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Rhizobacterial inoculants: The formulation as biofertilizer and its application on chili plants (*Capsicum annum* L.)

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ABSTRACT

Aims: The use of rhizobacteri as biofertilizer may help plants in obtaining nutrients from soil. A consortium inoculant (co-inoculant) consisting of nitrogen fixing and phosphate solubilizing bacteria is formulated to maintain its ability as booster of plant growth. This is easy to be stored and applied on plants. The aims of the study were to formulate rhizobacterial co-inoculant and its application on chili plants at greenhouse experiment.
Methodology and results: Isolates of *Burkholderia cepacia* KD 2.10, *Serratia marcescens* KAHN 15.12, and *Bacillus thuringiensis* SAHA 12.12 which have the ability in fixing nitrogen and solubilizing phosphate were used in this study. The three isolates did not show antagonistic activity and hypersensitivity reaction on chili plant. Biofertilizer as carrier material with talc-based powder was mixed with three isolates. This 10^9 CFU/g cell population of rhizobacterial consortium could be maintained up to six months of storage. Based on result of completely randomized design (CRD) using two factorials and four replicates, application of rhizobacterial co-inoculant significantly affected plant height, number of leaves, flowering age, dry weight of upper plant and root, and root length of chili plant.
Conclusion, significance and impact of study: Rhizobacterial co-inoculant was effective as biofertilizer to improve the growth of chili plants and it reduced the use of chemical fertilizer.

Keywords: biofertilizer, chili, co-inoculant, phosphate, tal

INTRODUCTION

Chili is one of the most popular commodities in Indonesia. The needs and market demand of chili are increasing at the moment. However, this condition is not balance with its production. This is due to limited available land for chili crop because of the lack of soil fertility, the existence of plant pests such as aphids and whiteflies as well as plant diseases such as anthracnose and mosaic virus. The farmers usually use chemical fertilizer to increase chili production. In fact, long-term application of it can harm the environment and kill beneficial soil microorganisms. Therefore, plant growth promoting rhizobacteria (PGPR) is an alternative biofertilizer which can obtain nutrients for plant growth but it does not give harmful effect to environment.

Biofertilizer is a substance containing inoculants with active ingredients of living microorganisms to improve the availability of nutrients for plants (Bhattacharjee and Dey, 2014). The role of PGPR as biofertilizer is done by providing and mobilizing nutrient absorption, synthesizing phytohormone which promote growth, and supressing pathogenic activity by producing various compounds or metabolites such as antibiotics and siderophore (Fraile *et al*., 2015).

Nitrogen (N) is major essential element required for plant growth in the largest amount. Although it is highly abundant (about 79% by volume) in the atmosphere, it is not fully utilized by plant (Cheng, 2008). Phosporus (P) is considered as the second most limiting nutrient in soil after nitrogen (N) because it is bound to soil colloid whereas plants can only use unbound inorganic phosphate (Pi), which is found in very low concentration in soil solution (Becquer *et al.*, 2014). Nitrogen-fixing rhizobacteria are able to use N_2 as a cellular N source in the process of nitrogen fixation by converting it into ammonium (NH_4) and nitrate (NO_3) (Gupta *et al*., 2015). Phosphate solubilizing rhizobacteria are able to release P bound to be absorbed by plant as free phosphate ions, $H_2PO_4^-$ and HPO_4^{2-} (Richardson and Simpson, 2011).

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The high quality of a biofertilizer product is determined by the number of population, viability of microbes in certain period of time and its efficacy on plants in various environmental conditions. Successful utilization of biofertilizer is not only determined by the superiority of inoculant, but also by the formulation process related to production of hygiene and suitability of carrier (Maheswari and Iswarya, 2015). Formulation defined as stages of biofertilizer production process that aims in order to make microbes to be easily applied, and increase cell survival by protecting it from dryness (Brar *et al.*, 2012).

