Bulgarian Journal of Agricultural Science, 25 (No 3) 2019, 480–485

Induction of total phenol and salicylic acid with *Pseudomonas* spp. suspension as indications increasing systemic resistance on vanilla plant

I. Ketut Widnyana*, I. Ketut Sumantra, Ni Putu Pandawani, Putu Eka Pasmidi Ariati

Mahasaraswati University of Denpasar, Department of Agrotechnology, Faculty of Agriculture, Bali 80233, Indonesia
**Corresponding Author:* widnyanaketut@unmas.ac.id

Abstract

Widnyana, I. K., Sumantra, I. K., Pandawani, N. P., & Ariati, P. E. P. (2019) Induction of total phenol and salicylic acid with *Pseudomonas* spp. suspension as indications increasing systemic resistance on vanilla plant. *Bulgarian Journal of Agricultural Science, 25*(3), 480–485

This study aims to determine the effect of *Pseudomonas* spp. in inducing the resistance of vanilla plants characterized by increasing total phenol content and salicylic acid in vanilla plant tissue after inoculation with *Pseudomonas* spp. suspension. In this study, vanilla cuttings were inoculated with *Pseudomonas* spp. suspension with a population density of 5 x10^6 CFU applied by root immersion, injection of the stem, and injection of vanilla leaves. The results showed that there was an increase of total phenol and salicylic acid content in vanilla plant tissue. The highest total phenol content was found in the *Pseudomonas* spp. W80 suspension treatment, which averaged 260.27 mg/g or an increase 106.89% compared to the distillated water treatment as control. The total phenol content average in the 2.6.D (2.6-dichloro-isonicotinic acid (INA) treatment is 240.80 mg/g or an increase of 91.41% compared to control. The highest salicylic acid content found in the treatment of *Pseudomon*as spp. W02 suspension is 37.65 ppm or 72.94% increase compared to control, followed by 2.6.D treatment of 32.55 ppm or 49.52% increase compared to control. Bacteria *Pseudomonas* spp. W02, W68, and W80 isolates has the ability to induce vanilla plant resistance with different abilities

Keywords: induction; resistance; vanilla; *Pseudomonas* spp.; total phenol; salicylic acid

Introduction

Vanilla stem rot disease caused by *Fusarium oxysporum* f.sp.*vanillae* is the most dangerous disease in vanilla plants in Indonesia. This disease causes very large losses and causes the death of plants until 50% – 100%, shorten the production life from 10 times the harvest to twice, even cannot produce, and low fruit quality (Hadisutrisno, 2004). *F. oxysporum* fsp. *vanillae* which is a soil-borne pathogen and can survive as a saprophyte in the soil, capable of destroying all parts of the plant at all growth phases, and producing a ground-breaking klamidospore for 7-10 years. This fact which causes vanilla stem rot disease is quite difficult to control (Semangun, 1996). Until now vanilla stem rot disease has been attacked in all regions of Indonesia, such as Java, Bali, North Sulawesi, South Sulawesi, North Sumatra, Nusa Tenggara Timur. In the world this disease also attacks in major vanilla-producing countries such as Puerto Rico, Brazil, Uganda, Tongga, Thailand and China. (Tombe, 2010). Transmission of this pathogen is mainly through cuttings that are used as a source of plant seeds. Vanilla cuttings used by farmers currently have an infection risk of 7-32% (Tombe, 1987).

The spread of the disease through the soil has a very important meaning, because the cuttings are planted on the soil of former plants affected, will also die because the base of vanilla stems will soon rot. This suggests that soil is a major source of infection. Although the infected soil is dried, it still contains pathogenic fungal spores (Semangun, 1996).

Vanilla stem rot disease until now still cannot be overcome effectively although some research has been done

(Nurcahyani et al., 2012). Biological control of stem rot disease has not been done. In general, there are several biological activities of microbial antagonists in suppressing pathogens by colonization of rhizosphere, antibiotics, enzymatics (hydrolysis), nutritional competition, volatile compounds, siderophores and by inducing systemic plant resistance (Chet, 1993).

