

## ***In vitro* characterization of selected indigenous Rhizobacterial strains as biocontrol agent of bacterial wilt disease on chili**

Trimurti Habazar, Yulmira Yanti, Reflinaldon, Yaherwandi, Chainur Rahman Nasution and Srimano Felia

### **ABSTRACT**

Bacterial wilt caused by *Ralstonia solanacearum* is the important dangerous diseases in chili. Until now this disease has been difficult to control; while the bacteria attack the xylem vessel they cannot be reached by any bactericide. Biocontrol is potential for controlling this disease. One group of important biocontrol agents is the plant growth promoting rhizobacteria (PGPR). Based on in planta screening method, we have found 13 indigenous rhizobacterial strains from healthy chili's rhizosphere, which have the ability to control bacterial wilt and to increase growth and yield of chili. This research is aimed at characterizing the direct mechanisms of selected indigenous rhizobacterial strains as biocontrol agents of *R. solanacearum in vitro*. The physiological characters of indigenous rhizobacterial strains as biocontrol agents had been observed, including production of antibiotic, siderophore, HCN, haemolysine, protease, and biosurfactant. For root colonization rifampicin mutants of indigenous rhizobacterial strains had been the results of in vitro analysis showed that those selected rhizobacterial strains had various physiological characters as biocontrol agents. Not all biocontrol tested characters have been produced by indigenous rhizobacterial strains. Only RZ2.1AP4 strains showed the positive on 3 characters. All indigenous rhizobacterial strains produced biosurfactant but only *Pseudomonas hibiscicola* strain RZ1.1AG4 showed the highest viscosity. All rifampicin mutants of indigenous rhizobacterial strains have colonized chili roots on rhizoplane and in the root tissues (endophyte) from nursery until 9 days after planting. RZ2.1AP2 and *Klebsiela michiganensis* strain RZ1.3AG4 showed the highest bacterial population on chilli roots. The bacterial population was higher in root tissue (endophytes) than on the root surface (rhizoplane) from seedling stage until 9 days after transplanting.

**Keywords:** biocontrol mechanisms, physiological characters, plant growth promoting rhizobacteria, *Ralstonia solanacearum*, root colonization

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### **INTRODUCTION**

Bacterial wilt caused by *Ralstonia solanacearum* is one of the important serious vascular diseases of chilli crop causing maximum crop losses (Basu, 2014), ranging between 15 to 55% around the world (El-Argawy and Adss, 2016). Control is difficult due to high variability of the pathogen, limited

possibility for chemical control, high capacity of the pathogen to survive in diverse environments and its extremely wide host range. Biological control is desirable because control with other methods gives variable results (Nguyen and Ranamukhaarachchi, 2010). Since biological control is a key component of integrated disease management,

it is important to search for plant growth promoting rhizobacteria (PGPR) active against specific pathogens and evaluate the antagonists for wider application (Nakkeeran *et al.*, 2006).

Several PGPR as antagonists have been evaluated to control *R. solanacearum* with variable success, such as *P. fluorescens* (Seleim *et al.*, 2011), and *Stenotrophomonas maltophilia* (Messiha *et al.*, 2007) on potato. *Bacillus*-based biocontrol agents are quite important to control bacterial wilt on mulberry (Ji *et al.*, 2008) and tobacco (Maketon *et al.*, 2008). Nguyen and Ranamukhaarachchi (2010) reported two bacterial antagonists isolated from soil (*Bacillus megaterium* and *Enterobacter cloacae*) that showed high potential for bacterial wilt disease suppression on chilli and also increased fruit weight, biomass and plant height. Furthermore, Yanti *et al.* (2017) reported 13 strains of rhizobacterial from chili rhizosphere showing high potential for bacterial wilt disease suppression. Two strains also increased growth and yield of chili.