B. cepacia KD 2.10 has been tested its ability to increase chili plant growth at greenhouse experiment (Miladiarsi *et al*., 2017). However, it is provided in liquid form of bacterial suspension which resulted in constraints in term of packaging and storage if it is used at large scale. Two other bacterial isolates, namely *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12 which had chitinolytic activity and were able to control *Colletotricum capsici* that attacked chilli plants (Nurdin *et al.*, 2016) were used as consortia with *B. cepacia* KD 2.10 in this study. Therefore, this study was conducted with objectives to formulate rhizobacterial co-inoculant and its application as biofertilizer on chili plants at greenhouse experiment.

MATERIALS AND METHODS

Materials

Rhizobacterial isolates of *B. cepacia* KD 2.10, *S. marcescens* KAHN 15.12, and *B. thuringiensis* SAHA 12.12 were obtained from Institut Pertanian Bogor Culture Collection (IPBCC), superior variety of red chili seeds, talc carrier material, commercial fertilizer, and chemical fertilizer of nitrogen, phosphorous, and potassium (NPK).

Characterization of rhizobacteria

Rhizobacteria were tested for Gram staining, phosphate solubilizing activity, and nitrogen fixing activity. Phosphate solubilizing activity was indicated by the formation of clear zone around bacterial colonies in solid Pikovskaya media after incubation at room temperature for 96 h, and phosphate solubilizing index (SI) was measured by subtracting the value of halo zone diameter toward the value of colony diameter. The resulted value was divided by colony diameter (Mursyida *et al*., 2015). Nitrogen fixing activity is shown by color change of solid nitrogen free bromothymol blue (NfB) media from green to blue after incubation at room temperature for 48 h (Ghevariya and Desai, 2014).

Antagonistic test between rhizobacterial co-inoculant

Antagonistic test was performed to determine antagonistic activity of rhizobacterial isolates that are mixed and added by some materials to form consortium. Isolate of target bacteria was grown on nutrient broth (NB) media until it reached a density cell of $\pm 10^8$ cell/mL. Inoculant product of 1% was poured into nutrient agar (NA) media in petridish and wait until it was solid. A well of 0.9 mm in diameter was made in the NA plate. About 1 inoculation loop of tested bacterial isolate was inoculated into 1 mL of 0.85% NaCl solution and mixed using vortex. A suspension of 1.5 µL was added into the well on the plate containing NA media which has been inoculated with the target bacteria. It was incubated at room temperature for 24 h. About 1000 ppm Kanamycin was used as positive control which could inhibit bacterial growth and sterile distilled water was used as negative control. Zone of inhibition formed around the well indicated antagonistic characteristic between bacteria tested and target bacteria.

Hypersensitivity test of red chili plant leaves

Hypersensitivity test was performed to find the pathogenic effect of isolates used on chili plant. Bacterial isolates were grown in NB media until they reached a density cell of $\pm 10^8$ cell/mL. The liquid culture was injected into mesophyll between the leaf veins of the chili. *Pseudomonas syringae* was used as positive control that is pathogenic bacterium in plant, whereas *Bacillus cereus* was used as negative control (Umesha *et al*., 2008). Observation of disease symptoms was carried out until 48 h after injection. The existence of areas that are necrotic on leaves within 48 h after inoculation is considered as positive result of pathogen on plant.

Formulation of rhizobacterial co-inoculant in carrier materials

Formulation was performed by talc carrier with composition of 50 g talc, 1 g peptone, 0.5 g carboxy methyl cellulose (CMC), 1.5 g technical sucrose, and 1 g yeast extract. The pH value of the formulation was set close to pH 7.0 with the addition of calcium carbonate ($CaCO_3$). Each material was mixed thoroughly, packed in heat resistant plastic of 250 g and sterilized at temperature of 121 °C and pressure of 1 atm for 30 min or equal to two sterilizations. Preparation of inoculum was performed by inoculating 50 mL of each isolate in NB media and further incubated at room temperature until it reached OD_{600} nm = 0.8. In addition, mixing of inoculum was done to produce inoculum of consortium. Later, inoculum of consortium was inoculated in each carrier, namely 5 mL inoculum for dose of 50 g talc, then thoroughly mixed in heat resistant plastic and incubated at room temperature.

