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Utilization of Vermiwash for the Production of Liquid Biofertilizers and Its Effect on Viability of Inoculant Bacteria and Green Bean Germination

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ABSTRACT

Contribution of biofertilizer in Indonesia as an alternative material for improving soil fertility and crop production is still limited. This may due to several major obstacles on its utilization and effectiveness regarding to it quality in term of its microbial viability of inoculants on formulations. This study aims to determine the effect of liquid biofertilizer formulations made from *vermiwash* carrier material on indigenous bacterial viability and its testing on germination of green beans 'Vima-1'. The study included: (1) optimization of pH for the growth of inoculants bacterial consortium in *vermiwash* formulations with various additives, (2) viability tests and (3) pathogenicity tests of biological fertilizers on green bean sprouts (vegetative phase). The results showed that the best pH for the growth of the consortium of three bacterial isolates was at 5.5. Liquid formulation with a carrier material in the form of *vermiwash* plus 1% PEG (VP3 treatment) supported the highest viability of the bacterial consortium during 8 weeks incubation. All treatments did not inhibit the growth of green bean sprouts and symptom of pathogenicity was absent in all treated plants.From all treatments, the tallest green bean sprout was detected at VP3 treatment.

INTRODUCTION

Fertility and soil health are determined by the interaction of a number of physical, chemical and biological properties of the soil which are becoming a crucial issue on maintain crop productivity in sufficient level. In order to support plant growth and its production, those soil fertility and health need to be maintained through adopting nutrient balance concept which allow nutrients coming from both organic of inorganic source (Fahrurrozi, Muktamar, Setyowati, Sudjatmiko, & Chozin, 2019; Khairuddin, Isa, Zakaria, & Rani, 2018; Santosa, Maghfoer, & Tarno, 2017). The reliance on intensive use of inorganic fertilizers are believed to contribute the decrease of soil quality and lead to environmental degradation (Savci, 2012; Rivaliati, Suntari, & Prayogo, 2017). Agegnehu, Van Beek, & Bird (2014) reported that continuous application of inorganic fertilizers alone without organic fertilizer application has taken apart in many region causing lowering yields and damaging environment (Fahrurrozi, Muktamar, Setyowati, Sudjatmiko, & Chozin, 2019; Khairuddin, Isa, Zakaria, & Rani, 2018; Santosa, Maghfoer, & Tarno, 2017). The use of inorganic fertilizers in the Indonesian agricultural system is still considered in high demand (Latifah et al., 2019). These practices has led agricultural land in Indonesia to begin to decline in terms of health, soil fertility and polution hazard (Savci, 2012; Latifah et al., 2019). On the other side the application of organic fertilizers beside providing plant nutrients, they also affects on improving soil structure, raising soil porosity, macro and micro nutrients, providing source of energy for micro and macro organism, and enhancing crop root development (Prayogo & Ihsan, 2018; Wang et al., 2016).

One typical well known of organic fertilizer sources is Biofertlizer. Biofertilizers are able to

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increase land productivity in a sustainable manner by assisting the availability of soil nutrients. They have been produced under the use of biological agents (Boraste et al., 2009; Panda, 2011). However, the contribution of biological fertilizer is positioned under to those potential value. The main constraints in the utilization of biological fertilizers are closely related to: (1) the effectiveness of biological fertilizers is not immediately visible, (2) the availability of biological fertilizers in the market are still limited, (3) knowledge and understanding of the use of biological fertilizer are still low. In addition, the minimum utilization of biofertilizers are relates to: (1) the effectiveness of biological fertilizers related to microbial viability, (2) lack of information and the role of biological fertilizers in increasing soil and plant productivity, (3) simplicity in obtaining products at affordable prices (Malusá, Sas-Paszt & Ciesielska, 2012; Malusá & Vassilev, 2014). The effectiveness of biological fertilizers was related with the capability of the microbes to survive and adapt in rhizorfer environment.

The results of research in 2005-2006 showed that not all commercial biofertilizers in the market, had the quality in accordance with the promised promotion (Husen, Simanungkalit, Saraswati, & Irawan, 2007). In fact, the effectivity of a biofertilizer is determined by the number of populations, the viability of microbes in a certain period and their efficacy in plants under various conditions in the field (Malusá, Sas-Paszt & Ciesielska, 2012; Malusá & Vassilev, 2014). Therefore it is important to have complete information in a biological fertilizer product about the viability of the microbes used. High viability of bacteria in biofertilizers will create superior products and can increase fertility and yields. However, so far the information on the viability of bacteria in packages or products was sometimes not available. At present, the used of sterilized vermiwash was predicted to have increase the viability of the microbe in bio fertilizers, since the number of those agents sometimes was found under minimum requirements.

