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INDUCTION OF PLANT RESISTANCE AGAINST *SOYBEAN STUNT VIRUS* USING SOME FORMULATIONS OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The efficacy of *Pseudomonas aeruginosa* formulations in inducing plant growth and systemic resistance (ISR) on soybean against infection of *Soybean stunt virus* (SSV) was evaluated under greenhouse conditions. *P. aeruginosa* isolate PaJ was formulated in the forms of powder, liquid, gel, compost, and capsule. Some growth parameters such as maximum plant height, fresh and dry weight of shoot, fresh and dry weight of root, and dry weight of 1,000 seeds were observed. Virus concentration and disease incidence were determined using DAS-ELISA. Results of this study showed that application of *P. aeruginosa* formulations significantly increased the plant growth. The maximum plant height, fresh and dry weight of shoot, fresh and dry weight of root, and the dry weight of 1,000 seeds on treated plants were significantly higher than those of un-treated control plants according to the Duncan's multiple range test ($P<0.05$). Disease incidence on treated plants ranged between 15% to 80%, while most (90%) of the untreated plants were infected. Peroxidase activity on treated plants increased by 4.89 to 6.49 times compared with the un-treated plants. These results suggested that application of *P. aeruginosa* could increase the resistance of soybean against SSV, promote and increase the plant growth and the yield. Hence, can be considered as one of the measures to control SSV on soybean.

Key words : Virus concentration, disease incidence

INTRODUCTION

Soybean stunt virus (SSV) has been known to be an important constraint for soybean production in Indonesia, reducing the soybean production by 41-71% (Adisarwanto and Wudianto, 1999). To control the disease, synthetic pesticides have been used particularly to reduce the insect vector population (Akin and Barmawi, 2005). Considering the negative impact of the improper use of synthetic pesticides and the increasing awareness for healthy food and environment, it is necessary to develop environmentally safe control methods. One of the methods which is relatively safe to the environment is the use of plant growth promoting rhizobacteria (PGPR).

Several strains of bacteria have been investigated and reported as PGPR on different plants, however *Bacillus* and *Pseudomonas* were the most widely reported PGPR (Kumar et al., 2009; Murphy et al., 2000; Raupach and Kloepper, 1998). The impact of rhizobacteria is commonly on plant growth and health (Kloepper et al., 1989). One of the mechanisms in which the PGPR can protect the plant from pathogen's infection was through induced systemic resistance (ISR) (Wei et al., 1991; Press et al., 1997). The signaling pathway of ISR is different from systemic acquired resistance (SAR), in which ISR was characterized by the increase in ethylene and jasmonic acid synthesis followed by the activation of ISR genes (Pieterse et al., 2002). These ISR genes are mostly coding for pathogenesis related proteins (PR-protein) acting as antibiotic (Shah et al., 1997). According to van Loon (1997) peroxidase enzymes belong to the group of PR protein from *PR*-9 which are accumulated

when the plant is infected with a virus. A plant that is resistant to the virus disease tends to have higher peroxidase activity compared with the susceptible ones (Gupta et al. 1990).

Pseudomonas aeruginosa has been reported to be a potential PGPR that inhibited the growth of plant pathogens on several crops (Anjaiah et al., 1998; Mansoor et al., 2007; Siddiqui and Shaukat, 2005; Adesemoye et al., 2008). The *P. aeruginosa* PNA1 isolated from the rhizosphere of chickpea in India suppressed *Fusarium* wilt of chickpea caused by *Fusarium oxysporum* f.sp. *ciceris* and *Pythium* damping-off of bean caused by *Pythium splendens* (Anjaiah et al., 1998). Other work also proved that *P. aeruginosa* effectively induced systemic resistance in rice against *Rhizoctonia solani,* the cause of sheath blight disease and increased the rice yield (Saikia et al., 2006). Root inoculation of rice with *P. aeruginosa* 7NSK2 protected rice leaves against blast disease caused by *Magnaporthe grisea* (De Vleesschauwer et al., 2006). However, no report is available on the activity of *P. aeruginosa* in inducing systemic resistance against SSV. This study was done in order to evaluate the effectiveness of some formulations of *P. aeruginosa* in inducing the plant resistance against SSV.