In vitro viability test of rhizobacterial co-inoculant in formulation

Viability test was performed to observe bacterial endurance in carrier materials during storage. Stability of inoculants was determined by observing inoculant population during the storage treatment of 6 months at room temperature. Indicator of inoculant stability in carrier was determined by the number of living cells after storage period. Population was measured by plate method using NA media.

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Test of formulation effectiveness of rhizobacterial co-inoculant on the growth of red chili plants

Formulation of rhizobacterial consortium was applied by dissolving 10 g formulation in 90 mL sterile distilled water and 10% of the total volume was applied once a week in soil around the chili plants in the polybag. This study used completely randomized factorial design (CRD Factorial). The first factor was biofertilizer with 3 levels, namely without biofertilizer (P0), rhizobacterial co-inoculant (P1), and commercial biofertilizer (P2). The second factor was chemical fertilizer with 2 levels, namely without NPK fertilizer (T0) and NPK fertilizer at a dose of 50% according to recommendation (T1). Each treatment was performed in 4 replications with total of 24 units of experiment. Parameters observed included plant height (cm), number of leaves, flowering age (DAP), dry weight of upper plants and roots (g), and root length (cm).

Data analysis

The data were analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) post hoc test at confidence level of 95%.

RESULTS

Character of rhizobacteria

Rhizobacteria *B. cepacia* KD 2.10 and *S. marcescens* KAHN 15.12 are classified as Gram-negative, whereas *B. thuringiensis* SAHA 12.12 is Gram-positive. KD 2.10, KAHN 15.12, and SAHA 12.12 rhizobacterial isolates could solubilize phosphate on Pikovskaya media (Figure 1) with solubility index (SI) value of 3.60, 1.16, and 1.00, respectively. The formation of clear zones shows the activity of solubilizing P around bacterial colonies. This is because of rhizobacteria are able to produce organic acids which are characterized by a decrease in pH in the media (Khan *et al*., 2007). These organic acids cause the occurrence of the binding process, namely the formation of bonds between organic acids and metal cations such as Ca^{2+}. This resulted in phosphate being released from $Ca_3(PO_4)_2$ (Rodriguez and Fraga, 1999). In addition, bacteria can also produce phosphatase enzymes that release phosphate groups so that P becomes dissolved (Sharma *et al*., 2013).

Isolate KD 2.10 and KAHN 15.12 could be grown in NfB media by fixing nitrogen that shown by color change from green into blue color. This color change occurs due to an increase in pH because the bacteria binds N_2 to form NH_4^+ compounds or other N compounds and free the compound into the media (Ghevariya and Desai, 2014). The N_2 binding process is catalyzed by the nitrogenase enzyme produced by bacteria (Cheng, 2008).

Antagonistic activity between rhizobacterial co-inoculant

All rhizobacterial isolates did not show antagonistic activity when isolates were used as tested bacteria and target bacteria, compared to positive control, kanamycin (1 mg/mL). Therefore they can be stored in the form of consortium. The formation of clear zone may occur due to certain compounds produced by tested bacteria which can inhibit the growth of target bacteria (Figure 2).

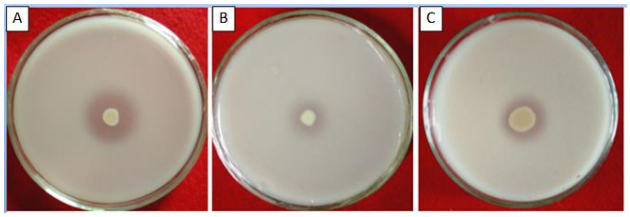

Figure 1: Qualitative results of phosphate dissolution on Pikovskaya media. (A) KD 2.10, (B) KAHN 15.12, (C) SAHA 12.12.

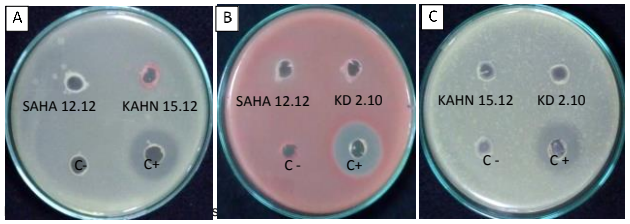

Figure 2: The antagonistic results between the consortium bacteria on NA media. (A) Isolates SAHA 12.12 and KAHN 15.12 did not show antagonistic towards KD 2.10. (B) Isolates SAHA 12.12 and KD 2.10 did not show antagonistic towards KAHN 15.12. (C) Isolates KAHN 15.12 and KD 2.10 did not show antagonistic towards SAHA 12.12. C-, negative control (sterile distilled water); C+, positive control (1 mg/mL kanamycin).

