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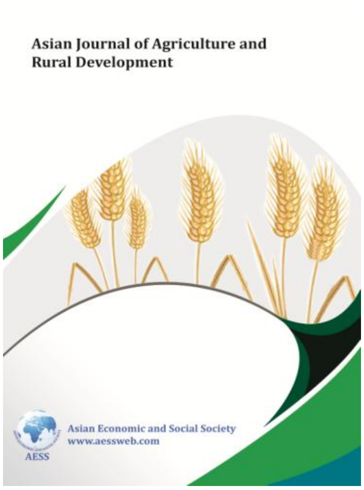

Rhizobacteria Selection to Enhance Spore Germination and Hyphal Length of Arbuscular Mycorrhizal Fungi in Vitro

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Abstract

In natural condition, Arbuscular Mycorrhizal Fungi (AMF) are surrounded by bacteria that help fungi symbiosis. The research aimed to get rhizobacteria that can act as Mycorrhiza Helper Bacteria (MHB) had been held at Soil Biology and Biotechnology Laboratory Faculty of Agriculture Unpad from February to March 2012. The experimental design used was completely randomized design with 11 treatments (bo= without rhizobacteria, b_1= *Pseudomonas diminuta*, b_2 = *Bacillus alvei*, b_3 = B. *mycoides*, b_4 = *P. malei*, b_5= *P. diminuta* + B. *alvei*, b_6 = *P. diminuta* + B. *mycoides*, b_7 = *P. diminuta* + *P. malei*, b_8 = B. *alvei* + B. *mycoides*, b_9 = B. *alvei* + P. *malei*, b_{10}= B. *mycoides* + P. *malei*) with 3 replications. Parameters evaluated were spore germination percentage and hyphal length of *Glomus sp* at 7, 14, 21, and 28 day after planting. The result showed that *P. diminuta* enhanced spore germination percentage and hyphal length of *Glomus sp*as much as 224 % and 330%respectively than control. So, *P. diminuta* can be used as MHB.

Keywords: AMF, *Glomus sp*, Hyphal length, MHB, Spore germination

Introduction

Almost all tropical plants can be infected by Arbuskula Mycorrhiza Fungi (AMF) and many of them are very responsive to the AMF. Only a few families and genera of plants that cannot form the structure of AMF, such Brassicaceae(root exudates are toxic to the AMF), Caryophyllaceae, Cyperaceae, Juncaceae, Chenopodiaceae, and Amaranthaceae (Cardoso and Kuyper, 2006). AMF increases absorption of nutrients, especially the immobile ones (P, Cu, and Zn), and increases absorption of required water for plant through extra-radical hyphae tissue. In addition, AMF increases plant resistance to pathogen attack, improve soil aggregation and form symbiosis with other soil microbes.

When AMF forms symbiotic associations with plant roots, the microbes will directly interact with other organisms in the soil, or indirectly affect the physiology of host plants such change root morphology and exudation patterns into the mycorrhizo sphere. One important organ for AMF to perform its function is external hyphae. The existence of external hyphae, besides increased nutrient uptake from the root zone of host plant, also expands area to interact with other microorganisms and as assimilate translocation pathway from the host plant to the soil.

According to Frey-Klett *et al*. (2007), under natural condition, mycorrhizal fungi are surrounded by complex bacterial community that help symbiotic fungi. These bacteria communities are found most in Glomus. Artursson *et al*. (2006) stated that there are two groups of bacteria that interact with AMF in mycorrhizosphere, i.e. saprophyte and symbionts, both of which can be neutral, harmful, or beneficial. Furthermore, Hildebrandt *et al*. (2005) noted only gram-positive bacteria (generally *Paenibacillus* ssp and *Bacillus*ssp) that associate with fungal hyphae.

Garbaye (1994) stated that bacteria with ability to enhance root colonization or promote hyphae growth, called Mycorrhiza Helper Bacteria (MHB). This MHB plays role in ectomycorrhiza and endomycorrhiza. Xavier and Germida (2003) obtained several bacteria which stimulate spore germination. According to Barea *et al*. (2005) rhizobacteria produce compounds that can increase root exudation rate, which in turn will stimulate the mycelia of AMF on rhizo sphere or facilitate root penetration by fungi.

Several rhizobacteria collection of Soil Biology and Biotechnology Laboratory Faculty of Agriculture Unpad isolated from various plants and different ecosystems belonging to genus Pseudomonas and Bacillus need to be tested for their ability to enhance germination and hyphal length as part of phase on generating bacterial inoculum and AMF consortium in order to improve soil physical character and plant growth.

