DOI: http://dx.doi.org/10.25181/jppt.v17i3.298
Jurnal Penelitian Pertanian Terapan Vol. 22 (1): 58-66
http://www.jurnal.polinela.ac.id/JPPT

pISSN 1410-5020
eISSN 2407-1781

Formulation of Soil Beneficial Microbes Solid Inoculant for Controlling Nematode in Coffee

Formulasi Inokulan Padat Bakteri Tanah yang Menguntungkan sebagai Pengendali Nematoda pada Tanaman Kopi

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ABSTRACT

Plant Growth Promoting Rhizobacteria Pseudomonas and Bacillus are well known biofertilizer due to their ability for solubilizing inorganic phosphate and producing phytohormones in soil. However, their potential to decreased diseases incidence caused by Pratylenchus coffeae has been documented. The objective of this laboratory experiment was to formulate solid biofertilizer contained Pseudomonas diminuta and Bacillus subtilis. The laboratory experiments consisted of three-step experiment: 1) selection of the molasses-based liquid media for P. diminuta and B. subtilis growth, 2) organic liquid inoculant formulation and 3) organic carrier-based inoculant formulation. The population of both bacteria in all steps was count by serial dilution plate method with specific medium. The result verifeid that cell density of P. diminuta and B. subtilis in liquid culture with 2% molasses were higher than cell count in the culture contained 4% molasses. Bagasse-based carrier inoculated with liquid culture of mixed bacteria has higher bacterial count compared to manure-based carrier. After 90-day incubation, bagasse-based solid inoculant maintained P. diminuta and B. subtilis density up to 10^8 CFU/mL and the acidity of solid inculaant was about neutral. The bacterial count and acidity of solid inoculant agreed with Indonesian standard for solid biofertilizer. The bagasse-based solid inoculant of both bacteria was effective to decrease the P. coffeae population in roots of coffee seedlings.

Keywords: *Bacillus subtilis*, cell density, culture acidity, *organic carrier, Pseudomonas diminuta*

Disubmit : 1 September 2021,**Diterima:** 15 Juni 2022 , **Disetujui :** 05 April 2022;

INTRODUCTION

Coffee is an important agricultural commodity in Indonesia. A half million tons of coffee produced every year in the estates and small holder farms mostly in the island of Sumatra, Java, Sulawesi, and Bali. One of the majors constrains to maintain or increase coffee production is infestation of Pratylenchus coffeae nematode (Budiman et al., 2020).; well known as plant endoparasitic root lesion nematode (RLN) In Indonesia serious and acute attack of P. coffeae on coffee tree enable to decrease coffee bean yield up to 78 % (Wiryadiputra and Tran, 2008).

The RLN limits water as well as nutrients absorption from the soil and hence causes plant growth retardation (Yu et al., 2012). Based on ultrastructural observations, severe nematode invasion resulting the breakdown of cortical parenchyma cells (Vieira et al., 2019). Invasion of P. coffeae reduced root growth of coffee plant seedlings (Rahayu and Sari, 2017) and results in leaf yellow rot disease in mature plants (Thiep et al., 2019)

To date, beneficial soil microbes are suggested to promote coffee plant growth as well as to control the pests and diseases (Duong et al., 2020). Inoculation of plant growth promoting rhizobacteria (PGPR) which increase growth of coffee and control the P. coffee is a way to increase coffee production. Pseudomonas spp. and Bacillus spp. are PGPR which are colonized coffee roots and has been succesfully isolated from the coffee rhizosphere (Muleta et al., 2013; Duong et al., 2020). Both rhizobacteria isolated from coffee rhizosphere play a prominent role to induce plant growth through phosphate solubilization and phytohormone production (Muleta et al., 2009).

The genus of Pseudomonas and Bacillus were reported for their ability to control invasion of P. coffeae (Asyiah et al., 2015). Inoculation of mixed inoculant of P. diminuta and B. subtilis isolated from coffee rhizosphere clearly enhance coffee growth traits i.e plant height, number of leaves and leaf area (Asyiah et al., 2020). Moreover, this experiment verified that decrease in P. coffeae in roots was related to the plant growth. Both rhizobacteria has also identified as mycorrhiza helper bacteria (MHB) which induce the formation of Arbuscular Mycorrhiza (AM) in i agricultural crops (Nanjundappa et al., 2019; Gupta and Chakraborty, 2020). Coffee plants with well-established arbuscular mycorrhiza (AM) symbiosis is reported to improve P status in soil and leaves (Moreira et al., 2019) and growth traits and root infection rate (Parapasan and Gusta, 2014) as well as to control invasion of P. coffeae (Schouteden et al., 2015).

