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Evaluation of the Inoculation Effect of Arbuscular Mycorrhizal Fungi on the Growth of Cocoa Seedlings (*Theobroma cacao* L.) in the Nursery

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Abstract: Cocoa farmers face many problems related to the acquisition of quality planting material for their farms to guarantee a good annual yield of cocoa beans. This is justified by the multiple constraints to which the cocoa tree is subjected to from the nursery. Faced with this, research is based on the diversification of production systems to reverse this trend. One of the promising methods is the promotion of the use of mycelial microorganisms such as Mycorrhizae to obtain vigorous plants resistant to various pathogens in the nursery. To achieve these objectives, the microbiological characterization of the different substrates used by nurserymen and the impact of fertilizers on the development parameters of young cocoa plants of the Tafo 79/501 variety were evaluated in the nursery. The results obtained indicate that the substrates used by nurserymen contain the AMF spores. However, the germination of these spores and the survival of mycorrhizal structures are compromised by the regular use of pesticides. Also, the height of the stem, the diameter at the collar of the stem, the fresh and dry biomass, the chlorophyll rate as well as the rate of root colonization of AMF are higher in cocoa plants whose substrate has been inoculated with AMF compared to other pots. Overall, biofertilizers are conducive to the harmonious development of cocoa seedlings in the nursery, which guarantees good productivity.

Keywords: Cocoa, Improvement, Nursery, Productivity, Biofertilizer, AMF, Tafo 79/501

Introduction

Projections by the United Nations show a 20% increase in the world population by 2050 [39]. In this register, Cameroon is not outdone. Actually, the forecasts of the third general population and housing census published in 2010 indicate an annual increase of 2.6%; we are therefore entitled to say that in Cameroon, the population could be around 48,625,874 inhabitants around the year 2050. The growth of the world population in general and that of Cameroon, in particular, have as a corollary global and national food needs. Satisfying the demand for agricultural products, therefore, requires either an increase in agricultural land or yields [41].

The quest for higher yields is very often associated with an intensification of agricultural practices, to the detriment of the preservation of animal and plant biodiversity [12]. Also, the majority of farmers practice less and less rotation and association of crops and the contribution of organic matter to crops is minimal [40]. The direct consequence is a decrease in the level of nutrients in the soil and an increase in the number of crop pests or diseases, leading to a decrease in productivity and production [4]. In response to this drop in yields, farmers use many chemical inputs, often incorrectly and excessively, contaminating soil, and groundwater [42].

Cocoa (*Theobroma cacao* L.) has its origins in Central and South America [28]. Its fruit is a bean which constitutes the raw material for the agro-food and cosmetic industries [12]. The various substitutes for this foodstuff are among others chocolate, wine, liqueurs, potato chips, lunch, caramel, butter, beauty milk, creams, and gels [3, 5, 15, 27]. Cocoa farming is an important activity in the world economy [33]. According to the International Cocoa Organization

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ICCO [18], the world production of cocoa beans is 4.2 million tons per year. Africa's contribution to this production is 73%, with Cameroon contributing only 5% of all this production.

As with all crops, one of the most valuable factors in cocoa production is the soil [1, 14]. For good agricultural productivity linked to the soil, the latter must undergo a correction plan and a restitution plan [21, 23]. The importance of soil fertilization in the technical route of cocoa cultivation is undeniable and numerous studies have been carried out to establish techniques that allow improved plant material to fully express its production potential [22].

The cocoa plantation restructuring plan in Cameroon involves vegetative and generative propagation. Vegetative propagation includes cuttings and grafting with efficient clones. This method is commonly used to rehabilitate old cocoa fields. As for generative multiplication, it is made from beans sown directly in the field or the nursery in polyethylene bags for a certain period.

The production of cocoa plants in the nursery remains very restrictive, especially when it is necessary to bring the fertilizing elements and make health protection. Nursery production necessarily requires mineral fertilization of the NPK nature [19]. However, the use of mineral fertilizers in cocoa trees has the major consequences of acidification and imbalance in the soil under fertilized cocoa [2, 11, 20, 43]. The production of cocoa plants in the nursery depends mainly on the availability of nutrients in the soil contained in each pot [1]. The decline in soil fertility, therefore, becomes an important biophysical constraint that limits agricultural production in general [26, 53].
The search for alternatives for maintaining the fertility of soils under cocoa trees is the subject of deep concern. Improving global cocoa production calls for the acquisition of quality planting material, which is the subject of much research right now around the world. Investigations have already been made on the genetic and environmental factors that can promote good growth and the quality of young cocoa plants in the nursery [50]. Apart from the main known cocoa varieties (Criollo, Forastero, and Trinitario), some clones have been developed by research stations in Cameroon with a view to varietal improvement to obtaining so-called resistant and productive varieties [6, 8]. Also, other research has focused on methods of fertilizing substrates used in nurseries based on microorganisms [16, 47, 52].

