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Original Article

Characterization of endophytic bacterial isolates from shallot as plant growth promoting rhizobacteria (PGPR)

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Abstract

The rhizosphere of shallot (*Allium ascalonicum* L.) might harbored by many bacteria that have potency as Plant Growth-Promoting Rhizobacteria (PGPR). Therefore, we isolated and characterized the endophytic bacteria from shallot and found the potency as PGPR. This study was done in two stages, the diversity of endophytic bacteria and the colonization of various endophytic bacteria as plant growth promoters. The results showed that the endophytic bacteria had a high diversity of morphological characters. Endophytic isolate B2 has potential as PGPR in increasing shallot growth indicated by the number of leaf 35 sheet, weight of plant 875 g, and weight of bulb 46.50 g.

Keywords: Bacteria, Endophytic, Shallot, PGPR

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Introduction

Shallot (*Allium ascalonicum* L.) is an important vegetable commodity that has high economic value in Indonesia (Limbongan and Maskar, 2003). This vegetable belongs to group of spices that function as food seasonings and traditional medicines. It is also a source of income and provide employment that contributes significantly to national economy (Badan Litbang Pertanian, 2006).

The bacteria and other microbes inhibiting rhizosphere and other part of plant were used to be regarded as useless, only causing damage and loses (pathogenic), or only act as soil decomposers. In this recent decade, they were no longer considered worthless, because of their role in plant growth and development, as well as enhancing the protection mechanism in plant against their natural enemies and environmental stress. Liu et al. (2017) even described their role around plant root as a 'gatekeeper' that is to filter soil bacteria from rhizosphere and rhizoplane.

Bacteria and plant interactions can occur in rhizosphere (rhizobacteria), in phyllosphere (epiphytes) and in plant tissues (endophytes) (Kobayashi and Palumbo, 2000). The endophytic bacteria live and develop in plant tissues to protect host plant from pest and pathogen that might damage plant. Radji (2005) stated that endophytic microbes are microbes that live in plant tissue at certain periods and are able to live by forming colonies in plant tissue without giving to damage their host. This bacterial component in interior of plant is largely harmless or beneficial to its host and is dynamic (Rosenblueth and Martínez-Romero, 2006). Hasegawa et al. (2006) suggests that endophytic bacteria that colonize plant tissue obtain nutrient and protection from their host plant. These bacteria can live in parts of plant such as root, stem, leaf and fruit (Simarmata et al., 2007;

Bacon and Hinton, 2006). According to Senthilmurugan (2013), endophytic microorganisms are microorganisms that originally came from rhizosphere region of plant. This organism will opportunistically enter plant root by utilizing natural wound and hole. The entry of these microorganisms was aided by the production of lytic enzyme that contributes to penetration and colonization. The microorganisms are native species for some plant and colonize plant tissue.

Plant tissue provides a safer and more uniform environment for bacterial development compare to rhizosphere and phylloplane (Buren et al., 1993; Chen et al., 1995). A study by Susanti et al., (2018) reveals that introduction of endophytic bacteria on shallot can reduce the intensity of damage by army worm, *Spodoptera exigua*, between 21 - 26% with the effectiveness of 38.5-52% compare to control. Therefore, here we wanted to further explore, isolate and characterize endophytic bacteria from shallot that function as Plant Growth Promoting Rhizobacteria (PGPR).

Material and Methods

The research was conducted from December 2016 to July 2017 at the Biological Control Laboratory, Faculty of Agriculture, Universitas Andalas and in Lipek Pageh and Alahan Panjang, Solok Regency, West Sumatera, Indonesia. The materials used were the soil around healthy shallot root, Nutrient Agar (NA) media, Nutrient Broth (NB), Pikovskaya's (PVK) media, aluminum foil, plastic wrap, tissue, label paper, soil, and manure sterile, sterile distilled water, and alcohol of 70%.

Bacterial sampling and isolation

The bacteria were isolated from shallot plant in Solok Regency. Shallot sampling was carried out in Lipek Pageh and Alahan Panjang villages. Two kinds of shallot samples were collected, viz. the vegetative phase or 11-35 days after planting, and the generative phase or 36-50 days after planting. Roots, bulbs, stems and leaves were taken, separately placed in paper bags, labelled, and kept in ice until further processing in laboratory. A total of 36 samples were collected and used for further steps.