MATERIALS AND METHODS

Isolation and identification of *P. aeruginosa*

Samples for isolation of *P. aeruginosa* were collected from rhizospheres of soybean in Denpasar, Bali in August, 2009. Samples of 10 g of rhizosphere (roots and soils) were shaken with 90 ml of 0.1% tryptic soy broth. Aliquot of 1 ml from each broth was added to King's B agar containing 20 g of peptone, 10 ml of glycerol, 2.25 g of asparagine, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4.7H_2O$, 15 g of agar and distilled water to make 1 liter of medium. The cultures were incubated at 30^oC for 48 h. Identification of isolates was done based on Oxoid Microbact GNB Kits (Oxoid Ltd., UK).

Formulations of *P. aeruginosa*

The *P. aeruginosa* isolate PaJ isolated from the root of soybean was used in this study with a population density of 1.9 x 10^8 cfu ml^{-1}. It was formulated in the forms of powder, liquid, gel, compost, and capsule. The cells of *P. aerginosa* were mixed with tapioca flour (1:3, v/w) for powder formulation (Pw); with extract solution of fresh leaves of rain tree (*Samanea saman*) collected from Denpasar, Bali (1:20, v/v) for liquid formulation (Lq); with polyacrylamide hydrogel and extract solution of fresh leaves of *S. saman* (1:1:100, v/w/v) for gel formulation (Gl); with straw, leaves of *S. saman*, rice bran and sugar (1:70:20:10:2, v/w/w/w/w) and incubated for 45 days for compost formulation (Cm); with wheat flour (1:3, v/w) and placed into a capsule (Brataco Chemical, Jakarta) with capacity of 700 mg for capsule formulation (Cp).

Application of *P. aeruginosa* formulations

The experiment was designed in randomized complete block design (RCBD) with six treatments of *P. aeruginosa* formulations namely Pw, Lq, Gl, Cm, Cp, and Kn (control). Each treatment was replicated six times, thus there were 36 experimental units in this experiment. Each experiment unit consisted of 10 soybean plants (cultivar Tangganus) which is susceptible to SSV. The seeds for *P. aeruginosa* treatments were soaked in *P. aeruginosa* suspension (10^8 cfu ml^{-1}) for 15 minutes, while the non treated seeds were soaked in sterile distilled water. The seeds were then germinated and grown in a seedling tray for two weeks. The soybean seedlings treated with *P. aeruginosa* formulation were transplanted in polyethylene bags filled with 3 kg of growth media per bag, consisting of soil and cow manure (3:1, w/w). The application of *P. aeruginosa* formulations were done as follows: Pw at 50 g per polyethylene bag, Lq at 50 ml per polyethylene bag, Gl at 50 g per polyethylene bag, Cm at 50 g per polyethylene bags, and Cp at 1 capsule per polyethylene bag. Seedlings without treatment of *P. aeruginosa* formulations (Kn) were planted in polyethylene bags

filled with the growth media. The youngest two leaves of each soybean plant were lightly dusted with carborundum and then rub-inoculated with SSV inoculums two week after transplanting. Inoculum consisted of SSV-infected soybean leaf tissue ground in 50 mM of KPO4, pH 7.0, containing 10 mM of sodium sulfite at a ratio of 1 g tissue : 5 ml buffer (Zehnder et al., 2000).

Plant growth

Growth parameters were observed until 110 days after SSV inoculation, such as plant height, fresh and dry weight of shoot, fresh and dry weight of root, and the dry weight of 1,000 seeds. Data analysis was done using F-test, followed by Duncan's multiple range test (DMRT) at 5% level of significance.

Detection of SSV infection and determination of chlorophyll content

Detection of SSV infection on inoculated plants was conducted by DAS-ELISA. To quantify the results, ELISA reader (Microplate Reader Model 550, Biorad, USA) at 405 nm wavelength was performed. Total chlorophyll content (SPAD unit) was determined with a chlorophyll-meter SPAD-502 (Konica Minolta, Japan).