Hypersensitivity of red chili plant leaves

All rhizobacterial isolates showed negative hypersensitivity reaction on chili leaves after 48 h of incubation based on negative control (*B. cereus*). *Pseudomonas syringae* as positive control showed yellow-brownish spot on chili leaves which reflects necrosis symptoms. Therefore they can be applied on chili plants (Figure 3).

Viability of rhizobacterial co-inoculant in formulation

Cell culture of 10^{10} CFU/mL which was homogenized into 50 g talc resulted in cell population of 10^9 CFU/g. Stability of rhizobacterial cell population in talc carrier can be determined through viability test. Result showed that viability of rhizobacterial consortium experienced a decline until six months of storage period (Figure 4). Decrease in the number of bacterial colonies was due to the

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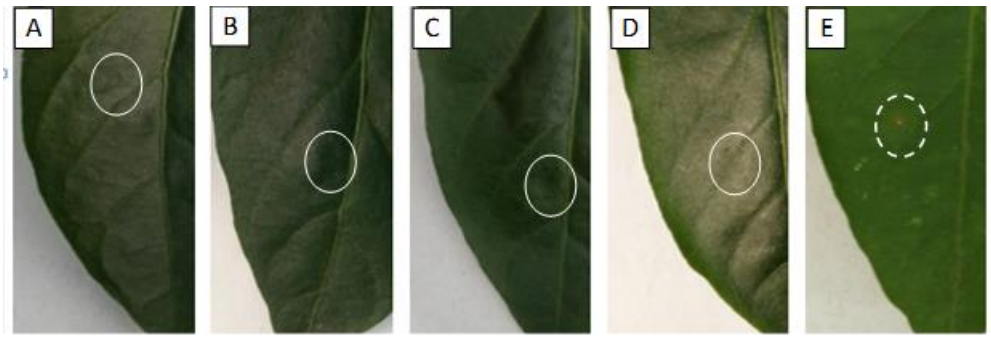

Figure 3: Hypersensitivity testing on chili leaves. (A) Isolate KD 2.10, (B) SAHA 12.12, (C) KAHN 15.12 did not show symptoms of necrosis, (D) *Bacillus cereus* as a negative control, and (E) *P. syringae* as a positive control (spotting necrosis).

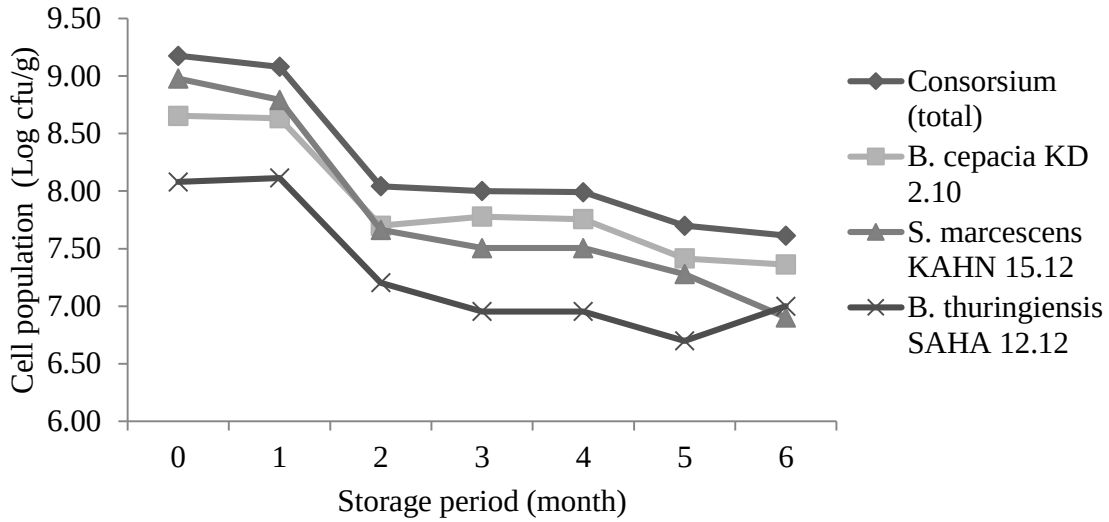

Figure 4: Viability of bacterial cells in carrier material during storage period at room temperature.