As microorganisms play an important role in the propagation and growth of plants, the present study is a contribution to the search for means of fertilizing soils in the cocoa nursery to obtain vigorous plants capable of withstanding various pathologies to guarantee good yields of cocoa beans. To that end, we carried out the microbiological analyzes of the substrate used and evaluated the impact of the inoculation of Arbuscular Mycorrhizal Fungi on the growth parameters of young cocoa plants aged three months in the nursery.

Materials and methods

Study site

This study was carried out from April to August 2016 at the Laboratory of Biological Control and Applied Microbiology of the Agricultural Research Institute for Development (IRAD) of Yaounde located in the Nkolbisson district, Mfoundi division, Centre region and Republic of Cameroon. The Centre region of Cameroon extends between latitudes 3°45'and 3°36' N, longitudes 11°10'and 11°45' E; and an average altitude of 760 m [24]. This region is located in the bimodal rain forest zone. It is therefore subject to an equatorial climate of the Guinean type characterized by the alternation of two rainy seasons and two dry seasons of unequal duration: the short rainy season (March-June) followed by the short dry season (June-August) and the long rainy season (September-November) followed by the long dry season (November-March). The average annual rainfall is 2000 mm and the average annual temperature is around 26.6 ° C.

The city of Yaounde is located in the savanna forest contact zone [44]; its characteristic vegetation is essentially a semi-deciduous secondary forest. This forest experienced a progressive degradation over time linked to intense urbanization activities; it contains some large trees such as *Terminalia altissima* A. Chev., *Musanga cecropioides* R. Br., *Ceiba pentandra* (L.) Gaertn., *Albizya zygia* (DC.) J.F. Macbr., *Astonia boonei* De Wild. and *Chlorophora excelsa* (Welw.) Benth. but remains mainly dominated by large shrubs like *Palisota mannii* C.B. Clarke and *Trema orientalis* (L.) Blume and herbaceous plants like *Panicum maximum* Jacq., *Ageratum conyzoïdes* L. and *Chromolaena odarata* (L.) R.M. King & Robinson [51].

Materials

The plant material consisted of the so-called Tafo 79/501 cocoa variety; this was obtained from SODECAO (Cocoa Development Company of Cameroon), Mengang production center (Nyong-et-Mfoumou division, Centre region, Cameroon).

The mycorrhizae used consisted of a mixture of two strains namely *Gigaspora margarita* and *Acaulospora tuberculata*. These strains came from the Laboratory of Biological Control and Applied

Microbiology of IRAD in Yaounde. The chemical fertilizer (NPK) was purchased from a plant protection product sales store at the Mfoundi market (Yaounde). As for the non-sterilized substrate used, it came from the forest undergrowth of Yaounde at a place called Messassi, a suburb of the Cameroonian capital.

Methods

Microbiological analysis of the substrate

It was based on the extraction of spores of AMF potentially present in the substrate as well as their counting.

Spore extraction was carried out using the rapid method [9]. 100 g of soil were introduced into a 1 liter Erlenmeyer flask; 300 ml of tap water was added and the mixture homogenized. After a latency of 15 seconds, the homogenate was decanted in 3 sieves whose mesh size was 250 µm, 125 µm, and 45 µm respectively. The previous two steps were repeated at least three times. The contents of each sieve were washed and the residue collected in a 69.4 cm^2 grid petri dish for spore counting using a WILD M3B 5Heerbrugg stereomicroscope, Switzerland.

The counting of the spores was carried out on 10 tiles with a total area of 2.5 cm^2. The total number of spores in each residue per 100 g was evaluated by the following formula: **N = [(69.4/2.5) x n]** where N = total number of spores and n = number of spores counted in the 10 cells [31].

Experimental design

The experimental set-up consisted of three completely randomized blocks (Figure 1). Each block had four treatments repeated three times. A total of 36 pots were made. The different treatments were distinguished according to the fertilizers inoculated in the pots. The first treatment (T_0) consisted only of the sterilized substrate; the second treatment (T_1) was made based on the unsterilized substrate which had not received any other substance; as for the third (T_2) and fourth treatment (T_3) respectively, they benefited from the impregnation with NPK (20-10-10) and the inoculation of two combined strains of AMF mentioned above.