Methods

Experiment conducted in Soil Biology and Biotechnology Laboratory Faculty of Agriculture Unpad and Laboratory of Food Crops Protection and Horticulture Institute West Java from February to March 2012.

The experiment used completely randomized design consisted of 11 treatments and 3 replications, as follows:

bo = *without rhizobacteria*,b_1= *Pseudomonas diminuta*, b_2= *Bacillus alvei*, b_3= *B. mycoides*, b_4= *P.malei*, b_5= *P. diminuta* + *B. alvei*, b_6 = *P. diminuta* + *B. mycoides*, b_7 = *P. diminuta* + *P. malei*, b_8 = *B. alvei* +*B. mycoides*, b_9 = *B.alvei*+ *P. malei* , b_{10}= *B. mycoides* + *P. malei*

Surface of fresh spores of *Glomus sp* was sterilized in solution of 20 g chloramines T per liter, 200 mg Streptomycin per liter and 1 liter of Tween 80 per liter for 20 minutes and then washed five times in sterile water. Bacteria strains were grown on Nutrient Agar (NA) medium at temperature of 28^{o}C for 48 hours. Moreover, bacteria suspension apt with treatment as much as 100 mL equivalent with 10^{-8}CFU ml^{-1} was spread on Petri dish surface (diameter 9) which containedagar liquid (0.8% Bacto agar Difco) with pH 7. Six spores of AMF that had been sterilized next transferred individually to Petri dish which had been inoculated with Rhizo bacteri isolates, and placed at hexagonal points with each side length of 3.5 cm. Petri dish taped with plastic wrap and incubated at temperature 24°C in dark condition for 28 days.

Parameter observations, spore germination percentage and length of external hyphae at 7, 14, 21, and 28 days after inoculation, were observed using an inverted microscope Zeis Prima Vert with magnification 100 times. Hyphae length was measured using Axio vision software. Data were analyzed using DAASTAT version 7.

Results and Discussion

Percentage of Spore Germination in Vitro

Percentage of spore germination without rhizobacteria and inoculated with rhizobacteria either single or double showed different patterns. Spore germination percent age for without rhizobacteria treatment indicated fixed value since the beginning until the end of observation. Meanwhile, the percentage of spore germination inoculated with rhizo bacteria showed improvement for each observation time (Table 1).

Table 1: Effect of Rhizobacteria on Spore Germination Percentage of AMF in Vitro at 7, 14, 21, 28 DAP

Rhizobacteria	Spore Germination Percentage (%)			
	7 DAP	14 DAP	21 DAP	28 DAP
Without rhizobacteria	15,89 a	15,89 a	15,89 a	15,89 a
B. alvei	15,89 a	24,12 b	27,83 a	35,25 b
P. diminuta + B. mycoides	15,89 a	15,89 a	27,83 a	27,83 b
P. malei	20,01 a	31,54 b	31,54 b	38,49 c
B. alvei + B. mycoides	20.01 a	27,83 b	31,54 b	31,54 b
B.mycoides	24,12 b	27,83 b	27,83 a	31,55 b
B.alvei +P. malei	27,83 b	27,83 b	27,83 a	27,83 b

P. diminuta + P.malei	27,83 b	27,83 b	31,54 b	35,25 b
P. diminuta+ B. alvei	27.83 b	27,83 b	31,54 b	31,54 b
B. mycoides +P. malei	27,83 b	27,83 b	38,49 b	38,49 c
P. diminuta	35,25 b	45,00 c	45,00 c	51,51 d

Explanation: Numbers followed by same letter are not significantly different based on Scot-Knott at real level 5 %.

Single and double rhizobacteria inoculation increased the percentage of spore germination about 51.79% to 121.77% compared with no inoculation of rhizobacteria at 7 DAP. At 14 DAP observation, both single and double inoculation of rhizobacteri increased the percentage of spore germination about 75.14% to 183.20% compared to the control, except for *B. malei* and *P. diminuta + B. malei*. On observation at 21 DAP, two treatments of single rhizobacteria and four treatments of double rhizobacteria increased the percentage of spore germination about 98.49% to 183.20% compared with no rhizobacteria inoculation. Rhizobacteria inoculation either single or double increased percentage of spores germination at the end of observation (28 DAP). The increase in percentage of spore germination ranged from 75% to 224%. *P. diminuta* increased the percentage spores germination as the highest.

