Application of arbuscular mycorrhizal fungi in combination with nitrogen fixing bacteria and other potential soil microbes as biofertilizer for soybean plant

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Abstract. The use of *mycorrhizae* as phosphate solubilizing fungus has been known since decade. *Mycorrhizae* could promote the growth of almost all species of plants. Biofertilizer product based on *mycorrhizae* fungus has been produced commercialization and easily found in the market. This study was aimed to understand the positive effect of *mycorrhizae* inoculation when it mixed with other microbes especially nitrogen fixing bacteria and growth hormone production bacteria. An experiment was set up at greenhouse to run the activities of microbes inoculation on soybean plants. Soybean was mainly used for testing the potential of soil microbes since the target of the experiment is increasing the production of soybean seed and decreasing the used of chemical fertilizer. The result of this study showed that multi strains inoculation was better than single inoculation. Single *mycorrhizae* inoculation produced slightly higher number of pods. However when the *mycorrhizae* was combining with other potential microbes the number of pods produced by the plants even significantly higher than only single inoculation. Updating the quality of biofertilizer is needed by including the biodiversity of soil microbes to gain the promotion of green farming program.

1. Introduction
Soybean is the most promise crops for food after rice. Since several years ago the Government of Indonesia has been announced the program of self sufficient of soybean. Many programs have been launched to develop the soybean production including their agriculture management. The used of biofertilizer is recommended to reduce the requirement of chemical fertilizer which mainly caused soil pollution. Beside of that, the used of high doses of chemical fertilizer may caused the unhealthy quality of food. Therefore the used of potential microbes as biofertilizer is recommended as it will maintain the environment and confirmed the sustainable of friendly agriculture system.

*Mycorrhizae* is one of the potential soil fungus which could be use as biofertilizer. This fungus has potential on fixing and solubilize the phosphorus available in the soil and it could be fulfill the requirement of phosphorous beside others plant nutrition such as micronutrition, minerals and water. Fakuara in 1988 reported that *mycorrhizae* could increase the absorption of nitrogen, kalium, calisium, magnesium, cupper and mangan including zink also produced the plant growth hormones. *Mycorrhizae* are very important fungal symbiont in many plants. They act as natural barriers to the soil borne pathogens. By the hyphae that cover the surface of root plant, the *mycorrhizae* could become the buffer to protect the root plants from the microbial root diseases. The protein of pathogen
will be destroyed by the bioactive compound produced by *mycorrhiza*. Moreover the *mycorrhiza* could absorbed heavy metal which are found in the surface of root [1].

The assessment of the beneficial of *mycorrhiza* has been study widely especially on plantation trees also crops. Kabirun [2] reported that *mycorrhiza* inoculation on upland rice could increase the rice growth, phosphate solubilizing and even increase the production of rice. Inoculation of *mycorrhiza* could increase the concentration of nitrogen up to 11.5 %, phosphate 14.9 % and kalium 12.2 % on upland rice [3].

The mechanism increasement of plant absorption by *mycorrhiza* was caused by the increase of metabolism system as a result of increasement of oksigen consumption and phosphatase enzyme [4]. The *mycorrhiza* produced phosphatase enzyme which required for fixing the plant minerals to become available for the plants [5]. Dighton [6] reported that the *mycorrhiza* hypae was very effective for minerals absorption since the ability to penetrate the soil aggregate is high since the diameter of hyphae is very tiny so it provide more possibility to absorb the minerals.

Legumes plants such as soybean is the most capable plant which could develop the symbiotic living with *Rhizobium*. This plant was recognize as an important sources of protein for mankind. *Rhizobium* sp has widely known as beneficial bacteria especially on the ability on processing the nitrogen fixing from the air. The legumes-*Rhizobium* symbiosis is the most efficient system for Nitrogen fixation, the bacteria will interact with leguminous plant in the host specific manner and form N- fixing root bacteria [7]. Study of the beneficial of *Rhizobium* has been done widely following by the collection of its biodiversity strains to be conserve as culture collection. Development of this bacteria also has been investigated and tested as biofertilizer for plants and crops. At this stage, the production of biofertilizer based on *rhizobium* and the application into the plants has been socialize continuously to the farmer.

*Pseudomonas* sp. bacteria was known as phosphate solubilizing bacteria which found associate on the rhizosphere of root plants. The bacteria was confirmed to be able to mobilized phosphate to become available for the plants. Combination with other bacteria, *Bacillus* sp. could support the growth of soybean in term of full canopy covered and greenish color of leaf. Further more it could be improved the growth of plant which performed by the height of plants and its was comparable with the height of plants which fertilized with SP-36 for 200 kg per hectar [8]. The production of soybean in acid soil (pH 5.5) inoculated with *Pseudomonas* sp. significantly increased from 2.06 ton per hectar to 2.20 ton per hectar [9].