Isolation of endophytic bacteria was carried out according to Zinniel et al. (2002). Stems, leaves, and roots were washed thoroughly before being cut to 2 cm length. Surface sterilization was carried out with 2% sodium hypochlorite containing 0.1% Tween-20. The leaves and stems were dipped for 30 seconds, while roots were soaked for 60 seconds. They were then washed 3 times with sterile distilled water and then dried with sterile paper towel. Samples were then macerated with a sterile mortar and pestle. The extract was serially diluted in 9 ml sterile distilled water and then plated into Nutrient Agar (NA), triplicated, and incubated for 48 hours. All bacteria were grown on plates at 27°C for 48-72 hours. The colonies were characterized 48 and 96 hours post incubation in NA for the following traits: color, form, elevation, margin, diameter, surface, opacity, and texture. The different colonies were then plated in NA to obtain pure cultures as the stock along the study.

Hypersensitivity reaction (HR) and Gram test

Hypersensitivity reaction (HR) and gram tests were conducted to further confirm that the endophytic bacteria found were not classified as plant pathogen. HR was carried out on tobacco plants as Klement et al. (1990). Bacteria that responded negatively to HR, viz. not show any symptoms on tobacco were regarded as non-pathogenic or belonged to endophytic bacteria. Gram tests were performed for 24 hours on NA media as Schaad et al. (2001). It was done with two procedures, namely 3% KOH test and gram staining.

Selection of phosphorus solubilizing (PSB)

Phosphorus solubilizing (PSB) activities of each confirmed endophytic isolate was measured in a 10 µl of cultures of Pikovskaya's (PVK) media (Pikovskaya, 1948 *in* Wang et al., 2017) containing 5 g of Tricalcium phosphate (TCP) or $Ca_3(PO_4)_2$ as the only phosphorus source. A small piece of pure isolate was inoculated on this media and incubated at 30°C for 7 days. The ability of the bacteria to solubilize insoluble phosphate was counted as the solubilization index (SI):

$$SI = \frac{\text{diameter of halo zone (mm)}}{\text{colony diameter (mm)}}$$

Confirmed endophytic bacteria was mass cultured in Nutrient Broth (NB) liquid media and incubated in rotary shaker for 24 hours at room temperature. Liquid cultures were then centrifuged at 5000 g for 5 minutes. The pellets were suspended in sterile distilled water to calculate bacterial population density. The suspensions were compared to each other with

McFarland solution on the scale of 8 (equivalent to 10^8 cells/ml) as recommended by Klement et al. (1990).

The confirmation of PGPR potency

Field experiment was conducted in a farmer's shallot field in Nagari Lipek Pageh, Solok Regency. Shallot bulbs were planted in polybags of 30 x 40 cm, contained 5 kg of sterile mixed of soil and chicken manure (2:1 v/v). The planted bulbs were of the same size, not deformed, bright red, and clean. The bulbs were cut 1/3 of the top and then soaked in suspension of endophytic bacteria for 15 minutes and air dried prior to planting. The study was arranged in Randomized Block Design with 28 treatments, i.e. the number of screened endophytic bacteria which were repeated in four replications.

The parameters measured were bacterial morphology and physiology, plant height (cm), number of leaves, and weights (g). Dry weight was measured after the bulbs were dried for 2 weeks at room temperature (14% water content), but wet weight was measured directly at harvest time.

Statistical analysis

Data were assessed using analysis of variance (ANOVA), and least significant difference (LSD) tests at a 5% probability to compare the differences among treatments.

