Phenylalanine Ammonia Lyase Activity of Soybean's Seedlings Introduced with Rhizobacteria, which had Ability to Induce Resistance of Soybeans Towards Bacterial Pustule (*Xanthomonas axonopodis* pv. *glycines*)

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Abstract

Xanthomonas axonopodis pv. *glycines* is a major constraint in soybean cultivation and cause bacterial pustule disease. One of mechanism of ISR on plant are changes of phenylalanine lyase enzymes activity. Four rhizobacterial isolates, which have ability to induce systemic resistance (ISR) of soybean were tested its ability to induce Phenylalanine ammonia lyase (PAL) activity. The aim of this experiment was to study the activity of PAL in rhizobacteria introduced soybean which have ISR against *Xanthomonas axonopodis* pv. *glycines*. The experiment has been done in triplicate. Four rhizobacteria isolates were introduced on soybean seed and two weeks old seedling. PAL activity was assayed on soybean seedlings gradually (0, 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 30 and 35 days after rhizobacterial introduction). Study showed that all rhizobacteria introduced to soybean's seedlings have ability to increase activity of PAL enzyme compared to control.

Keywords: Enzyme activity, induced systemic resistance, phenylalanine ammonia lyase, rhizobacteria, soybean, *Xanthomonas axonopodis* pv. *glycines*

Introduction

Xanthomonas axonopodis pv. glycines (*Xag*) is the causal agent of bacterial pustule of soybean (*Glycine max*), a serious disease in several major soybean-producing countries (Wrather et al., 2001). *Xag* causes bacterial pustules on susceptible soybean cultivars. The disease symptoms are due primarily to hypertrophy of host mesophyll cells (Jones and Fett, 1987), which can result in premature defoliation of infected plants. Yields are lowered because of reduced seed size (Weber et al., 1966), and under certain environmental conditions, serious economic losses can occur (Hwang et al., 1966).

The pustule bacterium over seasons in crop residue on the soil surface, on seeds, in the rhizosphere of wheat roots and other crops. Additional host plants include garden beans (*Phaseolus vulgaris*) cowpea (*Vigna*), redvine, buckwheat-vine or ladies-eardrops (*Brunnichia cirrhosa*), twinflower Dolichos (*Dolichos biflorus*), and hyacinth-bean (*D. lablab*) (Greifenkamp, 2002). The pathogen is very difficult to control. Research have shown that different diseases in fruit crops and soybean can be controlled using antagonistic bacteria (Wilson and Lindow, 1993), (Adijuwana et al., 2000). The need to minimize the use of pesticides and restriction in the application of antibiotics owing to their harmful effects for man and the environment, have resulted in microbial controls associated with integrated soybean crop management becoming a valuable alternative to fight bacterial pustule. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (De Weger et al., 1995; Gerhardson, 2002; Postma et al., 2003).

One group of important biocontrol agents is the Plant Growth Promoting Rhizobacteria (PGPR). PGPR are root-colonizing bacteria with beneficial effects including plant growth promotion and biological disease control. Some of these rhizobacteria are beneficial to the plant in direct or indirect way, resulting in protection of plants against pathogens and stimulation of plant growth. PGPR have various ability to induce systemic resistance in plant which provides protection against a broad spectrum of plant pathogens and is referred as induce systemic resistance (ISR). ISR pathway is induced when plant is challenged by pathogenic organisms (Bloemberg and Lugtenberg, 2001). The elevated resistance due to an inducing agent upon infection of pathogen; ISR is expressed upon subsequent or challenge inoculation with pathogen (Van Loon, 1997; Van Loon et al., 1998; Ramamoorthy et al., 2001).

Significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies (Shiddiqui, 2005), such as fluorescent pseudomonads against *Ralstonia solanacearum.* All three strains suppressed wilt of tomato and increased yield (Jagadeesh, 2001). *P. fluorescens* against *Xanthomonas oryzae* pv. *oryzae,* showed resistance to the rice bacterial blight pathogen (Vidhyasekaran et al., 2001), (Habazar et al., 2001). *Azospirillium brasilense* against *P. syringae* pv. *tomato,* prevented bacterial speck disease development and improved tomato growth (Bashan and Bashan, 2002). *Serratia* J2, *Pseudomonas, Bacillus* BB11 against *Ralstonia solanacearum* suppressed wilt of tomato and increase yield (Guo et al., 2004). *B. cereus, B. lentimorbus, B. pumilus* against *Xanthomonas campestris* pv. *campestris.* Incidence and severity of black rot of cabbage were reduced when antagonists were applied (Massomo et al., 2004). Seratia marcencens N. 2.5 againts *Xanthomonas axonopodis* pv. *allii* suppressed bacterial leaf blight on shallots in green house (Yanti, 2015). Psudomonad fluoresens suppressed bacterial leaf spot by *Xanthomonas axonopodis* pv. *vesicatoria* on tomato (Habazar et al., 2000a), bacterial pustule by *Xanthomonas axonopodis* pv. *glycines* on soybean (Habazar et al., 2000b). *Pantoea agglomerans, Enterobacter cloacae, Stenotrophomonas* sp., *Serratia marcescens,* five *Bacillus thuringiensis* strains, *Bacillus cereus, Bacillus weihenstephanensis* and *Bacillus* sp. suppressed xanthomonas leaf blight by *Xanthomonas axonopodis* pv. *allii* on onion (Habazar et al., 2010). We have found three rhizobacterial isolates, which have the ability to induce systemic resistance of soybean against *Xanthomonas axonopodis* pv. *glycines*. We have identified these isolates as *Bacillus* sp., *Bacillus thuringiensis* serovar *toumanoffi* and *Bacillus thuringiensis* strain TS2 (Habazar et al., 2011; Yanti et al., 2013).

The constitutive defenses of plants include structural barriers, such as the plant cell wall, as well as inhibitory compounds including phenolics (Nürnberger et al., 2004). Phenolic compounds can be formed in response to the ingress of pathogens and their appearance is considered as part of an active defense (Nicholson and Hammerschmidt, 1992). Evidence strongly suggests that esterification of phenols to cell-wall materials is a common theme in the expression of plant resistance and it has been suggested that crosslinking of such phenylpropanoid esters leads to the formation of lignin-like polymers (Fry, 1987). Phenylalanine ammonia lyase (PAL) is a key enzyme of phenyl-propanoid metabolism in plants. PAL activity in plant tissue may rapidly change under the influence of various factors, such as pathogen attack and treatment with elicitors (Lamb, 1990). The activities of PAL may rapidly be enhanced under the influence of elicitors or pathogen attack. Enhancement of PAL activities was reported in response to *Rhizoctonia solani* inoculation in cowpea pretreated with SA (Chandra, 2007). In this study, we investigated the activity of phenylalanine ammonia lyase enzyme in rhizobacterial introduced soybean which have ISR against *Xanthomonas axonopodis* pv. *glycines*.

Materials and Methods

Experimental Set Up

Isolates used in this research are Pl4.Rz1.1, St4E1.1, ST1E1.1, Pl2Rz4.2 which have best ability to increase growth rate of soybean and pathogen *X. axonopodis* pv. *glycines* (*Xag*) from Previous Research (Yanti et al*.,* 2013). Rhizobacteria isolates saved in microtubes were streaked on petri dishes contain Nutrient Agar (NA) and incubated for 46 hours. After that, all isolates cultured on petri dishes contain NA for 48 hours, then suspended until 10^7 (compared with *McFarland* solution scale 7) for treatments. *Xag* isolate grown on Medium NA with strike method. One pure colony then re-strike on Medium NA for 48 hour and then suspensed with sterile aquadest, homogenized with vortex and compared with *McFarland* solution scale 6 (population estimated 10^6 CFU/ml). Seeds of soybean (cultivar Anjasmoro) were obtained from Bogor District, West Java, Indonesia. This research used *Completely Randomized Design* with three replications, each replication used 11 seedlings planted one seedling per pot as destructive sampling.

Introduction of rhizobacterial isolates

Surface sterilized soybean seeds of variety Anjasmoro were soaked in 100 mL rhizobacterial isolates (10^8 cfu/ml) suspension for 1 h with occasional shaking to ensure uniform coating on the surface under aseptic conditions. Seeds soaked in sterilized distilled water were treated as control. The seed were then directly plant to sterile growth medium (soil type Latosol: cow dung manure = 3:1) without synthetic fertilizer application, and watered with tap water routinely until harvested. Rhizobacterial isolates then reintroduced after 7 days old, the second introducing count as 0 day.

