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Arbuscular Mycorrhizal Fungi and Biochar Improved Early Growth of Neem (*Melia azedarach* Linn.) Seedling Under Greenhouse Conditions

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Abstract

The objective of this research was to determine the effect of biochar on the seedling quality index and growth of neem tree seedlings and arbuscular mycorrhizal fungi (AMF) development grown on ultisol soil medium. Two factors in completely randomised experimental design was conducted under green house conditions and Duncan Multiple Range Test was used to analyse the data. The results showed that neem seedling quality index was improved by interaction of AMF fungi and biochar amandment. The growth of neem seedling was significantly increased by interactions of arbuscular mycorrhizal fungi and biochar. The combination treatment of Glomus etunicatum and biochar 10% gave best results of height and diameter, and significantly increased by 712% and 303% respectively, as compared to control plant, while the combination treatment of Gigaspora margarita and biochar 10% gave the best result of shoot dry weight, and root dry weight and significantly increase by 4,547% and 6,957% as compared to control plant. The mycorrhizal root colonization was increased with increasing biochar added, but decreases when 15% of biochar was applied. N, P, and K uptake of 12 weeks neem seedling old was higher and significantly increased as compared to control plant.

Keywords: AMF development, nutrient uptake , plant growth , seedling quality index, biochar

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Introduction

Indonesia is known as a country with megadiversity, however, in the present moment Indonesian tropical deforestation has extremely become an important global environmental issue due to the Indonesian forest degradation rate which had reached to 1.7 million ha per year (MoF 2011). The negative impact of deforestation leads to loss of beneficial microorganism propagules, loss of soil organic matter, loss of biological diversity, increased soil erosion, decreased soil fertility, damage of wildlife habitats, degradation of watershed areas, and finally deterioration in the quality of life. Various efforts have been conducted to rehabilitate degraded forest through various efforts, such as development of Indonesia plantation forest and national movement of land and forest rehabilitation (GNRHL). However, the successful of such effort still low due to several major constraints, which are related to the unfavourable environmental conditions mainly the site condition which dominated by marginal especially ultisol soil as well as seedling quality used.

In Indonesia, ultisol soil cover 45,794,000 ha, almost 25% of total land surface which wide spread in Sumatera, Java, Nusa Tenggara, Sulawesi, Kalimantan, Maluku, and Papua (Subagyo *et al.* 2004; Budi & Christina 2013). Ultisol soil has the characteristics low in nutrient content, high acidity with Al and Mn toxicity, low organic matter and biodiversity and very low mineralization and nitrification (Kochian *et al.* 2004; Budi & Christina 2013). Commonly, the top soil of ultisol soil were also used as growing media in the nursery and become a major constraints to seedling growth and quality (Budi *et al*. 2012; Budi & Christina 2013).

Production of forest seedlings is one of the most important stages in the establishment of forest stands as it strongly affects forest yield (Binotto *et al*. 2010). A successful, high yielding stand is closely dependent on the quality of planted seedlings, which should be capable of resisting adverse field conditions (Binotto *et al.* 2010).

Many efforts have been applied to improve seedling quality grown in ultisol soil medium (Budi *et al*. 2012; Budi & Christina 2013). The application of charcoal to acid soil significantly increased diameter and heigh growth of *Accacia mangium* and *Michelia montana* and increase the pH, soil organic C, N, P, K, Ca, Mg, decreased CEC, Al^{3+} of the soil (Siregar 2007). The used of organic amendment and inorganic fertilization (Ahmadloo *et al*. 2012; Wei *et al.* 2012),

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bio-fertilizer (Khrisna *et al*. 2008; *a*Saravanan *et l.* 2012), and bioinoculants (Meenakshisundaram *et al.* 2011) for improving seedling quality and plant growth have been well documented.

Arbuscular mycorrhizal fungi (AMF) are symbiotic soil fungi that colonize roots of about 80% of vascular plants (Vierheirlig 2004) and is one of the soil microorganism that form essential components of sustainable soil-plant system (Hause & Fester 2005; Budi *et al*. 2012). They improve plant fitness and soil quality (Barea *et al*. 2002; Duponnois *et al.* 2005; Wu *et al*. 2010), increased plant uptake of P and N mainly in acidic soil (Goussous & Mohammad 2009; Guissou 2009; He *et al.* 2009; Rotor & Delima 2010; Budi & Christina 2013). They also produced plant growth hormones (Herrera-Medina *et al.* 2007), and defending root against some plant pathogens (Bachtiar *et al.* 2010). However, little information is known about interaction effect of AMF and biochar on *Melia azedarach* seedling quality and growth grown in ultisol soil.

