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Induced Defense Related Enzyme Activities of Tomato Plant by Indigenous Endophytic Bacteria and Challenged by *Ralstonia Syzigii* Subsp. *Indonesiensis*

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Our previous research had screened 9 best indigenous endophytic isolates for their ability to control *Ralstonia syzigii* subsp. *indonesiensis,* the causal agents of bacterial wilt disease in tomato (*Lycopersicon esculentum*) in greenhouse condition. Those 9 strains were *Bacillus cereus* EPL1.1.3*, B. cereus* TLE2.3, *B. toyonensis* EPL1.1.4*, Serratia nematodiphila* TLE1.1, *B. anthracis* SNE2.2, *B. cereus* E1.AB1.2, *B. cereus* E1AB2.1, *Enterobacter cloacae subsp. dissolvens* TLE2.2 and *S. marcescens* KLE3.3. The purpose of this study is to test the ability of the endophytic bacteria strains in increasing defense-related enzyme activities of tomato. Bacterial strains were tested for its ability to induce the defense-related enzymes which were phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) in roots and leaves of tomato plants. *R. syzigii* subsp. *indonesiensis* inoculated to host plants 7 days after the endophyte bacteria strain inoculation. Enzyme activities were recorded at 0, 1, 3, 5, 7, 9, 12 and 15 days after pathogen inoculation (dpi). It was observed that PAL, PO, and PPO activities were significantly increased in all of the endophytic bacteria inoculated treatments compared to the control plant. Activities of PAL in the leaves were fast similar to the roots; but PO activities was higher in the roots compared to that in the leaves, whereas PPO activities were higher in the leaves than in the roots. PAL and PO reached the maximum level at a different times in the leaves (3 dpi and 15 dpi), in the roots (5 dpi and 12 dpi), whereas PPO in the leaves at 12 dpi and in the roots at 9 dpi.

Key words: endophytic bacteria, induce systemic resistance, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase

Penelitian sebelumnya telah diseleksi 9 isolat bakteri endofit indigenos terbaik yang mampu mengendalikan *Ralstonia syzigii* subsp. *Indonesiensis,* penyebab penyakit layu bakteri pada tomat (*Lycopersicon esculentum*) pada kondisi rumah kaca. Isolat tersebut yaitu *Bacillus cereus* EPL1.1.3*, B. cereus* TLE2.3, *B. toyonensis* EPL1.1.4*, Serratia nematodiphila* TLE1.1, *B. anthracis* SNE2.2, *B. cereus* E1.AB1.2, *B. cereus* E1AB2.1, *Enterobacter cloacae subsp. dissolvens* TLE2.2 dan *S. marcescens* KLE3.3. Penelitian ini bertujuan untuk menguji kemampuan strain bakteri endofit untuk meningkatkan aktifitas enzim ketahanan tanaman pada tomat. Strain bakteri endofit diuji kemampuannya untuk meningkatkan enzim ketahanan yaitu fenilalanin ammonia lyase (PAL), peroksidase (PO) dan polifenol oksidase (PPO) pada akar dan daun tomat. *R. syzigii* subsp. *indonesiensis* diinokulasikan ke tanaman inang pada umur 7 hari setelah introduksi strain bakteri endofit. Aktivitas enzim diamati pada at 0, 1, 3, 5, 7, 9, 12 dan 15 hari setelah inokulasi pathogen. Hasil pengamatan menunjukkan aktivitas PAL, PO dan PPO meningkat secara signifikan pada semua tanaman tomat yang diinokulasi bakteri endofit dibanding tanaman control. Aktivitas PAL pada daun dan akar tomat meningkat dengan cepat, namun aktivitas PO lebih tinggi pada akar dibanding pada daun, sedangkan aktivitas PPO lebih tinggi pada daun dibanding pada akar. PAL dan PPO mencapai aktivitas maksimum pada waktu yang berbeda pada daun (3 hari dan 15 hari setelah inokulasi) dan pada akar (5 hari dan 12 hari setelah inokulasi), sedangkan aktivitas PPO maksimum pada daun pada 12 hari setelah inokulasi dan 9 hari setelah inokulasi pada akar.

Kata kunci: bakteri endofit, induksi ketahanan sistemik, fenialanin ammonia lyase, peroksidase, polifenol oksidase

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Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop in the world next to potato. Production of tomato was estimated more than 151.7 million tons annually worldwide (FAOSTAT 2010). Bacterial wilt disease is one of the main pathogens that constraint tomato cultivation nowadays (Chen *et al.* 2009). Bacterial wilt disease on tomato is caused by *Ralstonia syzygii* subsp. *indonesiensis* (*Rsi*) (formerly *R. solanacearum*) (Safni *et al.* 2014)). A devastating disease worldwide, bacterial wilt limits the production of solanaceous crops such as tomato, pepper, eggplant, tobacco and potato as well as other important crops like peanut, banana, ginger, and geranium. Approximately 450 crop species have been reported as hosts of this pathogen (Grimault *et al.* 1994; Swanson *et al.* 2005). Bacterial wilt caused 15% to 55% crop losses around the world

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(El-Argawy and Adss 2016).

