Comprehensive test of rhizobacteria as biostimulant, vesicular arbuscular mycorhiza (VAM) and graded dose of NPK fertilizer on the growth of bok choy (*Brassica rapa* L.)

S Widawati1* and Suliasih1

1Micobiology Division, Research Center for Biology, Indonesian Institute of Sciences, Cibinong Science Center, Jl. Raya Jakarta-Bogor km 46, Cibinong 16911, Indonesia

*Email: widadomon@yahoo.com

(All authors contributed equally to this work)

Abstract. Collaboration test of rhizobacteria as biostimulant, vesicular arbuscular mycorhiza (VAM) and graded dose of NPK fertilizer was a preliminary experiment to re-fertilize marginal soils. The objective of this experiment was to obtain the suitable collaboration of rhizobacteria as biostimulant, VAM and NPK dose that effectively supports the productivity of the growth media (zeolite) and the growth of bok choy plants. The experiments design was RCD factorial with 2 treatments which were NPK dose (0 %, 25 %, 50 %, 75 %, and 100 %) and microbial biostimulant. Rhizobacteria acting as biostimulant used were nitrogen fixing bacteria (NFB) (*Azospirillum* sp., *Azotobacter* sp., *Rhizobium radiobacter* InaCCB835), phosphate solubilization bacteria (PSB) (*Serratia* sp., *Klebsiella variicola* InaCC B827, *Mangrovibacter plantisponsor* InaCC B841, *Tolumonas osonensis* InaCCB831) and VAM. Each treatment, including control, was repeated 3 times. Bok choy seeds from each treatment were planted individually on zeolite media and grown in a greenhouse for 40 days. The results showed that the treatment of MIXN1 (NFB + PSB + VAM + NPK 25%) and MIXN2 (NFB + PSB + VAM + NPK 50%) were effective collaborations of biostimulating rhizobacteria, VAM and NPK dose on supporting the fertility of growth media with the number of rhizobacteria of $10^7$ cfu g$^{-1}$ zeolite and supporting optimization of bok choy plant growth and the percentage of bok choy root colonization by VAM with a very high level of infection.

1. Introduction

Root microbes have various characteristics. They can be beneficial or detrimental to the growth other microbes and pathogenic for plants. Root microbes that are beneficial for soil stability, water and nutrient absorption, biological control, antibiosis and symbiosis and able to stimulate growth, provide essential nutrients for plant growth, produce indol acetic acid hormone (IAA), which is one of the plant growth promoting rhizobacteria (PGPR) indications [1] are called rhizobacteria as biostimulant. Microbes that showed biostimulant effect are nitrogen fixing bacteria (NFB), phosphate solubilizing bacteria (PSB) and vesicular arbuscular mycorhiza (VAM) fungi.

Rhizobacteria is a group of bacteria that live in the rhizosphere. Rhizobacteria are able to form productive, cooperative and harmonious collaboration with other microbes, affect the soil fertility, and plant growth through processes such as association or symbiosis. Rhizobacteria activities provide
essential elements necessary for plant growth, such as nitrogen (N) by nitrogen fixation from air and soil (NFB), phosphate (P) by releasing it from the bonds of Al, Fe, and Ca (PSB), and IAA growth hormone. Whereas VAM activity participates in nutrient cycles [2] and absorption and translocation of important nutrients [3] such as P [4] N, K, Mg, Cu and Zn ions [5], provides P (80%) for plant growth [6], helps plants survive adverse condition [7] such as drought [8].

Some researchers, regarding the properties of rhizobacteria (NFB and PSB) and VAM, reported that the collaboration between the two organisms is not only affecting plant physiology [9], but also increasing plant growth through nutrient absorption [10], nutrient uptake [11], root branches stimulated growth [12, 13], and plants protection from pathogenic fungi [14]. The presence of rhizobacteria in the soil is very advantageous for VAM organisms due to their high selectivity to VAM [15]. However, the compatibility in collaborating as biostimulants depends on exudates released by fungi in plant roots or exudates released from plant roots that grow on it [16], both culture on fertile or marginal soil.

The application of rhizobacteria and VAM as biostimulants to help marginal land recovery must be done gradually rather than hastily. Inorganic biostimulant such as NPK fertilizer is still required at the early phase of the marginal soil health recovery. Its success can be combined by utilizing the potential of the VAM fungi [16]. The augmentation of collaboration between effective microbes acting as biostimulant (rhizobacteria and VAM) and application of NPK fertilizer on marginal soils (low on nutrient content, organic matter, and microorganism activity), are expected to increase the efficiency of chemical fertilizers, thus chemical fertilizers usage which impacts on environmental pollution will be slowly reduced. Microbes as biostimulants provide macro and micro elements to promote plant growth as well.

Organic biostimulants have been greatly developed due to the vast interest of agricultural community in advancing sustainable organic farming [17]. This phenomenon transpires because they have realized the harmful outcome of excessive use of chemical fertilizers, including NPK. Overuse of NPK decrease the population of indigenous microbes which act as biofertilizers and result in acidic and dry soil. Righi et al. [18] reported that high dose of NPK damaged the soil and polluted the environment. It prompted the idea to find the right dose of NPK to be collaborated with microbes that act as biostimulant. Thus, the collaboration will support plant growth effectively without polluting the environment. The objective of this experiment was to obtain the suitable collaboration of rhizobacteria (NFB and PSB) as biostimulant, VAM and NPK dose that effectively supports the productivity of the growth media (zeolite) and the growth of bok choy plants.