Fluorescent *Pseudomonas* spp. has been studied for their plant growth-promoting effects through effective suppression of soilborne plant diseases. The modes of action play siderophore-mediated competition for iron, antibiosis, production of lytic enzymes, and induced systemic resistance (ISR) (Peter et al., 2007). Previous research results show that *Pseudomonas* spp. can improve yield and protect the wheat plants from *Phytium* spp. through the treatment of seed (Weller & Cook, 1986); protecting peanut crops from stem rot and root pathogens *Sclerotium rolfsii* (Ganesan & Gnanamanickam, 1986) and peas from pathogen *F. oxysporum* f.sp. *fisi* & *Phytium ultimun* (Benhamou et al., 1996); can induce the resistance of cucumber plants against *Colletotrichum orbiculare* through seedlings root treatment (Wei et al., 1991). *P. alcaligenes* KtS1, TrN2, and TmA1 have been shown to suppress Fusarium wilt disease in tomatoes (Widnyana et al., 2013). Widnyana and Javandira (2015) state that tomato seed treatment by *Bacillus sp*. for 10 to 30 min can spur the germination of tomato seeds. Similar research that soaks seed treatment with *P. alcaligenes* for swamp cabbage encourages 25% faster germination, increased yield by 24.4% (Widnyana et al., 2017). Rhizobacteria *Pseudomonas* spp. has a positive effect by occupying the plant root tissue surface and providing compounds that are beneficial to plants. Some of these bacteria enter the tissue as endophytes without causing damage or morphological changes in plants (Rosenblueth & Martinez-Romero, 2006).

In this study the cuttings of vanilla were given *Pseudomonas* spp. suspension through roots immersion, stems injection, and leaf injection in the hope that immune effects will occur in the induction of systemic resistance of vanilla plants to stem rot disease so as to get an effective, efficient and environmentally friendly disease control strategy.

Materials and Methods

Tools and materials

The tool used in this study i.e. petridish, test tube, erlenmeyer, autoclav, plastic bag, laminar air flow, centrifuge, millipore filter 0.45 µm MILLEX®-HA, and encase. The materials used are 70% alcohol, spirits, aluminum foil, King's B selective media, PDA (Potato Dextrosa Agar), Natrium Broth, 2.6-Dichloro-nicotinicacid , sterile planting media, vanilla plant cuttings, and aquadest.

Research methods

This research is an experimental research conducted in the lab and in the greenhouse by using a completely randomized design with a factorial pattern. The first factor is five inducing materials: *Pseudomonas* spp.W02 suspension, *Pseudomonas* spp.W68 suspension, *Pseudomonas* spp.W80 suspension, 2.6.D (2.6-Dichloronicotinic acid), distilled water (control). The second factor is the induction method that is: root immersion, injection on the stem, and injection on the leaves of vanilla. Each treatment was repeated three times so that there were 45 treatment units.

Planting short cuttings of vanilla

This treatment was started by planting 20 cm vanilla cuttings on a plastic pot containing 1000 g of sterile planting medium with soil and compost composition (2:1 ratio). The growing medium sterilized using autoclave for 2 x 2 hours to minimize contaminants (Widodo et al., 1993). Watering with distilled water is done every afternoon with 100 ml volume. Shoots and vanilla roots begin to grow after 6 weeks, and at 8 weeks of cuttings are ready for induction treatment.

Preparation of bacterial suspension *Pseudomonas* spp.

Natrium Broth medium (NB) 50 ml volume placed in erlenmeyer was inoculated with bacterial culture of *Pseudomonas* spp. incubated for 48 h at room temperature. There are 3 (three) isolates of *Pseudomonas* spp. bacteria used, are isolates W02, W68 and W80. The three isolates of *Pseudomonas* spp. bacteria are non pathogenic and have antagonistic ability against *F. oxysporum* f.sp. *vanilla*e. Furthermore, *Pseudomonas* spp. suspensions were filtered with millipore filter 0.45 µm MILLEX®-HA to obtain a suspension volume as needed.

Treatment of resistance induction with bacterial suspension *Pseudomonas* spp.