Integrated disease management using soil fumigation, resistant cultivars, and crop rotation has been suggested as a control strategy for bacterial wilt disease (Schonfeld *et al.* 2003). However, those control methods do not always effective, since *Rsi* can persist for a long period time while associated which plant debris. Thus, the management practices effectivity for *Rsi* is limited, especially when the disease had been occurred (Liu *et al.* 2012). *Rsi* control using bactericides has also proven ineffective and harmful to the environment (Yi *et al.* 2007). Tomato cultivars that resistant to *Rsi* were reported to be limited. The tomato cultivar Hawaii 7996 which resistant to bacterial wilt by polygenic resistance (Grimault *et al.* 1995) was suggested to resistance only to specific strain (Wang *et al.* 2000).

The use of beneficial microorganisms in biological control has been considered as a promising strategy for the management of soil-borne diseases (Chen *et al.* 2011; Lang *et al.* 2012; Liu *et al.* 2012). Some evidence had suggested that endophytic bacteria can contribute to plant disease control (Kloepper *et al.* 1992). Endophytic bacteria are prokaryotes that colonize the internal tissues of healthy plants without causing any disease symptoms (Wilson 1995). The other advantages using endophytic bacteria as biocontrol agents are that they are well adapted to live inside the plants and therefore can provide reliable suppression of vascular disease and do not cause environmental contamination (Wang and Liang 2014). The possible mechanisms of disease suppression by endophytic bacteria are include competition for space and nutrients, antagonism due to production of secondary metabolites and elicitation of induced systemic resistance (ISR) (Philippot *et al.* 2013; Pieterse *et al.* 2014), that confers an enhanced level of protection to a broad spectrum of pathogens (Pieterse *et al.* 2014). The protection of cucumber plants against cucumber anthracnose induced by *Pseudomonas fluorescens* strain 89B-61 was the first case demonstrating that endophytic bacteria could elicit ISR in plants (Wei *et al.* 1991; Kloepper, Ryu, 2006). Subsequent studies established that the ISR was induced by endophytic bacteria of genus *Bacillus, Pseudomonas* and *Serratia* in different plant-pathogen systems and molecular cell signaling mechanisms involved in the defense priming (Kloepper, Ryu, 2006; Pieterse *et al.* 2014). The use of plant's own defense mechanisms induced by endophytic bacteria in pests and disease management is a matter of current interest (Rajendran *et al.* 2006).

Induced resistance (IR) consists of two types, as follow: 1) Systemic acquired resistance (SAR), when a plant becomes infected by a pathogen, it can develop resistance to a broad and distinctive spectrum of pathogens (Durrant and Dong, 2004; Ryals *et al.* 1996). The pathogen-induced resistance can be established in the tissue surrounding the site of initial infection and also in the distant, uninfected parts of the plant (SAR) (Hammerschmidt, 2009; Ross, 1961a,b). SAR is frequently associated with the accumulation of so-called pathogenesis-related (PR) proteins. Some of these proteins have antimicrobial activity and, therefore, may contribute to the resistance (Van Loon *et al.* 2006). 2) Induced systemic resistance (ISR), colonization of plant roots by selected strains of nonpathogenic plant growth-promoting rhizobacteria (PGPR), such as various species of the genera Pseudomonas (Ahn *et al.* 2007a; Van Loon 2007; Van Loon *et al.* 1998), *Bacillus* (Kloepper *et al.* 2004), or *Bradyrhizobium* (Cartieaux *et al.* 2008), can induce a distinct broad-spectrum resistance response in both below- and above-ground parts of the plant. This type of IR was named "rhizobacteria-mediated ISR (De Vleesschauwer and Hoefte 2009; Van Loon, 2007; Van Loon *et al.* 1998). Induced systemic resistance activates multiple defense mechanisms that include increased activity of pathogenesis related (PR) proteins like peroxidase (PO) (Xue *et al.* 1998) and by the accumulation of low molecular weight substances called phytoalexins (van Peer and Schippers 1992). Peroxidase, lipooxygenase, and phenylalanine ammonia lyase are linked to the ISR pathway regulated by jasmonate and ethylene which are activated by saprophytic microorganisms including rhizobacteria (Van Loon 1998). PO and phenylalanine ammonia-lyase (PAL) are the key enzymes involved in phenylpropanoid metabolism (Vidhyasekaran *et al.* 1997). PAL is an important enzyme in the phenolic compound biosynthesis in tomato (Thordal-Christensen *et al.* 1997) and phytoalexins biosynthesis (Surekha *et al.* 2014). The enhanced resistance of acibenzolar-Smethyl-(ASM-) treated tomato plants against *Clavibacter michiganensis* ssp. *michiganensis* was associated with significant increases in peroxidase (PO) activities (Baysal *et al.* 2005). Polyphenol oxidase (PPO) has an important role in the oxidation of phenolic compounds into antimicrobial quinones through defense against pathogens (Barilli *et al.* 2010). Phenylpropanoid compounds are widely used by plants as part of their antimicrobial defense arsenal. For example, flavonoids, isoflavonoids, stilbenes,