Results and Discussion

Bacterial morphology

Endophytic bacteria which isolated from samples of shallot plant tissue obtained 36 isolates consisting of 4 isolates from roots, 14 isolates from stems, 17 isolates from tubers and 1 isolate from leaves. The dominant form of colonies was circular (22 isolates), while the other colonies were irregular (10 isolates), and others were filamentous (2 isolates). The dominant colony edge was entire (21 isolates), while the others were split (7 isolates), lobate (7 isolates), and serrate (2 isolates). The dominant colony surface was smoothly shiny (23 isolates), while the other colonies were wrinkles (13 isolates). The coloration of the colonies was ranging from white to beige. Twelve isolates were yellowish white, 9 isolates were yellow, 9 isolates were cream white and 6 isolates were beige. Therefore, most of the collected bacteria were morphologically showed circular colony with entire margin, not elevated (flat), the surface was smooth and shiny, and yellowish white in color. Morphological characters of endophytic bacteria isolates could be seen in Table 1.

The physiological characteristics of endophytic bacteria

The physiological characteristics of the collected isolates indicated that only 28 out of 36 collections that belongs to the endophytic bacteria (Table 2). Out of 36 collected bacteria, 28 were negatively responded to HR, and only eight isolates (A4, B12-B14, D1, U15-U16, U77) showed positively response. When tobacco leaves were inoculated with these 8 bacteria, necrotic symptom appeared within 2x24 hours after inoculation on the leaves, which indicated that they were pathogenic.

Based on gram test, most of the isolates collected were gram negative bacteria. Twenty-one isolates were shown to have low peptidoglycan and higher lipid contents on their cell wall or termed as gram-negative, while only 15 isolates were gram-positive i.e. of the opposite condition. Therefore, amongst the 28 selected endophytic isolates after HR test, 22 isolates were classified as gram-negative bacteria and only 6 bacteria that belonged to gram-positive bacteria. There were 13 out of 28 endophytic bacteria that shown the ability to solubilize phosphate viz. A1, A2, A3, B2, B4, B7, B11, U1,U2, U3, U4, U6, U14, The index of solubilization phosphate among these bacteria was ranging from 0.60 to 2.57 mm. Furthermore, amongst the collected endophytic bacteria, we found that there were able to solubilize phosphate (Figure 1).

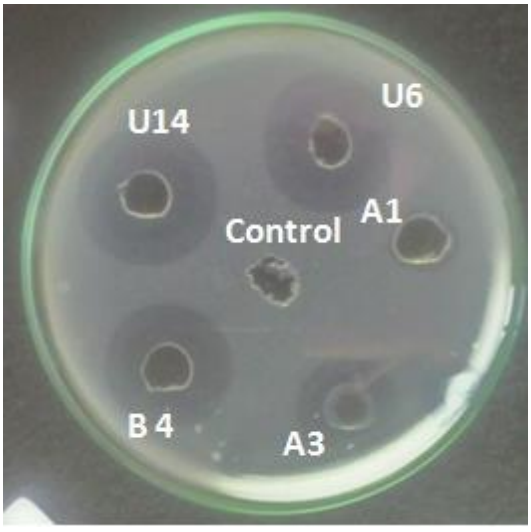

Figure-1: *In vitro* phosphate solubilization activity of shallot rhizosphere associated bacteria.

The PGPR potency

Based on 29 endophytic bacteria (including control) introduced into shallot, it can be seen that some treatments shown a big range of height to control and all of those bacteria did not affect plant height, number of leaves and number of tillers significantly. However, the endophytic bacteria of B2 and U6 increased wet weight of plant but only endophytic bacteria of B2 has increased wet and dry weight of bulb (Table 3). Endophytic bacteria of B2 and U6 were bacteria from Gram-positive and Gram-negative group. Both bacteria were able to dissolve the phosphate on Pikovskaya media with a phosphate solubility index of 0.6 and 1.60 (Table 2).

Table-1: Morphological characters of collected bacterial isolates from shallot leaves, stems, and roots.