in order to increase the effectiveness of P. diminuta and B. subtilis mixed inoculant in suppresing P. coffeae and coffee seedling growth, formulation of solid inoculant with propriate cell viability is needed. Solid inoculant in common is formulated from liquid inoculant by using carrier which have the ability to maintain bacterial cell viability. Bacterial inoculant produced by chemical media with defined composition is concidered the best one to ensure the optimal call proliferation and biological functions. Nonetheless, the chemical media price is high. Inoculant production by using locally available carriers become more popular and guarantee their availability throughout the year. The used of product of agricultural activity facilitate the lower price of microbial inoculant. In Indonesia, sugar cane molasses, bagasse, composted manure, and sawdust has been utilized as organic carrier materials for inoculant production.

Molasses and bagasse are by product of sugarcane-based industry. Molasse contain 18 % of low molecular weight saccharides as well as macro- and microelement (Lino et al., 2018) that provide organic carbon and nutrients for heterotrophic P. diminuta and B. subtilis. Bagasse is sugarcane press mud consiste of 60% carbon and small quantities of element N, P, S and Ca (Ameram et al., 2019). Composted cow and goat manures consiste of 17% and 32% organic C respectively and both manures contain more P, K, and Na but less N than fresh manure (Irshad et al., 2013). It is accepted that the low nutrients but high organic C is important to support bacterial viability due to physical protection to adverse condition (Nur et al. 2015). In order to formulate solid mixed inculant PGPR P. diminuta (PD) and B. subtilis (BS), the laboratory experiments have been performed to verify the effect of 1) sugarcane molasses level on liquid medium as well as composition of PD and BS on the cell count of both PGPR, 2) different organic carrier materials on population of both bacteria in solid inoculant and 3) solid formulation of both PGPR on the P. coffeae population in coffee plants roots

RESEARCH METHODS

The Research were conducted at Microbiology Laboratory of Universitas Jember as well as Universitas Padjadjaran in April – June 2017. The mycorrhiza helper bacteria *P. diminuta* and *B. subtilis*

were belong to Universitas Jember and Universitas Padjadjaran respectively. Both bacteria were maintaining in nutrient agar media (0.5% peptone, 0.3% yeast extract, 0.5% sodium chloride, 1.5% agar, 1 L distilled water, pH neutral) at 4oC. Molasses and bagasse was obtained from the Jati Tujuh Sugar Factory in West Java. Cow and Sheep manure were provided from local feed lot. The count of cell counts of PD and BS have been carried out by serial dilution method in selective media for Pseudomonas and Bacillus as well.

Both PGPR Liquid culture were prepared separately by inoculating 1 mL of bacterial pure culture into 500 mL nutrient broth, incubated 24 hr in 30oC shaker incubator at 100 rpm. In order to harvest bacterial cell, liquid culture of both bacteria were centrifuged in 4oC at 5,000 rpm for 10 minutes. Pelleted bacterial cell in the base of centrifuged tube were suspended in 10 mL sterilized distilled water and store at 4 ^{0}C.

Laboratory experiment consist of four trials (Fig 1) which include: 1. Selection of molasses-based broth; 2. Organic Liquid inoculant formulation, 3. Organic carrier-based inoculant formulation, and 4. in planta experiment