The resistance induction treatment with *Pseudomonas* spp. bacterial suspension was performed on cuttings of vanilla stems that had grown buds and roots (age 8 weeks) by following modified procedures from Hofland et al. (1996) through root immersion, by injection of the stem and by injection of leaves with bacterial suspension volume of *Pseudomonas* spp. of 10 ml each. Population density of bacterial suspension Pseudomonas spp. is $5x10^6$ CFU/ml where previously bacteria were grown for 48 hours on NB medium at room temperature.

Analysis of the content of total phenol and salicylic acid in vanilla plant tissue

The total phenol and salicylic acid content was analyzed by HPLC (*High Performance Liquid Chromatography*) method

by taking 100 g of plant part consisting of leaves, stems, and root of vanilla plant with the same proportion. Taking part of the vanilla plant was done 60 days after the cuttings of vanilla were induced with *Pseudomonas* spp. suspension. The result analysis to the total phenol and salicylic acid content will be compared quantitatively between control, induction treatment with *Pseudomonas* spp. suspension and induction with 2.6.D. (2.6-dichloro-isonicotinic acid (INA).

Results

The total phenols content in vanilla plant tissue

The result of statistical analysis shows that there is a very real interaction (P> 0.01) between applications of *Pseudomonas* spp. suspension to the total phenol content in vanilla tissue. The total phenol content in vanilla plant tissue after being treated with *Pseudomonas* spp. suspension is presented in Table 1. The highest total phenol content found in the *Pseudomonas* spp. W80 suspension treatment were injected via stem is 277.9 mg/g or an increase of 121% compared to distilled water treatment as a control, followed by *Pseudomonas* spp. W80 injected through leaves is an average of 261.8 mg/g or an increase of 108.1% compared to distilled water treatment.

Treatment of induction with chemicals 2.6.D can increase the total phenol content of 94.8% compared with distilled water treatment. The data shown in Table 1 shows that the suspension treatment of *Pseudomonas* spp. through root immersion, stem injection, and injection of the leaves proved able to increase the total phenol content in the vanilla plant tissue. Among the three isolates of *Pseudomonas* spp. which is used in the treatment, there is one isolate that can increase the total content of phenol exceeds the induction ability of 2.6.D is *Pseudomonas* spp.W80 isolates injected through the stem or vanilla leaf. When viewed the treatment mechanism of bacterial suspension *Pseudomonas* spp., the injection of suspension through the stem causes the highest increase of total phenol which is average 212.96 mg/g, followed by leaf injection is 212.08 mg/g, and through root immersion of 193.68 mg/g.

The two-way difference test between vanillary resistance induction treatment and its application to the total content of phenol in vanilla plant tissue, showed that the highest total phenol content was found in suspension treatment of *Pseudomonas* spp. injected via stem (277.95 mg/g), via leaf injection (261.80 mg/g), and root immersion (241.13 mg/g), and all were not significantly different from other treatments, as presented in Table 2.

Effect of *Pseudomonas* spp. suspension treatment to content of Salicylic Acid on Vanilla Plant Tissue

The result of salicylic acid content analysis on vanilla plant tissue after being treated with *Pseudomonas* spp.

Table 1. The total phenols content of vanilla plant tissue induced by *Pseudomonas* spp suspension (mg/g)

Material for induction	Procedure of resistance induction			Average
	Root immersion	Stem injection	Leaf injection	
Pseudomonas spp W02	199.8 j	209.4 h	199.4 j	202.87
Pseudomonas spp W68	179.1 k	206.6 i	218.7 g	201.47
Pseudomonas spp W80	241.1 e	277.9 a	261.8 b	260.27
2.6.D	222.6 f	245.1 d	254.7 c	240.80
Distilled water	125.8 l	125.8 l	125.8 l	125.80
Average	193.68	212.96	212.08	

Note: the numbers followed by the same letter are not significantly different in 5% Duncan Multiple Rings Test

Table 2. The 2-way difference test results between the resistance-inducing material treatment and the application method to the total content of phenol in the vanilla plant tissue

Treatment	Isolate of *Pseudomonas* spp.						2.6. D		Distilled water		LSD 5%
	Isolate W02		Isolate W68		Isolate W80						
Root immersion	199.75	b	179.09	c	241.13	c	222.60	c	125.80	a	3.89
	c		d		a		b		e		
Stem injection	209.39	a	206.62	b	277.95	a	245.09	b	125.80	a	1.81
	c		d		a		b		e		
Leaf injection	199.39	b	218.71	a	261.80	b	254.70	a	125.80	a	1.73
	d		c		a		b		e		
LSD 5%	2.12		4.39		3.02		2.82				

