacterial strains increased the activities of peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase, and lipoxygenase in tomato plants. Accoding to Van Loon (1998), peroxidase, lipoxygenase, and phenylalanine ammonia-lyase are linked to the ISR pathway regulated by jasmonate and ethylene signaling, which can be activated by saprophytic microorganisms including rhizobacteria.

ISR activates multiple defense mechanisms, including increased activity of chitinases, peroxidases (PO), and polyphenol oxidase (PPO), resulting in enhanced resistance against various plant pathogens (Kandan et al., 2003). ISR is also associated with increased synthesis of defense-related enzymes such as peroxidase (Rajinimala et al., 2003), elevated levels of acid-soluble proteins (Zdor & Aderson, 1992), and accumulation of phytoalexins in induced plant tissues (van Peer et al., 1991). Ramamoorthy et al. (2002) reported that pretreatment of tomato plants with *P. fluorescens* isolate Pf1, followed by challenge with *P. aphanidermatum*, induced earlier and higher accumulation of PAL, PO, PPO, and phenolics compared with non-inoculated controls. Similarly, Nakkeeran et al. (2006) demonstrated that *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 effectively controlled damping-off disease in greenhouse-grown tomato and enhanced defense-related enzymes (PAL, PO, and PPO) and phenolic compounds, thereby inducing systemic resistance in the host.

CONCLUSION

Results showed that there are reduced incidence and severity of the wilt disease on tomato introduced with the 2 strains compared with control, where *E. oryzendophyticus* RZ.2.2.AG2 shown better ability in reducing incidence (26%) and severity (2.5) compared with *B. thuringiensis* RZ.1.1.AG4. Further enzyme activity assay also support that both strains induced the plant resistance by increasing the PAL, PO and PPO activity, while the activity were significantly higher in the tomato roots than leaves. Tomato plants introduced with *E. oryzendophyticus* RZ.2.2.AG2 also had highest enzyme activity both in leaves and roots, supporting that both the strains mechanisms on controlling *Ralstonia syzygii* subsp. *Indonesiensis* by ISR on defense related enzyme activities.

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AUTHORS' CONTRIBUTIONS

YY. designed and developed the research methodology, and led the implementation of experiments both in the laboratory and in the greenhouse. HH was responsible for the bacterial culture propagation and processing, including the preparation of PGPR and pathogen inoculum. Y supervised the enzymatic testing, including the extraction and analysis of plant defense enzyme activities. R and YY contributed to the analysis of laboratory test data and writing the methodology section of the manuscript. All authors were actively involved in scientific discussions, review, and editing of the final manuscript.

COMPETING INTEREST

Authors are required to declare any competing interest such as financial or non-financial interests, professional or personal relationships that are directly or indirectly connected to the work submitted for publication. If there is no competing interest regarding your publication, you are also required to declare.

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