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# Adaptation of Oil Palm Seedlings Inoculated with Arbuscular Mycorrhizal Fungi and Mycorrhizal Endosymbiotic Bacteria *Bacillus subtilis* B10 towards Biotic Stress of Pathogen *Ganoderma boninense* Pat

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The effects of mycorrhizal endosymbiotic bacteria *Bacillus subtilis* B10 and composite of arbuscular mycorrhizal fungal spores in green house experiment were examined in order to evaluate their effectiveness and compatibility with oil palm seedlings in the presence of a fungal pathogen *Ganoderma boninense*, the most serious pathogen in oil palm (*Elaeis guineensis* Jacq) in Indonesia. A three factors experiment were conducted, with mycorrhizal inoculation (M0 and M1), bacterial *B. subtilis* B10 inoculation (B0 and B1), and *G. boninense* inoculation (G0 and G1) as the first, second, and third factors, respectively. The results showed that disease severity index, plant height, root dry-weight, and phosphorus uptake were affected by co-inoculation of mycorrhizal endosymbiotic bacteria *B. subtilis* B10 and composite of arbuscular mycorrhizal fungi. Co-inoculation of mycorrhizal endosymbiotic bacteria *B. subtilis* B10 and arbuscular mycorrhizal fungi did not only reduce the percentage of basal stem rot incidence, but also significantly increased plant height and phosphorus uptake by oil palm seedlings. Our results suggest that in oil palm seedlings mycorrhizal endosymbiotic bacteria *B. subtilis* B10 worked synergistically with arbuscular mycorrhizal fungi in increasing plant adaptation toward biotic stress of pathogen *G. boninese* and could be promising biocontrol agents.

Key words: arbuscular mycorrhizal fungi, *Bacillus subtilis* B10, biotic stress, *Ganoderma boninense,* mycorrhizal endosymbiotic bacteria, oil palm seedlings

Patogen *Ganoderma boninense* Pat penyebab penyakit busuk pangkal batang merupakan patogen paling mematikan pada tanaman kelapa sawit (*Elaeis guineensis* Jacq). Ko-inokulasi bakteri endosimbiotik mikoriza *Bacillus subtilis* B10 dan komposit dari spora fungi mikoriza arbuskular pada bibit kelapa sawit telah dievaluasi efektivitas dan kompatibilitasnya dalam meningkatkan daya adaptasi bibit kelapa sawit terhadap cekaman biotik patogen *G. boninense* Pat. Percobaan tiga faktor dalam rumah kaca telah dilakukan dengan faktor pertama adalah inokulasi fungi mikoriza arbuskular yang terdiri dari tanpa inokulasi (M0) dan dengan inokulasi (M1). Faktor kedua adalah inokulasi bakteri *B. subtilis* B10 yang terdiri dari tanpa inokulasi (B0) dan dengan inokulasi (B1), sementara faktor ketiga adalah inokulasi patogen *G. boninense* yang terdiri dari tanpa inokulasi (G0) dan dengan inokulasi (G1). Hasil penelitian menunjukkan bahwa ko-inokulasi bakteri endosimbiotik mikoriza *B. subtilis* B10 dan komposit spora fungi mikoriza arbuskular mempengaruhi indeks keparahan penyakit, tinggi tanaman, bobot kering akar dan penyerapan fosfor oleh bibit kelapa sawit. Ko-inokulasi bakteri endosimbiotik mikoriza *B. subtilis* B10 dan fungi mikoriza arbuskular tidak hanya menurunkan persentase kejadian penyakit busuk pangkal batang, akan tetapi secara signifikan juga meningkatkan pertumbuhan tinggi tanaman dan penyerapan fosfor oleh bibit kelapa sawit. Hasil penelitian ini menyimpulkan bahwa bakteri endosimbiotik mikoriza *B. subtilis* B10 bekerja sinergis dengan fungi mikoriza arbuskular dalam meningkatkan daya adaptasi bibit kelapa sawit terhadap cekaman biotik patogen *G. boninense* dan berpotensi sebagai agen biokontrol.