Bok choy (Brassica rapa L.) plants were selected as the test plant for this experiment. It belongs to the group of cabbages and has high economic value along with short harvesting age (40 days). Other favorable properties of bok choy plant are that it contains fiber, vitamin A, B, B2, B6, and C, calcium, phosphorus, copper, magnesium, iron, minerals, protein and folic acid which are classified as very high, is useful in preventing cancer, hypertension, heart disease, maintains healthy digestive system and prevents anemia in pregnant women [19].

2. Materials and Methods
2.1. Biostimulant and experimental design
They were two types of microbes (rhizobacteria and VAM) as biostimulants used in this experiment. They have been recognized to produce IAA, phosphatase, ACC-diaminase, nitrogenase, and as cellulose decomposers. The experiments design was complete randomized design (RCD) factorial with 2 treatments which were NPK dose (0%, 25%, 50%, 75%, and 100%) and microbial biostimulant (rhizobacteria and VAM). Rhizobacteria as biostimulant used were NFB (Azospirillum sp., Azotobacter sp., Rhizobium radiobacter InaCCB835) coded R, and PSB (Serratia sp., Klebsiella varicola InaCC B827, Mangrovibacter plantispawner InaCC B841, Tolumonas osonensis InaCCB831) coded S. Each treatment including control (K = no microbial biostimulant, only added with NPK fertilizer) was repeated 3 times to obtain 40 biostimulant collaboration treatments (RN0, RN1, RN2, RN3, RN4, SN0, SN1, SN2, SN3, SN4, RSN0, RSN1, RSN2, RSN3, RSN4, MN0, MN1,
2.2. The test on biostimulant effectivity on the growth of bok choy

Bok choy was used as the host plant. Before planting, bok choy seeds were sterilized by soaking them in alcohol 70% for 1 minute and rinsing with sterile distilled water for 3 times. Bok-choy seeds were then germinated on Petri dish covered with filter paper soaked with sterile aquades. The growth media used was 260 grams of zeolite per pot (13 cm high and 10 cm in diameter), covered and sterilized in an autoclave for 2 hours at 121 atm. The following procedures were done in a sterile room. NPK fertilizer (dose based on the combination of each treatment) was inoculated into cool sterile zeolite media. Plating holes were made on the sterile zeolite media after 4 days incubation. Rhizobacteria and VAM were then inoculated into the media (following the combination of each treatment). The VAM dose was 1 gram pot\(^{-1}\) (10 spores g\(^{-1}\)). The dose of single rhizobacteria (PSB and NFB) and the mixture was 1 mL pot\(^{-1}\) (10\(^8\) cfu mL\(^{-1}\)), adjusted for treatment combination and replication. Next, bok choy sprouts (1 seedling pot\(^{-1}\)) were planted into the planting hole and incubated until the first leaf appears, then the experimental pots were transferred to the greenhouse. Planting media moisture content was maintained at 24% with a spray of sterile distilled water containing nutrient solution "Hoagland" [20]. Harvesting and data collection were performed when the bok choy plant was 40 days old. The parameters observed included the growth of bok choy plants (plant height, root length, number of leaves), and wet weight of the plant that is the weight of the living plant and measured directly after harvesting before the plant wilted due to water loss [21], content of phosphate and nitrogen measured using a spectrophotometer according the method from soil research center book compiled by Sulaeman et al [22], the number of rhizobacteria population and infection analysis or the degree and intensity of root colonization by VAM (carried out after the harvest).

2.3. Analysis on the intensity of root colonization by VAM

Infection analysis or the degree and intensity of root colonization by VAM was done according the root staining method [23]. The roots were washed with aquadest until clean, then were immersed in 10% KOH (m/v) solution and heated (± 60 °C) for 30 minutes. The solution was removed and the roots were washed with distilled water and then immersed in 2% HCl (v/v) solution for 2-3 minutes. The solution was removed, then the roots were soaked in trypan blue 0.05% (m/v), heated (± 90 °C) in an autoclave at a pressure of 15 psi for 10 minutes. The solution is removed, the roots were immersed in a destaining solution, namely glycerol 50% (v/v). The analysis was done by cutting up roots in a size of ± 1 cm, randomly taking 10 pieces of roots, arranged in a row on the object glass, then viewed and calculated under a compound microscope with the slide method [6]. The degree / percentage of root colonization was calculated using the formula: Root infected (%) = \(\sum\) root infected : \(\Sigma\) all observed roots \times 100 %. Root infection rates are 5 classes: Class 1 if root infection is 0% - 5% (very low), class 2 if root infection is 6% - 25% (low), class 3 if root infection is 26% - 50% (moderate), class 4 if root infection is 51% - 75% (high), and class 5 if root infection is 76% - 100% (very high).

2.4. The analysis of rhizobacteria population count

The number of rhizobacteria population in the media was calculated using the plate count method [24] on 10\(^1\) to 10\(^7\) dilution system. As much as 5 grams of zeolite were taken compositely from the root area with 3 replications for each treatment. One gram of zeolite was put into a test tube containing 9 mL of sterile aquades (10\(^{-4}\) dilution) and homogenized on a vortex for 1 minute at 1000 rpm. As much as 1 mL of 10\(^{-5}\) dilution sample extract was transferred to 10\(^{-2}\) dilution tube. It carried on until the 10\(^{-7}\) dilution tube. As much as 0.1 mL of sample extract was taken from 10\(^{-3}\), 10\(^{-5}\) and 10\(^{-4}\) dilution tubes and put into a sterile Petri dish and selective media was poured over it. Pikovskaya and Nutrient agar
media were used to grow PSB and NFB population contained in zeolite media. Rhizobacteria population calculation is carried out after 3-7 days incubation at 28 °C. The colonies counted of PSB on Pikovskaya media was only on bacterial colonies with clear zone. While the NFB population in the nutrient agar media was calculated by composite counting of bacterial colonies which have pink, round, sparkling, flat colony edges (Azospirillum), round bacterial colonies, rounded colonies, convex colonies and clear white color (Azotobacter), prominent bacterial colonies, round, circular, convex, semitranslucent, and non-transparent milky white (Rhizobium).

