Peroxidase Enzyme Activity of Rhizobacteria-Introduced Shallots Bulbs to Induce Resistance of Shallot towards Bacterial Leaf Blight (Xanthomonas axonopodis pv allii)

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Abstract

Bacterial leaf blight disease caused by Xanthomonas axonopodis pv allii is an important disease in shallots. The research purpose was to study the activity of peroxidase enzyme in shallots which was introduced by the rhizobacteria isolates and was able to induce resistance to Xaa. The research was conducted by introducing eight isolates of rhizobacteria bacteria in shallots. The results showed that introduced rhizobacteria could increase the activity of peroxidase enzymes, and the isolate PK2Rp3 (Serratia marcescens strain N2.4) was the highest activity in both roots and leaves of 0.058 μm · mL⁻¹ and 0.053 μm · mL⁻¹.

Keywords: Peroxydase enzyme; rhizobacteria; red onion; Xanthomonas axonopodis pv allii.

Nomenclature

d day
min minute
PGPR plant growth promoting rhizobacteria

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1. Introduction

Bacterial leaf blight disease (BLBD) caused by Xanthomonas axonopodis pv. allii (Xaa) is an important disease that can affect various types of onions\(^1\). Xaa induces symptoms that consist of lenticular water-soaked leaf spots, which turn into dry chlorotic lesions and eventually coalesce. When the disease is severe, leaf dieback can occur, resulting in a reduction of bulb size. Therefore, crop yields could drastically decrease, and yield losses ranging from 10% to 50% have been reported in diseased fields in Colorado and California\(^2\). Damage caused by the symptoms of the disease can reduce yield and tuber quality (up to 100%) when the environmental conditions favor temperatures and high rainfall\(^1\).

Xaa has many hosts, not only from species Allium spp. L. such as onion (Allium cepa L.), garlic (Allium sativum L.), welsh onion (Allium fistulosum L.) shallot (Allium cepa L. var ascolonicum), but it also attacks legumes such as beans (Phaseolus vulgaris L.), soybeans (Glycine max L.), lime beans (Phaseolus lunatus L.) and Pisum sativum L.\(^3\). Pathogens spread of through irrigation water, agriculture equipment, crop residue, as well as contaminated seeds, including seed-borne pathogens (seed-borne)\(^1\).

This pathogen is very difficult to control, most control is done using pesticides. The use of pesticides should be minimized because of the dangers for humans and environment. Biological control is thus being considered as an alternative or supplemental way of reducing the use of chemicals in agriculture\(^4,5\).

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 the ability to induce systemic resistance in plant which provides protection against a broad spectrum of plant pathogens and is referred to as Induce Systemic Resistance (ISR). ISR pathway is induced when plants are challenged by pathogenic organisms\(^6\), reducing the severity of disease, induced plant resistance\(^8\), resulting in anti-herbivory compounds\(^9\), beneficial to absorption of nitrogen\(^10\) and increase the absorption of minerals for the plant\(^11\). The elevated resistance due to an inducing agent upon infection of pathogen, ISR is expressed upon subsequent or challenged inoculation with pathogen\(^12-14\).

Significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies\(^15\), such as fluorescent pseudomonads against Ralstonia solanacearum. All three strains suppressed wilt of tomato and increased yield\(^16\). P. fluorescens against Xanthomonas oryzae pv. oryzae, showed resistance to the rice bacterial blight pathogen\(^17\). Azospirillium brasilense against P. syringae pv. tomato, prevented bacterial speck disease development and improved tomato growth\(^18\). Serratia J2, Pseudomonas, Bacillus BB11 against Ralstonia solanacearum suppressed wilt of tomato and increase yield\(^19\). 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\(^20\). 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\(^21\). rhizobacteria isolates have found, which have the ability to induce systemic resistance of soybean against Xanthomonas axonopodis pv. glycines. These isolates identified as Bacillus sp., Bacillus thuringiensis serovar toumanoffi and Bacillus thuringiensis strain TS2\(^22\).