monolignols, and lignins serve as inducible phytoalexins or preformed phytoanticipins in many plant species (Dixon 2001), and the phenylpropanoid polymer lignin can act as an inducible physical barrier against pathogen ingress (Mitchell *et al.* 1999).

Our previous research had screened 9 best indigenous endophytic bacterial isolates to control *Ralstonia syzigii* subsp. *indonesiensis,* the causal agents of bacterial wilt disease in tomato, in green house condition. Those 9 strains were *Bacillus cereus* EPL1.1.3*, B. cereus* TLE2.3, *B. toyonensis* EPL1.1.4*, Serratia nematodiphila* TLE1.1, *B. anthracis* SNE2.2, *B. cereus* E1.AB1.2, *B. cereus* E1AB2.1, *Enterobacter cloacae subsp. dissolvens* TLE2.2 and *S. marcescens* KLE3.3 (Yanti *et al.* 2018). In this study, we investigated the activity of phenylalanine ammonia lyase, polyphenol oxidase, and peroxidase enzyme activity in tomato inoculated with selected endophytic bacteria which were effective to control *R. syzygii* subsp. *indonesiensis*.

MATERIALS AND METHODS

Endophytic Bacterial Preparation. Endophytic bacterial strains used in this research were *Bacillus cereus* EPL1.1.3*, B. cereus* TLE2.3, *B. toyonensis* EPL1.1.4*, Serratia nematodiphila* TLE1.1, *B. anthracis* SNE2.2, *B. cereus* E1.AB1.2, *B. cereus* E1AB2.1, *Enterobacter cloacae subsp. dissolvens* TLE2.2 and *S. marcescens* KLE3.3. Endophytic bacterial isolates (preserved in microtube from the previous study) were streaked on Petri dishes containing Nutrient Agar (NA) and incubated for 48 hours. After that, all isolates were re-cultured on Petri dishes containing NA for 48 hours, and suspended until 10^7 (compared with *McFarland* solution scale 7) for treatments.

Inoculation of endophytic bacterial isolates. Surface sterilized tomato seeds of 'Warani' variety were soaked in 100 mL endophytic bacteria for 10 minutes and control dipped to sterilized distilled water for 10 minutes. The seeds then were dried and planted to pot-tray contained sterilized soil and cow dung manure mixture (2:1 v/v) as growth media. Tomato seedlings were maintained for 3 weeks. Each treatment consisted of 25 seeds. The seedlings then were re-inoculated with the same endophyte bacteria by dipping the roots in the bacterial suspension, each seedling then planted to the polybags contain 1 kg of the same growth media as nurseries.

Pathogen Inoculum Preparations. Virulent strain of *R. syzigii* subsp. *indonesiensis* was multiplied by culturing the strains using triphenyl tetrazolium chloride (TZC) medium agar (Kelman *et al.* 1954). One pure *Rsi* colony was cultured on TZC agar medium for 48 hours by the striking method. The culture then was suspended in sterile distilled water and homogenized with the vortex. The *Rsi* populations used for inoculums were estimated at 10^6 CFU ml^{-1} (measured by comparing with *McFarland* solution scale 6).

Pathogen Inoculation. The pathogens were inoculated on 2 weeks old tomato plants. Before pathogen inoculation, the roots were wounded by cut the rhizosphere around 5 cm from the stem. The plants were inoculated as soil drench by pouring 30 mL of pathogen suspensions around wounded roots (Klement *et al.* 1990).

Plant Harvest for Enzyme Assay. Plants were harvested after 0, 1, 3, 5, 7, 9, 12, and 15 days post pathogen inoculation (dpi). The leaves and roots were separated and used further for enzyme extractions while still in fresh conditions.