Rhizobacteria influence on spore germination percentage in vitro at 7,14,21, and 28 days after planting entirely can be seen in Figure 1.

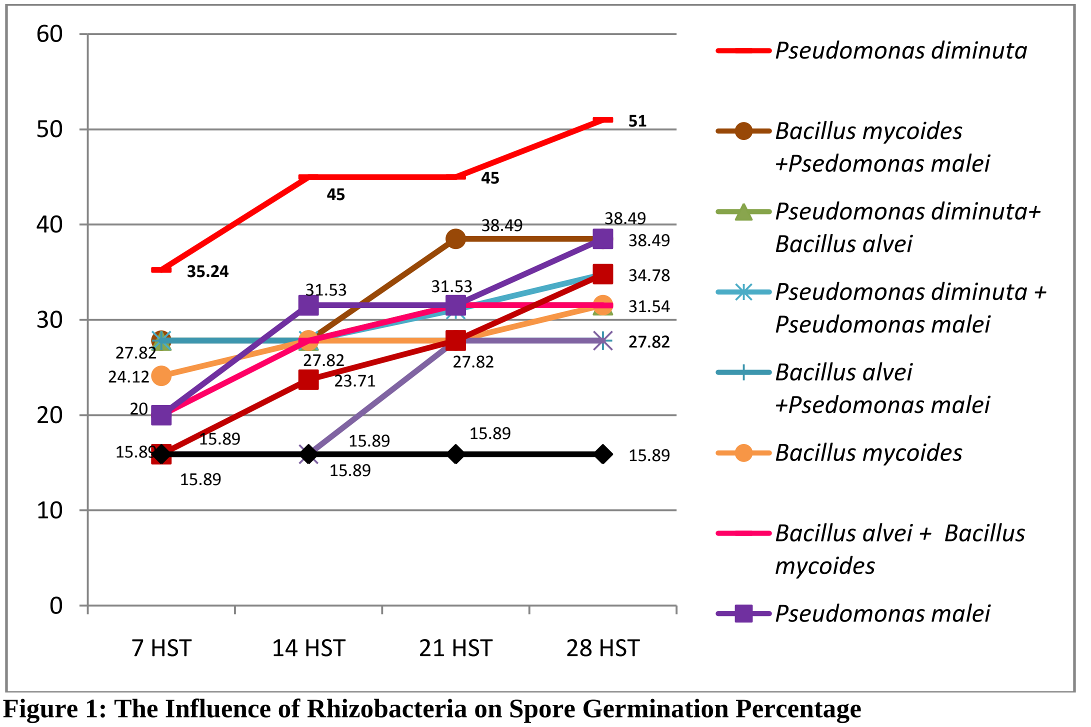

Figure 1: The Influence of Rhizobacteria on Spore Germination Percentage

Inoculation of single or double rhizobacteria increased spore germination of *Glomus* sp. At 7 DAP observation; the highest increase was generated by *P. diminuta*. This type of rhizobacteria consistently increases spore germination at 14, 21 and 28 days after planting observations. Other rhizobacteria given either single or double promoted spore germination at 14 and 21 days after planting. Finally, all rhizobacteria increased spore germination at 28 DAP with increase ranged from 75% to 224% compared with no microbe inoculation.

Figure 2 shows *Glomus sp* spore without rhizobacteria inoculation that has not germinated (left) and has germinated because of *P.diminuta* inoculation (right)

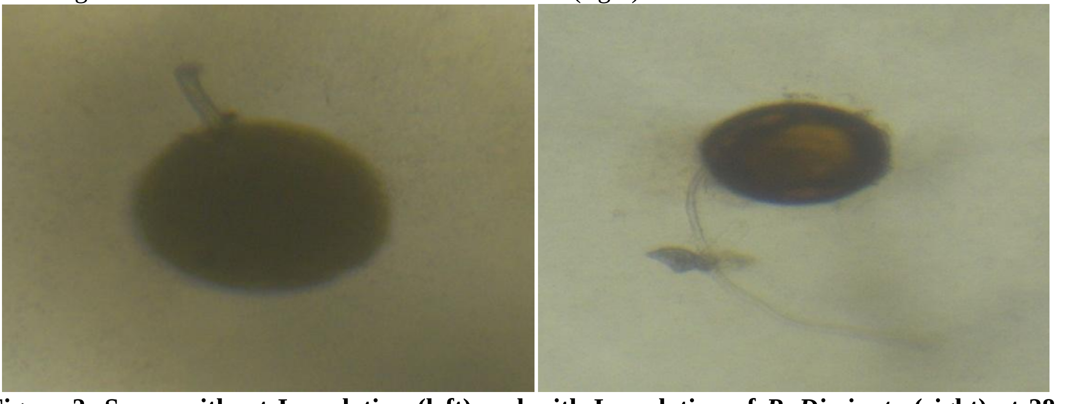

Figure 2: Spore without Inoculation (left) and with Inoculation of *P. Diminuta* (right) at 28 days after planting