Structurally constitutive plant defense includes barrier, such as cell wall, as well as inhibiting compounds such as phenolic compounds\(^23\). Peroxidase enzymes act as catalysts in monolignol polymerase that build plant cell walls\(^17\). Infiltration of lignin in the cell wall can increase the mechanical strength of plant cells against penetration of pathogens\(^24\). Peroxidase is an enzyme that plays a role in the resistance of plants including hypersensitivity reactions, the process of lignification, synthesis of phenol, glycoproteins, suberization and production of phytoalexin\(^25,26\). The purpose of this research was to study the activity of peroxidase enzyme in shallots crop which is introduced by the rhizobacteria bacterial isolates and are able to induce resistance to Xanthomonas axonopodis pv allii.
2. Material and methods

2.1. Plant material

Bulbs of shallot (cv Singkil Medan) were obtained from Alahan Panjang District, Solok, West Sumatera, Indonesia. Plants were grown and maintained in the greenhouse of the Faculty of Agriculture, University Andalas Padang. The research was conducted from March until October 2012.

2.2. Rhizobacteria isolates

Seven rhizobacteria isolates used in this study are the best isolates that can induce resistance to bacterial leaf blight disease (Xaa). Isolates were isolated from healthy roots of shallots from Solok districts in West Sumatra. Rhizobacteria isolates used were STP1Rp1 (Bacillus sp. QC-13), LL1Rp1 (Bacillus cereus strain Y2), STP1Rp2 (Bacillus thuringiensis strain DAB-Bt6), RD2Rp2 (Bacillus weihenstephanensis strain P2-14), LL1Rp2 (Bacillus thuringiensis strain HD29), ULG2Rp4 (Stenotrophomonas sp. YRL06) and PK2Rp3 (Serratia marcescens strain N2.4).

2.3. Introduction of rhizobacteria bacteria

Bulbs of onion cultivar Singkil were cut in three parts from the top and soaked with rhizobacteria bacterial suspension (10^8 cells · mL^{-1}) for 15 min to ensure that the suspension of bacteria uniform coating on surface is under aseptic condition. The bulbs soaked in sterilized distilled water were treated as control. The bulbs were directly planted in a sterile growth medium (soil and manure ratio 3 : 1), without synthetic fertilizer application, and watered with tap water routinely until harvested.

2.4. Pathogen inoculation

Pathogen was inoculated in 14-day-old plants, by leaves injuries method with a sterile needle. The suspension of bacteria Xaa (10^6 cells · mL^{-1}) was applied to the tip of the leaf that was already injured with a sterile needle. The plants were incubated with transparent plastic, observed each day until water soaking symptoms appear. Root and leaves were harvested 0 d, 1 d, 3 d, 5 d, 7 d, 9 d, 11 d, 13 d, 15 d, 17 d, 21 d and 30 d after pathogen inoculation.

2.5. Peroxidase activity assay

One gram of leaf and root was macerated, subsequently 2.5 mL of 0.5 % potassium phosphate buffer pH 7 and 0.1 g PVP (Polyvinyl pyrrolidone) were added. The suspension was homogenized and filtered with two layers of gauze pads, centrifuged at 6 000 rpm (60 rpm = 1 hertz) speed for 15 min at 4 °C. The supernatant was used for the measurement of peroxidase activity. Measurement of peroxidase activity was performed based on the method by Bateman. Extraction of the enzyme as much as 0.2 mL included in cuvette which contained 5 mL pirogalol (0.631 g pirogalol in 0.005 M phosphate buffer pH 6, final volume 100 mL) and then shaken. Cuvette was placed in the spectrophotometer set to needle showed the same absorbance at a wavelength of 420 nm. 0.5 mL of 1 % H₂O₂ was subsequently added into the cuvette, then shaken and immediately placed in a spectrophotometer. Absorbance changes were observed every 5 s, until no more changes occurred. Peroxidase activity is expressed in μg · mL⁻¹.