Kata kunci: *Bacillus subtilis* B10, bakteri endosimbiotik mikoriza, bibit kelapa sawit, cekaman biotik, fungi mikoriza arbuskular, *Ganoderma boninense*,

Oil palm (*Elaeis guinnensis* Jacq) is one of the highest yielding plants in oil producing crops and the most important plantation and commercial crop in Indonesia. However, some fungal diseases have reduced the annual production of oil palm in South East Asia (Idris *et al*. 2003). Fungal pathogen *Ganoderma boninense*, the causal agents of basal stem rot (BSR) in oil palm, can severely attack the plant leading to

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dramatic yield losses (Idris *et al.* 2003), and could kill more than 80% of the plants (Abdul Razak *et al.* 2004). Until now there is no effective treatment for *Ganoderma* infected palms, while preventive treatments showed varying degrees of effectiveness (Haniff *et al.* 2005). Nowadays, biological control has gained much attention as a way of reducing the use of chemical products in agricultural practices.

Arbuscular mycorrhizal fungi (AMF) live as an obligate symbiont in the roots of about 80% of land plants, including oil palm. The uses of AMF to improve fertilizer efficiency and/or to protect against root diseases have been getting more attention during the last decade. Their importance in controlling plant disease, nutrient cycling and soil quality are well reported (Rillig and Mummey 2006; Smith and Read 2008, Lioussanne 2010). In the rhizosphere, the AMF coexist with other soil organisms. Several bacteria associated with AM fungal spores (mycorrhizal endosymbiotic bacteria) have positive or negative effects on the AMF or host plants (Bharadwaj *et al.* 2008). Bakhtiar *et al.* (2010) found that *Bacillus subtilis* ZJ 06 had higher antagonistic activity against *G. boninense in vitro* and has a potential to be used as biocontrol agent against basal stem rot in oil palm.

The role of AMF in increasing plant resistance to pathogens is quite well known. Arbuscular mycorrhizal fungi might control plant pathogens activity by direct interactions with the pathogens or mediated by mycorrhizal endosymbiotic bacteria (Whipps 2004). The antagonism of bacteria inhabiting the mycorrhizal endosymbiont has also been suggested as a possible mechanism (Budi *et al.* 1999). Currently, the use of chemicals to control soil-borne pathogens is a major problem with modern crop production due to increasing concern for human health and environmental safety. The utilization of AMF together with mycorrhizal endosymbiotic bacteria to control plant pathogens, combined with the positive effects on AMF colonization and plant growth offer an important solution yet to be fully exploited. Therefore, the aim of this study is to evaluate growth response of oil palm seedling in the green house, when inoculated with arbuscular mycorrhizal fungi and mycorrhizal endosymbiotic bacteria in the presence of root pathogen *G. boninese*.

## MATERIALS AND METHODS

**Preparation of Arbuscular Mycorrhizal Fungal spores, *B. subtilis* B10 Suspension and Fungal Pathogen *G. boninense*.** The arbuscular mycorrhizal fungi (AMF) used in this study was composed of AMF spores isolated from rhizosphere of oil palm in previous study (Bakhtiar *et al.* 2010). The fungi was propagated in pot cultures of *Pueraria javanica* in zeolite sterile. The spores of AMF were collected and sterilized in two steps (Reimann 2005), first in 2% (w/v) of chloramine-T and tween 20 for two min and second in mixture of 20% (w/v) of streptomycin and 10% (w/v) of gentamycin (100 mL) for 10 min. All spores were stored in 50 mL of sterile tap water at 4 °C until used. Isolates of *B. subtilis* B10 were obtained from the mycorrhizal endosymbiotic of oil palm roots colonized by AMF and found to have the biggest inhibition against *G. boninense* in vitro (Bakhtiar *et al.* 2010). Suspension of mycorrhizal endosymbiotic bacteria *B. subtilis* B10 was prepared by inoculating 1 mL of bacterial suspension into 50 mL of nutrient broth. The bacterial cultures were incubated in a shaking incubator (150 rpm) at 28 °C for 12 h. Inoculum of root pathogen *G. boninense*, grown on rubber wood block, was provided by the Indonesian Oil Palm Research Institute (IOPRI).