2.5 Data Analysis
The parameters observed were plant height, root length, number of leaves, wet biomass, P and N content, number of rhizobacteria population, and percentage of root colonization by VAM. The data obtained were analyzed with ANOVA statistical variants followed by Duncan Multiple Range (DMRT) tests at the 5% level using SPSS version 23 [25].

3. Result and Discussion
3.1 The effectiveness of biostimulant on the growth of bok choy, N and P content
In general, the result showed that all treatments from the collaboration of rhizobacteria as biostimulant (NFB: Azospirillum sp., Azotobacter sp., Rhizobium radiobacter InaCCB835 and PSB: Serratia sp., Klebsiella variicola InaCC B827, Mangrovibacter plantspoiner InaCC B841, Tolomonas osonensis InaCCB831), VAM and NPK graded doses (0%, 25%, 50%, 75%, 100%) promote the stability of bok choy growth in zeolite media and help plant roots absorbing available phosphate and nitrogen. Statistically, the collaboration treatments of rhizobacteria as biostimulant, VAM and NPK showed various significant and non-significant effects on all parameters observed (table 1). The control treatment (KN0) showed significant effect on the height of the bok choy plant compared to other treatments, except for RN0, SN0, RSN0, MN0, MSN0, and KN4. While other parameters such as root length, number of leaves, and wet biomass were mostly not significantly different to the NPK graded doses (NPK 0% - 100%) treatment (control KN0, KN1, KN2, KN3, and KN4), except for MIXN1 (rhizobacteria + VAM + NPK 25 %) and MIXN2 (rhizobacteria + VAM + NPK 50 %) biostimulant treatments. The biostimulant, a collaboration of rhizobacteria, VAM and the right NPK dose, was effective and suitable to achieve the optimum growth of bok choy. This is in accordance with Kapulnik et al. [26] that stated that rhizobacteria biostimulant significantly increase plant growth.

The highest plant height, root length, and number of leaves consecutively were 23.77 cm, 14.58 cm, 15.90 leaves (MIXN1), 23.80 cm, 14.58 cm, and 16 leaves (MIXN2) (table 1). These growth parameter affected the value of wet biomass numbers, which were 17.46 grams per plant / pot (MIXN1) and 17.47 grams per plant / pot (MIXN2). The result of this experiment is in accordance with Ramadhani et al. [27] on sorghum plants, which stated that the fresh weight of sorghum treated with biological fertilizers and NPK 50% collaboration and the height of shoots and the number of leaves of sorghum treated with biological fertilizers and NPK 25% inclined to be better than when treated with biological fertilizers and NPK dose of 0%, 75% and 100%. Collaboration of rhizobacteria as biostimulant and NPK 50% resulted in higher plant height (17.47 cm) and lower wet biomass (17.47 grams / plant / pot) compared to the result of Utami and Setiawati [28] which was 17, 20 cm plant height and 31.88 grams /plant wet bok choy biomass in liquid media (hydroponics) treated with = 50% inorganic fertilizer and 50% biological fertilizer. Latif et al. [29] reported that the collaboration of 5 ml of biological fertilizer and NPK 50% resulted in the highest value of plant growth. The lowest result of bok choy growth in all observed parameters (11.13 cm, 6.87 cm, 5.33 leaves, 2.73 g) was obtained in the KN0 control treatment (NPK 0%) and the lowest number of leaves in plants treated with NFB, PSB, VAM as biostimulant at 0% NPK (RN0, SN0, and MN0) (table 1). While KN1 - KN4 control treatments showed better growth than KN0 control plants, optimum growth was not achieved even when there were available N and P from NPK treatments at 25% - 100%. It indicated
that the growth of plants in marginal land is not only affected by inorganic nutrition but also by intake of organic nutrients produced by bacteria and fungi as biostimulants. The treatment MIXN1 and MIXN2 were a collaboration of rhizobacteria as biostimulant VAM and NPK. This suggested that NFB (Azospirillum sp., Azotobacter sp., Rhizobium radiobacter InaCCB835), PSB (Serratia sp., Klebsiella varicola InaCC B827, Mangroviibacter plantisponsor InaCC B841, Tolumonas osonensis InaCCB831) and VAM as IAA, N and P agents have high effectiveness and competitiveness among inoculants to simultaneously promote plant growth optimization. Effectivities of Azospirillum and Azotobacter are especially superior in associating with horticultural crops such as bok choy, caysin and others compared to Rhizobium that only be symbiotic with Leguminoaceae plants. Likewise, Klebsiella varicola InaCC B827, not only stimulate the growth of higher plants but also the growth of horticultural plants. Similarly, VAM associate and infect the root system of host plants (horticulture, crops, agricultural plants, plantations) by producing hyphae intensively. Thus, the roots are able to increase the uptake of P to support plant growth. Previous experiments reported that bacteria which solubilizing phosphate while nitrogen fixation and producing IAA, can increase the wet biomass of caysin by 606.42 g / 4 plants / pot on marginal soils [30]. As stated by Afifi et al. [31] that the combination of Azospirillum brazilense, Bacillus megaterium and Bacillus circulans increased plant growth. The results and discussion of this experiment were supported by statement of Husen [32] that plant growth and productivity are highly dependent on the effectiveness of biofertilizers such as superior functional bacterial properties, population density, compatibility with host plants, and competitiveness between inoculants. Furthermore, Bashan and Bashan [33] concluded that microbes that possess such characteristics and abilities are classified into rhizobacteria as biostimulant or Plant growth promoting rhizobacteria (PGPR).