PGPR suppresses pathogens, mediate several mechanisms of biological disease control, most of which involve, 1) Directly, competition for niche and nutrient (Haas and Défago, 2005), the production of metabolites which affects the pathogen directly, such as antimicrobial compounds (Bashan and de Bashan, 2005), lytic enzymes (Van Loon, 2007; Lugtenberg and Kamilova, 2009; Glick, 2012; Bashan and de Bashan, 2005). 2) Indirectly by induced systemic resistance (ISR) in the host plant (Compant *et al.*, 2005a). It is noteworthy that different mechanisms may be found in a single strain and can act simultaneously (Kloepper *et al.*, 1992).

Competition for nutrition, such as iron is one of the resources that can limit growth of plant pathogens. Many bacterial isolates used for biocontrol have the ability to produce iron-chelating compounds, siderophores (Schippers *et al.*, 1987; Dowling and O'Gara, 1994). Competition for niche such as colonization had been shown by experiments of many authors as follows, mutants of biocontrol

strains that are deficient in the production of antimicrobial substances are almost as efficient in biocontrol as the wild type (Kempf and Wolf, 1989; Kraus and Loper, 1992; Maurhofer *et al.*, 1994). When the bacterial colony reaches a certain critical density, there is a change in the expression of genes that are regulating for instance the production of secondary metabolites and formation of sexual pili (Swift *et al.*, 1996). This shift in the expression of genes is referred to as quorum sensing. The mucigel formed both by the plant and the bacteria, might aid the bacterial spread on the roots. It has been shown that biosurfactants are important and commonly produced by rhizobacteria (Nielsen *et al.*, 2002). The physical characteristics of surfactins increase the benefits as biocontrol agents of *Bacillus* spp. (Bacon and Hinton, 2011). Fermentation studies were carried out with the *Bacillus* isolates that showed the highest antimicrobial and hemolytic activities, to follow up growth and production of bioactive and surfactant compounds (Monteiro *et al.*, 2005).

Rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period in the presence of the indigenous microflora (Lugtenberg and Dekkers, 1999; Parke, 1991; Weller, 1988; Whipp, 1997). PGPR colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Kloepper and Schroth, 1978; Kloepper *et al.*, 1999). Some of these PGPR can also enter root interior and establish endophytic populations (Gray and Smith, 2005). Reinhold and Hurek (1998) reported that endophytes either become localized at the point of entry or are able to spread throughout the plant and can live within cells, in the intercellular spaces or in the vascular system. Many of them are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers, and other organs (Bell *et al.*, 1995; Compant *et al.*, 2005b; Gray and Smith,

2005). The extent of endophytic colonization of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches (Gray and Smith, 2005).

The production of metabolites which affect the pathogen directly, are antimicrobial compounds like diacetylphloroglucinol, pyrrolnitrin, phenazine-1-carboxylate, HCN (Bashan and de Bashan, 2005), pyoluteorin (Bashan and de Bashan, 2005); lytic enzymes (Van Loon, 2007; Lugtenberg *et al.*, 2009; Glick, 2012) like hydrolytic enzymes such as chitinase, laminarinase, Q-1,3-glucanase, protease, and lipase (Bashan and de Bashan, 2005).

From our previous research, based on in planta technique we have found 13 selected PGPR strains to control *R. solanacearum* and increase growth rate of chili plants in planta Yanti *et al.* (2017). The aim of this research was to detect physiological characters of the selected PGPR strains as biocontrol agents of *R. solanacearum* in vitro (production of HCN, siderophore, biosurfactant, protease and antibiotic) and in planta (root colonization).

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

Pathogenic bacteria *R. solanacearum* and 7 selected PGPR strains were used from our culture collection (Yanti *et al.*, 2017). The selected PGPR strains were as follow: *P. hibiscicola* strain RZ1.1AG4, *K. michiganensis* strain RZ1.3AG4, *A. xylooxidans* strain RZ1.3AP1, *C. lactis* strain RZ1.4.AG4, *A. insolitus* strain RZ1.4AP4, RZ2.1AP2, and RZ2.1AP4. All selected PGPR strains were streaked on Nutrient Agar (NA) and incubated at room temperature for 48 hours. *R. solanacearum* isolates were streaked on Tetrazolium Triphenyl Tetrachloride (TZC) and incubated with the same methods. These inocula were prepared in order to be used in vitro tests of the selected PGPR strains and for their colonization on chili roots in planta.