**Growth Medium.** The growth medium was a 1:1:1 (w/w) mixture of red yellow podzolic soil (PMK) : sand : compost in total 1 kg per polybag. The acidic PMK soil was obtained from Gajrug, Rangkas Bitung, Banten. The growth medium was sterilized using autoclave and allowed to equilibrate for 7 d. The mixture of soil media was then placed in the polybag (1 kg/polybag in the pre-nursery step and 5 kg/polybag in the main-nursery step).

**Inoculation of AMF and *B. subtilis* B10 into Oil Palm Seedlings.** Seeds of oil palm germinated variety of Dy x P Dumpy type (supplied by IOPRI, Medan, North Sumatera) were grown in polybags containing 1 kg soil growth medium in the glasshouse (pre nursery step). The plant seedlings were inoculated with 200 spores of AMF/seed, followed by drenching 20 mL of mycorrhizal bacterial *B. subtilis* B10 suspension into the soil. Control polybags were prepared in a similar way but without suspension of bacteria and spores of AMF. In total, five replicates per treatment were arranged and each treatment consisted of two polybags. The plant seedlings were fertilized with half strength of IOPRI recommended dosage. Oil palm seedlings were regularly irrigated and rotated to minimize any border effects. After 12 weeks, all of the oil palm seedlings were transplanted into bigger polybags in the main-nursery step. At this step, some of the oil palm seedlings were inoculated with fungal pathogen *G. boninense*. The oil palm seedlings were

then harvested after 52 weeks. The variables recorded were disease incidence of basal stem rot, height of plant and root dry weight. The phosphorus (P) use efficiency of plants was calculated and expressed as gram shoot dry weight per gram P absorbed.

**Disease Severity Index (DSI).** Disease progression was described using the disease severity index (DSI) which depicts the severity of the disease based on the progress of the disease. The symptoms were indexed along with the following formula (Abdullah 2003): 0 = for healthy plants; 1 = for appearance of three necrotic leaves; 2 = for appearance of more than three necrotic leaves; 3 = for fruiting bodies appearance at the bole; 4 = for dying/dead plant. The DSI was calculated at the end of experiment (52 weeks) based on the following formula:

$$\text{Disease severity index (DSI)} = \frac{\Sigma(A \times B) \times 100}{\Sigma B \times 4}$$

where: A: disease class (0, 1, 2, 3 or 4)
B: number of plants showing that disease class per treatment.

**Experimental Design.** The three factors experiment was arranged in a Complete Randomized Design with four replications, resulted in a 2 x 2 x 2 treatment combinations, with mycorrhizal inoculation (M0 and M1), bacterial *B. subtilis* B10 inoculation (B0 and B1), and *G. boninense* inoculation (G0 and G1) as factors. Each experiment was performed on two polybags. The quantitative data were analyzed using ANOVA analysis and the means were separated using LSD test in SAS program.

## RESULTS

**Disease Severity Index of Oil Palm Seedlings after Inoculation with Arbuscular Mycorrhizal Fungi and Mycorrhizal Endosymbiotic Bacteria *B. subtilis* B10.** In general, 52 weeks after treatments, basal stem rot disease developed slower in the seedlings inoculated with AMF, whether or not the seedlings were co-inoculated with *B. subtilis* B10, than in the seedlings without AMF (Fig 1). A lower DSI would indicate disease suppression by the AMF and *B. subtilis* B10. The seedlings treated with mixed of *AMF* (M1) and *B. subtilis* B10 (B1) showed the lowest DSI (5%) (Fig 1). As expected, the seedlings without AMF inoculation showed the highest DSI, whether or not they were co-inoculated with *B. subtilis* B10. The DSI of the seedlings with and without *B. subtilis* B10 inoculation were 42.5% and 37.5%, respectively.