Enzyme extraction and assay

Assay of phenylalanine ammonia-lyase (PAL). One gram of root and leaves samples were homogenized separately in 3 ml of ice-cold 0.1 M sodium borate buffer, pH 7.0 (Appendix II), containing 1.4 mM 2-mercaptoethanol and 0.1 g insoluble polyvinyl pyrrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16000 g at 4 °C for 15 min. The supernatant was used as the enzyme source. Sample containing 0.4 mL of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. Optical Density (OD) value was recorded at 290 nm. The activity of phenylalanine ammonia-lyase was determined as trans-cinnamic acid as described by Dickerson *et al.* (1984). Enzyme activity was expressed as µmol trans-cinnamic acid min^{-1} g^{-1} protein.

Assay of peroxidase (PO). One-gram of root and leaves samples were homogenized separately in 2 ml 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 16.000 g at 4 C for 15 min and the supernatant was used as the enzyme source. 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 percent H2O2. The changes in OD were recorded at 30 sec intervals for 3 min at 420 nm. The enzyme activity was expressed as changes in the OD min^{-1} g^{-1} protein (Hammerschmidt *et al.* 1982).

Assay of polyphenol oxidase (PPO). Polyphenol oxidase activity was determined as described by Mayer *et al.* (1965). Freeze dried root and leaves samples of one gram each were separately homogenized in 2 ml 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16.000 g for 15 min at 4 C. The supernatant served as the enzyme source. The assay mixture comprised 0.2 ml of enzyme extract, 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml of 0.01 M catechol. The rate of increase in absorbance was recorded in 30 sec intervals up to 3 min at 420 nm. The enzyme activity was expressed as changes in absorbance $min^{-1}g^{-1}$ fresh weight of tissue.

RESULTS

In the present study, endophytic bacterial treatments increased the activities of various defense enzymes (PAL, PO, and PPO) either in the leave or the root of the plants compared to the control plants.

PAL Activity. The increased activity of PAL was recorded in leaves and roots of all endophytic bacterial strains-treated tomato seed and seedlings challenged with R. *syzigii* subsp. *indonesiensis*, and reached a maximum at 3 dpi and 5 dpi (Fig 1 and 2). Further, the PAL activity declined after this. In plants inoculated with pathogen alone and without endophytic bacterial strains, the PAL activity declined rapidly. PAL activities in the endophytic bacterial inoculated tomatoes were higher in the leaves than in the roots. In tomato roots, PAL activity in treatments with *B. toyonensis* strain EPL1.1.4 and *B. cereus* strain E1AB2.1 was increased 3-fold by 5 dpi of the challenger. Whereas in tomato leaves PAL activities in treatments with *B. cereus* E1.AB1.2 and *B. cereus* E1.AB2.1 was increased by 4-fold by 3 dpi of challenger. The activity slowly declined thereafter. Increased resistance of PAL was one of the signs of increased resistance of plants to pathogens.

All endophytic bacterial strains can increase the PAL activity on tomato leaves compare to both inoculated and un-inoculated control. Activity of PAL was found to increase sharply, peaking at 3 dpi compared to the control where the highest peak at 5 dpi (Fig 1). However, PAL activity of all the tomato inoculated with the endophytic strains decreased after 3 dpi, but still had higher activities than control. Comparing to the no *Rsi* inoculation control which only shown a low change of PAL activity, all the treatment showed a high increasing activity of PAL. The activity of the enzyme inoculated with *B. cereus* E1AB2.1 was the highest among all the treatments.

PAL activities of the tomato plants inoculated with endophytic bacteria strains were higher in the leaves compared to the roots. All strains also had PAL activity increased compared to control (Fig 2). However, the highest PAL activity was observed at 5 dpi, slower than the PAL activities in the leaves which had the highest peak at 3 dpi. As observed in the leaves, *B. cereus* E1AB2.1 also had the highest activities in the roots compared to other endophytic bacteria treatments. The control inoculated with *Rsi* also showed an increased activity of PAL, but lower compared to the plants treated with endophyte bacteria.

Tomato plants inoculated with endophyte bacteria following *Rsi* inoculation had an increase PAL enzyme activity since the first day of observation (Fig 3 and 4), indicated the increased resistance of tomato induced by endophyte bacteria strains. All of the PAL activity on leaves also seems to increase from 0 to the 5^{th} days of observations, but some strains show decrease activity between those day of observations. All strains shown decreased enzyme activity in the last day of the observations (15 days after endophyte bacteria inoculations), but its activities were higher compared to control (with and without *R. syzigii* subsp. *indonesiensis* inoculation). However, strain *E. cloacae* subsp. *dissolvens* TLE2.2 shown the highest activity of PAL on tomato leaves until the last day of observations.