Other microbes such as *Azospirillum* has been known as beneficial microbes since they has potential on nitrogen fixing as well as growth hormone production. *Azospirillum* known as a *rhizosphere* bacteria and normally attach with non leguminous plants such as rice, corn etc. However positive effect of combined inoculation with *Azospirillum* and *Rhizobium* have been reported for different legumes. Volpin and Kalpunik [7] reported that *Glycine max*, *Pisum sativum*, *Madicago sativa*, *Cicer arietinum*, *Psophocarpus tetragonolobus* inoculated with *Azospirillum* and *Rhizobium* showed enhancement in early nodulation, increases nodule on main root, total nodule number, nodule weight and nodule specific activity of nitrogen fixation. In this case *Azospirillum* stimulated efficiency of *Rhizobium* to nodulate plants root. So, combination of *Rhizobium* and *Azospirillum* inoculation was recommended to be applied on growing food crops.

*Agrobacterium* species from the family of *Rhizobiaceae* (update scientific name is *Rhizobium radiobacter*) was known as a phytopatogenic bacteria which is the casual agent of crown gall diseases and Plant Growth Hormone, *Rhizobiaceae* (PGPR) bacteria beside commonly used as a model bacteria for molecular study, especially used for inserting the foreign gene into plant cell and to produced transgenic plants [9],[10]. Gram negative bacteria has a big size of plasmid namely T1 – plasmid which contain the gene factor for virulency of bacteria cause plant diseases. Through the gene in T-DNA, production of auxin and indole-3-acetic acid were encoded. In this case the DNA transmission capabilities of *Agrobacterium* have been vastly explores to insert foreign gene into plant [11].
Arbuscular Mycorrhizal Fungi (AMF) live symbiotically with almost all families of trees, therefore AMF could be develop as biofertilizer for many plants and crops. Soybean is one of target crops for the study. The effectiveness of mycorrhizae inoculation and combination of AMF with other potential microbes especially nitrogen fixation bacteria hopefully will promote the growth of soybean and its production.

2. Materials and Methods

2.1. Microorganisms

The microorganism used in this experiment is the collections of soil microbes belong to the Laboratory of Plant Symbiotic Microbes, Research Center for Biotechnology LIPI. The list of microbes are, *Bradyrhizobium* sp. (BTCC B- 64), *Rhizobium* sp. (DCM), *Pseudomonas* sp. (BPF), *Endophytes diazotroph bacteria* isolated from *Shorea selanica* and *Arbuscular Mycorrhizal fungi* (BIOVAM – LIPI biofertilizer product) used in this experiment.

*Endophytes* microbes was isolated from forest trees (*Shorea selanica*) and it was selected for their potential for plant growth hormone production, indole acetic acid (IAA). Isolation of endophytes bacteria from *Shorea selanica* was done using MP media : Meat extract 5 g, Peptone 10 g, NaCl 5 g Nystatin 100 mg, Distilled water 1 liter [12].

BIOVAM, arbuscular *mycorrhizal fungi* was produced in pilot scale production unit located in Cicurug, Sukabumi, West Java, Indonesia by the Sari Daun Farmer community. The production of BIOVAM was conducted in the green house using specific soil collected from mountain area near Sukabumi. The product actually consist of *mycorrhizae* root infected which could be spread out when its inoculated to the early germinates seed. At a present time the farmer continuously produce the BIOVAM for their own needed.

2.2. Growth media

*Bradyrhizobium* and *Rhizobium* (DCM) were grown in Yeast Extract Mannitol Broth [13]; K$_2$HPO$_4$ 0.5 g, MgSO$_4$.7H$_2$O 0.2 g NaCl, 0.1 g, Mannitol 10 g, Yeast Extract 0.5 g, Aquadest 1 liter. Mass production of *Bradyrhizobium* sp. and *Rhizobium* sp. were done in YEM Broth and shake at 150 rpm for 4 days incubation periods.

*Pseudomonas* sp. (BPF) was grown on Pikovskaya media (Hi Media) : Ca$_3$(PO$_4$)$_2$ 5 g, NaCl, 0.2 g, KCl, 0.2 g, MgSO$_4$.7H$_2$O 0.1 g, NH$_4$SO$_4$ 0.5 g, Glucosa 10 g, Yeast Extract, 0.5 g MnSO$_4$.7H$_2$O 0.0025 g, FeSO$_4$.7H$_2$O 0.0025 g, Agar 20 g, Aquadest 1 liter. This media used to screen the phosphate solubilizing bacteria base on their ability to used calcium phosphate for their energy sources. *Pikovskaya Broth* was used for cultivation of phosphate solubilizing microorganism [14]. The inoculated plates were incubated 32 °C for 9 – 10 days. Mass production was done by growing the bacteria in Pikovskaya broth media with shake in 150 rpm.