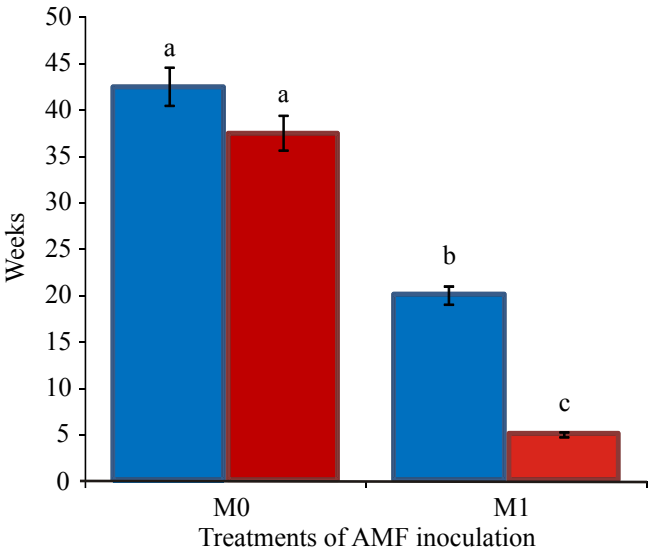


Fig 1 Disease severity index (DSI) at 52 weeks after planting and chalanged by fungal pathogen *G. boninense*. Means with the same letters between treatments are not significantly different (LSD 0.05). M0: without inoculation of AMF; M1: with inoculation of AMF; : without inoculation of *B. subtilis* B10; : with inoculation of *B. subtilis* B10.

Inoculation with only *B. subtilis* B10 could not suppress the growth of pathogens *G. boninense* and the DSI value was very high. Conversely, when the *B. subtilis* B10 was co-inoculated with AMF, the DSI value dropped dramatically (Fig 1). The leaves of infected plants were chlorotic and had white fungal mass on some parts of the plants, which then formed basidiocarp before the plants dried (Fig 2).

**Effect of AMF and Mycorrhizal Endosymbiotic Bacteria *B. subtilis* B10 on Height of Seedlings and Root Dry Weight.** The effects of mycorrhizal endosymbiotic bacteria *B. subtilis* B10, AMF or co-inoculation of them on the growth parameters and the differences in plant responses to the microbial inoculation among oil palm seedlings were observed. The presence of root pathogen *G. boninense* reduced the height of seedlings (Fig 3). Inoculation of AMF, as well as *B. subtilis* B10, significantly increased the seedlings height compared to those without AMF. Interestingly, the tallest seedling (156.88 cm) was obtained when AMF was co-inoculated with *B. subtilis* B10. It suggested that the mycorrhizal endosymbiotic bacteria *B. subtilis* B10 contributed to sustaining the plants' growth in the presence of pathogen *G. boninense*. Generally, the un-inoculated seedlings (controls) challenged with *G. boninense* grew stunted and were the smallests (Fig 3). Oil palm seedlings inoculated with AMF and *B. subtilis* B10 showed no significant difference in the root dry weight compared

to the control seedlings (Fig 4). All seedlings had lower root dry weight when challenged with pathogenic fungus *G. boninense*, either inoculated with AMF and or *B. subtilis* B10. Application of mycorrhizal endosymbiotic bacteria *B. subtilis* B10 to the oil palm seedlings with neither AMF nor *G. boninense* increased root dry weight over uninoculated ones. However, the root dry weight decreased when this bacteria were co-inoculated together with AMF. It means that the combination of AMF and the bacteria might not be compatible in increasing root dry weight of oil palm seedlings when the pathogenic fungus *G. boninense* was present.

**Phosphorus Uptake of Oil Palm Seedlings Inoculated with AMF and Mycorrhizal Endosymbiotic Bacteria *B. subtilis* B10 in the Presence of *G. boninense*.** In general, the presence of fungal pathogen *G. boninense* reduced the uptake of phosphorus (P) by the seedlings at 52 weeks after planting, except when seedlings were co-inoculated with AMF and *B. subtilis* B10 (Fig 5). Inoculation of *B. subtilis* B10 alone significantly increased P uptake to 27.67 g/plant when the seedlings did not treated with *G. boninense*. However, the P uptake decreased to 20.73 g/plant when seedlings were challenged with *G. boninense*. The result suggested that mycorrhizal

A B

Fig 2 Comparison of healthy and infected oil palm. (A) Healthy oil palm seedlings; (B) Infected plants with the symptoms of basal stem rot caused by *G. boninense*. The infected plants generated chlorotic leaves, formed *G. boninense* fruiting bodies (yellow arrow) and the plants became dry.