Pathogen Inoculation

Pathogen inoculated to the leaves of 14 days old seedlings which had been punctured before, then inoculum smeared with cotton on the underside of leaves and covered with clear plastic bag for 5x24 hour (Klement et al., 1990). Plants were harvested after 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 28 days post introduction (dpi). Freshly harvested plant then separated for leaves, stem and root parts for its separated PAL activity assay.

Enzim Extraction and Assay

Enzyme extraction steps were carried out at 4°C. 1 g fresh weight of leaf, stem or root tissue was macerated, and then added 2,5 ml buffer borate (pH 8,8) containing 54 mM mercaptoethanol and 1 gram polyvinyl pyrrolidone (PVP). The suspension was homogenized for 1 min and then centrifuged at 10 000 × g for 25 min. PAL was assayed directly in the supernatant after concentration. Subsequently, 200 mM Tris- HCl (pH 7.0) was used as assay buffer and 20 mM L-phenylalanine as substrate of the enzyme in the assay. PAL activity was assayed using a modified method of Sainders and McClure (1975). The reaction was carried out for 60 min at 37°C and the increase of absorbance in A290 nm was recorded at every 15 min interval. The rate of formation of *t*-cinnamic acid was taken as a measure of enzyme activity using an increase in absorbance of 0.01 at A290 nm as 3.09 nmol of cinnamic acid formed. The PAL activity was expressed in nkat/mg protein. Protein concentration was measured according to the standard method of Bradford (1976).

Results and Discussion

Results

PAL is considered as a key defense enzyme of the plants and its activity was studied after introduction of the rhizobacterial on roots, stems and leaves of soybean (0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 28). PAL activity showed that PAL enzyme Activity are higher in root (17,65 ppm) and leaves (15,23 ppm) compare in stem (11,94 ppm). The activity of PAL on soybean roots was found to increase from 0 day of introduction and was highest at 12 days (Figure 1). PAL activity showed an increase by all three rhizobacterial strains compare to the control (gradually decrease); then there was a decrease in PAL activity in the root tissues. At 28 days post introduction, PAL activity of introduced roots was remained higher than control.

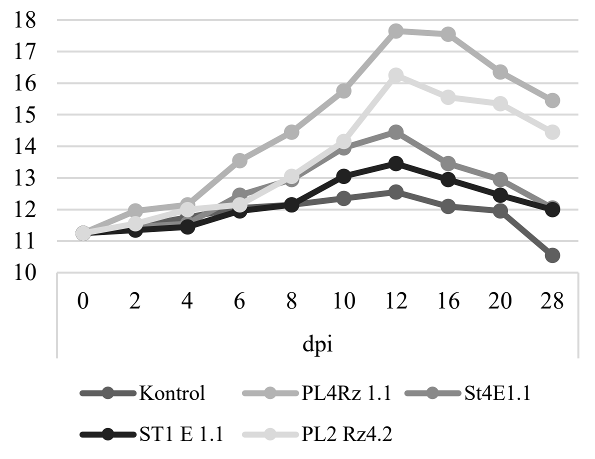

Figure 1 Phenylalanine ammonia lyase activity on root of rhizobacterial introduced soybean

The activity of PAL on soybean stems was found to increase from 0-12 day of rhizobacterial introduction (Figure 2). Similar trend of PAL activity was observed in all treatment included control. However, PAL activity showed a higher trend increase by all three rhizobacterial strains compare to the control.

The activity of PAL on soybean leaves was found to increase from 0-12 day of introduction, except on leaves of soybean which introduced by rhizobacterial isolate ST1 E1.1. was highest at 4 days (Figure 3). PAL activity showed an increase by all three rhizobial strains compare to the control (slightly increase until 4 dpi).