decreasing nutrients contained in the formulation along with the storage period. The viability on the sixth month was 4.1×10^7 CFU/g.

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Formulation effectiveness of rhizobacterial co-inoculant on the growth of red chili plants

The result of ANOVA showed biofertilizer significantly affected plant height, number of leaves, flowering age, dry weight of upper plant and root, and root length (Table 1). The treatment of rhizobacterial co-inoculant formulation (P1) gave the better effect than the control (P0) and commercial biofertilizer (P2) (Table 2). Application of chemical fertilizer did not give significant effect on plant height, number of leaves, dry weight of upper root, and root length of chili plants (Table 1). This showed that the treatment without NPK fertilizer (T0) has been able to support the response of these parameters properly. However, for the response of the flowering age and dry weight of upper plant, it has a significant effect. This showed that in terms of flowering age and dry weight of upper plant, NPK fertilizer of 50% (T1) was still better than without NPK fertilizer (T0) (Table 2).

The effect of interaction between biofertilizer and chemical fertilizer was not significant on plant height,

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number of leaves, flowering age, and root length of chili plants. However, it has a significant effect on the response of dry weight of upper plant and root of chili plants (Table 1). In general, combination treatment P1 produced a higher average growth. The treatment of P1T0 showed an increase in growth in response to plant height, number of leaves, dry weight of upper plant and root, and root length of chili plants, while P1T1 treatment was able to accelerate the flowering age (Table 2).

DISCUSSION

Rhizobacteria used in research were *B. cepacia* KD 2.10, *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12 obtained from soil around roots of plants. All rhizobacteria used in this study could solubilize phosphate (Figure 1). Rhizobacterial isolates were mixed into one consortium and formulated as biofertilizer and then applied to chili leaves (Figure 3). Before the isolates were mixed, antagonistic testing was conducted to ensure that the three isolates used were not antagonistic but compatible with each other. There is no formation of clear zones around the well shows that the rhizobacterial are not antagonistic so that they can be consorted in the make of biofertilizer formulations (Figure 2).

Rhizobacterial co-inoculant was applied in the form of powder formulation with the addition of carrier material. Formulation is carried out to facilitate the application of rhizobacterial inoculants, storage and distribution in the field. In addition, the carrier material also plays a role in maintaining the viability of rhizobacterial inoculants. The carrier material used, which is talc. Talc is a clay mineral composed of hydrated magnesium silicate which have been widely used because it is easy to be obtained, cheap, and contains minerals which do not produce toxic compounds after sterilization process; thus, it is safe for bacteria (Saharan *et al*., 2010).

Table 1: The results of ANOVA on observation parameters on chili plants.