PO Activity. In general, there was a tremendous increase in peroxidase activity from 0 to 15 days of observations in tomato plants due to endophytic bacterial inoculation compare to control plants. At 15 dpi, the highest peroxidase activity was recorded in the plant leaves receiving endophytic bacterial strain and at 12 dpi in the roots (Table 3 and 4). Though all the strains tested in this investigation induced biosynthesis of peroxidase, *E. cloacae* subsp. *dissolvens* TLE2.2 showed the highest induction of peroxidase in the leaves which was 74.07 % higher than the plant control, whereas *B. cereus* E1.AB1.2 in the roots which were 59.46 % higher than plant control. However, some strains have lower enzyme activity compared to control with *Rsi* inoculations, indicated that not all strains ability to control *Rsi* was by ISR mechanisms.

In this study, the activity of PO on tomato roots increased after endophytic bacteria inoculations and reached its maximum peak varied from 9 to 15 days after endophytic bacteria inoculations (figure 4). The control tomato plants inoculated with the *Rsi* pathogen produced lower PO enzyme than tomato plants inoculated with endophytic bacteria, but the

uninoculated control also shows increased PO activity, but the increase was low compared to *Rsi* inoculated control and with endophyte bacteria treatment. The tomato plants treated with some endophytic bacteria strains demonstrated higher activity than the control with no *Rsi* inoculations. However, some strains showed no different curve trends with the control without *Rsi* inoculation. The activity of PO on tomato roots reached its highest peak in all the treatments on 15 days after challenge inoculation and then slowly decreased. The highest activity of PO was observed in the tomato plants treated with *B. cereus* EPL1.1.3 strains following challenge inoculated with *R. syzygii* subsp. *indonesiensis*.

PPO activity. The activity of PPO increased in the leaves and roots up to 9 dpi and the activity declined thereafter. The PPO activity of the endophytic bacterial treatment was 57.58-118.18 % and 128.57-342 % greater than the control plants in the leaves (Figure 5) and roots (Fig 6), respectively. The PPO activity in the roots was higher than that in the leaves. The greater PPO activity in the roots was inoculated with *B. cereus* strain EPL1.1.3, and in the leaves was inoculated with *B. cereus* strain E1.AB1.2.

Plants synthesized higher levels of PPO when tomato seeds were treated with all endophytic bacterial strains. In tomato roots, PPO activity in treatments with *B. cereus* EPL1.1.3 was increased 342,86 % by the 9th day after inoculation of treated tomato plants with R. *syzigii* subsp. *indonesiensis*. Whereas in tomato leaves PPO activities in treatments with *B. cereus* strain E1.AB1.2 and *S. marcescens* KLE3.3 were increased 180 % by 9th days after inoculation of treated tomato plants with R. *syzigii* subsp. *indonesiensis*. The activity slowly declined thereafter (Fig 3).

All the endophytic bacteria strains could increase PPO activity in varying degrees compared to the control treatments (with and without *Rsi* inoculations) (Fig 5). *S. nematodophila* TLE1.1 strain show the ability to increase the PPO activity on tomato leaves to the highest peak at 12 days after inoculations, while other strains have the highest peak at 7 to 9 days after inoculations.

DISCUSSION

In the present study, seed treatment and seedling treatment of tomato plants treated with endophytic bacterial isolates and challenged with the pathogen showed an increase in PAL, PO and PPO activity compare to untreated plants. Our previous research had screened the best endophyte bacteria isolates (used in this study) which could to control *Rsi* in field conditions (Yanti *et al.* 2017). Thus, the induction of defense related enzymes corresponding to a reduction in *R. syzigii* subsp. *indonesiensis* infection in tomato supports the previous study that the resistance induced by the endophytic bacterial strains is systemic. The induced systemic resistance promoted by endophytic bacteria can enhance the synthesis of defense compounds in response to pathogen attack (Van Loon 2007; Wang and Liang 2014). PO, PPO, and PAL are linked to the ISR pathway regulated by jasmonates and ethylene and that is activated by saprophytic microorganisms including rhizobacteria (Van Loon *et al.* 1998). The plant hormones jasmonic acid and ethylene play a major regulatory role in the network of interconnected signaling pathways involved in ISR induction (Pieterse *et al.* 2012). Generally, endophytic bacteria are known of make the host plants more tolerant of pathogens by stimulating ISR, which protects aboveground plant tissues and acts through roots to leaves.

