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Role of arbuscular mycorrhizal fungi (AMF) in cocoa (*Theobroma cacao* L.) seedlings growth

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ABSTRACT: Arbuscular mycorrhiza (AM) fungi are mutualistic symbiotic with plant roots of around 80% vascular plants, including corn and cocoa. This study aimed to identify and inoculate of AM fungi to cocoa seedlings growth. Cocoa seedlings were planted on polybag with sterilized peat-moss for 20 weeks in green house. Two species of AM fungi, *Glomus constrictum* and *Acaulospora spinosa* were identified by using morphological and molecular markers. Results of molecular method showed that PCR technique with ALF01 and NDL22 primers were detected in all isolates with sequence analysis around 350bp PCR band. Interaction between AM fungi and low phosphate fertilizer had significantly effect on growth of cocoa seedlings concerning to seedling height, leaves number and stem diameter. *Glomus constrictum* and 2 g of NPK showed higher response in all parameters than without AM fungi and NPK fertilizer.

Keywords: identification, arbuscular mycorrhizal fungi, cocoa seedling

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Introduction

The cocoa (*Theobroma cacao* L.) bean is one of the most important agricultural export products of Indonesia which the third largest cocoa producer in the world after the Ivory Coast and Ghana. Indonesia's cocoa output is produced around 90% by smallholder farmers and productivity has decreased significantly in some areas. Therefore, development of cocoa plant growth needs to get attention for to be sustainable agriculture. Witjaksono (2016), concluded that intensification of cocoa should meet productivity, profitability, feasibility, environmentally friendly and compromise social economic forces.

The production of cocoa is low because of several factors such as aging trees, crop losses from pest and diseases, soil degradation, unmeasured an ineffective chemical fertilizer and limited skilled farmer (Darma and Soviana, 2014). The main problem is many cocoa trees have passed their most productivity age. One of an effective way of improving quality of cocoa is to provide seedlings to farmer through produce seedling with mycorrhizal fungi. Gianinazzi and Gianinazzi-Pearson (1986) explained that arbuscular mycorrhizal (AM) fungi are symbiotic associations formed between the members of phylum *Glomeromycota* and the roots of most terrestrial flowering plants. Important roles of AM fungi in natural and managed ecosystems are benefits to plant nutrient supply through mycorrhizal roots, antagonisms of parasitic organisms and non-nutritional benefit due to water relation (Brundrett et. al., 1996). Cocoa is one of plant has symbiotic mutualism with AM fungi (Miyakasa and Habte, 2001).

The method inoculating plant with AM fungi is to induce early infection in seedlings or cutting by increasing soil infectivity, particularly around the young emerging roots (Mosse, 1991). Therefore, inoculation AM fungi spores were effective in the cocoa seedlings and use the low phosphate fertilizer because the addition of low phosphate to agricultural soils has been used for improving plant growth and 9nutrition (Vassilev et al., 1996). Growth increases of AM plants over non AM plants counterparts are maintained if high total soil phosphate is poorly available (Bolan, 1991). The objectives of the research were to identify of AM fungi based on morphological and molecular markers and to study the effectiveness of low phosphorus fertilizer and AM fungi to cocoa seedlings growth.

Materials and methods

Morphological Markers

Nine species of AM fungi spores, *Glomus constrictum*, *Glomus* sp. No.1, *Glomus* sp. No.2, *Acaulospora spinosa*, *A. scrobiculata*, *A. laevis*, *A. delicatata*, *Scutellospora* sp. No1, were examined from weed rhizospheres (five kinds of weeds as *Vernonia cinerea*, *Euphorbia geniculate*, *Chloris barbat*a, *Hewittia sublobata*, *Tridax procumbens*) and selected two species of AM fungi, *G. constrictum* and *A. spinosa* which have high number and colonization by using trapping culture on corn were inoculated in cocoa. Soil samples were collected from corn and cocoa seedlings which grown on sterilized peat-moss under greenhouse condition.