Based on overall and statistics results, achieving optimum plant growth required available phosphate and nitrogen whose amount depends on the compatibility of the biostimulant and the host plant. Suitable biostimulant collaboration improved the culture media productivity and stimulated bok choy plant growth. Haryanto et al. [34] suggested that crops certainly need enough nitrogen to grow properly. Evidently, organic N was obtained through the process of plant association with NFB, while phosphate can be obtained through PSB activity through enzymatic activity involving the phosphatase, phytase, and nuclease that will produce available P. Furthermore, supply and intensive absorption of organic P was also be assisted by VAM fungi which directly colonize plant roots. Charisma et al. [35] concluded that VAM supported plants in the supply and absorption of P and increase plant growth which includes increasing size, volume, biomass and cell count [36]. Nutrients N, P, K, Mg and Ca stimulate synthesis and anticlinal division of cell wall resulted in the immediate increase of plants height [21].

Holevas [37] stated that available phosphate in the soil can essentially be absorbed by plants. However, in this experiment, pots which were inoculated with NPK 75% and 100% showed that the available N and P could not be absorbed by the roots. Hence, optimization of the bok choy growth, the activity and effectiveness of NFB, PSB, and VAM were inhibited and the development of NFB, PSB, VAM population were hindered. This is supported by the report of Novizan [38] that high concentrations of inorganic fertilizers caused failure in nutrients absorption resulting in plasmolysis of the root tissues. It was further strengthened by Jumiati [39] that the concentration of nutrient solutions affected plant metabolism such as the photosynthesis rate, enzyme activity, and ion absorption by plant roots. Subiksa [40] also suggested that the addition of excessive NPK caused VAM death, thereby reducing the activity, population and mycorrhizal infection. Conversely, if the P is scarcely
found in the media, the presence of VAM will increase [41]. This phenomenon was observed in the results of the P and N content analysis of all bok choy plants. The highest plant P amount were obtained in plants treated with biostimulants of MIXN2 (NFB + PSB + VAM + NPK 50 %), MIXN1 (NFB + PSB + VAM + NPK 50 %), and MSN2 (VAM + PSB + NPK 50 %), consecutively 3.19%, 3.17%, and 3.25% (table 1).

Table 1. The effectiveness test of rhizobacteria, VAM, NPK biostimulants on the growth of bok choy, P and N content of plants and analysis on the intensity of root colonization by VAM