The neem, *M. azedarach* Linn. (Meliaceae) are widely planted in West Java as a community forest (Yulianti *et al.* 2011), and it's a native trees of Asia including Indonesia (Orwa *et al.* 2009). This plant has a fairly coarse root system with very few root hairs which is a common characteristic of plant very responsive to arbuscular mycorrhizal symbiosis (Budi *et al.* 2012), therefore neem is one of the potential forest tree species for reforestation program.

The objectives of this study were to test the effect of biochar on AMF development and neem seedling growth and quality grown in ultisol soil medium under green house condition.

Methods

Soil and biochar Top soil (0–20 cm) was collected from Forest Research Experiment managed by Forestry Research and Development Agency (FORDA) in Jasinga West Java. Air dried and sieved (2 mm), and stored in the bag until used (Budi *et al*. 2012; Budi & Christina 2013). The physicochemical properties of soil were determined following standard method in Soil Laboratory, Faculty of Agriculture, Bogor Agricultural University (Budi & Christina 2013). The important chemical properties of soil medium are pH H_2O = 4.5, C-organic (Walkley & Black) = 2.64%, N-Total (Kjeldhal)= 0.24%; C/N = 11, P (Bray I) = 8.6, P (HCl 25%) = 206.6, and Al-dd = 1132 me 100 g^{-1}. Biochar was obtained from super market, break and sieved (2 mm) than stored until used. The physicochemical properties of biochar are pH H_2O = 8.9, C-organic (Walkley & Black) = 17.35%, N-Total (Kjeldhal) = 0.24%, C/N = 72.29, P (Bray I) = 1.5 ppm, P (HCl 25%) = 15.3 ppm and was determined in Soil Laboratory, Faculty of Agriculture, Bogor Agricultural University. The soil and biochar were autoclaved at 121 ˚C for 1 hour. The sterilized soil and biochar were mixed depending on the treatment and transfered to 15 × 20 cm polybag.

Seedling and AMF materials Seeds of *M. azedarach* were originally from the collection of Silviculture Laboratory, Faculty of Forestry, Bogor Agricultural University. Pre-sowing treatments of *M. azedarach* seeds were done according to the methode described by Azad *et al.* 2010). Pre-treated seeds were sown in plastic box containing sterilized sand and placed in green house for 1,5 month. AM spores of *Glomus etunicatum* and *Gigaspora margarita* were originally from the collection of Silviculture Laboratory, Faculty of Forestry, Bogor Agricultural University. Spores were surface sterilized according to the method described by Budi *et al.* (1999), spores (500–1000) were washed in sterile water by slow vortexing to remove loosely adhering particles, transferred to fresh sterile water, vortexed again, and rinsed with sterile water until this remained clear. They were then transferred using a sterile Pasteur pipette to a sterile Millipore filtering apparatus (Millipore SA) with a 0.8–mm filter. After a rinse with 50 ml sterile water, the spores where washed successively with 3 sterilizing solutions: (1) 96% ethanol, (2) a mixture of 2% Chloramine T (w v^{-1}), 0.02% streptomycin (w v^{-1}), 0.01% gentamycin (w v^{-1}) and 2 drops of Tween 20, (3) 6% calcium (Ca) hypochlorite (w v^{-1}), pumped through the Millipore column at a pressure of 5 hg cm^{-1} and stored in refrigerator until used.

AMF spores inoculation One and half months old uniform seedlings of *M. azedarach* seedlings were transplanted and inoculated with 50 surface sterilized AM spores in the planting hole depending on the treatment. Plants were grown for 12 weeks in the green house conditions (daily light intensity 2,330–2,500 lux) and watered as needed to maintain field holding capacity. No fertilizer was added during the experiment.

Harvesting and parameter measurement After 12 weeks establishment plants were harvested and analyzed for their height, diameters, and biomass production in term of shoot and root dry weight (dried in oven at 70 °C for 48 hour), shoot and roots ratio, and seedling quality index. N, P, and K concentrations were determined following standard procedure in Soil Laboratory, Faculty of Agriculture, Bogor Agricultural University. AM colonization were determined following the method described by Koske and Gemma (1989), after cleaned with 2.5% KOH, and stained with 0.05% trypan blue in acidic glycerol. Ten pieces of stained root segments were selected randomly from each root sample, and were arranged parallely to each other on a microscope slide and the mycorrhizal root infection (hyphae, vesicles, and abuscules) was determined microscopically with magnification of 100 ×. The root infection percentages (colonization) were calculated as a ratio of the infected to the total sections examined (Biermann & Linderman 1981). Seedling quality index was evaluated according to the method of Dickson *et al*. (1960).