3. Results and discussion

Peroxidase activity in roots and leaves of shallots was analyzed after being introduced by the rhizobacteria and inoculated with pathogenic bacteria (Xaa). Peroxidase activity increased from 0 dpi (days post inoculation) to 10 dpi compared to controls. The activity of peroxidase in the roots (Fig. 1) is higher than in the leaves (Fig 2). Isolates PK2RP3 is rhizobacteria isolate with the highest peroxidase activity at 15 dpi (0.058 μg · mL⁻¹) in the roots and 0.053 μg · mL⁻¹ in the leaves.
When plants are invaded by pathogen or damaged by mechanical injuries, major physiologic changes are induced, and plant-defense enzymes are generally activated. Peroxidase is one of the enzymes involved in the plant defense process. Establishment of defense due to enzyme activity is determined by the sensitivity of plants against the diseases. Structurally constitutive plant defense includes a barrier, such as cell wall, as well as inhibiting compounds such as phenolic compounds. Peroxidase is an enzyme that plays a role in the resistance of plants including hypersensitivity reactions, the process of lignification, synthesis of phenol, glycoproteins, suberization and production of phytoalexine.

Increased activity of the peroxidase in shallots that was introduced by rhizobacteria is one indicator of induced resistance to pathogens. Peroxidase activity was higher in the introduced plants with rhizobacteria than the control. The highest increase occurring in the introduced plants with rhizobacteria isolate PK2Rp3 was 0.058 μg · mL⁻¹, followed by isolate LL1Rp1 was 0.048 μg · mL⁻¹, isolate LL1Rp2 and STP1Rp2 was 0.047 μg · mL⁻¹, isolate RD2Rp2, STP1Rp1 and ULGRp4 was 0.046 μg · mL⁻¹, and control was 0.029 μg · mL⁻¹ (Fig. 1). This suggests that the introduction of shallot bulbs with rhizobacteria Bacillus, Stenotrophomonas and Serratia marcescens could increase plant resistance to disease of bacterial leaf blight. Isolate of PK2Rp3 had the highest peroxidase activity in both roots and leaves (0.058 μg · mL⁻¹). The isolate was Serratia marcescens which is a group of rhizobacteria and is able to induce plant resistance to pathogens. Serratia marcescens is known as rhizobacteria isolated from onion and can induce resistance in Arabidopsis plants against Cucumber Mosaic Virus. Serratia marcescens 90-166 which reduces Cucumber Mosaic Virus on tomato and cucumber, also reduces anthracnose and Fusarium wilt in cucumber. Bacterization of betelvine cut with Serratia marcescens NBR11213 induces phenylalanine ammonia-lyase, peroxidase, and polyphenoloxidase activities in leaf and root.

Fig. 1. Activity of peroxidase enzyme on shallots roots that was introduced by rhizobacteria and inoculated with Xanthomona axonopodis pv allii
Peroxidase activity is associated with the mechanism of lignification in the cell walls of plants and the production of phenolic compounds. Strong cell wall would block the entry of pathogens during infection. According to Silva et al.\textsuperscript{33}, peroxidase activity could inhibit pathogenic infection due to lignification that inhibits the pathogen entry. Introduction of \textit{Pseudomonas fluorescens} strain CHAO in tomato could suppress \textit{Fusarium oxysporum} f. sp. \textit{lycopersici} and increased the activity of the enzyme peroxidase\textsuperscript{34}. Accumulation of peroxidase enzyme, polyphenol oxidase, and phenylalanine anamine-lyase in roots of banana which was resistant to \textit{Fusarium oxysporum} f. sp. \textit{cubense} and introduced by \textit{Pseudomonas fluorescens} showed induced resistance on banana\textsuperscript{35}. Peroxidase enzyme activity was significantly increased in the treated cucumber plants with \textit{Bacillus subtilis} B57\textsuperscript{36}.

Fig. 2. Activity of peroxidase enzyme on shallot leaves that was introduced by rhizobacteria and inoculated with \textit{Xanthomonas axonopodis pv allii}

Peroxidase activity was increased even in the presence of pathogen alone (control) until 6 dpi, and then continuously decreased until 30 dpi due to the reaction of plants against pathogen infection, which left injuries after pathogen inoculation. According to Van Loon et al\textsuperscript{37} peroxidase enzyme was the group of PR-protein (pathogenesis Related-protein) of the class of PR-9 accumulated at the plant during infection of pathogen. In addition to an increase in peroxidase enzyme activity it was influenced by the presence of pathogen. Peroxidase activity as a marker of the induced resistance was localized and systemic\textsuperscript{38}.