Isolation of AM fungal spores from the soil samples was applied by using wet sieving and decanting method (Gerdemann and Nicolson, 1963). The spores were mounted with polyvinyl alcohol-lactic acid-glycerol (PVLG, 100 ml distilled water, 100 ml lactic acid, 10 ml glycerol and 16.6 g polyvinyl alcohol) and Melzer's solution (100 g chloral hydrate, 100 ml distilled water, 1.5 g iodine, and 5 g potassium iodide) on glass slides. The spores were observed by using a compound microscope of 10x-40x magnification under Olympus CX31 microscope based on spore size, color, wall structure and hyphal attachment (Schenck and Pérez, 1988; Morton and Benny, 1990), description in Brundrett et. al (1996) and information from the INVAM website (http://www.invam.caf.wvu.edu). The spores were counted under Olympus SZ51 microscope. The spores and roots pictures were taken by using Dino eye-piece camera 2.0.

The root samples were rinsed thoroughly with running tap water several times. Roots were cut into 1 cm, segments for cleaning by using 10% KOH then 5% HCl and staining with trypan blue solution (1 L glycerol, 950 ml distilled sterilized water, 50 ml acetic acid, and 0.2 g trypan blue). Colonization of root segments were observed for the presence or absence of functional structures (mycelium, vesicles and arbuscules) of AM fungi. Percent colonization was calculated using the

following formula:

$$\% \text{ Colonization} = \frac{\text{Total number of root segments colonized}}{\text{Total number of root segments observed}} \times 100$$

Molecular Markers

AM fungi spores were selected for DNA isolation from each isolate. The spores were surface sterilized using 0.1% (v/v) Tween 20 for 10 min and then thoroughly rinsed with sterilized distilled water. 2 µl 1xTE Buffer (1M Tris HCl pH 8.0, 0.5 M EDTA pH 8.0 and sterilized distilled water) was added into PCR tube and placed one spore then crushed it and added 3 µl 1x TE Buffer. The samples were incubated at 90°C for 10 min then centrifuged and taken 2 µl of supernatant (DNA template) for PCR step.

A primer was designed to amplify a 460-bp region of the variable D2 region of the LSU with primers ALF01/NDL22 (Clapp et al, 2001). PCR reaction mixtures contained 2 µl DNA template, 1 µl of each primer (10 µM), 10 µl 2x Phire Plant Direct PCR Master Mix (Thermo Scientific), and 6 µl sterile distilled water. The amplification was carried out using PCR machine (Biometra Thermocycler TProfessional Basic, Canada) programmed as follows: initial denature cycle at 98° C (5 min), annealing at 59°C (5 sec), extension at 72°C (20 sec) followed by 40 cycles of denature at 98°C (5 sec), annealing at 59°C (5 sec), and extension at 72°C (20 sec); the last cycle was a final extension at 72°C for 10 min. Amplification products were separated by gel electrophoresis on 1.2% agarose gel for 90 min at 80 V in 1x TBE buffer (Tri base, Boric acid, 0.5 M EDTA, distilled water) and PCR products were analyzed after 45 min run in 1x TBE. Also**,** sequencing was analyzed by First Base Laboratories, Malaysia

Inoculation AM Fungi to Cocoa Seedlings

Experiment was designed on randomized completely block design with two factors. First factor is mycorrhizal spore treatment (without mycorrhiza, with mycorrhiza spore of *Glomus constrictum* and *Acaulospora spinosa*). Second factor is level of NPK fertilizer i.e. 1 g and 2 g fertilizer in each pot and without fertilizer. Cocoa seedlings grown on sterilized peat-moss of greenhouse condition. The two species of AM fungi were inoculated at fifty spores into the roots cocoa seedlings then put NPK 19:9:20 fertilizer at 14 days after planting. Data were collected start from 1 to 4 months after inoculation (MAI). The parameters were examined such as plant height (cm), leaves number and stem diameter (cm).