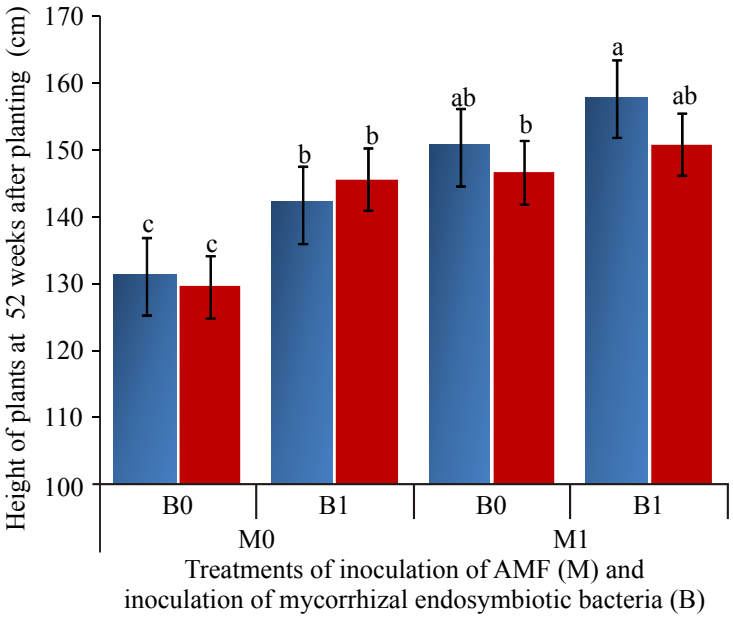


Fig 3 Height of plants at 52 weeks after planting, either challenged or not challenged by pathogen *G. boninense*. Means with the same letters between treatments are not significantly different (LSD 0.05). M0: without inoculation of AMF; M1: with inoculation of AMF; ■: without inoculation of *B. subtilis* B10; ■ : with inoculation of *B. subtilis* B10.

endosymbiotic bacteria *B. subtilis* B10 played an important role in improving P uptake. Interestingly, inoculation with AMF alone in the presence of *G. boninense* (M1B0G1) did not improve P uptake (14.64 g/plant). However,when the plants were treated with AMF together with mycorrhizal endosymbiotic

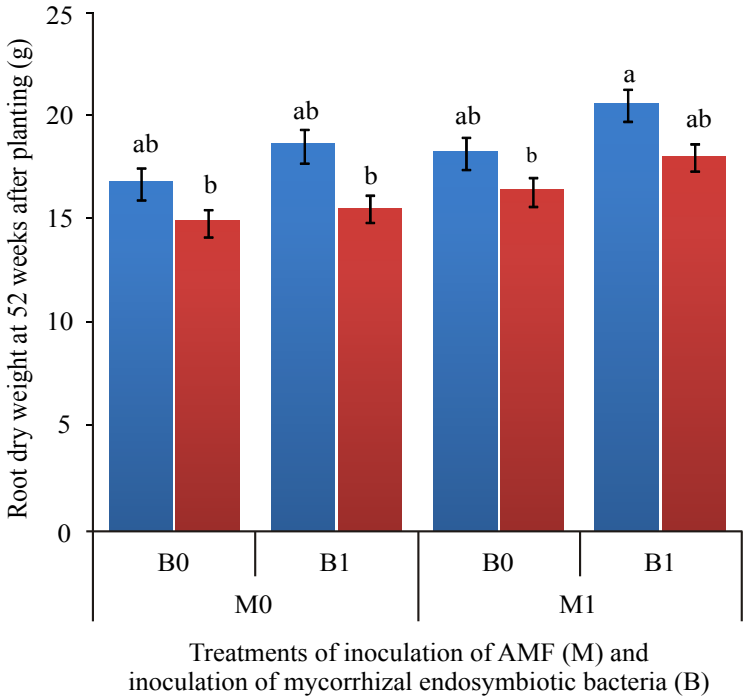


Fig 4 Root dry weight of oil palm seedlings at 52 weeks after planting, either challenged or not challenged by pathogen *G. boninense*. Means with the same letters between treatments are not significantly different (LSD 0.05). M0: without inoculation of AMF; M1: with inoculation of AMF; ■ : without inoculation of *B. subtilis* B10; ■ : with inoculation of *B. subtilis* B10.