4. Conclusion

Results showed that introduction of rhizobacteria could increase activity of peroxidase enzymes, and isolate PK2Rp3 (\textit{Serratia marcescens} strain N2.4) was the highest activity in both roots and leaves of 0.058 $\mu$g $\cdot$ mL$^{-1}$ and 0.053 $\mu$g $\cdot$ mL$^{-1}$.

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References

1. Roumagnac P, Pruvost O, Chiroloeu F, et al. Spatial and temporal analysis of bacterial blight of onion caused by Xanthomonas axonopodis pv allii. Phytopathology 2004;94(2):138–146.

2. Paulraj L, O’Garro LW. Leaf blight of onion in Barbados caused by Xanthomonas campestris. Plant Dis 1993;77:198–201.

3. Schwartz F, Gent D H. Xanthomonas leaf blight of onion [Internet]. Accessed on February 22nd 2006 from http://www.Extcolestate.edu/push/gorden.html. 2006.

4. de Weger L A, van der Bij J, Dekkers LC, et al. Colonization of the rhizosphere of crop plants by plant beneficial Pseudomonas. FEMS Microbiol Ecol 1995;17:221–228.

5. Postma J, Montanari M, van den Boogert P H J F. Microbial enrichment to enhance the disease suppressive activity of compost. Eur J Soil Biol 2003;39:157–163.

6. Bloemberg GV, Lugtenberg BJJ. Molecular basis of plant growth promotion and biocontrol by rhizobacterial. Current Opinion in Plant Biology 2001;4:343 – 350.

7. Kloepper JW, Ryu CM, Zhang S. Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology 2004;94:1259–1266.

8. Bakker PAHM, Pierterse CMJ, van Loon LC. Induced systemic resistance by fluorescent Pseudomonas spp. Phytopathology 2007;97:239–243.

9. Sullivan TJ, Rodstrom J, Vandop J, et al. Symbion-mediated change in Lolium arundinaceae defense: evidence from changes in gene expression and leaf composition. New Phytologist 2007;176:673–679.

10. Iha PN, Kamar A. Rhizobacterial colonization of Typha australis by a plant growth-promoting bacterium Klebsiella oxytoca GR-3. Journal of Applied Microbiology 2007;103:1311–1320.

11. Mallinowski DP, Allouch GA, Belesky DP. Leaf endophyte Neotyphodium caenophialium modifies mineral uptake in tall fescue. Plant and Soil 2000;227:115–126.

12. Nicholson RI, Hammerschmidt R. Phenolic compounds and their role in disease resistance. Annual Review of Phytopathology 2003;30:369–389.

13. Tagushi F, Shimizu R, Nakajima R, et al. Differential effects of flagellins from Pseudomonas syringae pv. tabaci, tomato and glycineca on plant defense response. Plant Physiol Biochem 2003;41:165–174.

14. Tretel-Azir P, Couderechet M, Vernet G, et al. Chitosan stimulates defense reactions in grapevine leaves and inhibits development of Botrytis cinerea. Eur J Plant Pathol 2006;114:405–413.

15. Shiddiqi ZA, PGPR: Prospective biocontrol agents of plant pathogens. In: Shiddiqi ZA, editor. PGPR: biocontrol and biofertilization. Dordrecht: Springer Verlag; 2005. p. 39–66.

16. Jagadeesh KS, Kulkarni JH, Krisharaj PU. Evaluation of role of fluorescent siderophore in the biological control of bacterial wilt in tomato using Tn5 mutants of fluorescent Pseudomonas spp. Current Science 2001;81:882–883.

17. Vidyasagarap P. Concise encyclopedia of plant pathology. Food Product Press and Howard Reference Press. London. 2004.

18. Bashan Y, Bashan LE. Protection of tomato seedlings against infection by Pseudomonas syringae pv. tomato by using the plant growth-promoting bacterium Azospirillum brasilense. Appl Environ Microbiol 2002;68:2637–2643.

19. Guo JH, Qi HY, Guo YH, et al. Biocontrol of tomato wilt by plant growth promoting rhizobacteria. Biol Contr 2004;29:66–72.