Vermiwash known as a foliar spray, is a liquid biological fertilizer that is collected from the fermentation of worm droppings. So vermiwash is a liquid product that is deposited from earthworms along with other micronutrients. Vermiwash contains sugar, amino acids and phenols along with plant growth-promoting hormones such as Indole acetic acid and humic acid. Fresh vermiwash contains many beneficial microorganisms, which can support plant growth and protect plants from various diseases (Gulsar & Rohini, 2006). Vermiwash is used in organic agriculture both as replacement and supplement for solids and for their unique capacity to provide nutrients effectively and quickly. Vermiwash is made from vermicompost. It is an organic material reformation with the help of nutrient-rich microorganisms and worms. Vermicompost is a media for worm maintenance which is a by-product of earthworm cultivation (Sinha, Herat, Chauhan, & Valani, 2009). The used of vermiwash was expected not only to support the microbe viability but also serve as carrier material that is important in manufacturing the biofertiizer. In this study, the bacteria used as inoculants are indigenous bacteria from various place such as : Junrejo in Batu region and two site in Malang region which are Kendalpayak and Jambe Gede, namely : free Nitrogen (N) fixing bacteria *(Bacillus licheniformis)*, soluble Phosphate (P) bacteria (*Pantoea* ananatis) and Exopolysaccharide (EPS) producing bacteria (*Pseudomonas plecoglossicida*) obtained from previous studies (Arfarita, Muhibuddin, & Imai, 2019). The information related with the compatibility of the carrier material with the respected microbes, the self life and their pathogenicity are still limited. The research was then to find out the effect of vermiwash on the viability of consortium of these beneficial microbes and germination of green bean.

MATERIALS AND METHODS

Soil Samples

Collection of soil samples was taken in April 2016 in the area of Junrejo (Batu region), Kendalpayak and Jambe gede (Malang region) from the rhizosphere of mung bean plants (*Vigna radiata* L.). Soil samples were taken by pulling out the plant together with its roots carefully. Plant shoot was cut and then the roots and the soil were put into plastic bags. They were kept in a cooling box and then immediatellybrought to the laboratory or stored at 4-8°C to isolate the bacteria.

Bacterial Innoculant Purification

The isolates used in this study were were: (1) *Bacillus licheniformis*, (2) *Pantoea ananatis*, and (3) *Pseudomonas plecoglossicida*. These bacteria were resulted from the exploration activity. Purification of isolates was carried out using the Streak plate method. The bacterial inoculum were inoculated on the media using an ose needle. The results of purification in the form of a single colony from each bacterium were taken using an ose needle and scratched back on the Nutrient Agar (NA) medium

then incubated for 24 hours at room temperature. The purity of the bacterial colony was observed with gram staining technique.

Optimum pH for Bacterial Growth

Observation of pH was carried out using Nutrient Broth (NB) media prepared in Erlenmeyer dishes with various pH levels, i.e.5.0, 5.5, 6.0, 6.5 and 7.0. All isolates were in consortium condition which were cultured on 250 ml erlenmeyer containing 100 ml NB media, and incubated at room temperature. The growth of the consortium bacteria was observed every 12 hours for 84 hours using a spectrophotometer at a wavelength of 600 nm (OD600). Each treatment was repeated 4 times.

Biofertilizer Liquid Formulation

Liquid biofertilizer formulation material were consisted of vermiwash, molasses, peptone water, PEG and glycerol 1%. The treatments were : (1) P0 (Peptone water + 3 bacterial isolates), (2) VP1 (Vermiwash + molasses + 3 bacterial isolates), (3) VP2 (Vermiwash + molasses + Glycerol + 3 bacterial isolates) and (4) VP3 (Vermiwash + molasses + PEG + 3 bacterial isolates). To all treatments, the pH of liquid biofertilizer formulation were adjusted based on observations to achieved optimum condition. The step of biofertililizer liquid formulation were as follow. First, the carrier material was sterilized, and leaved to cool then put in a plastic bottle. Secondly, then additional ingredients and liquid cultures of the bacterial consortium of 1 ml per 100 ml of liquid formulation were added. The solutions were mixed until homogenous conditions were achieved. All treatment bottles were stored in a dry place, at room temperature and prevented from direct sunlight.