| Code of treatment | Plant height (cm) | Root length (cm) | Number of leaves (strands) | Plant wet biomass (gram) | P content of plants (%) | N content of plants (%) | Root infected | Colonization percentage (%) | Root infection classis |
|-------------------|------------------|------------------|---------------------------|--------------------------|-------------------------|-------------------------|---------------|-----------------------------|----------------------|
| RN0               | 15.93           | 8.43             | 5.30                     | 5.63                     | 1.30                     | 1.56                     | -             | 0%                          | Very low             |
| RN1               | 20.43           | 10.47            | 9.00                     | 8.96                     | 1.60                     | 2.78                     | -             | 0%                          | Very low             |
| RN2               | 21.20           | 12.40            | 10.67                    | 10.18                    | 1.62                     | 2.99                     | -             | 0%                          | Very low             |
| RN3               | 19.23           | 10.80            | 8.67                     | 8.31                     | 1.57                     | 2.69                     | -             | 0%                          | Very low             |
| RN4               | 17.73           | 9.70             | 7.33                     | 7.82                     | 1.42                     | 1.86                     | -             | 0%                          | Very low             |
| SN0               | 15.13           | 7.70             | 5.34                     | 5.90                     | 1.35                     | 1.26                     | -             | 0%                          | Very low             |
| SN1               | 19.27           | 10.40            | 9.67                     | 8.08                     | 1.59                     | 1.77                     | -             | 0%                          | Very low             |
| SN2               | 19.90           | 12.03            | 10.00                    | 10.48                    | 2.84                     | 2.02                     | -             | 0%                          | Very low             |
| SN3               | 18.77           | 9.93             | 9.67                     | 8.55                     | 1.60                     | 1.51                     | -             | 0%                          | Very low             |
| SN4               | 19.00           | 9.87             | 8.00                     | 7.84                     | 1.54                     | 1.30                     | -             | 0%                          | Very low             |
| RSN0              | 16.17           | 7.90             | 8.33                     | 7.73                     | 1.44                     | 1.57                     | -             | 0%                          | Very low             |
| RSN1              | 20.30           | 12.37            | 10.33                    | 8.72                     | 1.68                     | 2.78                     | -             | 0%                          | Very low             |
| RSN2              | 21.23           | 12.83            | 11.33                    | 12.24                    | 1.79                     | 1.31                     | -             | 0%                          | Very low             |
| RSN3              | 21.77           | 10.00            | 10.33                    | 8.20                     | 1.65                     | 2.71                     | -             | 0%                          | Very low             |
| RSN4              | 18.47           | 9.27             | 8.67                     | 8.72                     | 1.52                     | 1.88                     | -             | 0%                          | Very low             |
| MN0               | 17.33           | 9.62             | 5.31                     | 6.82                     | 1.38                     | 1.27                     | +             | 87h                         | Very high            |
| MN1               | 20.47           | 10.67            | 10.00                    | 9.54                     | 1.60                     | 1.79                     | +             | 84g                         | Very high            |
| MN2               | 20.53           | 12.43            | 11.33                    | 12.75                    | 2.84                     | 2.42                     | +             | 83g                         | Very high            |
| MN3               | 18.67           | 10.27            | 8.33                     | 8.97                     | 1.63                     | 1.42                     | -             | 0%                          | Very high            |
| MN4               | 18.43           | 9.28             | 7.67                     | 7.88                     | 1.56                     | 1.38                     | -             | 0%                          | Very high            |
| MRN0              | 17.87           | 9.33             | 9.33                     | 5.84                     | 1.26                     | 1.52                     | +             | 80f                         | Very high            |
| MRN1              | 19.90           | 11.63            | 10.67                    | 11.28                    | 1.52                     | 2.71                     | +             | 79f                         | Very high            |
| MRN2              | 21.97           | 12.83            | 11.00                    | 15.22                    | 1.61                     | 2.99                     | +             | 76f                         | Very high            |
| MRN3              | 19.27           | 10.50            | 10.33                    | 10.24                    | 1.57                     | 2.80                     | -             | 0%                          | Very low             |
| MRN4              | 18.13           | 10.27            | 8.67                     | 8.79                     | 1.52                     | 1.89                     | -             | 0%                          | Very low             |
| MSN0              | 17.33           | 9.17             | 9.00                     | 7.53                     | 1.38                     | 1.25                     | +             | 62h                         | High                 |
| MSN1              | 19.97           | 10.33            | 9.67                     | 12.84                    | 1.79                     | 1.95                     | +             | 66h                         | High                 |
| MSN2              | 20.17           | 11.97            | 11.00                    | 15.08                    | 3.18                     | 2.59                     | +             | 64c                         | High                 |
| MSN3              | 19.70           | 10.35            | 10.33                    | 9.77                     | 1.58                     | 1.44                     | -             | 0%                          | Very low             |
| MSN4              | 18.87           | 9.79             | 8.90                     | 8.94                     | 1.50                     | 1.34                     | -             | 0%                          | Very low             |
| MixN0             | 23.35           | 12.47            | 10.00                    | 11.82                    | 1.42                     | 1.63                     | +             | 90f                         | Very high            |
| MixN1             | 23.77           | 14.58            | 15.90                    | 17.46                    | 3.17                     | 3.18                    | +             | 91i                         | Very high            |
| MixN2             | 23.80           | 14.57            | 16.00                    | 17.47                    | 3.19                     | 3.25                    | +             | 87h                         | Very low             |
| MixN3             | 22.20           | 12.90            | 10.67                    | 13.91                    | 1.59                     | 2.80                     | -             | 0%                          | Very low             |
| MixN4             | 19.66           | 11.57            | 9.33                     | 9.78                     | 1.52                     | 1.89                     | -             | 0%                          | Very low             |
| KN0               | 11.13           | 6.87            | 5.33                     | 2.73                     | 1.16                     | 1.24                     | -             | 0%                          | Very low             |
| KN1               | 19.23           | 10.20            | 10.50                    | 7.43                     | 1.64                     | 2.02                     | -             | 0%                          | Very low             |
| KN2               | 19.67           | 10.60            | 9.33                     | 7.15                     | 1.47                     | 1.59                     | -             | 0%                          | Very low             |
| KN3               | 18.33           | 9.67            | 8.67                     | 7.66                     | 1.59                     | 1.40                     | -             | 0%                          | Very low             |
| KN4               | 17.17           | 8.43            | 6.33                     | 4.81                     | 1.42                     | 1.31                     | -             | 0%                          | Very low             |

Note: The number followed by the same letter are not significantly different at (p<0.05) level of Duncan’s test.

The plant P amount from this experiment was lower than the results of Ramadhani et al. [27] ie 5.33% in the VAM + PSB + NFB treatment with 25% NPK and 4.39% with 50% NPK. Similarly, compared to result of Dewi and Setiawati [42] which received plant N content of 4, 39% in the treatment of inorganic + fertilizer biological at a dose of 50%. While the lowest P and N contents
were observed in the KN0 (control without biostimulant) treatment, namely 1.16% and 1.24%. Although the content of P and N were lower compared to other studies, the optimization of growth, especially wet biomass was achieved well. This phenomenon showed that the content of P and N in plants were sufficient to meet the standard needs of plants for growth. As stated by Jones et al. [43] the minimum plant N content for bok choy growth are 2, 5% - 4, 5%. Furthermore, Jones [44] explained that plant N content of 3 - 4, 5% was sufficient to support bok choy growth.

3.2 Analysis on the intensity of root colonization by VAM

Analysis of bok choy root colonization by VAM covered external hyphae, internal hyphae, and vesicles. The results showed that VAM was able to colonize the roots of its host plant and form a new colonization structure in the root (figure 1). Sieverding [45] reported that new structures formed at the roots of host plants such as hyphae, vesicles, arbuscules, and spores due to VAM colonization. This occurred in NPK treatments with doses of 0%, 25%, and 50% that collaborated with the biostimulant treatment of VAM fungus. Whereas the treatment of rhizobacteria as biostimulants and NPK without VAM as biostimulants showed that the intensity of root colonization by VAM was negative (0%) (table 1).