Experimental design A 3 × 4 factorial experiment with 3 level of arbuscular mycorrhizal inoculation (inoculated and uninoculated with *G. etunicatum* or *G. margarita*), and 4 level of biochar soil amandment (control, and ultisol soil ammanded with 5, 10, and 15% biochar) was arranged in a completely randomized design in a polybag culture with 5 replicates. All data was analyzed by analysis of variance procedure (ANOVA) using SPSS 15.0 for Windows Software (SPSS Inc. USA). The difference between

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treatment means was assesed using duncan multiple range test (DMRT) at 5% level.

Results and Discussion

Effect of biochar on arbuscular mycorrhizal fungi development Present study demonstrated that mycorrhizal root colonization was increased with the increased of biochar amount added to the soil but decreased when 15% of biochar was applied (Table 2). There was no mycorrhizal colonization recorded in the non-inoculated seedlings. The ultisol soil amended with 5, 10, and 15% biochar increased the mycorrhizal root colonization of *G. etunicatum* by 204, 247, and 162%, respectively and 38, 103, and 62%, respectively, for *G. margarita* mycorrhizal root colonization. Those data demonstrated that there is negative effect of biochar on mycorrhizal development when applied in high dosage (15%). In these cases, it appears that the negative effect of the charcoal addition on AMF were largely due to nutrient absorbtion effect by the biochar (Warnock *et al.* 2007).

Several studies demonstrated that the addition of biochar materials in to soil corresponded with significant increases or decreases in plant root colonization by mycorrhizal fungi depend on the amount of biochar applied. Ishii and Kadoya (1994) found the enhancement of mycorrhizal root colonization by 540% when the river sand medium was mixed with 2% biochar. In ectomycorrhizae fungi Harvey *et al.* (1976) reported that root colonization was increased by 2,900% when biochar was mixed with a montana forest soil. The possible mechanisms positive effect of biochar on mycorrhizal development are (1) alteration of soil physico-chemical properties, (2) indirect effects on mycorrhizae through effects on other soil microbes, (3) plant–fungus signaling interference and detoxification of allelochemicals on biochar, and (4) provision of refugia from fungal grazers (Warnock *et al.* 2007). In contrast to those data showing positive effects of biochar on mycorrhizal fungi Rondon *et al.* (2007) demonstrated the decreased of mycorrhizal root colonization by 38% when biochar from *Eucalyptus deglupta* was apllied to the soil in the rate of 6%. It has been previously shown that the AM root colonization affected by soil P avaibility, as stated by Vierheirlig (2004), that mycorrhizal root colonization is reduced or absent in plants with a high P status, whereas in low P levels enhanced root colonization. Smith and Read (2007) reported that mycorrhizal root colonization correspond with leakage of plants excudates to the soil and linked with P status. Plants grown at hight P condition resulting decrease of the cell membran permeability and in turn resulting in a lower leakage of amino acids and sugar. The biochar used in this study not only contain high available P but also high pH that could contribute to the increase of soil pH and soil P avaibility and in turn decrease mycorrhizal root colonization when applied in excess dosage (>15 %). Data on Table 1 also demonstrate that there are different responses of *G. etunicatum* and *G. margarita* to biochar application.

Effects of arbuscular mycorrhizal fungi and biochar on *M. azedarach* growth The *M. azedarach* seedlings inoculated with AMF *G. margarita* had higher height, diameter, shoot and root dry weights, and shoot roots ratio than control plants regardless of biochar levels added, in the contrary those plants inoculated by AMF *G. etunicatum* showed lower height and diameter than control plant (Table 2), meaning that there is different effectitivity between *G. etunicatum* and *G. margarita* on *M. azedarach* growth, and in line with previously studied (Cavagnaro *et al.* 2001). *M. azedarach* inoculated with *G. margarita* significantly had higher height, diameter, shoot and root dry weight than plants grown in soil medium amended with 5, 10, and 15% of biochar (Table 2), and their height had much greater 291, 336, and 147%, respectively and 244% much higher than control plants (Figure 1). Earlier studies also showed such a trend for tropical and non tropical plant growth (Duponnois 2005; Marco *et al.* 2006; Arpana & Bagyaraj 2007; Nagarathna *et al.* 2007; Ortas 2010; Budi *et al*. 2012; Budi & Christina 2013), due to the improvement of soil nutrient absorption. Soil used in this study was characterized by P deficiency due to high (= 1132 me 100 g^{-1}) Aluminium (Al-dd). In such soil characteristic, the P uptake by diffusion alone often exceeded by plant P demand that create depletion zone in the rhizosphere (Junk & Claasseen 1989). In this study showed that mycorrhizal seedlings absorbed more N, P, and K than non mycorrhizal seedlings (Table 3) indicating the functional aspect of mycorrhizae in absorbing nutrient from soil and support the previous finding results (Budi & Christina 2013).