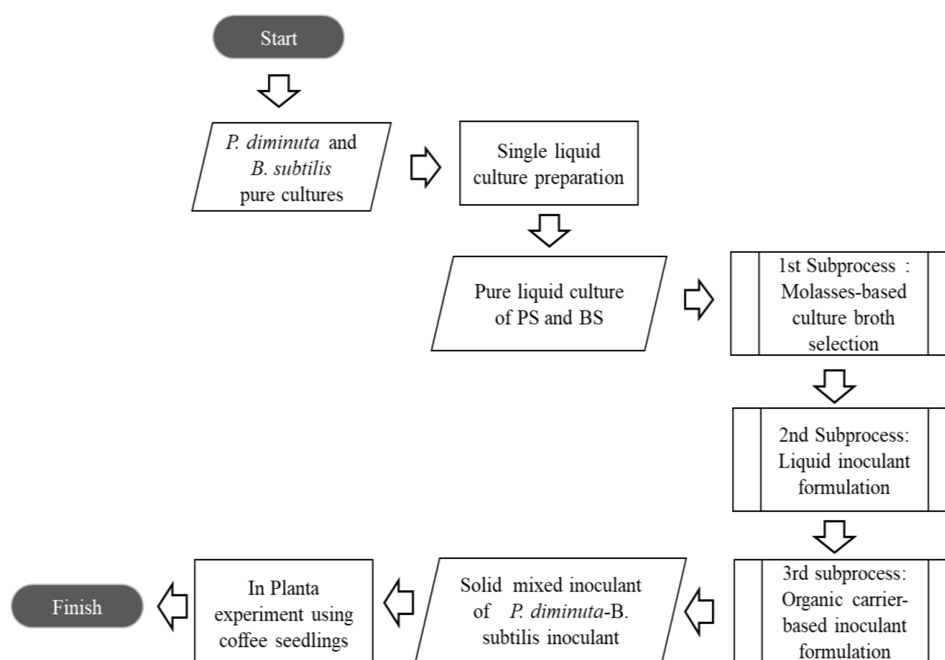

Figure 1. Experimental processes for formulation of P. diminuta and B. subtilis mixed solid inoculant

Selection of molasses-based broth

Selection of liquid media for PD and BS growth were carried out using 2% and 4% molasses broth enriched with 10% of vermi-cast extract and 30% of potato broth. The control media was nutrient broth. The nutrient composition of molasses was organic carbon 56.79%, C/N 19.58, 0.29% N, 0.18% P2O5 and 0.39% K2O. A total of 1,000 mL broth was autoclaved for 15 minutes and pour into 2 L fermenter aseptically. All media were inoculated with 5% of either PD and BS liquid mother culture and incubated for 3 days at room temperature (24-27oC) with stirring at 100 rpm. All treatment was repeated three times. The Cell density and pH of growth media were analysis every 24 hours during 3 days. Population of both bacteria was counted by using serial dilution plate method on nutrient agar. All data were subjected to analysis of variance (F-test) and followed by Duncan Multiple Range Test (DMRT) at $p \leq 0.05$

Liquid Inoculant Formulation

The trial was arranged in completely randomized design with 5 treatments and 4 replications. Sterilized broth contained 2% of molasses enriched with 10% of vermi-cast extract and 30% of potato broth (selected from the first trial) were inoculated with P. diminuta and B. bacillus at the ratio of 1:1, 1:2, 2:1, 2:3, and 3:2 (v/v). A total of 250 mL media in 750 mL Erlenmeyer flask was autoclaved for 15 minutes and left overnight at room temperature prior to the inoculation.

Liquid culture of both bacteria was mixed thoroughly with the composition described above. A total of 1% of different bacterial composition was poured into liquid media in the Erlenmeyer flask aseptically. All cultures were incubated for 7 days prior to cell culture enumeration and pH measurement. All data were subjected to analysis of variance ($p<0.05$) and DMRT with $p \leq 0.05$. Based on bacterial population, the best initial bacterial composition was stored for 90 days at room temperature in order to determine the bacterial population. This composition will be used for further experimental step.

Carrier-based Inoculant Formulation

The experimental design was completely randomized design with 6 treatments and 3 replications. the treatments were the combination of carriers (100-mesh enriched bagasse, cow manure and goat manure) with two initial cell density of mixed liquid inoculant (108 and 109 CFU/mL). All carriers were enriched with 5 mL/kg micronutrients, 0.5% chitin and 1% yeast extract. Based on the 2nd experiment, liquid inoculant was prepared by inoculating molasse-based broth at pH 7.4 with the PD and BS in volume ratio of 2:3. The culture then incubated for 3 days at room temperature. The final cell density of liquid inoculant decribed above was 109 CFU/ml.

The moisture content of carrier materials were 20% before sterilization. The carrier materials have been autoclaved for 15 minutes, stored for 24 h at room temterature, and mixed with 10% of liquid inoculant of mixed bacteria. A total of 50 g of solid inoculants were put into individual capped bottle, stored in the dark cabinet for 3 days prior to the cell count. After PD and BS enumeration, we verified that the cell viability of bagasse-based inoculant was higher than two other carrier material. This solid inoculant was stored at room temperature for another 90 days. Cell enumerations were carried out at day 30, 60 and 90. Cell count were subjected to analysis of variance ($p<0.05$) and DMRT with $p \leq 0.05$.