![Figure 1. VAM colonization of Brassica rapa L. roots](image)

Qualitatively and quantitatively, the results of the analysis of root colonization by VAM showed that not all roots inoculated with VAM as biostimulants were positively infected. Positive results were found in the roots treated with VAM biostimulant with a combination of treatment coded MN0 (87%), MN1 (84%), MN2 (83%), MSN0 (62%), MSN1 (66%), MSN2 (64%), MRN0 (80%), MRN1 (79%), MRN2 (76%), M1XN0 (90%), M1XN1 (91%) M1XN2 (87%). Bok choy plants that were treated with VAM as biostimulant and NPK fertilizer of 75% (N3) and 100% (N4) showed negative result (were not infected by VAM). The treatment codes with negative results (0%) were MN3, MN4, MSN3, MSN4, MRN3, MPN4, MixN3, and MixN4. According to Smith and Read [46], sufficient N and P nutrient caused a negative response to mycorrhizal colonization. The highest percentage (91%) of root colonization by VAM was found in the roots of bok choy treated with VAM as biostimulant in collaboration with rhizobacteria and NPK 25% (MIXN1). While the lowest percentage (0%) of root colonization by VAM was found in the roots of bok choy plants treated with VAM as biostimulant in collaboration with PSB, NFB, PSB + NFB, NPK 75% and NPK 100%. Thus, administering low doses of NPK fertilizer (0% and 25%) produced very high level of infection. Song [47] suggested that deficiency of N, P and K (NPK) caused an increase of root infection by VAM. Moreover, Nurhidayati et al. [48] also suggested that optimizing the action of mycorrhizae in infecting roots required low N nutrient conditions N. In contrast, administration of high doses of NPK (75% and 100%) resulted very low infection levels of bok choy root colonization by VAM. This is consistent with the results of
Subiksa [40], that productive media reduced the activity and infection of mycorrhizae, mycorrhizal populations, and even killed some mycorrhizae due to the addition of NPK. The same thing was stated by Ramadhani et al. [27] that sorghum roots without NPK (0%) displayed the highest percentage of VAM colonization. When the NPK concentration was higher, the colonization of sorghum roots by VAM was lower, even root colonization by VAM was not formed. Thus, when plants lack nutrients, the roots actually detect, differentiate, and associate with the right fungi and increase the transfer of nutrients to the root by providing carbohydrates [49]. These nutrients are needed by VAM to colonize roots. The presence of N and P from NPK with doses of 25%, 50%, 75%, 100% formed new structures on different plant roots such as hyphae, vesicles, arbuscules, and spores. As stated by Sieverding [45], new structures such as hyphae, vesicles, arbuscules, and spores are formed at the roots of host plants because of VAM colonization at the plant roots. In addition to nutrients contained in the culture media, soil temperature and pH affect the formation of new structures (hyphae, vesicles, arbuskula, and spores) resulting from VAM colonization of plant roots [6, 50, 51].

3.3. Analysis on rhizobacteria population count

In general, the average NFB population count in all biostimulant treatments was in the range of $10^7$ cfu g$^{-1}$ of zeolite. While the average PSB population count in the biostimulant treatments was in the range of $10^7$ cfu g$^{-1}$ of zeolite and a few were in the range of $10^6$ cfu g$^{-1}$ zeolite, namely RSN0, RSN1, RSN3, and MSN4. This number of population was still effective in fertilizing the culture media because the minimum requirement for a fertile soil is $\leq 10^7$ cfu g$^{-1}$ of soil [52]. Furthermore, the results of this experiment showed that culture media that were not inoculated with PSB as biostimulants, after harvesting no PSB populations were found in these media. Similar finding was observed in the NFB treatment. NFB and PSB populations were not found on control culture media (without bacterial and fungal inoculation). It indicated that the culture media were not contaminated by bacteria that was not inoculated into the culture media. Similary, the result of Widawati and Rahayu [53] showed that the growth media (sterile sand) was not contaminated by bacteria that were not inoculated. Furthermore, Widawati et al. [54] reported that in sterile sand media without inoculants, there was no formation of root nodule in red bean (Phaseolus vulgaris L.) and black-eyed plants (Vigna unguiculata L.).

Figure 2. The number population of NFB and PSB in planting media (Zeolite) after harvest