Statistical Analysis and Data Processing

The data were analyzed by using analysis of variance which were compared by Tukey HSD test at 0.05 level if the treatment showed significant effect and graph with mean. All the data were analyzed using SPSS version 20.

Results and discussion

Morphological identification

Identification of AM fungi was determined using morphological markers based on color, size and ornament of spore. Morphology characteristics of spore were observed and distinguished from genera and species of AM fungi, then identified as two species of AM fungi, *A. spinosa* (**Figure 1 and Table 1**) and *G. constrictum* (**Figure 2 and Table 1**).

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Table 1 Characteristics of the AM fungal taxa found in the root of corn and cocoa by using morphological characteristics at 3 MAI

AMF species	%Colonization in root		Characteristics of spore
	Corn	Cocoa	
Acaulospora spinosa	87	65	Globose, range from 100-335 µm in diameter, yellow to orange, two-three layers of spore wall, combined thickness 4-10 µm, sporiferous saccule present (120-135µm).
Glomus constrictum	70	58	Globose, 150–330 µm, dark brown to dark, one-two layers of spore wall, combined thickness 7-15 µm.

Morphological characteristic including variations in size, shape, wall thickness, wall layer, position and abundance, hyphal branching patterns, the diameter and structure of hyphae (especially near from entry-points), and the staining intensity of hyphae (dark or faint). Brundrett et al. (1996) has been reported that characteristic root colonizing patterns which can be used to identify different genera of Glomalean fungi. The genera of *Glomus* relatively straight hyphae ramify along the root cortex (root anatomy permits), often producing 'H' branches which result in simultaneous growth in two directions. Staining of these hyphae is branching patterns. Hyphae in the outer cortex generally are more irregularly branched, looped or coiled than for *Glomus.* Internal hyphae are thin-walled, often stain weakly and thus may be very hard to see.

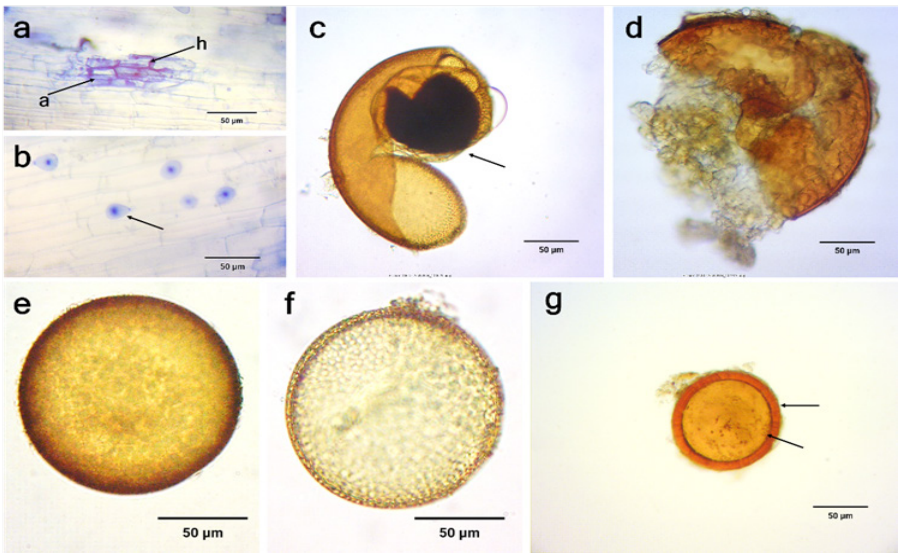

Figure 1 Characteristics of Acaulospora spinosa (a) colonization of hyphae and arbuscules (b) vesicles (c) ornaments of sporiferous saccule and (d-g) spore walls.h = hyphae and a = arbuscules