Viability Test of Bacteria in Biological Fertilizer Formulations During the Eight-Week incubation

The viability test was carried out using the spread plate method on PCA (Plate Count Agar) media from the results of a series of dilutions of liquid fertilizer liquid formulations. Growing bacteria were calculated using the TPC (Total Plate Count) method in cfu/ml. Observations were made once a week for 8 weeks period of incubation.

Pathogenicity Test of Green Bean Plant Sprouts

Pathogenicity tests were carried out on green bean sprouts 'Vima-1'. Green bean seeds were germinated for 2-3 days (± 1 cm long). The uniform growth of the sprouts were selected before being cultivated in a test tube containing sterile Yoshida solution. Liquid formulation of biofertilizer inoculated as treatment P0, VP1, VP2 and VP3, repeated 4 times. Observations of the sprout were conducted after there was only one green bean left whereas the tip of sprout leaves reached the cotton cover. Examination of pathogenicity test were made visually and qualitatively based on the symptom of pathogenicity such as decay, necrosis and abnormalities of growth compared to control plants. Sprout growth variables that were plant length and root length.

Data Analysis

The data of the observations were analyzed using ANOVA in Genstat ver 20.00 (VSNI-UK) with 95% level of significances. If the effects of treatment were significant, the mean comparison between treatments were performed based on Least Significant Different ($\alpha \leq 5\%$). Multivariate analysis were performed using Canonical Variate Analysis (CVA) toward the effect of different time of pH observation and the effect of the treatments to bacterial viability.