In our study, the inoculation of tomato plants with all endophytic bacterial isolates and challenged with the pathogen resulted in the highest synthesis of PAL in the leaves 93.30-493.30 % in the roots 153.33-313.33 % higher than the untreated plants. Whereas in the diseased control showed the enhancement of PAL lower, in the leaves 46.70 % and in the roots 29.63 % than untreated control. The same trends have been reported by Nakeeran *et al.* (2006), the increased activity of PAL was recorded in PA23-treated hot pepper seedlings challenged with *P. aphanidermatum*, but the maximum PAL activity reached a maximum at 12 days, later than our result 3 dpi in the leaves and 5 dpi in the roots. Further, the PAL activity declined after this. In plants inoculated with pathogen alone, the PAL activity declined rapidly, and in untreated plants the PAL activity more lower. PAL plays an important role in the biosynthesis of phenolics and phytoalexins (Daayf *et al.* 1997). In the previous studies, the enhanced activities of PAL in plant tissues are positively associated with ISR and plant disease suppression (Li *et al.* 2012, 2015; Prathuangwong and Buensanteai 2007). Thus, higher induction of PAL might have reduced the disease incidence and increased disease control in all the endophytic bacteria treated plants.

Increased PAL activity is a key response to pathogen challenge in many plant species and is closely correlated with resistance (Pallas *et al.* 1996). PAL regulates

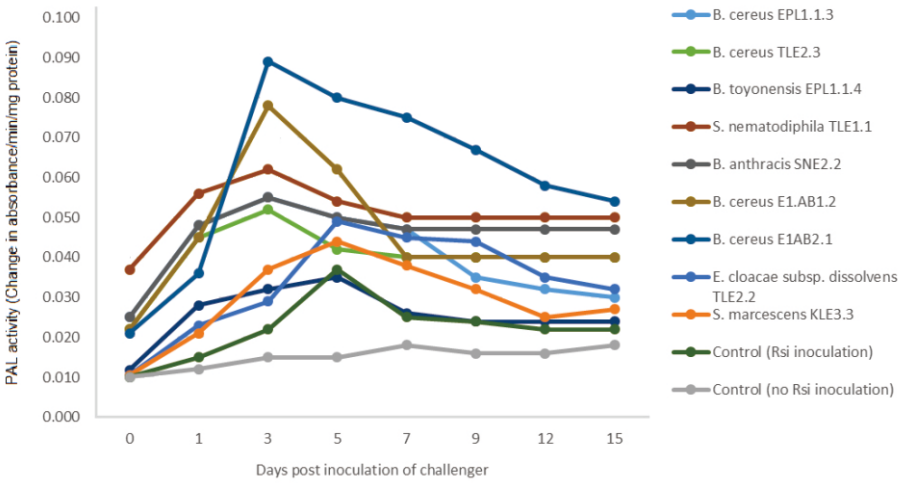

Fig 1 Phenylalanine ammonia lyase activity in tomato leaves inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *Indonesiensis.*

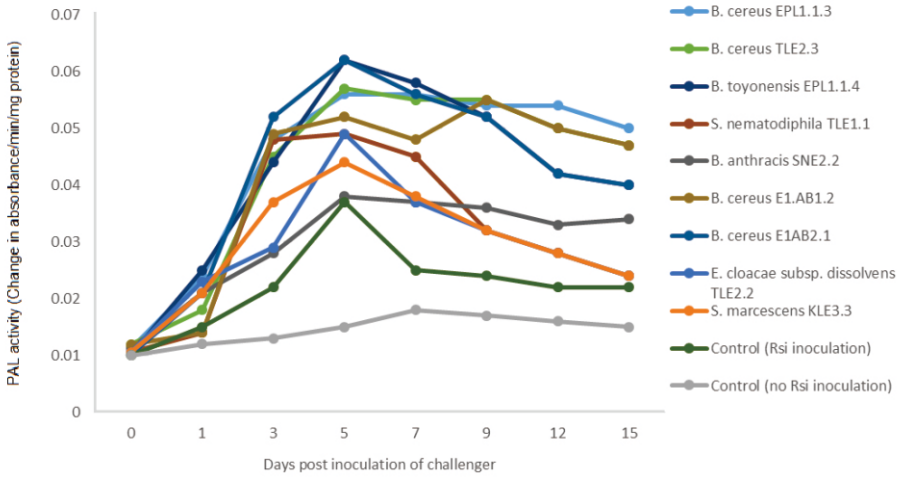

Fig 2 Phenylalanine ammonia lyase activity on tomato roots inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *Indonesiensis.*