### In vitro characterization of selected PGPR strains as biological agents

Hydrogen cyanide detection was performed according to the method developed by Bakker and Schippers (1987). Briefly, selected PGPR

strains were streaked onto tryptic soy agar (TSA) supplemented with 44 % (w/v) glycine and the Petri plates were inverted. A piece of filter paper impregnated with 5 % picric acid and 2% sodium carbonate was placed in the lid of each Petri plate. Petri dishes were incubated at 28 °C for 96 hrs. Decolouration of the filter paper to orange-brown indicated production of cyanide. Protease activity was expressed as clearing zones on LB-medium amended with 2 % skim milk powder (Nestle) after incubation at 28 °C for 48 hrs.

### Siderophore Production

Production of siderophore was determined by Chromazurol Sulphonate (CAS) agar method (Alexander and Zuberer, 1991). Briefly, the bacterial inoculum was spotted into the center of a CAS agar plate. After incubation at 28 °C for 5 days, siderophore production was assayed by the change in the color of the medium from blue to orange.

### Hemolytic assay

Hemolytic activity was determined by agar diffusion technique. One microliter of PGPR suspension was placed on the surface of plates containing blood agar medium and incubated at 37 °C for 72 hrs (Monteiro *et al.*, 2005). The plates were visually observed for clearing zone (hemolysis) around the colony.

### Production of antibiotic by selected PGPR strains against *R. solanacearum*.

The selected PGPR strains were grown on Potato dextrose broth (PDB) medium. The pH of the medium was adjusted to 7.0 before sterilization. The PGPR culture were incubated at 28 °C for 6-7-days. The bacterial suspension was centrifuged at 4000 rpm for 20 min. The supernatant was filtered through a 0.2 mm pore size membrane filter (NC 20-cellulose nitrate). Sterile culture filtrate (CF) was used without dilution and tested against *R. solanacearum*. The agar well diffusion assay, as reported by Tagg and McGiven (1971), was used for determination of antagonistic activity of the crude extract. *R. solanacearum* plated on NA. After medium has solidified, three separated holes (5 mm) were made by borer in every plate. 200 µL of culture filtrate was poured into these holes. The last hole was

filled with sterile distilled water as a control and the whole procedure was repeated twice for each species. The plates were kept in the refrigerator at 3 °C for 24 hrs to promote diffusion of the culture filtrate. Petri dishes were then transferred to an incubator at 28 °C until inhibition zones around the holes appeared as a result of inhibition of growth of *R. solanacearum* demonstrating the antibacterial metabolites present in the culture filtrate secreted by the selected PGPR strains (Klement *et al.*, 1990; Zeller and Wolf, 1996).

#### **Biosurfactant**

Detection of biosurfactant production by the selected PGPR strains was tested using the drop collapse technique as described by Jain *et al.* (1991). To determine the surface tension, the bacteria were grown in liquid KB-medium for 48 h. The cells were pelleted and the surface tension of the cell-free culture supernatants was measured with a viscometer.