RESULTS AND DISCUSSION

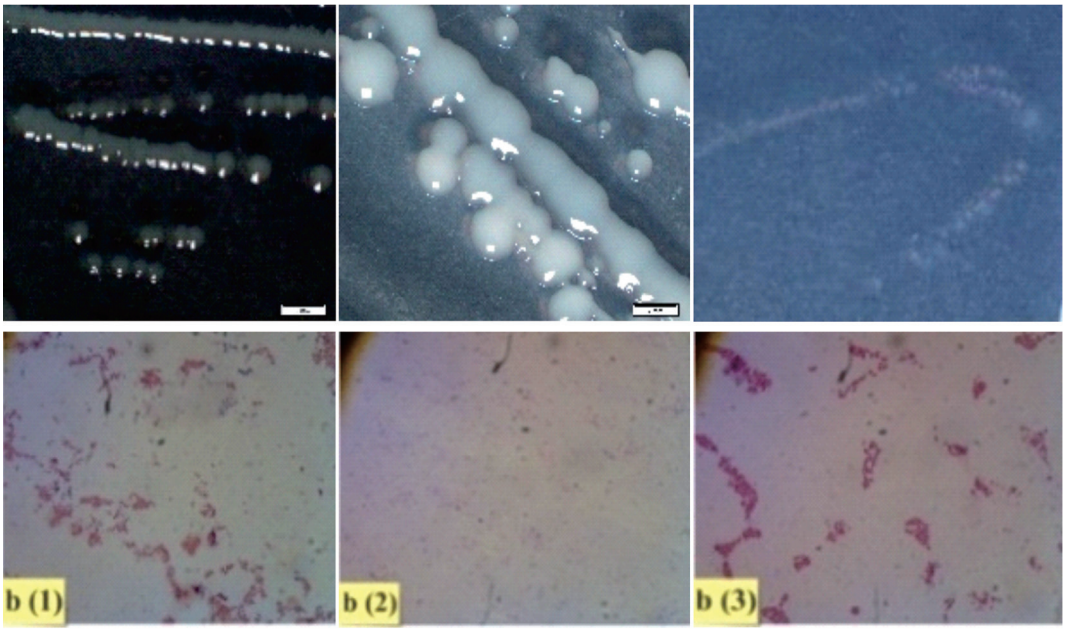
Bacterial Inoculant Purification

The result of isolation and purification of all three types of bacteria on NA media showing the morphology of N fixing bacteria (*Bacillus cereus* or SNF 5), soluble P bacteria (*Pantoea ananatis* or SPP 1) and soil aggregate stabilizer bacteria (*Pseudomonas plecoglossicida* or SPE 20) were presented in Fig. 1. The bacterial morphology was obtained from gram staining of bacteria at 24-36 hours after streaking. The observations under the microscope confirmed that the isolates were in a pure state, which was characterized by the uniformity of cell morphology. Pure bacterial isolates that successfully grown formed a single colony, before their cell morphology and purity could be observed with gram staining technique (Fig. 1). Bacterial isolation was also carried out on selective media for each type of bacteria to confirm and maintain the purity of the isolates. Before inoculated in various liquid biofertilizer formulations, 3 bacteria types were grown on NB (Nutrient Broth) media under consortium formation to achieve the targeted density. The clear golden yellow of NB media color would turn into cloudy color when the inoculated bacteria have overgrown. This is due the bacterial deposits material from their activities on the media

after 2 days of inoculation and incubated at 37°C. As a result of the warm temperature, the acceleration of bacterial growth is marked by cloudiness of the media. At certain levels of growth/turbidity, then this liquid culture is ready to be used for biological fertilizer starter materials.

pH Optimization

Optimization of pH for the bacterial growth of the consortium in liquid media was measured using a spectrophotometer which expressed in OD (Optical Density) at OD_{600} value. The pH treatment were significantly affected bacterial growth (Table 1). Each bacterial isolate prefers different pH conditions. The optimum pH required by the three bacteria to grow in a consortium was at pH 5.5. Fig. 2 shows the log phase of the growth of the bacterial consortium in the liquid formulation was at 0-12 hours, the stationer phase were detected at 12-60 hours and then the similar log phase were repeated again at 60-72 hours. In general, bacterial growth is influenced by soil pH because each microbe will be growing well during favorable environmental conditions. Changes in environmental conditions will affect the growth and life of bacteria at the beginning of growth. Unfavorable enviromenatl changes might contribute to the death of bacteria, since they cannot carry out metabolic processes (Haruta & Kanno, 2015; Ratske & Gore, 2018).



Remarks: Above: Colony morphology: a (1) SNF 5, a (2) SPP 1 and a (3) SPE 20. Below: Cell morphology: b (1) SNF 5, b (2) SPP 1 and b (3) SPE 20

Fig. 1. Purification of three bacterial isolates

Table 1. Average pH medium and optical density across the treatments over various time of observation

pH medium	Average of OD_{600} at time of observation (hours)-						
	0	12	24	36	48	60	72
5	0.141	0.603 b	0.667 c	0.795 c	0.836 d	0.917 d	1.252 c
5.5	0.124	0.483 a	0.567 a	0.772 b	0.815 c	0.905 c	1.542 d
6	0.111	0.544 a	0.602 b	0.661 a	0.742 a	0.745 a	1.12 a
6.5	0.114	0.658 a	0.729 e	0.761 b	0.795 b	0.862 b	1.064 a
7	0.111	0.631 a	0.711 d	0.772 b	0.871 e	1.043 e	1.154 b
LSD 5%	NSig	Sig	Sig	Sig	Sig	Sig	Sig

Remarks: Values in the same column followed by the different letters differ significantly based on LSD % (Sig: Significant, NSig: Not significant)

Generally, the pattern of bacterial consortium growth in liquid medium under different pH value indicated by OD_{600} value were following sigmoid models (Fig 2).The increasing number of bacteria during the log phase was detected before entering stationary which were confirmed at the 72 hours. It showed that the best pH for bacterial growth was observed at 5.5. This means that cell production will increase for 72 hours. This character might serve as a basic reference for the long shelf life of biological fertilizer products. The increasing number of bacteria after 72 hours effected bacterial growing media (Nutrient Broth) which were decrease in pH to the level of 4.5. The decreasing pH medium occurs because of the formation of organic acids and other metabolites that affected bacterial cell metabolic activities (Arif et al., 2010). This will determine the storage period and bacterial viability in biological fertilizer formulations. In this study, bacteria were isolated within range of pH in which relevant to other studies, were recorded that the range of pH for cultivating those above bacterial consortium were at pH 4 to 11 (Table 2).