Isolate	Colony Characteristics				
	Shape	Margin	Elevation	Surface	Color
A1	Circular	Entire	Raised	Smooth Shiny	Yellow
A2	Filamentous	Serrate	Flat	Smooth Shiny	Cream white
A3	Irregular	Lobate	Raised	Smooth Shiny	Yellowish White
A4	Circular	Entire	Raised	Smooth Shiny	Yellowish White
B1	Circular	Entire	Flat	Smooth Shiny	Yellowish White
B2	Circular	Entire	Umbonate	Wrinkled	White reaches
B3	Irregular	Lobate	Flat	Smooth Shiny	Yellowish White
B4	Circular	Entire	Convex	Smooth Shiny	Yellowish White
B5	Circular	Entire	Umbonate	Wrinkled	White reaches
B6	Circular	Entire	Flat	Smooth Shiny	Beige
B7	Circular	Entire	Flat	Smooth Shiny	Beige
B8	Circular	Entire	Umbonate	Wrinkled	White reaches
B9	Circular	Entire	Convex	Smooth Shiny	White reaches
B10	Irregular	Undulate	Flat	Smooth Shiny	Yellow
B11	Circular	Entire	Convex	Smooth Shiny	Beige
B12	Circular	Lobate	Flat	Smooth Shiny	Yellow
B13	Irregular	Lobate	Flat	Smooth Shiny	Beige
B14	Circular	Entire	Flat	Smooth Shiny	Beige
D1	Circular	Entire	Flat	Smooth Shiny	Yellow
U1	Circular	Entire	Flat	Smooth Shiny	Yellow
U2	Irregular	Lobate	Flat	Smooth Shiny	Yellowish White
U3	Circular	Entire	Flat	Smooth Shiny	Yellowish White
U4	Irregular	Lobate	Flat	Wrinkled	Yellow
U5	Irregular	Undulate	Flat	Smooth Shiny	White reaches
U6	Irregular	Undulate	Flat	Smooth Shiny	Yellowish White
U7	Circular	Entire	Umbonate	Smooth Shiny	Yellowish White
U8	Circular	Entire	Flat	Smooth Shiny	Yellowish White
U9	Circular	Entire	Flat	Smooth Shiny	Yellowish White
U10	Circular	Undulate	Flat	Smooth Shiny	White reaches
U11	Irregular	Lobate	Flat	Smooth Shiny	White reaches
U12	Filamentous	Serrate	Convex	Smooth Shiny	Yellow
U13	Circular	Entire	Flat	Smooth Shiny	White reaches
U14	Irregular	Undulate	Flat	Smooth Shiny	Yellowish White
U15	Irregular	Undulate	Flat	Smooth Shiny	Yellow
U16	Circular	Entire	Flat	Smooth Shiny	Yellow
U77	Circular	Entire	Flat	Smooth Shiny	Beige

Table-2: Physiological characteristics of endophytic isolates from shallots tissues

Isolate	Hypersensitive reaction (HR)	Gram test	Phosphate Solubilization (mm)
A1	Negative	Positive	0.60
A2	Negative	Negative	1.77
A3	Negative	Negative	1.40
A4	Positive	Negative	nt
B1	Negative	Negative	-
B2	Negative	Positive	0.60
B3	Negative	Negative	-
B4	Negative	Negative	2.00
B5	Negative	Positive	-
B6	Negative	Negative	-
B7	Negative	Negative	2.57
B8	Negative	Negative	-
B9	Negative	Positive	-
B10	Negative	Negative	-
B11	Negative	Negative	1.71
B12	Positive	Negative	nt
B13	Positive	Negative	nt
B14	Positive	Negative	nt
D1	Positive	Negative	nt
U1	Negative	Negative	2.25
U2	Negative	Negative	2.16
U3	Negative	Positive	0.80
U4	Negative	Negative	1.30
U5	Negative	Negative	-
U6	Negative	Negative	1.60
U7	Negative	Positive	-
U8	Negative	Negative	-
U9	Negative	Negative	-
U10	Negative	Negative	-
U11	Negative	Negative	-
U12	Negative	Negative	-
U13	Negative	Negative	-
U14	Negative	Positive	1.50
U15	Positive	Negative	nt
U16	Positive	Negative	nt
U77	Positive	Negative	nt

Note: not tested

The population of endophytic bacteria in shallot tissue varied, with in the stem 10 times higher than in the bulb, and in the bulb was 100 times higher than in the leaves. Different plant organs were associated with different endophytic bacterial communities in terms of diversity and composition. However, the population of endophytic bacteria in plant tissue was relatively low. According to Liu et al. (2017), in the root endosphere indicated the number of bacterial cell 10^4-10^8 per gram of root tissue, the microbiome was significantly less diverse than microbiomes in the rhizosphere and bulk soil (c.10^6-10^9 bacterial cell g^{-1} soil). Therefore, the population of our studied bacteria were higher. The number of studied endophytic bacteria that can be isolated from the living tissue of the leaves, stem and root of shallot was somewhat similar from one tissue to another, where they can contain 3 – 4 isolates. However, Pranoto et al. (2014) reported that there were 13 endophytic bacteria found on tea, five isolates were derived from leaves, four isolates from stem, and four isolates from root.