AMF Hyphae Length *in Vitro*

Inoculation of rhizobacteria either single or double succeeded to increase the length of hyphae at 7, 14, 21, and 28 days after planting observations by 86.67% to 300% compared to controls (Table 2).

Table 2: Influence of Rhizobacteria on AMF Hyphae Length *in Vitro*at 7, 14, 21, and 28 DAP

Rhizobacteria	Hyphae Length (mm)			
	7 DAP	14 DAP	21 DAP	28 DAP
Withoutrhizobacteria	0,015 a	0,028 a	0,041 a	0,057 a
B. alvei	0,028 b	0,046 b	0,065 b	0,079 b
B. mycoides	0,028 b	0,037 a	0,079 b	0,011 d
P. diminuta + B. mycoides	0,036 c	0,053 b	0,080 b	0,098 c
P. diminuta + P. malei	0,051 d	0,058 b	0,074 b	0,095 c
B. mycoides +P. malei	0,061 e	0.096 c	0,117 c	0,126 e
B. alvei + P. malei	0,068 f	0,096 c	0,110 c	0,152 g
B. alvei + B.mycoides	0,068 f	0,104 c	0,127 d	0,141 f
P. diminuta	0,071 f	0,129 d	0,159 f	0,228 i
P. diminuta +B. alvei	0,092 g	0,099 c	0,141 e	0,179 h
P. malei	0,093 g	0,110 c	0,133 d	0,149 g

Explanation: Numbers followed by same letter are not significantly different based on Scot-Knott at real level 5 %.

Inoculation of rhizobacteria either single or double increased hyphae length of *Glomus sp* grown in vitro at 7 DAP. The increase occurred between 87% and 520% compared with no inoculation. The highest increase was due to inoculation of *P. Malei.*

Rhizobacteria inoculation both single and double increased hyphae length of *Glomus sp* grown in vitro at 14 DAP, except for *B. mycoides*. The increase of hyphae length ranged from 64.29% to 360%. *P. Diminuta* promoted the increase hyphae length at most.

Single or double rhizobacteria inoculation increased hyphae length of *Glomus sp* grown in vitro at 21 DAP between 59% until 288% compared to without inoculation. *P. Diminuta* demonstrated as the best rhizobacteria species on triggering hyphae length.

At the last observation (28 DAP) single and double rhizobacteria inoculation increased the hyphae length of *Glomus sp* grown in vitro. Isolates of rhizobacteria could increase the hyphae length of *Glomus sp* between 38.60% and 300% each because of *B. alvei* and *P. diminuta* inoculation compared to without inoculation.

The hyphae length of *Glomus sp* given either single or double rhizobacteria was higher than that without rhizobacteriaat7, 14, 21, and 28 days after planting observations. Among the tested rhizobacteria, *P. diminuta* increased the hyphae length of *Glomus sp* as the highest of 300% compared with no inoculation.

The rhizobacteria influence on hyphae length of *Glomus sp* in vitro 7, 14, 21, and 28 DAP entirely can be seen in Figure 3.