Source	Sum of squares	df	Mean square	F	Sig.
A. Plant height					
Biofertilizer (P)	1192.188	2	596.094	16.844	0.000[a]
Chemical Fertilizer (T)	22.042	1	22.042	0.623	0.440
Interaction P*T	133.771	2	66.885	1.890	0.180
Error	637.000	18	35.389		
Corrected Total	1985.000	23			
B. Number of leaves					
Biofertilizer (P)	1494.250	2	747.125	17.871	0.000[a]
Chemical Fertilizer (T)	8.167	1	8.167	0.195	0.664
Interaction P*T	43.583	2	21.792	0.521	0.602
Error	752.500	18	41.806		
Corrected Total	2298.500	23			
C. Flowering age					
Biofertilizer (P)	680.333	2	340.167	9.108	0.002[a]
Chemical Fertilizer (T)	260.042	1	260.042	6.963	0.017[a]
Interaction P*T	20.333	2	10.167	2.272	0.765
Error	672.250	18	37.347		
Corrected Total	1632.958	23			
D. Dry weight of upper plant					
Biofertilizer (P)	293.486	2	146.743	435.870	0.000[a]
Chemical Fertilizer (T)	3.375	1	3.375	10.025	0.005[a]
Interaction P*T	22.998	2	11.499	34.155	0.000[a]
Error	6.060	18	0.337		
Corrected Total	325.918	23			
E. Dry weight of root					
Biofertilizer (P)	19.758	2	9.879	34.114	0.000[a]
Chemical Fertilizer (T)	0.260	1	0.260	0.899	0.356
Interaction P*T	2.436	2	1.218	4.206	0.032[a]
Error	5.212	18	0.290		
Corrected Total	27.666	23			
F. Root length					
Biofertilizer (P)	172.000	2	86.000	49.935	0.000[a]
Chemical Fertilizer (T)	2.667	1	2.667	1.548	0.229
Interaction P*T	10.333	2	5.167	3.000	0.075
Error	31.000	18	1.722		
Corrected Total	216.000	23			

[a],Significant effect on F-5%.

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Table 2: The effect of treatments on chili plants 8 weeks after planting.

Treatments	Average of Treatments					
	Plant height (cm)	Number of leaves	Flowering age (DAP)	Dry weight of upper plant (g)	Dry weight of root (g)	Root length (cm)
Factor of biofertilizer (P)						
P0	85.38[a]	70.63[a]	52.13[a]	8.08[a]	3.85[a]	15.50[a]
P1	102.56[c]	89.88[c]	39.38[b]	16.44[c]	6.00[c]	22.00[c]
P2	92.56[b]	78.75[b]	43.38[b]	13.86[b]	5.41[b]	19.50[b]
Factor of chemical fertilizer (T)						
T0	92.54	80.33	48.25[a]	12.42[a]	5.19	19.33
T1	94.46	79.17	41.67[b]	13.17[b]	4.98	18.67
Interaction between P and T						
P0T0	81.88	69.75	54.25	7.43[a]	3.68[a]	15.25
P0T1	88.88	71.50	50.00	8.73[b]	4.03[a]	15.75
P1T0	104.75	92.25	43.75	17.38[e]	6.55[c]	23.25
P1T1	100.38	87.50	35.00	15.50[d]	5.45[b]	20.75
P2T0	91.00	79.00	46.75	12.45[c]	5.35[b]	19.50
P2T1	94.13	78.50	40.00	15.28[d]	5.48[b]	19.50

The number followed by different letters in the column showed significantly different results based on Duncan test at α = 0.05. P0, without biofertilizer; P1, rhizobacterial co-inoculant; P2, commercial biofertilizer; T0, without NPK; T1, 50% NPK; P0T0, without biofertilizer and NPK (control); P0T1, without biofertilizer with 50% NPK; P1T0, formulation rhizobacterial co-inoculant without NPK; P1T1, formulation rhizobacterial co-inoculant with 50% NPK; P2T0, commercial biofertilizer without NPK; P2T1, commercial biofertilizer with 50% NPK.

The number of population and the ability of rhizobacteria to survive in the formulation is a requirement in making biofertilizers. The stability of rhizobacterial populations can be known through viability tests. The results of the viability test showed that population of rhizobacteria in formulation decreased until six months of storage with 4.1×10^7 CFU/g of cell population (Figure 4), which it was the minimum requirement of quality standard for biofertilizer in the regulation of the Minister of Agriculture Republic of Indonesia No.70/Permen-tan/SR.140/10/2011. Based on this regulation, biofertilizer should have microbial colony of ≥ 10^7 CFU/g with carrier material of flour or powder.

The result of viability test during storage period will determine the expiration period of biofertilizer. The number of population which was still at the minimum limit of population on standard requirement for biofertilizer in the stage of viability test will be the benchmark of expiration period of fertilizer. Therefore, formulation of this rhizobacterial consortium can only be used for 6 months. Stability of formulation is also supported by the additional materials used, namely CMC, peptone, sucrose, yeast extract, and $CaCO_3$. CMC functions as additive material to make formulation easily attach on the surface of plant organs. The addition of peptone, sucrose, and yeast extract also plays role as additional carbon source which can maintain cells to survive and to be efficient in biofertilizer as well as the addition of $CaCO_3$ as nutrient and calcium source for bacterial growth and to neutralize pH in carrier material media (Ardakani *et al.*, 2010; Fraile *et al*., 2015).