Significant differences were found in the population of NFB and PSB in zeolite media inoculated with NPK doses of 0%, 25%, 50%, 75% (3.75 g per 300 g zeolit/pot), and 100% (5 g per 300 g zeolit/pot). The results of this experiment showed that the growth of NFB on culture media could
endure NPK dose of 100%. Whereas PSB could only survive at 50% NPK dose. Nevertheless, PSB could survive if it was collaborated with rhizobacteria and VAM as biostimulants. Correspondingly, the results of Ramadhani et al. [27] showed that the population of *Azospirillum* sp. (NFB) at 75% and 100% NPK and *Klebsiella* sp. (PSB) at 50% NPK, did not survive unless in collaboration with VAM and rhizobacteria as biostimulants. In this experiment the 50% NPK dose with biostimulant treatment (RSN3, MIXN3, and MIXN3) resulted in the highest PSB population count of $0.5 \times 10^7$, $1.25 \times 10^7$, $0.85 \times 10^7$, $3.25 \times 10^7$ cfu g$^{-1}$ zeolite and in the treatment of RN3, RSN3, MRN3, MIXN3 resulted in the highest NFB population of $7.40 \times 10^7$, $7.05 \times 10^7$, $8.70 \times 10^7$, $10.30 \times 10^7$ cfu g$^{-1}$ zeolite. Whereas the 25% NPK dose increased the PSB population as much as $7.30 \times 10^7$, $1.0 \times 10^7$, $2.60 \times 10^7$ cfu g$^{-1}$ zeolite obtained in pots with treatment codes SN2, RSN2, MSN2, MixN2 and the highest population of NFB amounted to $10.3 \times 10^7$ cfu g$^{-1}$ zeolite in a pot with the MIXN3 treatment code. The lowest population count of NFB ($2.20 \times 10^7$ cfu g$^{-1}$ zeolite) and PSB ($0.5 \times 10^7$ cfu g$^{-1}$ zeolite) were obtained from zeolite media RN4 and RSN3. The number of rhizobacteria population count was affected by the viscosity of the NPK fertilizer. NPK doses of 75% and 100% treatments resulted in the non-optimal activity and effectiveness of NFB and PSB. The results of this experiment showed that the zeolite media bokchoy required the number population of rhizobacteria $\geq 10^7$ cfu g$^{-1}$ zeolite to become productive and suitable for planting which was achieved at 25% and 50% NPK doses. Similarly, the experiment of Ramadhani et al. [27] showed that the NFB and PSB population reached the optimum number ($10^7$ cfu g$^{-1}$ zeolite) at 25% NPK and 50% NPK. Hence, the higher the soil microbial population is, the higher the biochemical activity in the soil and the higher the soil productivity index (quality) [55].

4. Conclusion
The NPK dose of 25% - 50% NPK was suitable to maintain the growth of rhizobacteria (bacterial population $\geq 10^7$ cfu g$^{-1}$ zeolite) in the culture medium and colonization of bokchoy roots by VAM with very high infection levels (91%). The NPK dose of 50% inhibited the optimization of bok choy growth, rhizobacteria population growth, root colonization by VAM, and the amount of plant P and N. The collaborations of rhizobacteria (NFB and PSB), VAM and NPK doses of 25% (MIXN2) and 50% (MIXN3) were suitable and effective in supporting the productivity of the growth media (zeolite) and the optimization of bok choy growth (plant height, root length, number of leaves, wet biomass) and plant P and N.

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5. References
[1] Abbas S H, Sohail M, Saleem M, Tariq M, Aziz I, Qammar M, Majeed A, and Arif M 2013 *Sci. Techn. Develop.* 32(4): 277–280
[2] Yaseen T, Burni T, and Hussain F 2012 *Int. J. Agron. Plant Product.* 3(9): 334-345
[3] Guo Y, Ni Y, and Huang J 2010 *Trop. Grasslands* 44: 109-114
[4] Sharda J N and Koide R T 2010 *Botany* 88: 165-173
[5] Smith S E and Read D J 2008 *Mycorrhiza* 3rd ed (London: Academic Press)
[6] Liu A, Wang B and Hamel C 2004 *Mycorrhiza* 14: 93-101
[7] Bharadwaj D P, Lundquist P O and Alstrom S 2008 *Asian J. Exp. Sci.* 22: 89-93
[8] Davies F T Jr, Puryear J D, Newton R J, Egilla J N and Saraiva Grossi J A 2002 *J. plant nutrition* 25 (11): 2389–2407.
[9] Vivas A, Azcon R, Biro B, Barea J M and Ruiz-Lozano J M 2003 Can. J. Microbiol. 49: 577-588

[10] Barea J M 1997 Mycorrhiza-bacteria interactions on plant growth promotion: Plant Growth Promoting Rhizobacteria ed Ogoshi A, Kobayashi K, Homma Y, Kodama F, Kondo N, and Akino S (Paris: OECD Press) pp 150-158

[11] Barea J M Azcon R and Azcon-Aguilar C 2002 Antonie van Leeuwenhoek 81: 343-351

[12] Gamalero E, Trotta A, Massa N, Copetta A, Martinotti M G and Berta G 2004 Mycorrhiza 14: 185-192

[13] Mills H A and Jones J B 1996 Plant Analysis Handbook II: A Practical Sampling, Preparation, Analysis, and Interpretation Guide (Georgia: Micromacro Publishing) pp 6-81

[14] Barea J M, Andrade G, Bianciotto V, Dowling D, Lohrke S, Bonfante P, O’Gara F and Azcón-Aguilar C (1998) Appl. Environ. Microbiol. 64: 2304–2307.

[15] Artursson V 2005 Bacterial-fungal interactions highlighted using microbiomics: Potential application for plant growth enhancement dissertation (Sweden: Swedish University of Agricultural Sciences)

[16] Setiadi Y 1988 Technical Notes I (5):1-10

[17] Lumbantobing E L N, Hazra F, Anas I, Uji 2017 J Tanah Ling. 10(2): 72-76.

[18] Righi S, Luciialli P, and Bruuzzi L 2005 J. Environ. 82: 167-182.

[19] Tania N, Astina dan Budi S 2012 J. Sains Mahasiswa Pertanian 1(1): 10-15.