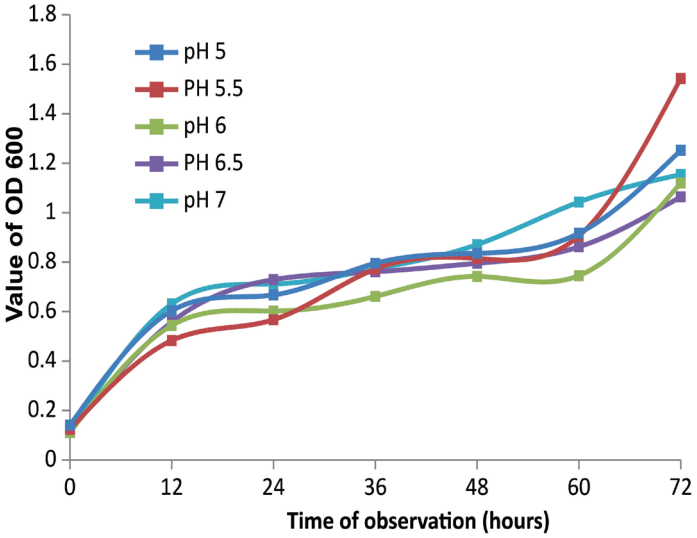


Fig. 2. Bacterial consortium growth in liquid medium under different pH value indicated by OD_{600} value

Table 2. Optimum pH for each bacterial isolate

	Bacteria	Range of pH	References
N fixing	*Bacillus cereus* (SNF 5)	4.9 – 11.0	(Finlay, Logan, & Sutherland, 2000)
Soluble P	*Pantoea ananatis* (SPP 1)	4.0 – 11.0	(Dastager, Deepa, Puneet, Nautiyal, & Pandey, 2009)
Soil stabilizer agregats	*Pseudomonas plecoglosiccida* (SPE 20)	4.0 – 11.0	(Sankaralingam, Eswaran, Boomi, Sundaram, & Shankar, 2014)

Using multivariate analysis (Canonical Variate Analysis : CVA) the effect of pH according to different the time of observation were analysed. CVA presented in Fig. 3 shows that between pH 5, pH 5.5, pH 6, pH 6.5, and pH 7 treatments were significantly different (P<0.05). This is indicated by a circle of confidence intervals (95%) that are not intersecting one to another. In all treatments treatment had a positive correlation to the axis of Canonical Variate 1 with a percentage variation of 82.31% which is higher than those axis of Canonical Variate 2 of 13.78%. So it can be concluded that in each observation parameter in one treatment has a relationship with each other which are mutually binding, where different time of pH succesfully significant clustered one treatment to the others.

Viability Test

The liquid formulation of biofertilizer treatment VP3 (vermiwash carrier with 1% PEG added ingredients) had the highest viability after 8 weeks storage (Table 3). Higher bacterial viability on VP3 treatment had even been detected from the first week storage compared to other treatments. The stationary phase of this treatments was longer than the viability of the other treatments. At week 0, bacteria in liquid formulation medium started to adapt to the environment and growing media, but then at week 1 to week 2 the bacteria in the treatment entering a log phase or exponential phase. Liquid fertilizer formulation in three treatments, (P0, VP2 and VP3) reached the peak phase of the third week except the treatment of VP1 which reached the peak phase at week 4 which then slowed or entering a stationary phase (Fig. 4).

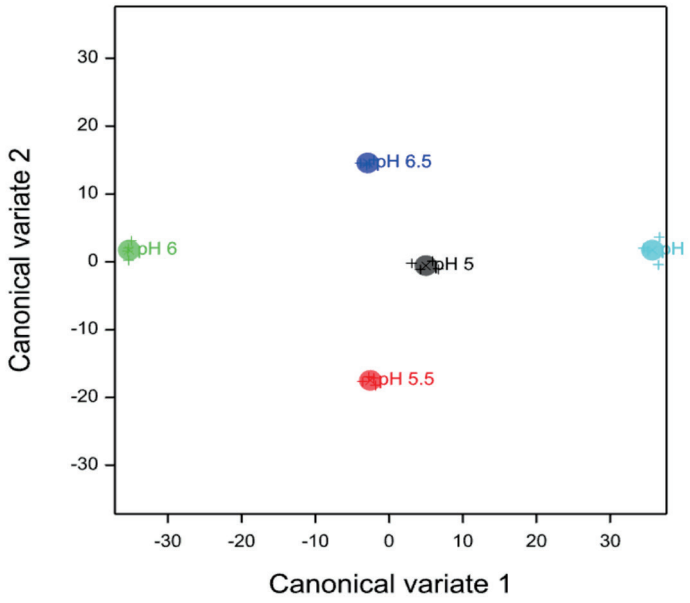


Fig. 3. CVA of the effect of pH at different the time of observation to OD 600 value