#### **Root colonization of rifampicin-resistant (Rif) mutants of selected PGPR strains.**

The selected PGPR strains were grown overnight in NB broth at  $28 \pm 2$  °C in a rotary shaker at 240 rpm. After centrifugation at 5000 rpm for 5 min, the pellet of each strain was dissolved in 10 mL of sterile 0.1 M citrate buffer (pH-5.0). After washing two times with citrate buffer, the pellet was dissolved in 100 ml of citrate buffer containing  $60 \mu\text{g mL}^{-1}$  rifampicin (Sigma, USA) and incubated for 2 hrs in a rotary shaker at  $28 \pm 2$  °C. After the incubation, the cells were pelleted down and washed twice with 0.1 M phosphate buffer (pH 6.7) and the pellet was resuspended in 100 ml of phosphate buffer and serially diluted up to  $10^{-4}$  in phosphate buffer and 100 mL each of the dilutions of  $10^{-3}$  and  $10^{-4}$  were plated onto nutrient agar plates containing  $60 \text{ mg mL}^{-1}$  rifampicin, amount sufficient to inhibit the growth of other bacteria. The mutants were purified and checked for stability by transferring them four times from TSA-rif to TSA and TSA-rif plates (Shanmugam *et al.*, 2011).

PGPR inoculated chili roots were partially collected at seedling stage (14 and 21 days after sowing) and 3, 5, 7, and 9 days after planting (DAP). For rhizoplane colonization,

one gram of roots was added in sterile normal solution (NSS), and 0.1 mL of serially diluted extract was placed on TSA-rif plates and incubated at  $37 \pm 1$  °C for 72 hrs. For endophyte colonization, one gram of roots was surface sterilized and crushed in NSS, and 0.1 mL of serially diluted extract was placed on TSA-rif plates and incubated at  $37 \pm 1$  °C for 72 hrs. These plates were placed on a Quebec colony counter to count the bacterial colonies. Colonies falling within the 30-300 range were selected and multiplied by the reciprocal dilution factor to obtain the number of bacterial colonies (Klement *et al.*, 1990) represented as colony forming units (CFU) per gram of root (Akhtar *et al.*, 2010).

#### **RESULTS**

##### **Characteristics of the selected PGPR strains**

Our previous study demonstrates that based on in planta technique, 13 selected PGPR strains inoculated chili could control *R. solanacearum* 100 % without wilt symptom and improve the growth of *R. solanacearum*-inoculated chili plants (Yanti *et al.*, 2017). The present study had demonstrated biocontrol characters of the selected PGPR strains as follows: all selected PGPR strains showed negative for the characters siderophore, HCN, and antibiotic (Table 1).

The negative characters of selected rhizobacterial strains for siderophore and HCN support the report of Maji and Chakrabarty (2014) that only one from five rhizobacterial strains produced siderophore and HCN and was not responsible for the growth inhibition of *R. solanacearum* Tom 5. Our result also showed negative character for antibiotic production by of selected rhizobacterial strains, could support our previous research on in planta screening to explore the biocontrol agents of plant pathogens (Habazar *et al.*, 2011; Yanti *et al.*, 2017). Hemolytic activity of the PGPR strains presented by lipopeptides can be used for selecting lipopeptide-producing microorganisms. Therefore, hemolytic activity tests were performed to investigate the possible role of these compounds in the antimicrobial activity of the selected PGPR strains. Only two strains

showed hemolytic activity; they were *P. hibiscicola* strain RZ1.1AG4 and RZ2.1AP4. Most of the selected PGPR strains showed protease activity. Only one *Pseudomonas hibiscicola* strain RZ1.1AG4 produced

biosurfactant; it enhanced the viscosity higher than the others.

The lack of correlation between in vitro and in vivo effectiveness of biological control had already been observed by Ran *et al.* (2005), who reported that fluorescent *Pseudomonads*

**Table 1.** Characteristics of selected PGPR strains as biocontrol agent of *R. solanacearum*.

PGPR strains	HCN production	Protease activity	Siderophore production	Biosurfactant production		Anti-biotic production	Hemolytic activity
				Viscosity ( $\eta$ = Poise)	Enhanced (%)		
<i>P. hibiscicola</i>	-	+	-	0.7991	49.09	-	+
<i>K. michiganensis</i>	-	-	-	0.5977	11.51	-	-
<i>C. lactis</i>	-	+	-	0.6329	18.09	-	-
<i>A. xylooxidans</i>	-	+	-	0.5753	7.33	-	-
<i>A. insolitus</i>	-	+	-	0.5801	8.23	-	-
<b>RZ2.1AP2</b>	-	+	-	0.5521	3.00	-	-
<b>RZ2.1AP4</b>	-	+	-	0.5816	8.51	-	+
<b>Control</b>				0.5360	0.00		