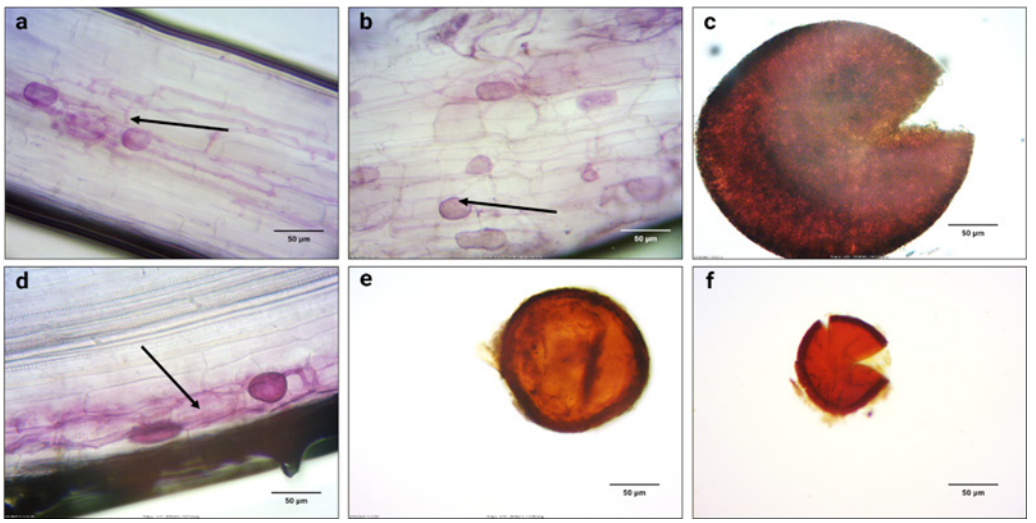

Figure 2 Characteristics of *Glomus constrictum* (a) colonization of hyphae, (b) vesicles and (d) arbuscules, and (c, e, and f) spore walls

They are often made more conspicuous by rows of lipid droplets. Intracellular oil-filled vesicles, that are initially rectangular, but often become irregularly lobed due to expansion into adjacent cell, are a characteristic feature of most isolates. These vesicles have thin walls and do not persist in roots. usually relatively dark. Oval vesicles, which usually from between root cortex cells, are present in some cases. These vesicles persist in the roots and often develop thickened and/or multilayered walls. Whereas the genera of *Acaulospora* entry-point hyphae have characteristic

The diversity of AM fungi has significant ecological consequences because individual species or isolates vary in their potential to promote plant growth and adaptation to biotic and abiotic factors. Schuessler et al. (2001) explained that diversity of AM fungi within the group of fungi that are usually present in all soils from the phylum Glomeromycota. The symbiosis is called "arbuscular" because all the fungi involved form specialized tree-like structures (arbuscules = tree-like) inside root cells. Other structures produced by fungi are intra and extra radical spores which are germinating structures, formed on the extra radical hyphae (some species also may form spores inside the roots), intra and extra radical hyphae, for some genera, intracellular fungal storage structures called vesicles and auxiliary cells branching from extra radical hyphae. Thus, structure give benefits to plant (Brundrett et. al., 1996).

Molecular Markers

Identification of AM fungi by using molecular technique using DNA extraction and Polymerase Chain Reaction (PCR). Many molecular techniques have been used to study the AM fungi, improving the knowledge on phylogenetic, cytogenetic, functional and ecological aspects and the selection of one or more techniques is a function of the research objective (Renker et al., 2006; Dickie and Fitz John, 2007; Gamper and Leuchtmann, 2007). Approaches polymerase chain reaction (PCR)-based on seem to offer the best current prospects for detecting most of the AM fungi present in an ecosystem.

The majority of DNA extracted from AM fungi spore is of plant origin and, therefore, specific PCR primers must be used in order to obtain fungal DNA fragments. Using universal primers that are functional with nearly all eukaryotes (Harney et al. 1997) does not appear to be a useful approach. Specific primers are also of great advantage for PCRs from spores. As the *Glomales* cannot be cultivated axenically, their spores harbor numerous other organisms (Clapp et al. 1999). In this research, DNA extraction method was used with one spore of AM fungi, PCR amplification was successful using ALF 01 and NDL 22 primer pairs tested.