In Planta Experiment

The experimental research has conducted in randomized block design with four treatments and six replications. The three-days old coffee (cv Arabica) seedings have been grown in substrate of soil and sand with balance composition. The experimental treatments were growth substrate inoculated with 1. P. coffieae, 2. P. coffieae with mychorrhiza Glomus sp, and 3. P. coffieae with Glomus sp, and MHB. The control treatment was substrate without P. coffieae. A total of 5 g Glomus inoculant and 20 g of MHB were added for one seedling. The MHB solid inoculant composed of P. diminuta dan B. subtilis was formulated following the method in the Carrier-based Inoculant Formulation section described above. The seedlings have been growing in green house for four months prior to counting nematode population in roots and measuring the plant height.

RESULTS AND DISCUSSION

Selected molasses-based liquid media

Molasses concentration affected the cell density of both bacteria in liquid media during three-day incubation at room temperature (Table 1). At day 1, bacterial cell count in nutrient broth (NB) was significantly lower than in molasses-based broth. Increased of cell density in NB over molasses-based broth was clearly shown at day 2 and 3. The acidity of molasses-based liquid culture was higher compared to NB.

The cell density of liquid media with 2% and 4% molasses did not differ, about 10^8 CFU/ml either for *P diminuta* or *B. subtilis*. In search of more economic growth media, bacterial liquid inoculant with 2% molasses is considerable to be used for next experiment.

Table 1. Effect of molasses concentration on cell density and acidity of *P. diminuta* and *B. subtilis* liquid culture at 1 to 3 days after incubation

Molasses concentration	*P. diminuta*		*B. subtilis*	
	Cell density 10^8 cfu/mL	Acidity	Cell density 10^8 cfu/mL	Acidity
Day 1				
Control (NB*)	3.2 b	5.5 a	5.3 a	5.5 a
Molasses 2%	7.6 a	6.0 a	15.6 b	6.1 a
Molasses 4%	7.2 a	5.5 a	13.1 b	6.2 a
Day 2				
Control	12.8 a	6.0 a	3.0 c	6.2 a
Molasses 2%	1.8 b	6.4 a	8.2 a	7.1 a
Molasses 4%	3.9 b	6.2 a	5.0 b	7.3 a
Day 3				
Control	14.7 a	6.0 b	9.1 c	6.9 a
molasses 2%	3.4 b	7.0 a	1.8 a	7.8 b
molasses 4%	1.8 c	6.4 b	7.1 b	7.8 b

Numbers in a column followed by the same letter were not significantly different based on DMRT
*Nutrient broth

Composition of bacteria in molasses-based liquid inoculant formulation

Based on analysis of variance, the ratio of *P. diminuta* to *B. subtilis* (v:v) determined the cell density of both bacteria didn’t affect the acidity of liquid culture with 2% molasses. Higher bacterial population at 7 days after incubation was in the liquid inoculant formulated with equal as well as 2:3 compositions of PD and BS. This composition increased the cell density up to 2 x10^9 cfu/mL compared to another treatment. The acidity of all inoculants were neutral. The liquid inoculants with PD to BS volume ratio of 2:3 were then stored for next 90 days at room temperature.

Table 2. Effect of initial *P. diminuta* to *B. subtilis* volume ratio on cell density and acidity of 2% molasses-based liquid inoculant at 7-day incubation.

PD:BS[1] (v:v)	Cell density (10^8 CFU/mL)		Acidity
	PD	BS	
1 : 1	15.4 a	31.2 a	7.7 a
1 : 2	3.6 b	10.1 b	7.6 a
2 : 1	8.2 b	2.7 c	7.2 a
2 : 3	20.6 a	26.2 ab	7.3 a
3 : 2	5.9 b	5.6 c	7.2 a

Numbers in a column followed by the same letter were not significantly different based on DMRT
[1]PD, *P. diminuta*; BS, *B. subtilis*

After 90-day storage, cell count of both bacteria was reduced significantly although the populations were still as high as 108 CFU/mL (Table 3). The acidity of 2% molase-based liquid inoculants was increased compared to 7-day storage (Table 2).