Note: the same letter behind the average number in the same column indicating the treatment of the plant-inducing material does not give a significant effect in the LSD5% test level to the total phenol content in the vanilla plant tissue; the same letter below the mean number on the same line shows no significant effect in the LSD5% test level between the treatment effects of the *Pseudomonas* spp. suspension treatment to the total phenol content of vanilla plant tissue

Table 3. Salicylic acid content of vanilla plant tissue induced by *Pseudomonas* spp. suspension (ppm)

Material for induction	Procedure of resistance induction			Average
	Root immersion	Stem injection	Leaf injection	
Pseudomonas spp. W02	39.31 c	42.58 a	31.06 f	37.65
Pseudomonas spp. W68	25.35 h	25.13 h	26.26 h	25.58
Pseudomonas spp. W80	27.80 g	36.02 d	33.84 e	32.55
2.6.D	40.43 b	33.46 e	25.63 h	33.17
Distilled water	21.77 i	21.77 i	21.77 i	21.77
Average	35.650	33.371	26.216	

Note: the numbers followed by the same letter are not significantly different in the 5% Duncan Multiple Rings Test

suspension with HPLC method is presented in Table 3. The highest salicylic acid content is found in the *Pseudomonas* spp.W80 suspension treatment which is 37.364 ppm or increased 71.63% compared to distillated water as control, followed by 2.6.D treatment of 32.500 ppm or increased 49.52 % compared to distilled water. Furthermore on the suspension treatment *Pseudomonas* spp.W68 of 31.990 ppm or 46.94% increase compared to distilled water, and the lowest is on the treatment of suspension *Pseudomonas* spp. W02 is 25.762 ppm or only increased 18.34% compared with distilled water treatment.

When viewed from the treatment procedure of *Pseudomonas* spp. suspension, the highest salicylic acid content is found in the immersion of root is 35.650 ppm, then followed by injection of suspense through stem that is 33.371 ppm and injection of suspense *Pseudomonas* spp. through the leaves of 26.216 ppm.

The result of 2-way difference test between the treatment of the induction material and its application to the salicylic acid content in vanilla plant tissue showed that in the root immersion treatment, the highest salicylic acid content was found in the treatment of 2.6 D (40.43 ppm) not significantly different with *Pseudomonas* spp. W02 suspense treatment (39.31 ppm), but significantly different from all other treatments, as presented in Table 4. In the treatment of stem injection, the highest salicylic acid content was found in *Pseudomonas* spp. W02 treatment of 42.58 ppm and significantly different from all other treatments.

Discussion

Phenolates are categorized into simple phenols (orsinol, 4-methylresorinol, 2-Methyl resossinol, resorcinol, catechol, hydroquinone, pyrogalol and floroglusinol) and phenolic acids (galat, protokatekuat, gentisat, p-hydroxybenzoate, siringat, vanilate and salicylate). Lignin is a phenol polymer present in plant cell walls, along with cellulose causing stiffness and robustness of the plant stems. There is a close relationship between lignin and the presence of phenolic acid in the same leaves of the tree.

Research conducted by Benhamou et al. (1996), which induces resistance of tomato plants with citosan. When applied by spraying and dyeing the root it is proven to

Table 4. The 2-way difference test results between the resistance-inducing material treatment and the application method to the salicylic acid content in the plant tissue vanilla

Treatment	Isolate of *Pseudomonas* spp						1.6 D		Distilled water		LSD 5%
	Isolate W02		Isolate W68		Isolate W80						
Root immersion	39.31	b	25.35	a	27.80	b	40.43	a	21.77	a	2.41
	a		c		b		a		d		
Stem injection	42.58	a	25.13	a	36.02	a	33.46	b	21.77	a	2.11
	a		d		b		c		e		
Leaf injection	31.06	c	26.26	a	33.84	ab	29.00	c	21.77	a	2.03
	b		c		a		c		d		
LSD 5%	1.83		1.46		8.05		2.54		0.40		