Rhizobacterial co-inoculant formulation was applied on chili plants at greenhouse. Before applied, it was carried out hypersensitivity test to ensure that the rhizobacteria used were not pathogenic to chili plants. The rhizobacterial isolates showed negative results, ie there were no yellow-brownish spot on chili leaves. Therefore they can be applied on chili plants and be safe to use as biofertilizers. Yellow-brownish spots are a symptom of necrosis which indicates a hypersensitivity reaction. The gene of hypersensitive reaction and pathogenicity (hrp) which is commonly found in Gram-negative pathogenic bacteria such as *P. syringae* affects the ability of bacteria to cause hypersensitivity reactions in leaves (Zhu *et al*., 2000). The three rhizobacterial isolates used in this research have been tested previously by Nurdin *et al*. (2016) and Miladiarsi *et al*. (2017) in tobacco leaves which showed negative result with no necrosis symptoms.

The effect of rhizobacterial co-inoculant formulation as PGPR agent on chili plants observed 8 weeks after planting at greenhouse. Based on result, formulation of rhizobacterial co-inoculant gave the best effect to all parameters. It was affected by the ability of rhizobacteria in fixing nitrogen and solubilizing phosphate; therefore, the needs of chili plants on nutrients can be obtained. Moreover, stability of bacterial cell population in carrier material also supported the success of formulation stability for a long time and it can survive on soil environment; thus, the potency of bacteria as PGPR agent in providing and mobilizing the absorption of nutrients succeed in promoting the growth of red chili plants at greenhouse experiment.

Results of previous study showed that *B. cepacia* KD 2.10 used in this formulation was able to solubilize P nutrient of 102.83 mg/L and had nitrogenase activity of 0.0614 µmol/(mL·h) (Miladiarsi *et al*., 2017). Moreover, *S. marcescens* KAHN 15.12 produced IAA of 3.75 ppm and was able to solubilize potassium. In addition, *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12 were also able to degrade chitin *in vitro* in

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controlling pathogenic fungus *C. capsici* which causes anthracnose disease in chili (Astriani *et al*., 2016; Nurdin *et al*., 2016). Therefore, formulation of this rhizobacterial co-inoculant does not only play role as biofertilizer, but also has potential to be a biological control.

Formulation of rhizobacterial co-inoculant without NPK fertilizer gave the best results for all observed characters except flowering age (Table 1 and 2). This shows that the provision of rhizobacterial co-inoculant formulation can reduce the use of NPK fertilizer. The combination treatment of the rhizobacterial co-inoculant formulation with NPK fertilizer at 50% dose can accelerate the flowering age compared to rhizobacterial co-inoculant formulation without NPK fertilizer. This is because when entering the generative phase, more plants need P and K nutrients. P nutrients are needed to stimulate flower and fruit formation, while K nutrients are needed to strengthen the tissue in plants so that flowers do not fall easily (Wahyudi, 2011) . In addition, the planting media used is less fertile so that the availability of nutrients is not enough for chili plants. Therefore, to improve soil fertility, fertilization needs to be done.

CONCLUSION

Rhizobacterial co-inoculant consisted of *B. cepacia* KD 2.10, *Serratia marcescens* KAHN 15.12, and *Bacillus thuringiensis* SAHA 12.12 did not show antagonistic activity and hypersensitivity reaction on chili plant. Cell population of rhizobacterial consortium in powder formulation of 10^9 CFU/g could be maintained until six months of storage with cell population of 10^7 CFU/g. Formulation of rhizobacterial consortium significantly affected the response on all parameters observed and was effective in increasing the growth of chili plant in greenhouse experiments. It is recommended to perform further study on testing formulation of rhizobacterial co-inoculant until fruit production stage and its application at field scale and optimization of production media to be developed at large scale.

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