Table 3. Cell density and acidity of liquid inoculant 2% molase-based liquid inoculant with initial *P. diminuta* to *B. subtilis* volume ratio of 2:3

Storage duration (days)	*P. diminuta* (10^8 CFU/mL)	*B. subtilis* (10^8 CFU/mL)	Acidity
30	12.5 a	9.3 a	8.1 a
60	6.7 b	6.1 b	8.2 a
90	7.6 b	4.2 b	8.3 a

Numbers in a column followed by the same letter were not significantly different based on DMRT

Solid inoculant formulation

Based on DMRT, enriched-bagasse inoculated with 20 % of 10^8 CFU/ml mixed bacteria liquid inoculant has highest *P. diminuta* population (Table 4). Nonetheless, the *B. subtilis* population of described formulation was lowest compared to other formulation. Meanwhile the population of *B. subtilis* of bagasse-based inoculant with 10^9 CFU/ml of bacteria was highest over other formulation.

Table 4. Cell density and acidity of solid inoculant with different carrier and initial cell density of liquid culture inoculated to the carrier after 3-day incubation.

Carrier	Initial cell density of liquid inoculant	Cell density (CFU) x 10^8 at 3 day		Culture Acidity
		P. diminuta	*B. subtilis*	
Bagasse	10^8	8.5 a	2.7 b	7.5 c
	10^9	2.4 b	4.4 a	7.6 c
Cow manure	10^8	2.3 b	2.0 c	9.7 a
	10^9	3.0 b	4.2 a	9.8 a
Goat manure	10^8	3.4 b	2.8 b	8.5 b
	10^9	2.5 b	1.1 c	8.5 b

Numbers in a column followed by the same letter were not significantly different based on DMRT at $p \leq 0.05$

The reaction of bagasse-based solid inoculant was slightly higher than neutral while the pH of another formulation increased over 8. Based on the data in Table 5, the best solid inoculant formulation to support cell density and acidity was enriched bagasse with 10^8 or 10^9 CFU/ml of initial liquid inoculant. Those formulation was then kept at room temperature for 90 days prior to cell count and pH measurement at 30,60 and 90 days of storage.

Duncan test found that cell density of *P. diminuta* and *B. subtilis* at 60 days were decreased but the population were regained at 90 days. The decreased of pH were found from day 7 to day 90 of storage. All formulation maintained the bacterial population up to 10^8 CFU/g and keep the pH about neutral.

Table 5. Cell density and acidity of P. *diminuta* dan *B. subtilis* in bagasse-based solid inoculant up to 90-day storage at room temperature

Cell density of liquid inoculant	days of storage	Cell density (10^8 cfu/g)		Culture Acidity
		P. diminuta	*B. subtilis*	
10^8	30 days	7.6 a	6.6 a	7.4 a
10^9		6.3 a	3.0 b	7.4 a
10^8	60 days	1.9 b	1.2 c	7.1 a
10^9		1.4 b	1.2 c	7.1 a
10^8	90 days	6.4 a	5.4 a	6.9 a
10^9		3.7 b	6.3 a	6.8 a

Numbers in a column followed by the same letter were not significantly different based on DMRT at $p \leq 0.05$

Based on your title it should be more complete if there are results of testing the formulation activity against nematode population suppression on coffee plants

In planta test verified that inoculation of mycorrhiza *Glomus* sp and MHB enabled to decrease the count of *P. coffeae* in roots of coffee plants seedling (Table 6). The soil was sterilized before experiment so that the nematode was not observed in control plants. The reduction of nematode population in roots was up to 86.3 % in coffee plants received Glomus and MH. Nonetheless, the plant height was not changed.

Table 6. Effect of *Glomus* sp. and bagasses-based solid formulation of MHB on plant height and the population of nematode in roots of coffee plants.

Biological agent's treatments	Plant height	Root nematode counts
Without *P. coffeae*	15.4 a	0 c
Without Glomus and MHB + *P. coffeae*	17.3 a	71.4 a
Glomus + *P. coffeae*	15.8 a	34.3 b
Glomus + MHB + *P. coffeae*	17.3 a	9.75 c

Numbers in a column followed by the same letter were not significantly different based on DMRT

The experiment showed that the PGPR *P. diminuta* and *B. subtilis* separately proliferated in the molasses-based broth supplemented with organic substances; and their population attained 10^8 CFU/ml during 3-day incubation. We found also that the best initial composition of PD and BSinoculated to the molasses-based broth was 2:3 (v/v). This composition doubled the cell count until 90 day-incubation compared to single culture of both bacteria.