Endophytic bacteria that have been obtained from the shallot tissue were not classified as plant pathogen, indicated by the results of hypersensitive reaction test (HR) on tobacco leaf tissue. There were 28 isolates showed no symptoms of necrosis in tobacco leaves (negative) so that they were potentially as biological agents for shallot. The introduction of 28 endophytic bacterial isolates on shallot did not affect plant height and number of tillers significantly. However, B2 and U6 isolates were able to increase wet weight of plant, but only B2 isolates that increased wet weight and dry weight of bulb. Among all parameters, the dry weight of the bulb could be considered as the most important one, for this was directly related to economic value. Furthermore, combining altogether the growth parameters shown by the introduction of endophytic bacteria, it can be seen that one treatment with the isolate of B2 gave the best result (Table 3). B2 however, was the one that showed the best result among others, with significant wet plant weight, wet bulb and dry bulb weight. This isolate was insignificantly different in all parameters compared to control, however the value shown was quite higher than control. Therefore, this isolate was considered to be potentially explored further.

The ability of endophytic bacteria to dissolve phosphate is one mechanism to improve plant growth. The phosphorus (P) element is used by plant to develop cells and roots so that if they are not sufficiently available for plant, it will disrupt the increase in wet weight (Suwandi, 2009).

Table-3: The effect of endophytic bacteria on shallot height, number of leaves, number of tillers and weights