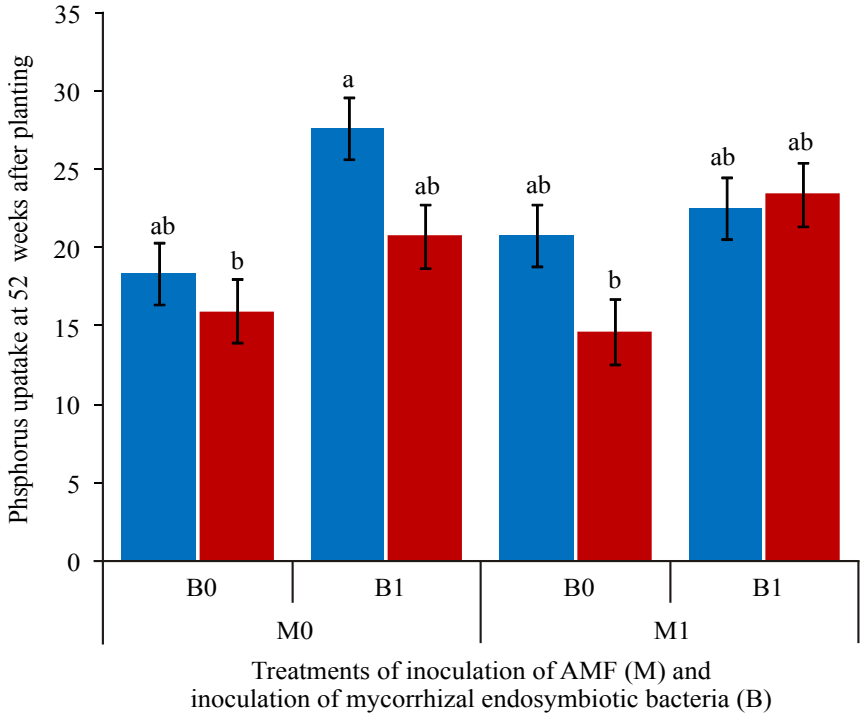


Fig 5 Phosphorus uptake by oil palm seedlings at 52 weeks after planting, either challenged or not challenged by fungal pathogen *G. boninense*. Means with the same letters between treatments are not significantly different (LSD 0.05). M0: without inoculation of AMF; M1: with inoculation of AMF; ■ : without inoculation of *B. subtilis* B10; ■ : with inoculation of *B. subtilis* B10.

bacteria *B. subtilis* B10 (M1B1G1), the amount of P uptake was increased (23.40 g/plant).

## DISCUSSION

The mycorrhizal endosymbiotic bacteria *B. subtilis* B10 used in this study were earlier shown to have an antagonistic activity towards the fungal pathogen *G. boninense* (Bakhtiar *et al.* 2010). We showed further by in vivo test of this bacteria in the presence of AMF on oil palm seedlings and revealed strong antagonism against *G. boninense*. The possible mechanisms responsible for this biocontrol activity includes competition for nutrients or colonisation sites (Johansson 2004), where siderophores play a role, niche exclusion (Bharadwaj *et al.* 2008), production of antifungal metabolites (Xiao *et al.* 2008) and several antibiotics (Raaijmakers and deSouza 2002), and induced resistance in plants (Nandakumar *et al.* 2001). In our study, the combination of AMF and *B. subtilis* B10 showed stronger suppression towards *G. boninense* compared to single application of AMF or *B. subtilis* B10 alone. In in vitro test, single application of *B. subtilis* B10 indicated strong suppression against *G. boninense.* However, the results of in vivo test showed that this bacteria alone could not increase plant adaptation to biotic stress of *G. boninense*, except when this bacteria was inoculated together with AMF (Fig 1). It might be because the bacteria *B. subtilis* B10 alone could not enter the root of oil palm seedlings unless it was together with AMF, which has the ability to infect the root of plants. Other possible reason for this, several factors will affect the ability of bacteria in giving the benefit to the plant. Some bacteria might give different results in in vitro and in vivo tests (i.e. when inoculated into plants). Saharan and Nehra (2011) reported that environmental factors (abiotic and biotic) may affect the growth and microbial effects of in the field. These environmental factors could be climate, weather conditions, character of soil, composition of indigenous microbes in the soil and competition among natural microbes in the soil. Cruz and Ishii (2012) also stated that the activity of probable endobacteria (PE) isolated from spores of AMF *Gigaspora margarita* in vitro, may not reflect their activity in the soils, especially for the bacteria living on the surface of the spore.