Note: the same letter behind the average number in the same column indicating the treatment of the plant-inducing material does not give a significant effect in the LSD 5% test level to the salicylic acid content in the vanilla plant tissue; the same letter below the mean number on the same line shows no significant effect in the LSD 5% test level between the treatment effects of the *Pseudomonas* spp. suspension treatment to the salicylic acid content of vanilla plant tissue

trigger the resistance of tomatoes to F.*oxysporum* f.sp. *radicis-lycopersici*. The growth and development of pathogens become inhibited due to the rapid accumulation of new macromolecules such as β-1.3 glucans, phenol compounds and lignin. There is a correlation between the levels of plant resistance with phenol content in the plant tissue.

Salicylic acid is a compound penolik that naturally occurs in plants, a major component in plant resistance locally or systemically. Colonization of tobacco plant roots with *P. fluorescens* strains CHAO, and leaf inoculation with Tobaco Mozaic Virus TNV causes an increase in salicylic acid content in leaves (Maurhofer et al., 1994). Salicylic acid is transported to the leaves from the point of inoculation to induce systemic resistance.

The bacteria with the filtrate it produces when it enters the plant tissue will get a response from the plant tissue in the form of a hypersensitive reaction, which is a sudden reaction in response to an infection. Hypersensitive reactions lead to increased respiration of plant tissues (Agrios, 2005). In normal conditions the respiratory process follows the path of glycolysis and the Krebs cycle and oxygen uptake is performed by the enzyme cytochromoxidase. The stimulating effect of induction by bacteria cause cytochromoxidase activity increases so that oxygen uptake increased in the initial respiration. In further respiration due to higher respiratory rate of oxygen uptake by relying on cytochromoxidase to be insufficient, consequently the secondary metabolic activity of the tissue is increased and oxygen uptake is assisted by polyphenoloxidase and its respiratory trajectory leads more to the pentose cross. This leads to more form phenol compounds, such as phytoalexin which is toxic to the pathogen. With the increased activity of polyphenol oxidase, some of the phenol compounds are oxidized into quinone compounds that are much more toxic than phenol, so plants become resistant to pathogens. This is consistent with the statement of Peer et al. (1991) which states that the *Pseudomonas sp*. strain Wcs 417 r inoculated in carnation plants will cause the plant resistant to disease wilt *F.oxysporum* f.sp *dianti* because the content of phenol compounds in the plant increases. In addition to the stimulation to form phytoalexin or phenol compounds, bacterial suspension inoculation may also release the phenol compound bound in carbohydrates of the plant tissue, so that the previously inactivated phenol compounds will become active. For example the *Venturia inaequalis* fungus when applied to the apple leaf will produce β-glycosidase, this compound can release phenol (Floretin) from carbohydrates so that the crop resistance increases (Agrios, 2005).

Conclusions

• From the research that has been implemented can be concluded as follows:

• Treatment of bacterial suspension *Pseudomonas* spp. in the cuttings of vanilla plants either through roots, stems or leaves can induce the systemic resistance of vanilla plants as indicated by increasing total phenol and salicylic acid content in vanilla plant tissues

• The highest total phenol content was found in bacterial suspension treatment of *Pseudomonas* spp. W80 isolate was 260.27 mg/g or an increase of 106.89% compared to distilled water treatment as control; in the treatment of 2.6.D (2.6-dichloro-isonicotinic acid (INA) the total phenol content 240.80 mg/g or an increase of 91.41% than control

• The highest salicylic acid content is found in the bacterial suspension of *Pseudomonas* spp. W02 isolate that is 37.65 ppm or increase 72.94% compared to control, followed by treatment 2.6. D (2.6-dichloro-isonicotinic acid (INA) equal to 32.55 ppm or increase 49.52% than control

• Bacteria *Pseudomonas* spp. W02, W68, and W80 isolates has the ability to induce vanilla plant resistance with different abilities

Acknowledgments

Acknowledgments submitted to the government of Indonesia, especially to the Ministry of Technology and Higher Education on financing provided so that this research can be implemented and to the Rector of Mahasaraswati University of Denpasar and the Dean of the Agriculture Faculty for the given research opportunity.