EB	Plant height (cm)		No. of tillers		No. of leaves		Weight of plant (g) wet		Weight of bulb (g) wet		Weight of bulb (g) dry	
A1	38.25 ± 5.51	ab	4.75	a	26.5	ab	450±1,58	bcde	26.75 ± 9.19	bcd	20.75 ± 11.79	c
A2	43.25 ± 3.27	a	4.25	a	26.8	ab	375±1,91	de	27.00 ± 15.32	bcd	20.75 ± 9.82	c
A3	43.00 ± 7.72	a	5	a	20	a	650±0,41	abcde	32.75 ± 4.86	abcd	23.00 ± 2.80	bc
B1	34.25 ± 4.69	ab	5.25	a	22.3	a	600±0,25	abcde	39.75 ± 17.73	abcd	28.00 ± 6.10	abc
B2	38.00 ± 3.30	ab	6.25	a	35	a	875±1,31	a	62.75 ± 21.28	a	46.50 ± 1.00	a
B3	37.25 ± 11.2	ab	5.5	a	31.5	a	725 ± 0,58	abc	39.00 ± 21.28	abcd	27.50 ± 6.81	abc
B4	42.00 ± 2.99	a	6.25	a	31.3	a	725±0,91	abc	54.75 ± 19.41	abc	37.50 ± 8.93	abc
B5	40.00 ± 5.07	a	4.75	a	27.8	ab	400±1,48	cde	29.75 ± 10.59	abcd	24.75 ± 5.00	abc
B6	41.50 ± 4.00	a	4.5	a	23	abc	475±0,48	abcde	35.00 ± 10.59	abcd	25.00 ± 2.33	abc
B7	39.75 ± 5.56	a	5.75	a	31	a	700±2,87	abcd	55.00 ± 13.22	abc	43.50 ± 9.49	ab
B8	26.00 ± 2.75	b	5	a	26	ab	500±0,71	abcde	33.50 ± 5.57	abcd	29.75 ± 8.35	abc
B9	39.00 ± 6.83	a	4.5	a	25.8	ab	525±1,29	abcde	33.00 ± 5.58	abcd	25.25 ± 4.29	abc
B10	36.25 ± 4.69	ab	4.75	a	21.8	a	400±0,95	cde	21.75 ± 7.93	cd	19.50 ± 4.20	c
B11	44.25 ± 12.25	a	4.75	a	31.5	a	475±0,71	abcde	25.00 ± 5.46	bcd	19.25 ± 3.78	c
U1	41.25 ± 7.89	a	5.75	a	29	ab	725±2,59	abc	54.00 ± 21.28	abcd	30.25 ± 8.34	abc
U2	38.00 ± 7.26	ab	5.25	a	20	ab	550±0,96	abcde	39.25 ± 21.28	abcd	30.00 ± 8.83	abc
U3	41.50 ± 7.79	a	6.25	a	28.8	ab	650±0,85	abcde	54.00 ± 15.72	abc	37.50 ± 4.80	abc
U4	36.50 ± 3.38	ab	5.5	a	20.5	abc	575±2,25	abcde	55.25 ± 22.11	ab	30.50 ± 11.39	abc
U5	38.00 ± 2.75	ab	4.75	a	31.5	a	575±0,5	abcde	25.25 ± 3.03	bcd	19.50 ± 3.31	c
U6	42.75 ± 8.23	a	5	a	24.8	abc	750±1,31	ab	29.50 ± 6.51	abcd	22.75 ± 2.23	bc
U7	42.00 ± 6.85	a	4.75	a	25	ab	475±1,44	abcde	28.25 ± 8.65	bcd	23.50 ± 7.21	bc
U8	38.00 ± 2.99	ab	4.25	a	28.3	ab	625±0,91	abcde	36.50 ± 3.92	abcd	25.75 ± 12.28	abc
U9	46.25 ± 11.62	a	5.25	a	21.8	a	525±0,65	abcde	36.75 ± 6.82	abcd	30.25 ± 8.77	abc
U10	41.75 ± 5.29	a	5.5	a	33	a	475±0,75	abcde	23.00 ± 8.62	bcd	19.75 ± 8.24	c
U11	41.50 ± 5.97	a	5	a	20.5	abc	350±1,32	e	20.00 ± 10.71	d	15.25 ± 4.13	c
U12	39.00 ± 13.3	a	5	a	26.8	ab	425±1,76	bcde	20.25 ± 3.64	d	18.75 ± 17.33	c
U13	40.00 ± 4.11	a	4	a	24.3	abc	400±1,97	cde	18.50 ± 4.54	d	15.50 ± 3.59	c
U14	44.50 ± 14.65	a	4.5	a	28.5	ab	575±0,71	abcde	33.25 ± 24.26	abcd	23.50 ± 7.21	bc
Control	37.00 ± 2.38	ab	4.5	a	20.5	abc	400±0,87	cde	28.25 ± 3.94	bcd	21.75 ± 9.20	bc

The numbers followed by the same letters are insignificant at $p<0.05$ of Tukey test.
EB = Endophytic bacteria

Available phosphorus and nitrogen produced by a mixture of phosphate solvent bacteria and nitrogen fixing bacteria are used to increase the formation of new cells in the meristematic tissues of plant, thus helping the plant's growth and development process (Tania and Budi, 2012) which ultimately increases plant wet weight. Furthermore, PGPR can increase plant growth through direct or indirect mechanisms, in addition to providing certain minerals such as phosphate needed by plants through dissolution mechanism (Suwandi, 2009). According to Oteino et al., (2015), the inoculation of plant with Phosphate Solubilizing Bacteria (PSB), grown under soluble phosphate limiting conditions, resulted in greater plant growth, than un-inoculated plant. It is proposed that these inocula release of soluble phosphate and that the soluble phosphate was subsequently assimilated by the plant.

Conclusion

There were 36 endophytic bacterial isolates found from shallots with a dominant colony shape in the form of a circle. The margins of the colonies were dominant entirely and the surface of the dominant colonies was smooth shiny. They were 28 endophytic bacterial isolates feasible further as PGPR agents and only the isolate of B2 has potency as PGPR in increasing shallot growth indicated by the number of leaf 35 sheet, weight of plant 875 g, and weight of bulb 46.50 g.

Contribution of Authors

Rahma H: Conceived idea, conducted experiment and write up of article
Nelly N: Helped in experiment and article write up
Susanti N: Helped in experiment, compilation of results and statistical analysis

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