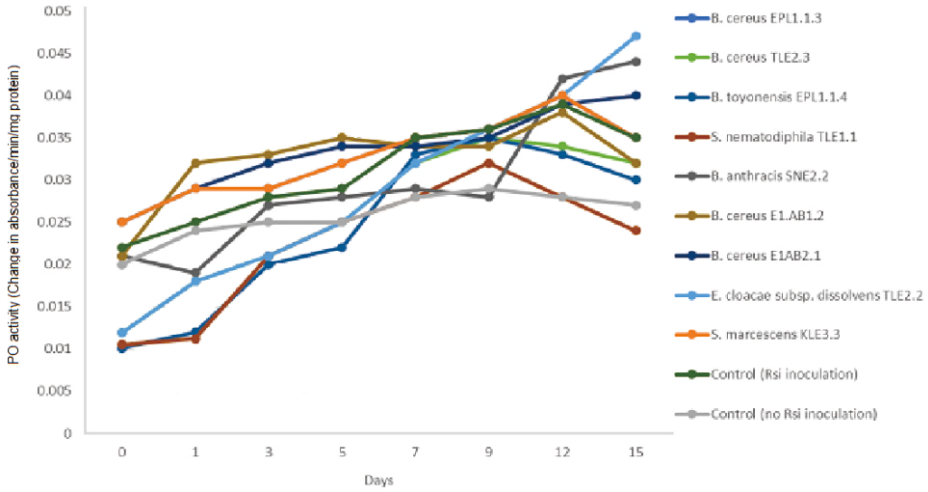

Fig 3 Peroxidase activity on tomato leaves inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *Indonesiensis.*

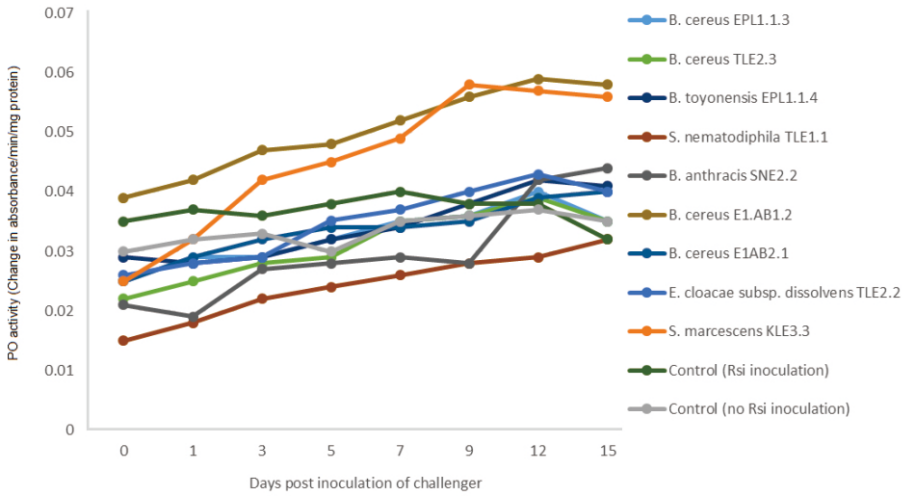

Fig 4 Peroxidase activity on tomato roots inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *indonesiensis.*

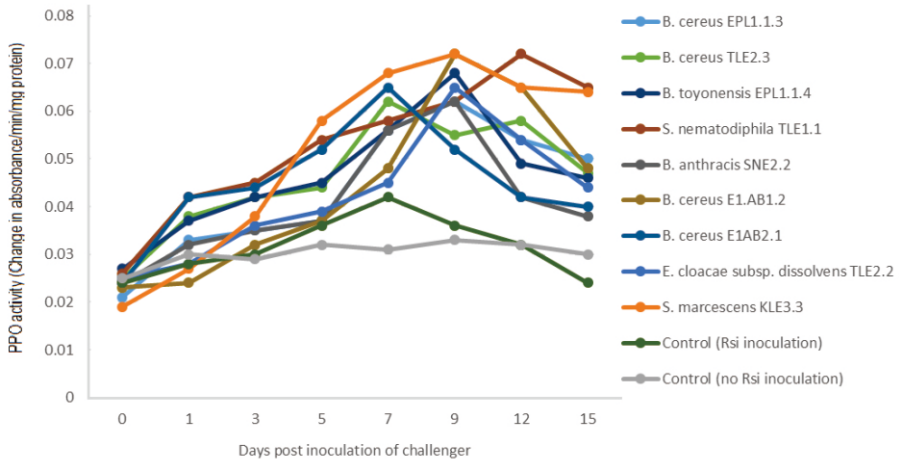

Fig 5 Polyphenol oxidase activity on tomato leaves inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *indonesiensis.*