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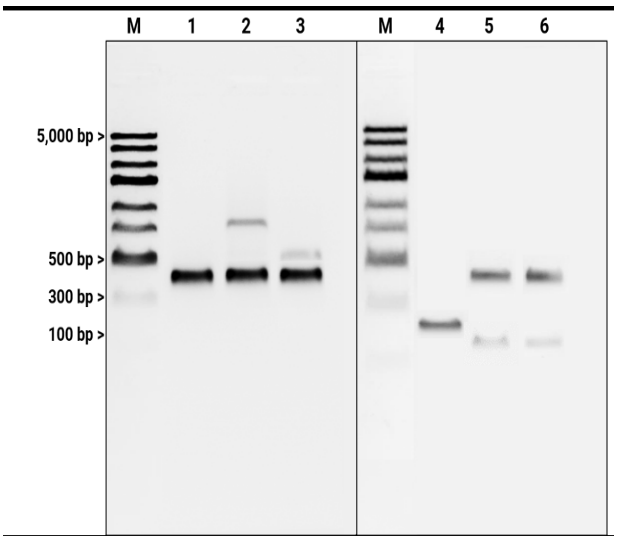

Figure 3 Result of PCR amplification of genomic DNA from using the universal primer ALF01 and the specific primer NDL22; Lane 1-3. PCR product of Arbuscular Mycorrhizal fungi, Lane 1 = *Glomus* sp. Isolate 1 Lane 2 and 3 = *Acaulospora* sp.

PCR technique with ALF01 and NDL22 primers were detected in all isolates with sequence analysis around 350bp PCR band. (**Figure 3**). Comparison of sequencing to NCBI databast revealed that *Glomus* sp. and *Acaulospora* sp. were identified as *G. constrictum* and *A. spinosa*, respectively.

This study had continued to enable characterization and identification of the species of AM fungi by means of molecular techniques. The most valuable species of arbuscular mycorrhizal fungi that colonize the roots of cocoa in nature will be used in fruit-growing practice, in particular those species and strains that improve plant nutrition and growth, plant yield and plant quality.

Inoculation of AM Fungi to Cocoa Seedlings

Preliminary test of colonization in the greenhouse was examined and found that *G. constricum* in the root of cocoa seedlings were higher than *A. spinosa* because *G. constricum* inoculation helps plant root to uptake nutrients. AM colonization, and hence the potential operation of the AM pathway, occurs behind the root apex. P is translocated rapidly to the roots (probably as polyphosphate), overcoming the slow diffusion that occurs in the soil solution.

Inoculation of AM fungi and low phosphate fertilizer in the cocoa seedlings not significantly in all the growth of parameters such as plant height, leaves number and stem diameter on 1 and 2 MAI whereas all parameter showed the significant effect after 3 and 4 MAI (Table 3). *G. constrictum* and 2 g of NPK fertilizer were more effective to increase the growth of cocoa seedling than other treatments. Application of NPK fertilizer increased the seedling growth of cocoa and AM fungi inoculation was higher results than others (**Table 4**).

Table 3 Significant effect of low phosphate fertilizer (19:9:20) and AM fungi inoculation on height, leaves number and stem diameter and of cocoa seedlings from 1 to 4 MAI

Duration (MAI)	Height (cm)	Leaves number	Stem diameter (mm)
1	ns	ns	ns
2	ns	ns	ns
3	*	*	*
4	*	*	*

Note: ns = non significant, * = significant

Table 4 Effect of low phosphate fertilizer (19:9:20) and AM fungi inoculation on height, leaves number and stem diameter and of cocoa seedlings on 4 MAI

Treatment	Height (cm)	Leaves number	Stem diameter (mm)
NPK dosage (g.seedling^{-1}):			
0	33.06 c	9.04 c	6.99 c
1	34.08 b	9.87 b	7.86 b
2	35.86 a	11.03 a	8.62 a
HSD test (95%)	0.967	0.439	0.421
AMF inoculation :			
Without AMF	33.06 C	9.08 C	6.02 B
Acaulospora spinosa	34.08 B	9.80 B	6.23 A
Glomus constrictum	36.86 A	11.06 A	6.65 A

Note: The number in one column followed by the same letter is not significantly different in HSD Test for 95%.