Biochar amendment significantly increased height, diameter, shoot dry weight, root dry weight of 12 weeks *M. azedarach* seedling when 15% of biochar added regardless AMF inoculation (Table 2). The plant height, diameter, shoot and root biomass was increased by 39, 54, 209, and 336% when soil amended by 15% of biochar respectively as compared to control plants (Table 2). Biochar is anorganic product obtanied by incomplete combustion of natural organic materials. Several study demonstrated that the application of biochar to soil could increase in the availability of major cations and phosphorus as well as in total nitrogen concentrations (Glaser *et al.* 2002; Lehmann *et al.* 2003). In this study biochar soil amendment 15% had much greater nutrient N, P, and K uptake than those plants inoculated by AMF *G. etunicatum* alone but lower as compared to the plant inoculated by *G. margarita* indicating there are different efectivities between *G. etunicatum* and *G. margarita* (Table 2), and this finding confirms previous study (Cavagnaro *et al.* 2001; Karandasho & Bucher 2005). Nutrient N, P, and K uptake was increased in non-inoculated mycorrhizal plant grown in soil medium amended with 15% biochar, indicating increasing nutrient avaibility absorbed by the plant and in turn increased their growth and development. Numerous studies demonstrated that the application of biochar as soil amendment could increase the soil pH and CEC (Mikan & Abrams 1995; Topoliantz *et al.* 2005), there by influencing the reaction and transformation of nutrient avaibility particularly P for plant growth and development. Phosphorus is a major macronutrient for all organisms and serves multiple functions as a key structural element in nucleic acids, phospholipids, and several enzymes and coenzymes, in addition it is involved in energy metabolism, activation of metabolic intermediates, signal transduction cascades, and enzyme regulation (Karandashov & Bucher 2005), and constituting up to 0.2% of the dry weight of the plant cell (Shtark *et al.* 2010). Soil used in this study was characterized by low pH (4.5), P deficiency due to high (= 1,132 me 100 g^{-1}) Al-dd, whereas the biochar has high pH

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Table 1 The effect of biochar on arbuscular mycorrhizal root colonization

Mycorrhizae	Biochar % (v v^{-1})	% Mycorrhizal roots colonization
Control	0	0.0a
Glomus etunicatum	0	9.99b
	5	30.33cd
	10	34.67c
	15	26.13c
Gigaspora margarita	0	25.0c
	5	34.5d
	10	50.67f
	15	40.4e

Table 2 The effect of biochar and arbuscular mycorrhizal fungi on the growth of neem 12 weeks after planting

Mycorrhizae	Biochar % (v v^{-1})	Height (cm)	Diameter (cm)	Shoot dry weight (g)	Roots dry weight (g)
Control	0	3.3a	1.04a	0.047a	0.028a
	5	3.2a	1.16a	0.047a	0.033a
	10	3.4a	1.16a	0.048a	0.037a
	15	4.6b	1.6b	0.145b	0.122b
Glomus etunicatum	0	3.1a	1.02a	0.032a	0.032a
	5	16.46d	3.42c	0.763c	0.699c
	10	26.8f	4.4e	1.993de	1.041d
	15	20.0e	3.62cd	1.687d	0.942d
Gigaspora margarita	0	11.34 c	2.84c	0.717c	0.641c
	5	20.2e	3.9d	1.803d	1.768e
	10	23.86ef	4.12e	2.184e	1.976e
	15	21.6e	3.36cd	1.993de	1.911e

Means followed by the same letter (s) are not significantly different at $p < 0.05$, duncan's multiple range test.