Measurement of peroxidase activity

Measurement of peroxidase activity on plants was carried out at 10 days after inoculation according to the method developed by Simon and Rose (1970). Total protein levels were measured by Bradford reagent using bovine serum albumin (Wako Pure Chemical Industries Ltd., Japan) as standard, through regression equation. The enzyme activity was determined using a spectrophotometer (Spectronic 20 Genesis, Spectronic Instrument, USA) with absorbance at 500 nm wavelength.

Data for plant growth parameters, disease incidence, and chlorophyll content were analyzed for analysis of variance (ANOVA) and the treatment means were separated by Duncan's Multiple Range Test (DMRT) (= 0.05) using SAS software version 6.12 (SAS Institute, Gary, NC, USA).

RESULTS AND DISCUSSION

The SSV infection inhibited plant growth significantly as indicated by the low plant height. The plants treated with *P. aeruginosa* formulations was significantly ($P<0.05$) higher than that of plants without *P. aeruginosa* treatment. Except for the powder formulation (Pw), all other *P. aeruginosa* formulations were able to increase plant height, fresh and dry weights of roots, fresh and dry weight of shoots, and dry weight of 1,000 seeds (Table 1). This result is in agreement with the result of Parmar and Dadarwal (1999), where PGPR treatment of groundnut plants increased the dry weight of root, dry weight of biomass, and total nitrogen in the plant significantly.

The mechanism by which PGPR promotes plant growth is related to the production of plant hormones and increase of the nutrients supply for the plants. The phytohormones produced by PGPR are auxin, ethylen, cytokinin and gibberelin which are needed for the plant growth. In addition, the PGPR can also dissolve phosphate in the soil and produce several organic acids such as formic acid, acetic acid, propionic acid, lactic acid, glycolic acid, fumaric acid, and succinic acid. These organic acids can form organic chelate (stable complex) with aluminum (Al), iron (Fe) or calcium (Ca) that fix the phosphorous (P) so that the ion of H_2PO_4 is released from the bond and will be available for the plants (Podile and Khishore, 2007). *P. aeruginosa* strain PNA1 also produced phenazines, phenazine-1-carboxylix acid, and oxychloraphine, and inhibited mycelial growth of *Fusarium oxysporum* f.sp. *ciceris* (Anjaiah et al., 1998). Three pathogenesis-related peroxidases with molar

mass 28, 36 and 47 kDa were detected in the rice plants treated with *P. aeruginosa* and challenged inoculated with *Rhizoctonia solani* as pathogen (Saikia et al., 2006). The accumulation of salicylic acid in the root system of rice plants after treatment of *P. aeruginosa* was also observed by Saikia and co-workers (2006).

Table 1. The maximum plant height, fresh and dry weights of shoot, fresh and dry weights of roots, and dry weight of 1,000 seeds.

Treatment	Maximum plant height (cm)	Shoot Fresh weight (g)	Shoot dry weight (g)	Root Fresh weight (g)	Root dry weight (g)	Dry weight of 1,000 seeds (g)
Kn	43,89 a*	85,43 a	23,28 a	36,87 a	5,63 a	110,63 a
Pw	44,00 a	122,70 f	30,00 f	71,15 f	10,96 e	107,87 a
Lq	57,25 e	145,15 e	34,93 e	70,12 e	10,40 f	124,13 d
Gl	62,37 d	192,48 b	56,43 b	134,50 b	16,36 b	131,13 b
Cm	66,56 b	186,92 c	55,62 c	83,51 d	14,54 d	130,50 c
Cp	63,63 c	174,15 d	45,87 d	97,27 c	15,03 c	123,38 e

*Values in the same columns followed by the same letters are not significantly different (P> 0.05) according to the Duncan's Multiple Range Test (DMRT).