sometimes succeeded as a biocontrol agent in vitro or under controlled conditions but failed under pot experiments and field conditions. The same result has been reported by Nguyen and Ranamukhaarachchi (2010) on bacterial wilt disease incidence in capsicum was higher with antagonist strain TR15 and in tomato with strain TR2 and 52.1 and 56.3%. respectively with TR10 and TR7). Thus, these antagonists showed high antagonism in vitro, but were not effective in vivo under greenhouse conditions. According to Ramadasappa *et al.* (2012), from 297 antagonistic bacteria were isolated through dual culture inoculation technique, out of which forty-two antagonistic bacteria were found positive for *phlD* gene. This gene is the key gene as it is essential for synthesis of antibiotic 2, 4-diacetylphloroglucinol (DAPG) (Raaijmakers and Weller, 2001; Landa *et al.*, 2002). Only ten isolated *phlD* bacteria were able to suppress infection of bacterial wilt disease in tomato plant and the PGPR (*phlD*) isolates s188, s215 and s288 was observed to be effective plant growth promoter (Ramadasappa *et al.*, 2012).

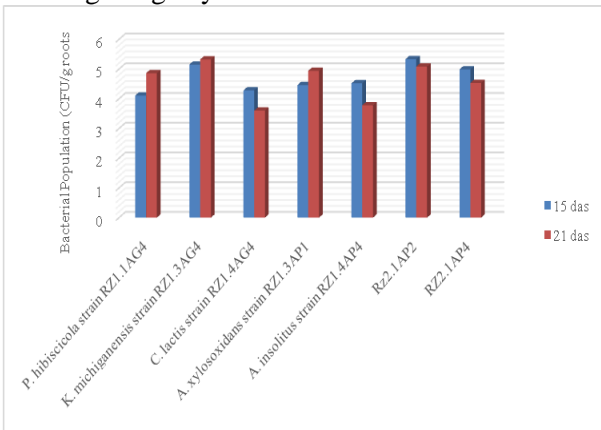
The widely recognized mechanisms of biocontrol mediated by PGPR were analyzed by (1) competition for an ecological niche or a substrate, (2) production of inhibitory chemicals, and (3) induction of systemic

resistance (ISR) in host plants to a broad spectrum of pathogens (Bloemberg and Lutenberg, 2001). This study showed, that direct mechanism of selected PGPR strains have not played important role to control *R. solanacearum* on chili. This bacterium infects plants through root wounds or at sites of secondary root emergence, then colonizes the xylem vessels and spreads rapidly to aerial parts of the plant through the vascular system. In xylem vessels, the bacterial population can multiply extensively and rapidly reach very high levels ( $>10^{10}$  cells/cm of stem in tomato) (Araud-Razou *et al.*, 1998; Vasse *et al.*, 1995). In this case, the pathogens could not have direct contact with biocontrol agents such as selected PGPR strains, because they have not the same niche. The main facets of altered host metabolism were the induction of a structural response at the sites of pathogen entry and abnormal accumulation of electron-dense substances in colonized areas. PGPR is known to suppress diseases by the inhibition of pathogens via diffusible or volatile products, induction of resistance in plants, aggressive root colonization, and stimulation of plant growth (Weller, 1988; Siddiqui and Mahmoud, 1999; Siddiqui, 2006). In addition, induced systemic resistance by PGPR is also considered a mechanism for the biocontrol of plant pathogens (Wei *et al.*, 1996).