[20] Hoagland D R and Arnon D I 1938 Circ. Calif. Agric. Exp. Stn. 16

[21] Ohorella Z 2012 J. Agroforestri VII (1) 43-49

[22] Sulaeman, Suparto dan Eviati 2005 Analisis Kimia Tanah, Tanaman, Air pupuk (Bogor: Balai Penelitian Tanah) pp 73-88

[23] Turk M, Albayrak S, Balabanli C, and Yuksel O 2009 J. Food Agric. Environ. 7 (3): 339-342

[24] Vincent J M 1970 A Manual for the Practical Study of Root-Nodule Bacteria IBP Handbook No 15 (Oxford: Blackwell Scientific Publications) pp 164

[25] SPSS 1996 SPSS 7±0 for Windows 95 (Chicago: SPSS Inc)

[26] Kapulnik Y, Kigel J, Onkon J, Nur I and Henis Y 1981 Plant and Soil 6: 165-170

[27] Ramadhani I, Suliasih, Widawati S, Sudiana I M, and Kobayashi M 2018 The effect of the combination of arbuscular mycorrhiza and rhizobacteria and doses of NPK fertilizer on the growth of Sorghum bicolor (L.) Moench. International proceeding of ISBIOREV

[28] Utami K P and Setiawati M R 2018 J. Penel. Saintek. 25(1): 1-9

[29] Latif F M, Elfarisna and Sudirman 2017 J. Agrosains Teknol. 2(2): 105-120

[30] Widawati S and Suliasih J. Biodiversitas 7 (1) 10-14

[31] Afifi M M I, El-Sayed G A M, Manal A H, El-Gamal and Massoud O N 2014 Middle East J. Appl. Sci. 4: 1065-74

[32] Husen E 2009 Telaah Efektivitas Pupuk Hayati Komersial dalam Meningkatkan Pertumbuhan Tanaman. (Bogor: Balai Penelitian Tanah) pp 105-117

[33] Bashan Y and Bashan L E 2002 Appl. Environ. Microbiol. 6: 2673 – 2643

[34] Haryanto 2006 Teknik budidaya sayuran pakcoy (sawi mangkok) (Jakarta: Penebar Swadaya)

[35] Charisma A M, Rahayu Y S, Isnawati 2012 LenteraBio 1(3): 111–116

[36] Rohmah F, Rahayu Y S, Yuliani 2013 Lentera Bio. 2(2): 149- 153.

[37] Holevas C D 1996 J. Hortic. Sciences 41: 57

[38] Novizan 2005 Petunjuk Pemupukan yang Efektif (Jakarta: Agro Media Pustaka) pp 116

[39] Jumiati E 2009 Pengaruh berbagai konsentrasi EM4 pada fermentasi pupuk organik terhadap pertumbuhan dan hasil tanaman bawang merah (Amaranthus tricolor L.) secara hidroponik. Skripsi. UNS. Surakarta.

[40] Subiksa I G M 2002 Pemanfaatan Mikoriza untuk Penanggulangan Lahan Kritis. Makalah Program PPS IPB Bogor http://www.rudyct.com/PPS702-ipb/04212/igm_subiksa.htm diunduh tanggal 28 Agustus 2019
[41] Simanungkat R D M 1987 Pengaruh jamur mikoriza vesikuler-arbuskular (MVA), sumber P dan sterilisasi tanah terhadap pertumbuhan padi gogo di tanah kahat P Seminar Bioteknologi Pertanian PAU-Bioteknologi (Bogor: Institut Pertanian Bogor) pp 16
[42] Dewi A K dan Setiawati M R 2018 Agrologia 6(2): 54-60
[43] Jones J B, Wolf B, and Mills H A 1991 Plant Analysis Handbook: A Practical Sampling, Preparation, Analysis, and Interpretation Guide (USA: Micro-Macro Publishing, Inc) pp 213
[44] Jonas J B Jr 2001 Laboratory Guide for Conducting Soil Tests and Plant Analysis (Boca Raton: CRC Press) chapter 3 pp 56
[45] Sieverding E 1991 Vesicular-Arbuscular Mycorrhiza Management In Tropical Agrosystems (Deutsche: GTZ, GmbH Eschbom) pp 371
[46] Smith S E and Read D 2008 Mycorrhizal Symbiosis. Third Edition (New York: Academic Press, Elsevier) pp 13-145
[47] Song G, Chen R, Xiang W, Yang F, Zheng S, Zhang J, and Lin X 2015 Plant. Soil. Environ. 61(3): 127-136.
[48] Nurhidayati T, Kristiani I P, dan Dini E 2003 J. Sains dan Seni Pomits 3(2): 2337-3520.
[49] Kiers E T, Duhamel M, Beesetty Y, Mensah J A, Franken O, Verbruggen E, Fellbaum C R, Kowalchuk G A, Hart M M, Bago A, Palmer T M, West S A, Vandenkoornhuyse P, Jansa J, Bucking H 2011 Science 333: 880–882.
[50] Medeiros C A B, Clark R B and Ellis J R 1994 Mycorrhiza 4: 185-191
[51] Clark R B 1997 Plant Soil 192: 15-22
[52] Obaton M 1977 Effectiveness, Saprophitic and Competitive Ability three Properties of Rhizobium Essensial for in-cresing the Yield of Inoculated Legumes: Biological Nitrogen Fixation in Farming Systems of the Tropics ed Ayanaba A, Dart P J (new York: John Wiley & Sons) pp 127-133
[53] Widawati S dan Rahayu S H 1999 J. Mikrobiol. Lingk. 3(1): 40-47
[54] Widawati S, Abdulkadir S, Purwaningsih S 1997 J. Biol. Nasional II (1): 46-53.
[55] Karlen D L, Hurley E G, Mallarino A P 2006 Agron. J. 98: 484-495.