Based on the DAS-ELISA test, the concentration of SSV in the plant tissues treated with *P. aeruginosa* formulations (except for Pw) was obviously higher than that of untreated plant tissues. The high virus concentration and the high disease incidence in the untreated plants, resulted in significant growth retardation. The disease incidences on the plants treated with *P. aeruginosa* formulations were 80%, 15%, 20%, 20%, and 10%, respectively, while the disease incidence in the untreated plants was 90% (Table 2). The untreated plants showed stunt and mosaic symptoms followed by leaf malformation, while no severe symptom was observed in the plants treated with *P. aeruginosa* formulations. Similar results were demonstrated by Damayanti and Katerina (2008) in which rhizobacteria treatment on hot pepper inoculated with *tobacco mosaic virus* (TMV) and *chili veinal mottle virus* (ChiVMV) exhibited milder symptom expression compared with control plants. The individual strains and strain mixtures of plant growth-promoting rhizobacteria such as *Bacillus pumilis* strain INR7, *Curtobacterium flaccumfaciens* strain ME1, *Burkholderia gladioli* strain IN26, and *Bacillus subtilis* strain GB03 reduced significantly the incidences of anthracnose and angular leaf spot diseases on cucumber (Raupach and Kloepper, 1998).

Table 2. DAS-ELISA test for SSV infection and chlorophyll content in the leaf.

Treatment	DAS-ELISA values at 405 nm	Disease incidence (%)	Chlorophyll content (SPAD Unit)
Kn	1.472*	90 a**	29.98 a**
Pw	0.922	80 f	41.70 f
Lq	0.547	20 de	43.24 e
Gl	0.519	10 b	51.97 b
Cm	0.611	15 c	51.63 c
Cp	0.572	20 d	44.69 d

*ELISA absorbance values were obtained from duplex measurement of composite samples per treatment. Positive results of ELISA are twice the absorbance of healthy plants. The absorbance value of SSV = 0.087
**Values in the same columns followed by the same letters are not significantly different (P > 0.05) according to DMRT.

This study proved that the chlorophyll content in the leaves of soybean plants treated with PGPR formulations was significantly higher than that of untreated ones. The increase in chlorophyll content in the leaves of treated plants may be due to increase of the ACC-deaminase enzyme in the PGPR-treated plants, which slow down chlorophyll degradation, or probably due to the increase of the photosynthetic rate in the plants treated with PGPR formulations. On the other hand, the untreated plants severely infected by SSV, showed low content of chlorophyll and, decrease in chlorophyll efficiency, and leaf growth. As the treatments with *P. aeruginosa* formulations promoted the plant growth, as well as induced the plant resistance to SSV, the plants were able to reduce unit mg^{-1} $minute^{-1}$ viral infection. The peroxidase activity at 10 days after inoculation increased from 0.0698 on untreated plant to 0.4112 - 0.5232 unit mg^{-1} $minute^{-1}$ on treated plants.

This result suggested that treatments with *P. aeruginosa* formulations in forms of gel, capsule, liquid and compost were able to increase the peroxidase activity about 4.89 to 6.49 times if compared with untreated one (Fig.1). Kishore and co-workers (2005) reported the peroxidase activity increased 1.5-2 times, both at 7 days after induction, and 12-72 hours after inoculation. Silva and co-workers (2004) stated that the high peroxidase activity is usually associated with the slow infection rate due to the lignifications and the formation of hydrogen peroxide that directly inhibit the growth of pathogen, or the formation of free radicals which had antibiotic activity.

The low resistance may be due to the weakness of the cell surface of soybean because of the low cell lignifications so that the penetration of SSV occurred easily, or the low expression of genes related to the plant resistance caused by the enzymes including peroxidase and chitinase. Cell lignification is the process of lignin formation in the cell wall, and this process involves peroxidase enzyme. This phenomenon supported by the study done by Murphy and co-workers (2000) that tomato plants treated with resistance inducing agents showed higher peroxidase activity in comparison with untreated plants.