2.3 Green house experiments

The experiment was conducted at green house condition in Cibinong Treatment s were arranged using soil media in polybag. Each microbes used in this experiment were prepared ahead before treatment. The bacteria were grown in the broth media normally used for mass production and incubated by shaking according to the propose age. The fungus used in this experiment is a product of arbuscular *mycorrhizae* biofertilizer (BIOVAM) which have packed as commercial LIPI product.

Seed of soybean was inserted by nitrogen fixing bacteria (*Bradyrhizobium* sp.) using vacuum technology. Planting activities was done by mixing the soybean seed with the *mycorrhizae* until the fungus covered the outer part of seed. The other bacteria (endophytic, phosphate solubilizing bacteria and other rhizobium bacteria were inoculated directly in a hole of soil then inoculated seed then were put inside the hole.

Observation of the plant growth were done starting at early stages (1 month) and continue further from two months old plants until the plant is 3 months old. All parameter were observed as prepared...
parameter such as height of plants, fresh weight of upper plants, dried weight of upper plants, fresh weight of lower plants, dried weight of lower plants, number of pod, weight of seed etc.

3. Results and Discussion

Biofertilizer are the product consisting selected and beneficial living microbes which are added to soil as microbial inoculants. They are gaining importance because of ecofriendly, non-hazardous and non toxic nature. Several organisms such as *Rhizobium*, *Pseudomonas*, phosphate solubilizing microorganism, *Mycorrhiza*, endophytic diazotroph and other soil potential microbes are presently being used as biofertilizer.

A greater part of soil phosphorous, approximately 95 to 99 % is present in the from of insoluble phosphate and hence cannot be utilized by plants [15]. Great proportion of phosphorus in chemical fertilizer becomes unavailable to the plants after its application in the soil [16].

Plant growth-promoting bacteria (PGPB) play an important role in supplementing phosphorous to the plant, allowing a sustainable use of phosphate fertilizer. Microorganism are involved in a range of process that effect the transformation of soil phosphorus (P). Phosphate-Solubilizing Bacteria (PSB) is slowly emerging as important organism for soil improvement [17].

The important of vascular arbuscular *mycorrhiza* as a tool for improving the growth and productivity in diverse group of plants was recognized [12]. *Mycorrhiza* is one of soil fungus which have potential on fixing phosphate to be available to the plants beside of that it help on inhibit the soil borne pathogen to infect the root since the hyphae of *mycorrhiza* cover the surface of roots [18]. *Mycorrhiza* known since decade as a potential microbes for biofertilizer. There are numbers of *mycorrhiza* base product found in the market and the quality of them are varies. The quality of biofertilizer become very important to confirm the successfully of the application on supporting the growth of plants. In case of BIOVAM product, the indicator needed to be confirmed was root infected percentage of inoculums before used it. Therefore in every step of production process, the percentage of root infection was analysed.

![Figure 1. Percentage of infection rate of mycorrhiza during the process of establishment with their host plant at the age 1-3 months](image)

Figure 1 explained the condition of root infection rate during the production periods. The percentage of *mycorrhiza* infection rate indicated the compatibility of potential fungus to live symbiotically with the host plant. BIOVAM is biofertilizer consists of *mycorrhiza* species and performed highly significant percentage of infection rate compared to control plant. This data confirmed that the *mycorrhiza* species used as inoculants were compatible with host plants (corn). The standard of root infection percentage is between 35 – 40 %. The highest percentage is
normally showed after 3 months inoculation. It was observed that control plant did not gave high percentage of infection rate which explained that the growth media used for mass production did not very much consist of *mycorrhizae*. The indigenous of *mycorrhizae* present in the soil, sometimes infected the root but often the potential on fixing phosphate was not significant. Method of application the *mycorrhizae* is simple, by inoculate the product content of chopped infected root. The hyphae of *mycorrhizae* fungus will then infected the new root development of host plant and further more established the symbiosis living with host plant.

Phosphorus deficiency leads to formation of small leaves, weak stem and slow development. Several mechanism like low soil pH, ion chelation and exchange reaction in the growth environmental have been reported to play a role in phosphate solubilizing by PSMs [17]. By inoculate the BIOVAM, the deficiency of phosphorus will not happened and the growth of plants were supported.