Table 3. Bacteria viability in various period of incubations

Treatment	Average bacterial viability (log CFU/ml) (weeks)								
	0	1	2	3	4	5	6	7	8
P0	6.25 b	10.61 a	11.68 a	13.52 a	12.87 a	12.67 a	12.33 a	12.32 a	12.03 a
VP1	6.32 b	11.70 b	11.79 b	13.46 a	13.77 b	13.11 b	12.52 a	12.35 ab	12.17 b
VP2	6.57 c	11.65 b	12.11 c	13.64 a	13.57 b	13.22 bc	12.49 b	12.48 b	12.46 c
VP3	5.85 a	11.78 b	13.01 d	14.03 a	13.52 b	13.36 c	13.21 c	13.05 c	12.89 d
LSD 5%	Sig	Sig	Sig	NSig	Sig	Sig	Sig	Sig	Sig

Remarks: Values followed by different letters in the same column differ significantly based on LSD 5% (Sig: Significant, NSig: Not significant)

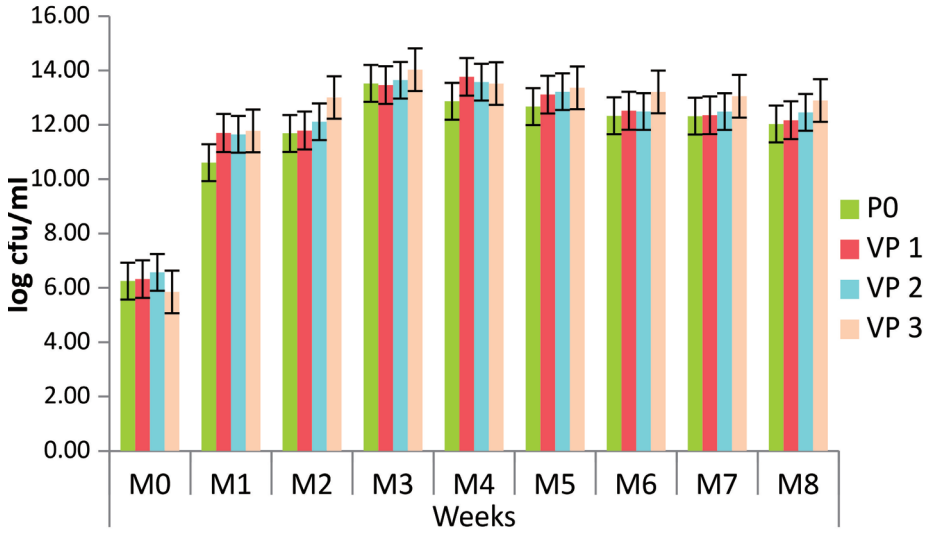


Fig. 4. Bacterial viability test for 8 weeks period of incubation

The observation on week 0 showed that bacterial growth in all formulations were almost similar (not significantly different). It is important to determine the initial population in each treatment which must be equal. Starting from the 1st week to the last week of observation except in the 4th week, bacterial viability in the treatment of VP3 (carrier material with an additional ingredient of 1% PEG material) has the highest viability. The liquid treatment formulations consisted of the three treatments, which is: P0, VP2 and VP3 reached the peak phase at the third week after incubation resulted bacterial viability at 13.52 log CFU/ml, 13.64 log CFU/ml and 14.03 log CFU/ml, respectively. VP1 treatment reached the peak phase at the 4th week after incubation, producing bacterial viability at 13.77 log CFU/ml. This pattern showed that rich complex nutrients in growing substances of the carrier material are digested or decomposed slowly by bacteria. The peak phase of VP1 treatment is different due to the less complex bacterial growth media. Uthayasooriyan, Pathmanathan, Ravimannan, & Sathyaruban (2016) also concluded that different type of culture media affect bacterial population.

All treatments have almost the same stationary phase, but from the 4 treatments (P0, VP1, VP2 and VP3), treatment with additional ingredients in the formulation has a greater viability than other treatments. The added ingredient, i.e. glycerol (1%) in VP2 and PEG (1%) in VP3 are able to maintain the stationary phase until the 8th week. Glycerol (1%) is the simplest glyceride compound with hydroxyl which is hydrophilic and hygroscopic. These compound various types of lipids including triglycerides. Glycerol (1%) serves to increase the elasticity of bacterial cell wall hemicellulose (Pop et al., 2015). PEG (1%) is also known as carrier material that is often used as an ingredient of a formulation to increase solubility inert, not easily hydrolyzed and inhibited fungal growth (Zhao et al., 2019).