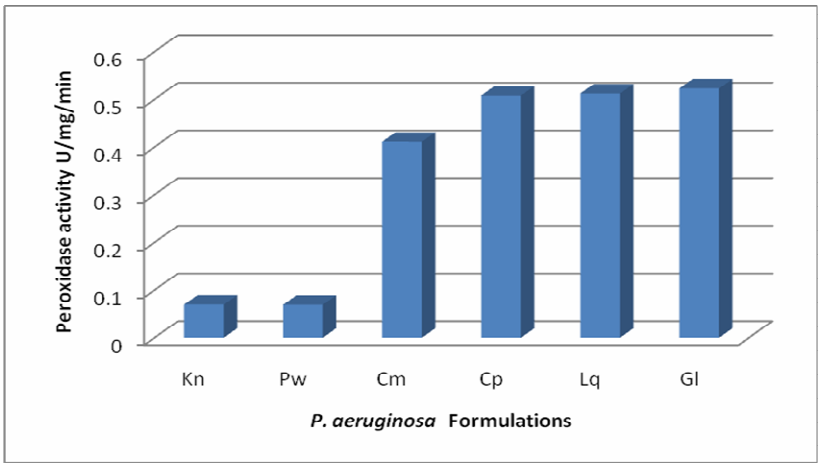

Figure 1. Peroxidase activity of SSV-infected plants without (Kn) and with *P. aeruginosa* formulations treatments (Pw: powder, Cm: compost, Cp : capsule, Lq: liquid, and Gl : gel).

Peroxidase has an important role in the process of papilla formation, especially in the papilla lignifications. Papilla is located at the layer of cells consisting of various materials concentrated in the plasma membrane and cell wall. This organ is formed as a response to host resistance against interference with the cell surface, such as penetration by pathogens and mechanical damage (Sheng and Huang, 2001). Peroxidase plays a role in resistance through the production of hydrogen peroxide.

Hydrogen peroxide can be directly toxic to the microorganisms and can also play a role in strengthening the cell wall with the formation of lignin precursors through the activity of peroxidase enzyme. The peroxidase enzyme is one of the enzymes that play a role in the process of plant resistance to pathogens (Seevers et al*.,* 1971). Peroxidase, lipoxigenase and phenylalanine ammonia lyase are associated with a series of ISR and jasmonate regulated by ethylene and activated by saprophytic microorganisms including rhizobacteria. The ISR activated by the application of rhizobacterial isolate is not specific to certain pathogens (Van Loon et al*.,* 1998). The pathogen non-specificity of the ISR is rather an advantage compared with the strong specificity of the traditional biological control procedure, where the antagonist that acts usually against one or few pathogens was used (Wei et al*.,* 1991). Murphy and co-workers (2000) reported that under natural conditions of high levels of vector-virus pressure, PGPR treatment resulted in reduced *tomato mottle virus* (ToMoV) incidence and disease severity and a corresponding increase in fruit yield. Three commercially available PGPR, i.e. Economas and Florezen (contained *Pseudomonas fluoresces*), and Trichozen (contained *Trichoderma viridae*) were tested for control of rice sheath blight caused by *Rhizoctonia solani* at Andhra Pradesh Rice Research Institute, India (Kumar et al., 2009). Their study showed that the treatment with PGPR resulted in the disease incidence reduction ranging from 14% to 38%. Van Loon and co-workers (1998) concluded that the main advantage of the use of PGPR induced systemic resistance is that, it can be applied only once, then the natural resistance mechanism will work for a long period.

Our present study under greenhouse conditions showed that the application of *P. aeruginosa* formulations could increase the resistance of soybean against *soybean stunt virus*, promoted plant growth and increased the yield. These results can be used as a reference to develop a control strategy for SSV on soybean.

CONCLUSION

The application of *P. aeruginosa* formulations in the forms of liquid, gel, compost and capsule effectively promoted plant growth, increased soybean resistance against *soybean stunt virus* (SSV) and increased the yield. Treatment with *P. aeruginosa* also increased significantly the chlorophyll content in the soybean leaf, reduced the viral concentration in the plant, and increased the peroxidase activity. This study suggests that the application of *P. aeruginosa* formulations can be considered as one of the effective measures to control SSV on soybean.

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