Table 3 N, P, K nutrient uptake by neem seedling

Mycorrhizae	Biochar % (v v^{-1})	Nutrient uptake (g $plant^{-1}$)		
		N	P	K
Control	0	0.84b	0.07a	1.53b
	5	0.64a	0.04a	1.17a
	10	0.79b	0.05a	1.21a
	15	4.72c	0.25b	4.99c
Glomus etunicatum	0	0.64a	0.05a	1.1a
	5	13.75d	1.65c	22.57d
	10	64.28g	5.02f	88.57h
	15	45.85f	3.91d	84.91g
Gigaspora margarita	0	18.16e	1.43c	28.96e
	5	46.69f	3.45d	75.85f
	10	54.46h	4.21e	86.43g
	15	59.05i	4.43e	77.12f

Means followed by the same letter (s) are not significantly different at $p < 0.05$, duncan's multiple range test.

(8.9) that could increase the pH of soil medium and increased nutrient avaibility.

The effect of biochar and AMF on *M. azedarch* growth depend on the ammount of biochar added and AMF species used. Interaction between biochar and AMF both *G. etunicatum* and *G. margarita* significantly increased plant height, diameter, shoot dry weight, and root dry weight and was much greater than control plant (Table 2). Interaction between AMF with biochar at the rate of 5% and 10% increased plant height, diameter, shoot dry weight, and root dry weight but at the rate of 15% decreased plant height, diameter, shoot dry weight, and root dry weight as compared to plants wether inoculated by AMF or treated by biochar at the rate of 15% alone (Table 1). There were synergetic effect between biochar and mycorrhizal fungi *G. etunicatum* and *G. margarita*, as shown in Table 2, the interaction between biochar and both fungi increased all parameter observed, and the best results were shown by interaction of *G. etunicatum* and biochar in the amount of 10%, which increased plant height and diameter by 712 and 323%, respectively. The N, P, and K uptake of mycorrhizal plant *G. etunicatum* amended with biochar at the rate of 15% was lower than mycorrhizal plant amended with biochar at the rate of 10%, indicate that nutrient avaibility especially P in soil amended by biochar was high and in turn decrease the mycorrhizal function as previously discussed. In addition, the ammount of biochar added also influence to the amount of beneficial microorganism in the soil (Ezawa *et al.* 2002). The pore of biochar will serve a protective haven for billions of beneficial microorganism, and plant roots may grow

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adjacent to and possibly into these lumps of biochar allowing these roots to obtain nutrients within the lumps of biochar (Warnock *et al.* 2007). Physically the diameter of biochar pore size less than 16 µm (Kawamoto *et al.* 2005; Hockaday *et al.* 2007) while the diameter of bacteria between 1–4 µm (Swift *et al.* 1979), and the bacteria can grow well in side the pore and protected from predator againts like ant and protozoa (Ezawa *et al.* 2002). Those beneficial microorganism could contribute in increasing plant growth and development. Mycorrhizal plant could have dense extramatrical hyphae extend up to 25 cm from the root (Smith & Read 2007), that facilitate in increasing the volume of soil explored for nutrient that are not accessed by root.

Effects of arbuscular mycorrhizal fungi and biochar on *M. azedarach* seedling quality index This research demonstrated that the seedling quality index of *M. azedarach* was improved by application of biochar to ultisol soil growing medium (Figure 1). Seedling quality is defined as "fitness for purpose" (Mattsson 1996). Several variables were used to evaluate sedling quality which were grouped in 2 atributes: (1) material atributes that consist of morphology, bud dormancy, water relations and nutrition and can be rapidly assessed by any number of direct or indirect methods and (2) performance attributes that consist of frost hardiness, vigour and root growth potential that are assessed by subjecting whole seedlings to certain environmental regimes and evaluating their growth response (Mattsson 1996).

Dickson *et al.* (1960) developed the dickson quality index (DQI), is a simple tool to evaluate seedling quality that combined several parameters growth including total dry matter, shoot height (*H*), stem base diameter (*D*), shoot dry matter (*SD*), and root dry matter (*RD*), and expressed as a shown in Equation *[1]*:

$$QI = \frac{\text{Total seedling dry weight (g)}}{\frac{H\ (\text{cm})}{D\ (\text{mm})} + \frac{SD\ (\text{g})}{RD\ (\text{g})}} \qquad [1]$$

note: *QI*= the seedling quality index

Previous study indicated that seedling quality index can be improved by several treatments. Bayala *et el*. (2009) demonstrated that 5 seedlings of *Acacia angustissima* (Mil.) Kuntze*, A mangium* Wild, *Gliricidia sepium* (Jacq.) Alp.*, Leucaena* hybrid (L × L)*,* and *L. leucocephala* (Lam.) de Wit increased their quality index by application of manure to normal growing medium. The seedling quality index of *Cupressus arizonica* var arizonica *Greene* and *C. sempervirens var. horizantalis* (Mill.) Gord were improved by addition of cattle manure and decomposed litter to soil medium (Ahmadloo *et al.* 2012). The used of organic amendmend and inorganic fertlizer also improved the seeedling quality index of *Larix olgensis* Henry.