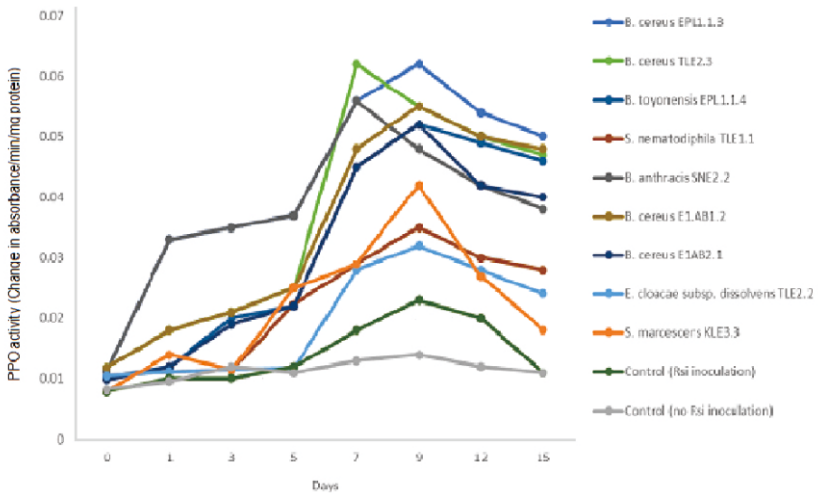

Fig 6 Polyphenol oxidase activity on tomato leaves inoculated with endophytic bacteria and challenged with R. *syzigii* subsp. *indonesiensis.*

secondary metabolism in plants, leading to the biosynthesis of phenylpropanoids as well as the signaling molecule, SA. In the previous studies, the enhanced activities of PAL in plant tissues are positively associated with ISR and plant disease suppression (Li *et al.* 2012, 2015; Prathuangwong and Buensanteai 2007). These results support that PAL might important for the induction of disease resistance to *Rsi*.

Fast all of endophytic bacterial strains tested in this investigation induced biosynthesis of PO higher in the leaves (11.11-74.7 %) and in the roots (5.41-59.46 %) than untreated control, whereas enhancement of PO in the diseased control were lower in the leaves (29.63 %) and the roots (2.7 %) compare than untreated control. According to Bradley *et al.* (1992) that PO is implicated in a variety of functions, such as defense mechanisms and lignification (Blee *et al.* 2003). Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West 1989). Increased activity of peroxidase has been implicated in several physiological functions that may contribute to resistance including exudation of hydroxyl cinnamyl alcohol into free radical intermediates (Gross, 1980) and lignification (Walter 1992). Peroxidase is also associated with the deposition of phenolic compounds into plant cell walls during resistance interactions (Graham and Graham 1991). PO is implicated in a variety of functions, such as defense mechanisms (Bradley *et al.* 1992) and lignification (Blee *et al.* 2003). Nikraftar *et al.* (2013) concluded that PO might be involved in phenolics production in tomato plants, as an effective resistance mechanism in tomato pathosystem. Hernández-Blanco *et al.* (2007) found that an alteration of secondary cell wall integrity by inhibiting cellulose synthesis has led to specific activation of plant defense response against the soil-borne bacterium like *Rsi*. Higher PO activity has also been reported correlated with disease resistance in many plants (Vidhyasekaran 1988). Chen *et al.* (2000) also noted that PO and PPO activity at a later stage may contribute to cross linking of hydroxyproline rich glycoproteins (HRGPs), lignifications that will act as barriers against pathogen entry. The induction of defense related enzyme activity in our study may correlate well with the accumulation of lignin in the cell wall of tomato roots where the pathogens reside. These defense responses may include the elaboration of cell wall thickenings usually accompanied by the deposition of lignin, a polymer of aromatic phenolics (Fattah *et al.* 2011). This cell wall thickenings activity providing structural support and barrier against invading pathogens (Carpita and McCann 2000) such as *Rsi* and play a physical barrier role to stop the pathogen spread through the plant.

In summary, the reduction of bacterial wilt disease infected tomato plants by *Rsi* by inoculation of endophytic bacteria from our previous study (Yanti *et al.* 2017) confirmed by this current study were due to the increased activity of defense-related enzymes and their relative gene expressions in plants treated with endophytic bacteria. Hence, the mechanism exhibited by our strains in controlling R. *syzigii* subsp. *indonesiensis* was assessed. All the strains were tested to study their ability to induce defense molecules such as PAL, PO, and PPO in tomato plants. Therefore, our results suggest that early molecular signaling in defense-related genes expressions may play an important role in-induced systemic resistance by endophytic bacteria in tomato. To elucidate the intricate interactions among the plant, antagonist, and pathogen and to improve our understanding of the mode of action of the selected endophytic bacteria strains, more mechanisms related to the induced systemic resistance by endophytic bacteria strains analyses are recommended.

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