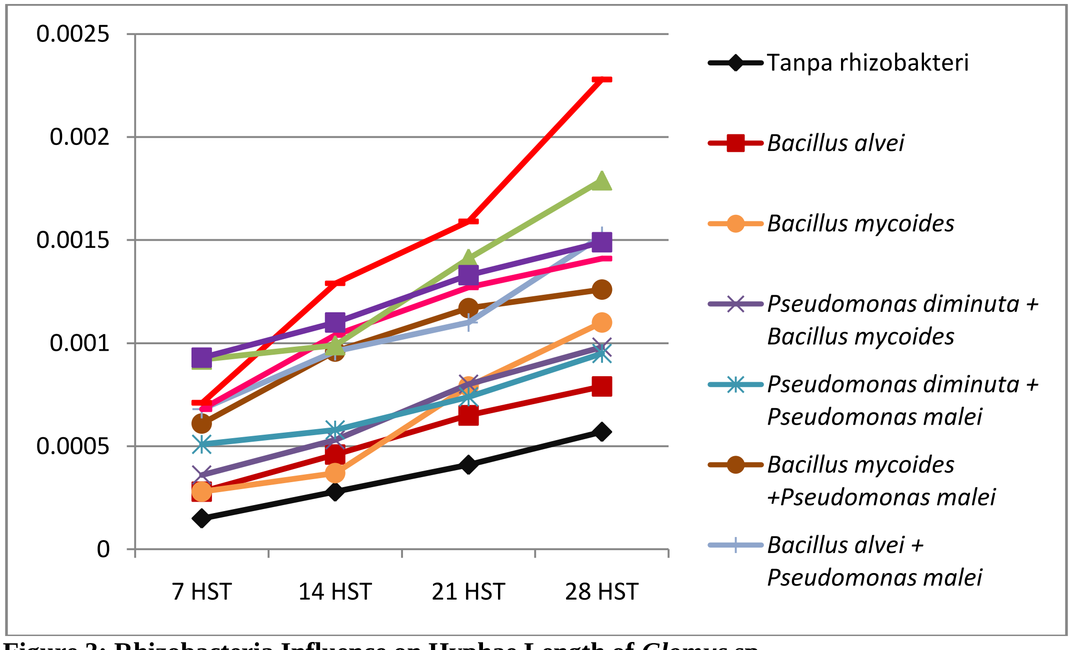

Figure 3: Rhizobacteria Influence on Hyphae Length of *Glomus* sp.

Rhizobacteria inoculation can increase the percentage of spore germination and hyphae length of *Glomus sp* in vitro, this is along with research result of Pivato *et al*. (2009) that found without bacteria inoculation, spore germination isonly1.04% and hyphae length is 57 mm, and when inoculated with bacteria strains included to Oxalobacteraceae and Comamonadaceae family, it obtains spore germination of *Glomusmosseae* between 12.93% to 44.12% and hyphae length 7.66 to 17.62 mm. The bacteria strains, which are able to manage increase on spores germination and hyphae length, belong to family Oxalo bacteraceae that are isolated from mycorrhizal plant roots. The effectiveness of bacteria strains in improving spore growth depends on the species; it is proved that the same bacteria strains produce not significantly different spore germination and hyphae length of *Gigasporaroseae*.

In this experiment, the increase in spore germination and hyphae length by rhizobacteria could be seen from the differences in auxin (IAA) level produced by rhizobacteria. *P. Diminuta* produced the lowest IAA (80.33 ppm) compared to other rhizobacteria either single or double (Table 3).

Table 3: Analysis of IAA Released by Rhizobacteria

Rhizobacteria	IAA
	-ppm-
P.diminuta	80.33
B. alvei	191.27
B. mycoides	100.44
P. malei	98.22
P.diminuta+ B.alvei	165.58
P.diminuta + B. mycoides	191.27
P.diminuta +P.malei	176.78

B.alvei+B.mycoides	209.02
B.alvei + P.malei	160.95
B.mycoides+P. malei	132.25

Hormone can stimulate in low concentration and inhibit in high concentration. This is in line with research of Kaneko and Tanimoto (2009) who obtained IAA at nanomolar concentration of 10^{-9} M given to in vitro agar medium to stimulate hyphal length of *Gigaspora margarita* and vice versa at micromolar concentrations (10^{-7} - 10^{-3} M) which inhibits. Hyphae length on application of 10^{-9} M IAA is 5.5 cm after 10 days incubation and decreased with increasing concentrations up to 10^{-4} M IAA, which is 0.3 cm. Likewise, spore germination experience improvement by the presence of IAA at nanomolar doses but at micromolardoses inhibit germination of *G. fistulosum*.

Based on the percentage of spore germination and hyphal length at four time observations, it was obtained that *P. diminuta* succeeded in increasing the two parameters as the highest compared to other rhizobacteriawhich was given either single or double, so it can be concluded that *P. diminuta*can act as MHB.

Conclusion and Recommendations

Conclusion

P. diminuta succeeded in increasing the percentage of spore germination and hyphae extension of *Glomus sp* planted in vitro thus categorized as MHB.

Recommendations

P. diminuta should be examined by inoculating it together with *Glomus sp* in vivo condition to see its effect on improvement of soil characteristics (physical, chemical and biology), also on growth and yield of plant.

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