The VP3 treatment has a higher viability value than the VP2 treatment. The carrier material in the formulation shows that vermiwash plays a role in maximizing the ability of bacteria to live during the storage period. Giyanto, Suhendar, & Rustam (2009) stated that vermiwash carrier material in the form of organic liquid waste has the potential as a multiplication medium for biological agents because it contains a good composition of nutrients for bacterial growth such as carbohydrates, proteins, water, amino acids, fats, mineral salts and other nutrients. In addition to the beneficial carrier material, proper storage of the liquid fertilizer formulation during the storage period is very influential. The immersion in the liquid formulation of biological fertilizers can stabilize bacterial growth. Immersion in the formulation functions in homogenizing bacteria with nutrients in the formulation, so that there will be no deposition of bacteria, nutrients and bacterial metabolites in the bottom of the container.

CVA presented in Fig. 5 shows that between P0 and VP3 treatments were significantly different (P<0.05), However VP1 and VP2 were positioned close to each other. This is indicated by a circle of confidence intervals (95%) that are intersecting or not intersecting. In the P0 and VP3 treatment had a positive correlation to the axis of Canonical Variate 1 with a percentage variation of 98.48% higher than the axis of Canonical Variate 2 of 1.20% where the treatment of VP1 and VP2 had no positive correlation to this axis. The observation of the effect treatments toward bacterial viability based on different time observation has a strong relationship with which means had mutually binding and it was succesfully gropued using one to another using CVA multivariate analysis (Fig. 5).

Pathogenicity Test

Green bean seeds that have been inoculated with biological fertilizers with various formulations showed no diseases symptom in the parameters of necrosis, decay, deformity and growth. Pathogenicity test of liquid fertilizer liquid formulation in vegetative phase (germination) of green beans after 3 DAP was presented in Table 4. It can be seen that there was no significant effect (P<0.05) of the treatment to the lenght of the bean sprout root, but those effected to the height of the bean sprout (Table 4). The physical performances of the green bean sprout were presented at Fig. 6. Germination growth in the VP3 treatment showed the highest value in the parameters of the length of the sprouts. The lenght of the sprout of this treatment were almost 2 x compared to the control treatments. The VP3 treatment has the highest sprout length (cm) compared to other treatments whereas the value was significantly different to other treatment. This evidence showed that the liquid formulation of biofertilizer with a carrier material of vermiwash with the addition ingredients (PEG 1%) is easily absorbed by plants. During vegetative phase nutrient in biological fertilizer liquid formulation is suspected being required by crop to carry out metabolic processes especially for cell division to form plant organs that support photosynthesis (Rizqiani, Ambarwati, & Yuwono, 2007). The acceleration of metabolism allows plants to carry out photosynthesis optimally in obtaining food, aside from food reserved in seeds. The available water conditions are more easily absorbed by plants resulting in increasing in crop growth which affected to the total the fresh weight (Sudjana, 2011). The high bacterial population in VP3 treatment is due to the possibility of bacterial metabolites needed by plants is also much higher compared to other treatments. The provisioning of VP3 biofertilizer further, stimulates the growth of green bean sprout in the early vegetative phase.

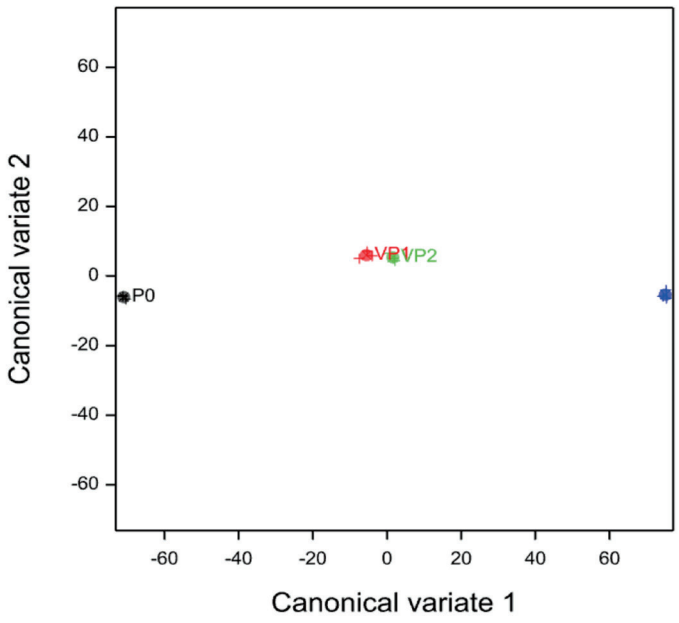


Fig. 5. CVA of the effect of treatments at different the time of observation to bacterial viability