20. Massimo SMS, Mortensen CN, Mabagala RB, et al. Biological control of black rot (Xanthomonas campestris pv. campestris) of cabbage in Tanzania with Bacillus strains. J. Phytopathol. 2004;152:98–102.

21. Yanti Y, Resti Z. Induksi ketahanan tanaman bawang merah dengan bakteri rhizoplan indigenus terhadap penyakit hawar daun bakteri (Xanthomonas axonopodis pv allii) [Induced resistance in shallot bulbs with indigenous rhizobacterial towards bacterial leaf blights (Xanthomonas axonopodis pv allii)]. In: Loekas S, Endang M, Ruth FR, Abdul M, editors. Prosiding Seminar Nasional Pengelolaan OPT Ramah Lingkungan. Purwokerto. 10-11 November 2010; 235-241. [Bahasa Indonesia]

22. Habazar T, Yanti Y, Resti Z. Pengembangan teknik penapisan rizobakteria indigenus secara in planta untuk pengendalian bakteri patogen tanaman [Developing of in planta screening technique of indigenous rhizobacterial to control bacterial plant pathogens] [Report]. Andalas University. 2011. [Bahasa Indonesia]

23. Nurnberger T, Brunner F, Kemmerling B, et al. Innate immunity in plant and animal: striking similarities and obvious differences. Immunol Rev 2004;198:249–266.

24. Huang, J S. Plant pathogenesis and resistance: biochemistry and physiology of plant-microbe interaction. Netherlands: Kluwer academic Publisher; 2001.

25. Nicholson RL, Hammerschmidt R. Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol 1993;30:369–389.

26. Wojtaszek P. The oxidative burst plants early response against infection. Biochemical Journal 1997;322:681–692.

27. Bateman DF. Increase in peroxidase deseated plant tissue in source book of laboratory exercise in plant pathology. San Francisco: WH Freeman and Co; 1967.

28. Edwards U, Rogal T, Bloecker M, et al. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16 S ribosomal RNA. Nucl Acids Res 1989;17(19):7843–7853.

29. Ryu CM, Fang MA, Hu CH, et al. Bacterial volatiles promote growth in Arabidopsis. Proc Natl Acad Sci USA 2003;100:4927–4932.

30. Raupach GS, Kloepper JW. Biocontrol of cucumber disuases in the field by plant growth promoting rhizobacterial with and without methyl bromide fumigation. Plant Disease 2000;84:1073–1075.

31. Liu L, Kloepper W, Tuzun S. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth promoting rhizobacterial. Phytopathology 1995;85:695–698.

32. Lavania M, Chauhan PS, Chauhan SVS, et al. Induction of plant defense enzymes and phenolics by treatment with plant growth-promoting rhizobacterial Serratia marcescens NBR1213. Current Microbiology 2006;52:363–368.

33. Silva HSA, Rometo RS, Macagun D. Rhizobacterial and induction of systemic resistance in tomato plant: non-specific protection and increase in enzyme activities. Biological Control 2004;29:288–295.
34. Ardepili ZO, Ardebili NO, Hamdi SMM. Physiological effect of *Pseudomonas fluorescens* CHAO on tomato (*Lycopersicum esculentum* Mill) plants and its possible impact on *Fusarium oxysporum f.sp lycopersici*. *Australian Journal of Crop Science* 2011;5(12):1631–1638.

35. Saravanan T, Bhaskaran R, Muthusamy M. *Pseudomonas fluorescens* induced enzymatological changes in banana roots again *Fusarium wilt* disease. *Plant Pathology J* 2004;3(2):72–80.

36. Chen F, Wang M, Zheng Y, et al. Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber *Fusarium wilt* by *Bacillus subtilis* B579. *World J Microbiol Biotechnol* 2010;26:675–684.

37. Van Loon, L.C. Induced resistance in plant and the role of pathogenesis-related protein. *Eur J Plant Pathol* 1997;103:753–765.

38. Martinez C, Baccou JC, Bresson E, et al. Salysilic acid mediated by the oxidative burst in key molecule in local and systemic response of cotton challenged by an avirulent race of *Xanthomonas campestris malvacearum*. *Plant Physiol* 2000;122:757–766.