**Root colonization**

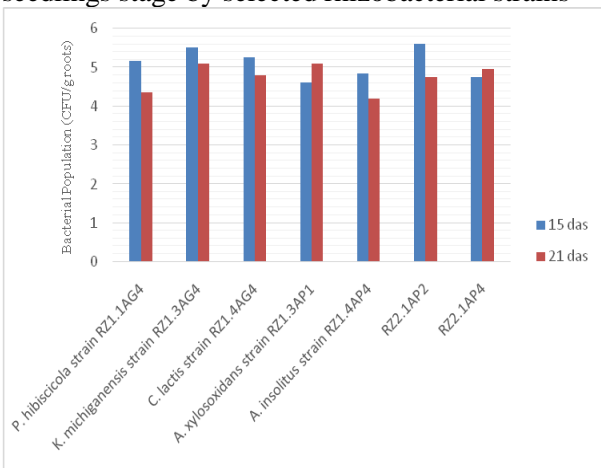
All strains show this ability to colonize both external and internal (endophyte) of chili seedling's root, and decreasing trends based on days after introduction on chili seeds. Although, some strains still show their ability to maintain populations, such as RZ2.1AP1 and *Klebsiela michiganensis* strain RZ1.3AG4. According to Patriquin *et al.*, (1983) and Okon, (1985) PGPR colonizes plant roots externally and internally. The selected PGPR strains colonized chilli roots at seedling's stage until 21 days after sowing at variable amounts:  $\log_{10}3.6$  CFU  $g^{-1}$  root -  $\log_{10} 5.33$  CFU  $g^{-1}$  root CFU/g roots on rhizoplane (Fig 1).

**Figure 1.** Rhizoplane colonisation of chili roots at seedlings stage by selected rhizobacterial strains



and  $\log_{10}4.20$  CFU  $g^{-1}$  root -  $\log_{10}5.61$  CFU  $g^{-1}$  root in internal root tissues (Fig. 2).

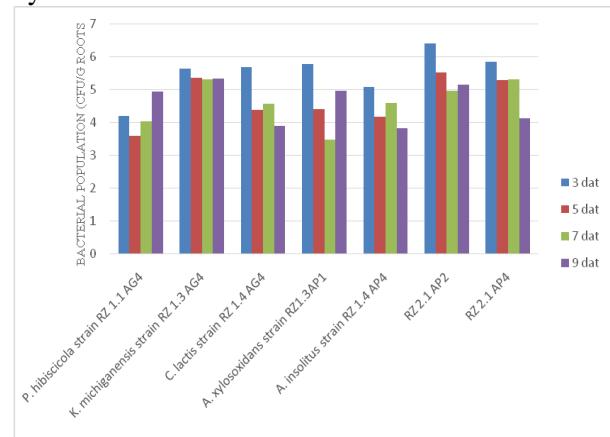
**Figure 2.** Internal colonisation of chili roots at seedlings stage by selected rhizobacterial strains



Most of the bacterial population was higher in internal root tissues than rhizoplane. This result is different from that in a report of Mia

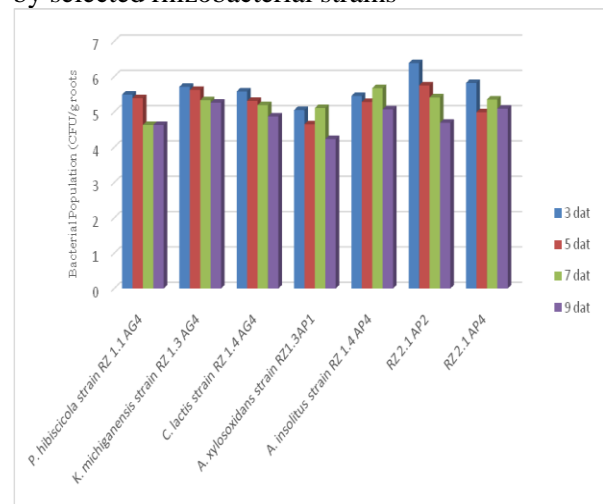
*et al.* (1999) that PGPR could colonize the roots externally and to a lesser extent internally. After transplanting, generally the population densities of selected PGPR strains were increased on chili's rhizoplane until 3 DAP and after that decreased until 9 DAP. Numbers of cfu of selected PGPR strains on the rhizoplane were varied between  $\log_{10}3.60$  -  $\log_{10}6.43$  CFU  $g^{-1}$  root (Fig. 3).