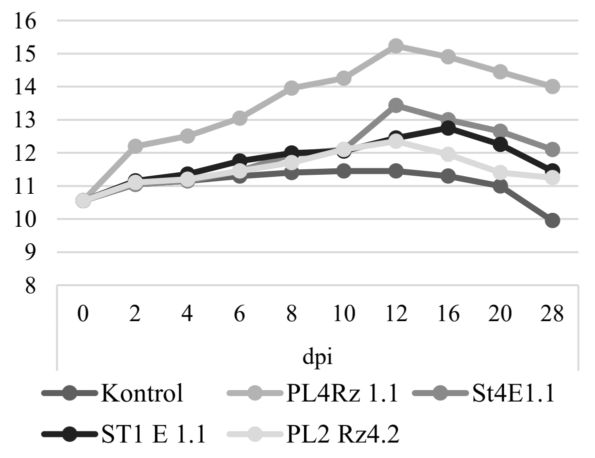

Figure 2 Phenylalanine ammonia lyase activity on stem of rhizobacterial introduced soybean

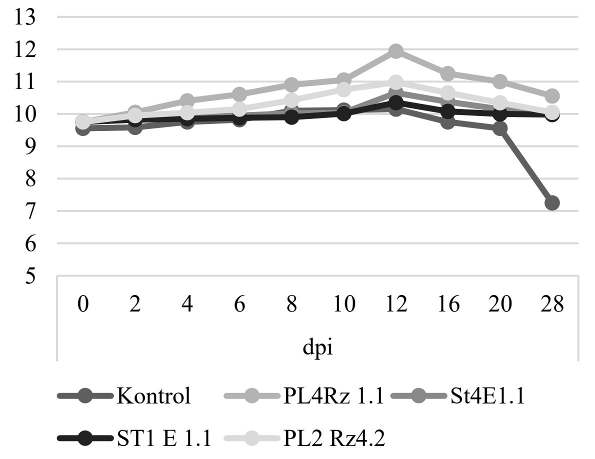

Figure 3 Phenylalanine ammonia lyase activity on leaves of rhizobacterial introduced soybean

Discussion

Elicitation of plants with elicitor molecules or biocontrol agents results in the activation of a series of defense responses, including cell wall reinforcement by deposition of lignin and induction of an array of defense enzymes (Desender et al., 2007). Cell wall strengthening, through the deposition of lignin, preceded by the induction of the synthesizing enzymes played an important role in the defense response of Lycopersicon esculentum in reaction to a variety of elicitors (Mandal and Mitra, 2007).

In the present study, it was demonstrated that three rhizobacterial isolates could induction of defense enzymes such as PAL on roots, stems and leaves of soybean. However, PAL activity was found to be higher on root and leaves of rhizobacterial introduced soybean seedlings. PAL activity in plant tissue may rapidly change under the influence of various factors such as treatment with elicitors (Dixon and Lamb, 1990). PAL mRNA is accumulated in both the incompatible interaction between tobacco cells and Pseudomonas syringae pv. tomato and the compatible interaction with P. syringae pv. syringae, in response to flagellin (Tagushi et al., 2003). Vanitha and Umesha (2011) Found that Pseudomonas fluorescens which have PGPR ability can increase PAL, Peroxidase, Polyphenol oxidase and lipoxygenase in tomato seedlings.

In this investigation, the rapid increase in PAL activity in rhizobacterial introduced soybean roots, stems and leaves supports published reports and correlates with the accumulation of phenolics and lignin (Mandal, 2010). Yanti et al., (2013) in Previous research found that Pl4Rz1.1 isolates have best ability to control Xag and also promote growth rate and increase yields of soybean. This present research confirmed that PAL activity are one of the isolate's mechanism to induced soybean's resistance. Jain et al., (2014) research also shown higher PAL activity on Glycine max L. treatment with Rhizobacterium.

Phenylalanine ammonia lyase (PAL) activity has been observed to be induced during plant -pathogen and plant - pest interactions and is known to play an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Mishra et al., 2014). This research's result consistent with this fact, and increases in PAL parallel with disease resistance induced by PGPR. Kurth et al. (2014) observed that PAL activity increased in pedunculate oak leaves after inoculation with Microsphaera alphitoides and Streptomyces sp. strain AcH 505.Chen et al. (2000) showed that high levels of PAL were induced in cucumber roots inoculated with Pythium aphanidermatum. Plants may accumulate phenolics as a function of PAL activity as a mean of passive defence and the magnitude of the accumulation primarily depends on the supply of the primary precursor, L-phenylalanine (Basha and Chatterjee, 2007). This explain why PAL activity are increase by PGPR introduction even though Xag has not inoculated. Innoculation by rhizobacteria isolates may induced resistance of soybean, increased defensive related compound before pathogen attacked.

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