Several studies on the use of microorganisms for improving seedling quality index were well documented. The application of *Azospirillum*, phosphorus solubilising bacteria, and *Azatobacter* could increased seedling quality index of medicinal plants (Khrisna *et al.* 2008). Saravanan *et al.* (2012) used *Azospirillum*, frankia, and phosphorous solubilising bacteria for improving seedling quality index of *Casuarina equisetifolia* (Forst.). This experiment demonstrated that arbuscular mycorrhizal fungi *G. margarita* can improved significantly the seedling quality index of *M. azedarach*. As shown in Figure 1, AM fungi *G. etunicatum* was not affect the seedling quality index, but when combined with biochar in all level dosage significantly increased seedling quality index of *M. azedarach*, indicating

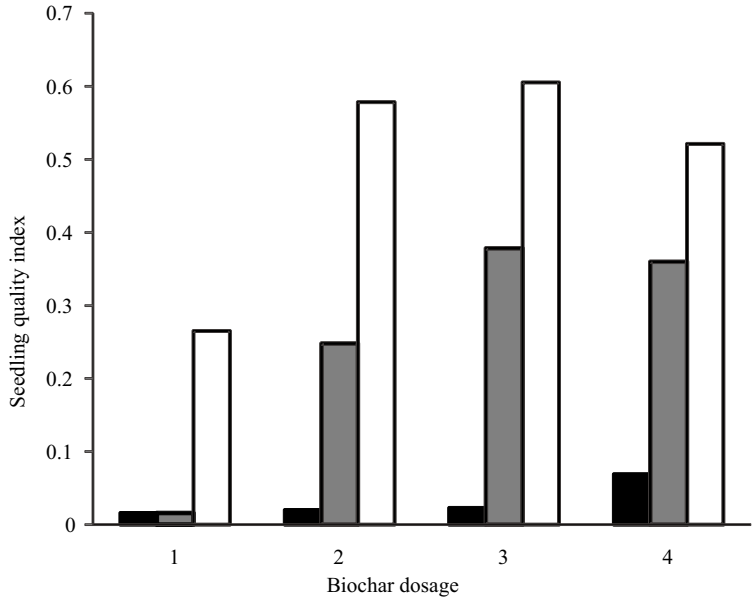

Figure 1 Seedling qualityi index as affected by arbuscular mycorrhizal fungi and biochar dosage (1= control; 2 = 5%; 3 = 10%; 4 = 15%). Control (■), G. *etunicatum* (▣), G. *margarita* (□).

there is a synergetic effect between biochar and AMF to improve seedling performance. *Gigasporaceae* are known to invest more extensively to extra radical mycelia growth (Hart & Reader 2002), and this could have resulted in improved nutrient acquisition, as indicated in Figure 1, *G. margarita* was more effective than *G. etunicatum* and was consistent with previous results (Budi *et al.* 2012; Budi & Christina 2013).

As previously disscussed that interaction between biochar and AMF significantly increased heigh, diameter, shoot dry weight, and root dry weight due to improvement of soil growing medium and better absorption of mineral nutrition (Table 2), consequently also can improved seedling quality index. This suggest good potential for growth and survival in the field.

Conclusion

This study clearly shows an efficient biological response of *M. azedarach* seedlings towards different rate of biochar added and AMF used, with the rate of 15% conferring greater benefits compared to all other doses used regadless AMF incoculation in obtaining best seedlings performace, but when combined with AMF, the rate of 10% was the best one. AMF *G. margarita* clearly shown best results compared biochar at all level dosage added and *G. etunicatum* and *G. margarita* had synergetic effect with biochar for increasing seedling performance. This findings reveal the prospective and potential use of *M. azedarah* inoculated by arbuscular mycorrhizal fungi combined with biochar for the succesful of forest land rehabilitation as well as post mine land rehabilitation which were characterized by low soil fertility.

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