**Figure 3.** Rhizoplane colonisation of chili root's by selected rhizobacterial strains



The numbers of cfu of selected PGPR strains in the root tissue were between  $\log_{10}4.23$  -  $\log_{10}6.38$  CFU  $g^{-1}$  root (Fig. 4).

**Figure 4.** Endophytic colonisation of chili root's by selected rhizobacterial strains



The same trend on seedlings stage, some strains still showed its ability to maintain its populations, such as RZ2.1AP1 and *Klebsiela michiganensis* strain RZ1.3AG4. The same was the result on seedlings stage; most of the population densities of selected PGPR strains in chili's root tissue (endophyte) were higher than on the rhizoplane. The present results

demonstrate that chili plants treated with selected rhizobacterial isolates as seed and seedlings treatment followed by application of pathogens protected chili plants against the pathogens. Intense colonization is one of the reasons for the reduction in bacterial wilt on chili. The present results confirm the colonization of chili roots by selected rhizobacterial isolates and additionally support the current concepts that rhizosphere bacteria are active inducers of plant disease resistance. The results showed that selected rhizobacterial isolates are a very good root colonizer, offers a general biocontrol system that works against the pathogens of root tissues. Similar result had been reported by Gomathinayagam *et al.* (2006) the rapid colonization of seeds and roots of rice IR50 variety by *P. chlororaphis* and by Anderson and Guerra (1985) with the reports of effective colonization of roots and other parts by *P. putida* and *P. fluorescens*, as the restriction of pathogens infection of bacterized plants is attributed to the blocking of penetration of pathogens to the outer root tissues by the root colonizers (Piga *et al.*, 1997).

Based on the properties of selected PGPR strains shown only a little effect of direct activities related to biocontrol, whereas from previous research (Yanti *et al.*, 2017), those strains were best biocontrol activity against *R. solanacearum* and caused no wilt symptoms. Based on these results, it can be concluded that the ability of all selected PGPR strains to control *R. solanacearum* are not related to directly mechanisms, but perhaps by indirect mechanisms such as induced systemic resistance (ISR). According to Kloepper *et al.*, (1999), induced systemic resistance might be one of the most important operating mechanisms when dealing with biocontrol of systemic plant pathogens. PGPR protect plants from soil-borne pathogens by antagonistic mechanisms (Bais *et al.*, 2004); Lugtenberg and Kamilova, 2009). Such bacteria colonizing on the roots can also induce systemic resistance in aerial plant parts, which are spatially separated from the inducing PGPR. This mechanism of induction of systemic resistance by root colonizing rhizobacteria in

aerial plant parts is referred to as induced systemic resistance (ISR) (Rudrappa *et al.*, 2010). Further research will recommend to characterize the mechanisms of induced systemic resistance as response chilli against inoculation of the selected PGPR strains, such as physiological and biochemical aspects.

Our result showed that all selected rhizobacterial strains had been detected at rhizoplane and in the root tissues as endophyte. According to Kloepper *et al.* (1980), rhizobacteria are rhizosphere competent bacteria that aggressively colonize plant roots, are able to multiply and colonize all the ecological niches found on the roots at all the stages of plant growth, in the presence of competing microflora. During the process of root colonization bacteria multiply in the spermosphere (region around the seed) in response to seed exudates rich in carbohydrates and amino acids, then these get attached to root surface and colonize the developing root system. Reinhold and Hurek (1998) reported that endophytes either become localized at the point of entry or are able to spread throughout the plant and can live within cells, in the intercellular spaces or in the vascular system.

The conclusion of this research is as follows: not all biocontrol characters by direct mechanisms have produced by the selected PGPR strains and all selected PGPR strains colonized rhizoplane and internal (endophyte) of chili's roots.

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