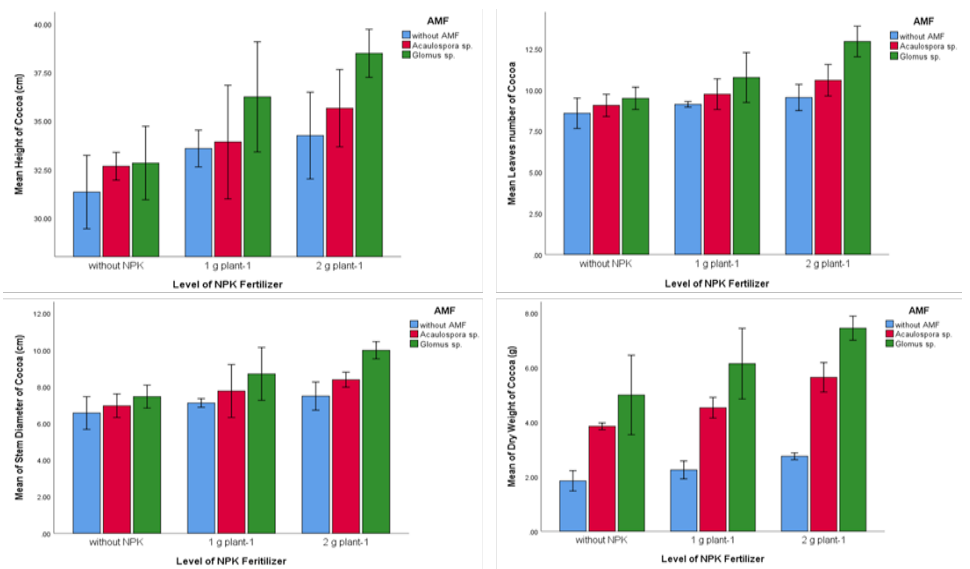

Figure 4 Mean of cocoa seedlings growth in NPK (19:9:20) and AMF inoculation treatments to seedling height, stem diameter, leaves number and dry weight

Mean of cocoa seedlings growth such as height seedling, leaves number, stem diameter and dry weight affected by NPK fertilizer and AMF inoculation treatments (Figure 4). They have significant effect showed the number which when fertilizer such as low phosphorus is one of the diffusion limited major nutrient, which is essential for plant growth. It has a defined role in plant metabolism such as cell division, development, photosynthesis, nutrient transport within the plant, transfer for genetic characteristic and regulation of metabolic pathways (Theodoru and Plaxton, 1993).

Dry-weight of cocoa seedlings with NPK fertilizer and mycorrhizal treatments stimulated significantly the production of biomass. Phosphorus (P) is important macronutrient for plant, making up about 0.2% of dry weight plant. P uptake by plants from soil is also necessary for nutrition transport (Schactman et al. 1998). Arbuscular mycorrhizal fungi had positive effects on growth, biomass production and nutrients concentrations analyzed in cocoa seedlings four months after AMF inoculation. The AMF positive effect was likely attributed to the improvement of nutrient assimilation and water uptake (Sweatt and Davies, 1984; Bethlenfalvay et al., 1988) and to the increase of root length density (Bryla and Duniway, 1997). Similar results have been reported for other plant species (Al-Karaki et al., 2004), who observed that the inoculation with AM fungi provided an important enhancement to yield of two wheat cultivars.