References

Agrios, G. N. (2005). Plant Pathology. 5th ed. Elsevier Academic Press, Gainesville, USA.

Bakker, P. A., Pieterse, C. M., & Van Loon, L. C. (2007). Induced systemic resistance by fluorescent Pseudomonas spp. *Phytopathology*, *97*(2), 239-243.

Benhamou, N., Belanger, R. R., & Paulitz, T. C. (1996). Induction of differential host responses by Pseudomonas fluorescens in Ri T-DNA-transformed pea roots after challenge with Fusarium oxysporum f. sp. pisi and Pythium ultimum. *Phytopathology, 86,* 1174-1185.

Chet, I. (1993). *Biotechnology in plant disease control*. Wiley Liss, New York.

Ganesan, P., & Gnanamanickam, S. S. (1987). Biological control of Sclerotium rolfsii Sacc. in peanut by inoculation with Pseudomonas fluorescens. *Soil Biology and Biochemistry*, *19*(1), 35-38.

Hadisutrisno, B. (2004). Tactics and strategies for crop protection against Fusarium wilt disease. In: *National Symposium,* 2-3

March 2004, Purwokerto, Indonesia.

Hoffland, E., Hakulinen, J., & Van Pelt, J. A. (1996). Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas* species. *Phytopathology*, *86*(7), 757-762.

Maurhofer, M., Hase, C., Meuwly, P., Metraux, J. P., & Defago, G. (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas* fluorescens strain CHA0: influence of the gacA gene and of pyoverdine production. *Phytopathology, 84,* 139-146.

Nurcahyani, E., Sumardi, I., Hadisutrisno, B., & Suharyanto, E. (2012). Development of stem rot disease suppression vanilla (*Fusarium oxysporum* f.sp *vanillae*) sorting through in vitro fusaric acid. *J. HPT Tropic, 12*(1).

Van Peer, R., Niemann, G. J., & Schippers, B. (1991). Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp. strain WCS 417 r. *Phytopathology*, *81*(7), 728-734.

Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions*, *19*(8), 827-837.

Semangun, H. (1996). Introduction to Plant Pathology. UGM Press, Yogyakarta.

Tombe, M. (1987). Stem rot disease in vanilla plant and its prevention efforts. News Research Development, Ministry of Agriculture.

Tombe, M. (2010). Environmentally friendly technology in integrated control of stem rot disease vanilla (BBV). Agency for Agricultural Research and Development, Ministry of Agriculture, Bogor.

Wei, G., Kloepper, J. W., & Tuzun, S. (1996). Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology, 86*, 221-224.

Weller, D. M., & Cook, R. J. (1986). Increased growth of wheat by seed treatments with fluorescent pseudomonads, and implications of Pythium control. *Canadian Journal of Plant Pathology*, *8*(3), 328-334.

Widnyana, I. K., & Javandira, C. (2016). Activities *Pseudomonas* spp. and *Bacillus* sp. to stimulate germination and seedling growth of tomato plants. *Agriculture and Agricultural Science Procedia*, *9*, 419-423.

Widnyana, I. K., Ngga, M., & Sapanca, P. L. Y. (2018). The Effect of Seed Soaking with Rhizobacteria *Pseudomonas* alcaligenes on the Growth of Swamp Cabbage (*Ipomoea reptans* Poir). *Journal of Physics: Conference Series, 953*(1), p. 012007.

Widnyana, I. K., Suprapta, D. N., Sudana, I. M., & Temaja, I. G. R. M. (2013). Pseudomonas alcaligenes, potential antagonist against fusarium oxysporum f. sp. lycopersicum the cause of fusarium wilt disease on tomato. *Journal of Biology, Agriculture and Healthcare*, *3*(7), 163-169.

Widodo, Sinaga, M. S., Anas, I., & Machmud, M. (1993). Use of *Pseudomonas* spp. fluorescent group for the control of gada root disease (*Plasmodiophora brassicae* Wor.) on caisin (*Brassica campestris* L. Var *chinensis* Rupr.). *Olson Bul. HPT, 6* (2), 94-105.

Received: August, 15, 2018; *Accepted:* February, 18, 2018; *Published:* June, 30, 2019