Molasses is an important agro-industrial by-product that contains high organic carbon (sugar) as well as macronutrients N, P and K which is essential for bacterial proliferation. The ability of both bacteria to proliferate in local molasses agree with some other experiments. The *Bacillus coagulans* grow in 1% molasses although higher viable cell count is recorded in chemical commercialized substrate (Ahamd Sanadi et al., 2017). Molasses liquid culture support *B. subtilis* and *B. megaterium* spore formation up to 10^6 spore/ml (Hindersah et al. 2020). The viable cell count of molasses-based liquid inoculant of PD and BS was lower than cell density in nutrient broth. The composition of nutrient broth is considered as the best for aerobic heterotroph bacteria. In search of cheap media for scaling up both bacteria, the uses of enriched molasses resulted in viable cell count about 10^8 CFU/ml, approximately 10% lower than cell count in nutrient broth. The result agrees with the growth of *B. coagulans* in Man, Rogosa and Sharpe broth which is too high to count compared to Bacillus count in 1% molasses (Ahamd Sanadi et al., 2017).

At 3-day old the viable count of PD and BS in 2% molasses broth were 3.4 x 10^8 and 1.0 x 10^8 CFU/ml respectively with the pH of 7.8. During 90 days of storage the acidity of its liquid inoculant was about 8; slightly alkaline. The culture reaction become more saline; demonstrated that the molasses-based broth didn't induce organic acid production by both bacteria. Acidity was slightly increased might be due to enzymatic proteins decomposition that leads to release ammonium, a compound that increases pH. Before experiment, the molasses contained 0.29% total N.

Solid formulation showed that bagasse-based solid inoculant had higher viable cell count at day three regardless of liquid inoculant concentration mixed with the carrier. The pH was about neutral which conforms to bacterial growth requirements. During the storage, the cell density was constant at 10^8 CFU/ml. In general, optimum pH for bacterial proliferation is neutral. It is reported that each species and even strain of Pseudomonas and Bacillus required different pH for optimal growth. The optimum acidity for the proliferation of *P. aeruginosa* is 8 with slightly decrease of growth at pH 7 (Sankaralingam et al., 2014). The *P. diminuta* grows optimally at a pH within one or two pH units of the neutral pH of 7 (Isnawati and Trimulyono, 2017). In liquid culture, the maximum viable count of B. subtilis strain BZR 336 and B. subtilis BZR 517 are observed at pH 8 and 6-8 respectively (Sidorova et al., 2020).

The experiment of bagasse-based mixed bacteria formulation was terminated at 90-day incubation when the cell density was slightly reduced compared to 7-day incubation. The result suggested that high organic carbon of bagasse and manure served as physical protectant to maintain cell count during 90-day storage. In general, the shelf life of carrier-based biofertilizer is shorter than liquid biofertilizer since liquid media induces not only cell proliferation but also spore and cyst formation (Brar et al., 2012).

The green house experiment verified that the used of bagasse for MHB solid formulation consist of *P. diminuta* and *B. subtilis* was effective to decrease the population of P. coffeae in roots of coffee cv Arabica without any effect on plant growth. Nematode population was less in roots of coffee inoculated with Glomus and MHB which is the evidence of both bacterial abilities to repress nematode.

Molasses and bagasse is by product of sugar factory in Java. They are affordable: cheap and available almost throughout the year. To meet the demand of longer shelf life solid biofertilizer, the shelf life test has to be continued for up to 6 months. The common expired date of commercialized carrier-based biofertilizers were 4-6 month after production date. Moreover, further experiment to determine the role both rhizobacteria for increasing mycorrhizal colonization in coffee roots is needed.

CONCLUSION

A liquid media contained 2% molasses induced for the growth of *P. diminuta* and *B. subtilis* up to 90 days with initial bacterial volume composition of 2:3. This liquid mixed culture was more supportive the growth of both bacteria in bagasse-based solid inoculant compared to cow and goat manure-based inoculant. Utilization of bagasse enriched with micronutrient, chitin and yeast extract as the carrier for solid inoculant formulation of both bacteria resulted in both bacterial count up to 10^8 CFU/ml during 90-day storage. However its populations were slightly lower than at day 30. The acidity (pH) of solid inoculant was decreased from 30-day to 90-day storage but the pH was still about neutral. Final viable count of bagasse-based mixed inoculant is in accordance with the regulation of Indonesian Ministry of Agriculture. This experiment suggested that molasses and bagasse were the promising carriers for *P. diminuta* and *B. subtilis* inoculant to decrease *P. coffeae* population in coffee roots.

ACKNOWLEDGEMENT

The research was funded by Ministry of Agriculture of Indonesia.

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