The enhancement in cocoa growth due to AM fungi might be attributed to higher mineral and water uptake. The inoculation of cacao seedlings in the nursery with efficient vesicular arbuscular mycorrhizal (VAM) fungi is a desirable objective because it could promote faster growth and hence a shortening of this stage, usually 4-5 months. Inoculation could also benefit transplant survival because there is a good correlation between the cacao seedling growth rate and vigor, and their survival after transplanting. Azizah and Ragu (1986), reported that mycorrhizal inoculation to cocoa seedlings give a positive response with the significantly increased compared without inoculated seedlings. Benefits AM fungi to the plant as a result of such an interaction with a soil fungus include increased mineral nutrient absorption, water-use efficiency, and disease resistance, and the interaction may also facilitate plant-to-plant communication (Azcon-Aguilar and Barea, 1997; Parniske, 2008).

Mycorrhizal treatment plant roots absorb phosphorus from the soil solution is much faster than the rate in which phosphorus moves in solution by diffusion. This results in a phosphorus depletion zone around the root. It is here AMF play the most significant role. The external hyphae of AMF travel much beyond the P depletion zone and scavenge a large volume of soil and supply P to the plants. The improved P nutrition in plants has been explained mainly by the extension of AM fungal hyphae beyond the root system which allows for the exploration of spatially unavailable nutrients (Smith and Read, 1997). In exchange, the AMF receive carbohydrates from its host plant. Mycorrhizal hyphae of *Glomus* sp. and *Acaulospora* sp. can transport P from distances of up to several centimeters from host plant roots (Li et al, 1991).

Mycorrhizae also have biochemical and physiological characteristics which differ from those of roots which can enhance P availability and can mobilize P (Bago and Azcón -Aguilar, 1997). Of particular interest is the transfer of soil nutrients mediated by the fungal symbiont to the plant host. Colonization of plant roots by fungi increases the surface area from which plants can change nutrients. Thus, root-colonizing fungi facilitate the absorption of crucial and often limiting soil nutrients, which results in increased photosynthetic ability and enhanced growth, productivity, and overall plant health (Clark and Zeto, 2000). The advantages of AM fungi can efficiently absorb and transport mineral nutrients, such as phosphorous and zinc from soil to the host plant, through an extended, indicate hyphal network, reproduction and health of the plant (Subramanian and Charest, 1997). During symbiosis, mycorrhiza obtain carbohydrate and other growing factors from host plant as the source of energy for its growth and development, while the plant itself can increase P and other nutrients uptake by the viability of mycorriza hyphal in roots (Muchovej, 2002).

Localized concentration of P in fertilized systems could also influence mycorrhizal activity. Lu et al. (1994) suggested that colonization was controlled locally by the P concentration in the portion of the root system being colonized.

Therefore, colonization of roots in a P band may be restricted by high P concentration in the local root tissue while the colonization of roots growing further from the band may not be influenced as much, until the overall plant P concentration is substantially increased.

The efficiency of P uptake by AMF has been related to both the spatial distribution of the AMF extra radical hyphae in the soil and to the capacity of P uptake by unit length of the hyphae. Cocoa seedling inoculated with AMF produced and the highest growth in all of NPK dosage levels. Phosphorus uptake in many crops is improved by associations with arbuscular mycorrhizal fungi.

Conclusion

Identification of AM fungi based on morphology characteristics of spore were observed and identified as *A. spinosa* and *G. constrictum*. PCR technique using ALF01 and NDL22 primers were manipulated to investigate all isolates with approximately 350bp of band. Sequencing analysis revealed that both species were identified as *G. constrictum* and *A. spinosa*. The most valuable species of arbuscular mycorrhizal fungi that colonize the roots of cocoa in nature will be used in fruit-growing practice. *G. constrictum* and 2 g of NPK(19:9:20) treatment were more effective to increase the growth of cocoa seedling than other treatments.

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