Our results suggest the possibility of integrated use of AMF and their associated bacteria in biological control of soil-borne pathogens. This finding is supported by Selim *et al.* (2005), who focused on a strain of *Paenibacillus* isolated from the rhizosphere of sorghum proven to be compatible with arbuscular mycorrhiza development but antagonistic towards soilborne fungal pathogens. This strain was found to produce small peptides which were responsible for the antagonistic effect against pathogens but were harmless to the symbiotic fungi.

In this study, the application of AMF together with mycorrhizal endosymbiotic bacteria *B. subtilis* B10 enhanced the height of oil palm seedlings but not root dry weight. The effects of co-inoculation on plants depend on the particular combinations used (Andrade *et al.* 1998; Barea *et al.* 1998). The interactions have ranged from the very compatible (producing positive effects), the less compatible (producing neutral effects), to the incompatible (producing detrimental effects), or demonstrated better results from single inoculations rather than dual inoculations (Vestberg *et al.* 2004). Based on our study, the combination of AMF and mycorrhizal endosymbiotic bacteria *B. subtilis* B10 produced positive effects on the height of oil palm seedlings but might be less compatible or neutral on root dry weight. It should also be noted that mycorrhizal association does not always improve plant productivity (Bhromsiri and Bhromsiri 2010). The competition for nutrients uptake by other microbes, may produce plant temporary nutrient starvation and result in detrimental impacts on the plant. These negative effects have occasionally been indicated, particularly when AMF and PGPR were used together (Raimam *et al.* 2007).

In our study, P uptake was improved when oil palm seedlings were inoculated with AMF and mycorrhizal endosymbiotic bacteria *B. subtilis* B10 together. The P uptake did not increase when AMF were inoculated alone without mycorrhizal endosymbiotic bacteria *B. subtilis* B10. Arbuscular mycorrhizal fungi increase host tolerance to pathogen by increasing the uptake of essential nutrients rather than phosphorus which are otherwise deficient in the non-mycorrhizal plants (Gosling *et al.* 2006). It is often brought to mind that AMF may improve P nutrition and enhance N uptake in their host plants. Rhizosphere microbes, such as N fixing bacteria or P solubilizing bacteria, may interact synergistically with AMF and thereby benefit plant development and growth (Johansson *et al.* 2004; Suparno 2009). This findings are supported by Jayasinghearachchi and Seneviratne (2005) that the solubilization of rock phosphate is enhanced by formation of mixed biofilms between phosphate-solubilizing saprotrophic fungi and a *Bradyrhizobium elkanii* strain. Taken together, these recent findings

strongly suggest that mycorrhiza-associated bacteria complement the roles of the external mycelium by mobilizing nutrients from minerals.

The present results showed that mycorrhizal endosymbiotic bacteria *B. subtilis* B10, which were isolated from AMF spore in the oil palm rhizosphere might be compatible with AMF in increasing oil palm height and phosphorus uptake, as well as showing an antagonistic effect against soilborne fungal pathogens *G. boninense*. Co-inoculation of AMF and mycorrhizal endosymbiotic bacteria *B. subtilis* B10 did not only reduce the percentage of basal stem rot incidence, but also significantly increase the plant height and phosphorus uptake of the oil palm seedlings. Our results suggest that the interaction between AMF and mycorrhizal endosymbiotic bacteria *B. subtilis* B10 in oil palm improves phosphorus uptake and plant growth, and protect the host against fungal pathogens.

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