Root nodules of *leguminous* plants reported could be the host of other endophytes bacteria of diverse genera and species which are unrelated to *rhizobia* symbiotic nitrogen fixing bacteria [19]. These non-*rhizobia* nodule endophytes improve plant growth and nodulation when co-inoculated with *rhizobium* compare to inoculation with *rhizobium* alone [20].

![Figure 2. Soybean plant height at 1 month and 2 months old plants](image)

**Figure 2.** Soybean plant height at 1 month and 2 months old plants

| Treatment | Plant Height (cm) |
|-----------|-------------------|
| 1 CO      | 120               |
| 2 CN      | 110               |
| 3 Miko    | 90                |
| 4 Miko RH | 80                |
| 5 Miko DCM| 60                |
| 6 Miko Endo| 50              |

Height of plants indicated the healthies of plants during growing periods. Figure 2 showed the data of soybean plants height at 1 to 2 months old plants. It is observed that the combination of some potential microbes especially combination between *mycorrhizae*, *Rhizobium* (DCM) and endophytes for growth hormone production (treatment no. 11) similarly with the combination of two strains of *Rhizobium* and two strains phosphorus solubilizing bacteria (treatment no 18) provide the best results on supporting the growth of plants compared to control plant (CO) and control nitrogen (CN). The inoculation with five different microbes resulted approximately 120 cm height of plants, however the normal height of plant is about 50 - 75 cm. This positif effect of inoculation open the possibility to develop new composition of microbes.
Figure 3. Dried weight of upper plant and root plant on soybean plant

Note:
1. CO 7. Miko. BPF 13. Miko. Endo. BPF.
2. CN 8. Miko RH. DCM. 14. Miko. Endo. RH. DCM.
3. Miko 9. Miko RH. Endo 15. Miko. BPF. RH. DCM.
4. Miko RH 10. Miko. RH. BPF 16. Miko. Endo. BPF RH.
5. Miko DCM 11. Miko. DCM. Endo. 17. Miko. Endo. BPF. DCM.
6. Miko. Endo 12. Miko. DCM. BPF. 18. Miko. Endo. BPF. RH. DCM.

The effective combination of microbes use as biofertilizer also observed by analysis the dried weight of upper part of plants. Figure 3 showed the effective combination of microbes on supporting the growth of soybean plants. The best combination of microbes is the combination among five different species of microbes (treatment no. 18), eventhough combination of mycorrhizae and rhizobium only, performed high dried weight of plants. The other microbes such hormone production bacteria (endophytes) and phosphate solubilizing bacteria are also effective on promoting the growth of plants.

Figure 4. Number of pods, wet weight of pods and weight seed of 2 plants of soybean plant

Note:
1. KO 7. Miko. BPF 13. Miko. Endo. BPF.
2. KN 8. Miko RH. DCM. 14. Miko. Endo. RH. DCM.
3. Miko 9. Miko RH. Endo 15. Miko. BPF. RH. DCM.
4. Miko RH 10. Miko. RH. BPF 16. Miko. Endo. BPF RH.
5. Miko DCM 11. Miko. DCM. Endo. 17. Miko. Endo. BPF. DCM.
6. Miko. Endo 12. Miko. DCM. BPF. 18. Miko. Endo. BPF. RH. DCM.
In regard to the production of soybean which is indicated by the number of pods, weight of pod and weight of seed of two plants showed that combination among the microbes especially five potential microbes confirmed the effective inoculation, although the minimum combination of microbes consist of *rhizobia, mycorrhizae*, phosphate solubilizing bacteria and endophytic bacteria were also significantly increased the production of seed. Combination of microbes eventually promises the ability to stimulate the growth of soybean and increase the seed production. However, these data confirmed that the most important is the quality of selected microbes uses for building up the biofertilizer product. The quality concerned with the viability of the microbes during biofertilizer processing, packaging of product and transportation to the agriculture area including storage period also unchanged the physiological characteristics especially the stability of each potential which lead to the plant growth supporting ability.

4. Conclusion
As a conclusion application of mycorrhizal fungi gave the positive effect to the growth of soybean even more when the mycorrhizae was combined with other potential microbes. Our study explained that *Rhizobium* strains isolated from forest trees could be used in crossed inoculation to soybean. Further more mixed inoculation among the potential microbes are applicable to be used for promoting the growth of soybean. More study are warranted to identify and understand the significant mechanism underlying the formation of microbes combination and its benefits as biofertilizer. The assesment of microbial biodiversity open a new knowledge for the new innovation of biofertilizer.

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Appendix 1

Figure 5. Root infection by *mycorrhizae* fungus (foto by Dr.Kartini Kramadibrata and Lisye Nurjanah)