Table 4. Pathogenicity test of liquid fertilizer liquid formulation during vegetative phase (germination) of green beans aged 3 DAP (days after planting)

Treatment	Parameter of green bean sprout	
	Root lenght of sprout (cm)	Height of sprout (cm)
Control	2.64 a	4.74 a
P0	2.46 a	4.71 a
VP 1	2.71 a	5.00 ab
VP 2	2.90 a	5.56 b
VP 3	3.58 a	7.74 c
LSD	NSig	Sig

Remarks: Values followed by different letters in the same column differ significantly based on LSD 5% (Sig: Significant, NSig: Not significant)

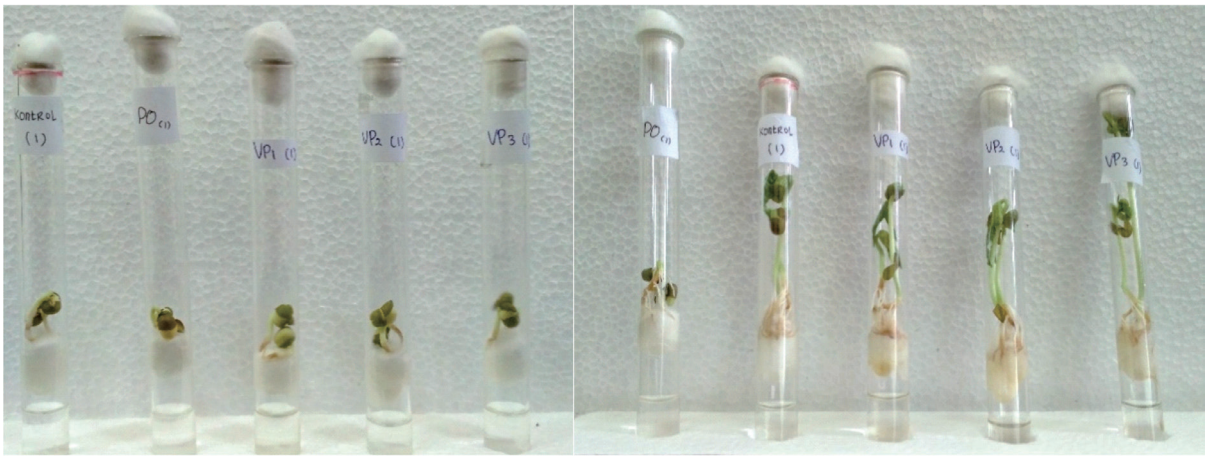


Fig. 6. (Left) The performance of acceleration of green bean sprout growth at the age of 3 HSS (days after seeding) and (Right) 5 DAP (days after planting)

However, in term of root length there were not significantly different among the treatment because the growing media (Yoshida solution) are able to provide sufficient nutrients, therefore the roots do not extend to obtain nutrients which was far from root distribution zone. Yoshida solution is a solution commonly used in plant physiology tests with advantages easily obtained, quickly absorbed by plants, more accurate and complete nutrient composition (Prasetiyono, Tasliah, Aswidinnoor, & Moeljopawiro, 2003). In addition, the low root length may be due to a greater supply of nitrogen which causes auxin to increase and inhibit root growth. Inhibition of root growth is caused by ethylene which is formed from the presence of auxin production and most ethylene in plants can slow root extension. Changes in ethylene level, and then their intreaction with the hormonal production can regulate the lifespan of crop (Iqbal et al., 2017; Qin & Huang, 2018).

CONCLUSION

Optimum pH for the growth of the three consortiums of bacterial isolates in the liquid fertilizer liquid formulation was 5.5. The highest bacteria viability was detected at VP3 treatment with *vermiwash* carrier material added with 1% PEG after 8 weeks incubations treated with liquid fertilizer formulation with three bacterial isolates, there was no symptoms of the disease in the vegetative phase (*germination phase)*of green bean sprout. The highest sprouting (root and height) was detected at VP3 treatment.

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