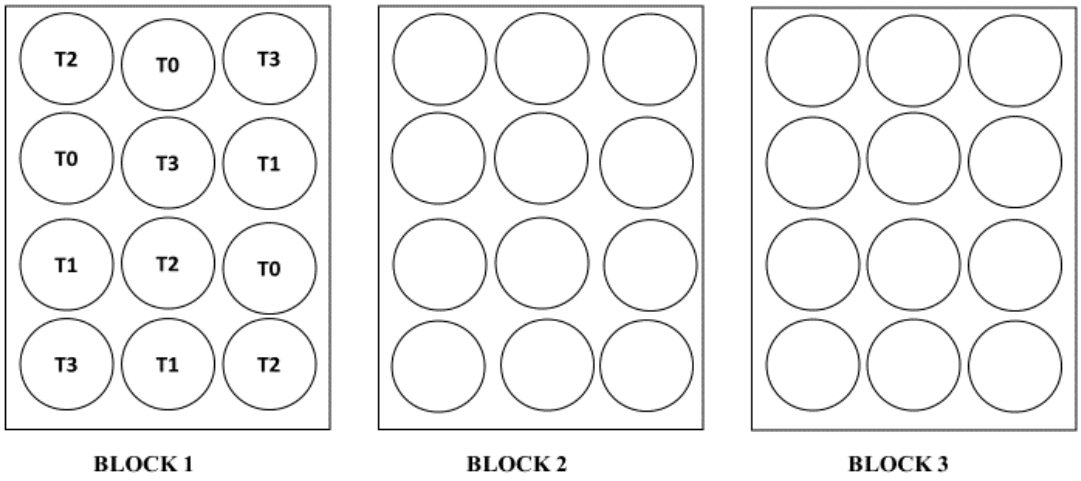

Figure 1: Experimental design

Preparation of pots

The pots were made with polyethylene plastic with a 2x17x65 mm characteristic. The beans were pre-germinated in a bin for 18 days before being potted. 10 g of the inoculum containing the AMF spores (*Gigaspora margarita* and *Acaulospora tuberculata*) were brought into 9 pots when transplanting the pre-germinated plants into the pots. The NPK chemical fertilizer was spread in 9 pots intended for this treatment at a dose of 10g/pot in a fractional manner (2g every week). For the sterile substrate, 9 pots were transplanted for this purpose. The soil was autoclaved at 121 ° C for 1 hour with 2 repetitions within 24 hr. Finally, 9 pots were made up for the negative control, the pots of which received no treatment from sowing until the trial was stopped after 3 months.

Parameters studied on seedlings

Certain agronomic parameters were evaluated on plants three months old before and after the harvest of young cocoa plants in the nursery.

Pre-harvest parameters

The following agro-morphological and physiological parameters were taken into account before harvesting the young cocoa plants: the height of the stems which was evaluated using a graduated ruler, the diameter at the collar of the stem which was noted using a

Vernier caliper and the chlorophyll rate of the leaves which was recorded by a chlorophyll meter.

Post-harvest parameters

The following parameters were taken into account after the seedlings from the pots were harvested: fresh biomass, dry biomass, and the rate of root coloring.

Fresh biomass

The aerial (leaves and stem) and underground (roots) parts were separated for each plant and weighed separately on a hypersensitive balance. The different values of the corresponding biomass were read directly on the balance screen.

Dry biomass

The aerial (leaves and stem) and underground (roots) parts were wrapped in newspaper and transferred to the biotechnology center of the University of Yaoundé I for parboiling. As with fresh biomass, the dry biomass values corresponding to each plant were read directly on the balance screen.

Acid fuchsine root coloring of young cocoa plants

The purpose of staining the roots with acid fuchsine was to search for the presence of hyphae, arbuscular, vesicles, and spores in the roots of cocoa plants. For this, the cocoa plants were uprooted from different pots. These roots were amputated from the aerial part of each plant, then cleaned and stored separately in 50% alcohol, depending on the origin of their substrate. The roots were broken into 1 to 2 cm pieces and washed with tap water, then transferred to 10% potash to thin the roots. These were then rinsed three times with tap water and introduced into 30% hydrogen peroxide at room temperature for 10 minutes [9]. The coloring of the cocoa roots was carried out using a 0.01% acid fuchsine solution. This solution colors the root tissues and the characteristic structures of AMF in the red of the root. The roots have been discolored to allow observation of the characteristic structures of AMF which remain colored. To do this, 1g of each root was removed using fine scissors for observation under a microscope [31]. The rate of root colonization (T (%)) was estimated by the following formula: **T (%) = [(nc/NT) x 100]** where nc = number of root fragments colonized and NT = total number of root fragments observed [47].

Statistical analysis

The Excel spreadsheet was used to record the data collected. SAS (Statistical Analysis System) software was used for the analysis of the data itself; mean values were followed by standard deviation. Variance analysis (single-factor ANOVA) was used for multiple comparisons of means. When the overall differences were significant, analyzes were pursued in pairs using Tukey Kramer's HSD test.

Results

Spore extraction and counting

Microbiological analysis of the substrate used revealed the presence of several spore structures within it. Figure 2 shows some morphotypes of these spores. Table 1 below shows the results on the microscopic count of spores from the substrate used. It appears from this table that the analyzed substrate contains several spore structures. The spores identified thus present eight morphotypes that we shorten as M1, M2, M3, M4, M5, M6, M7, and M8; these differ according to their color, size, and shape. The different spore morphotypes identified in the substrate used correspond to the genera *Glomus*, *Gigaspora*, and *Entrophospora*. Finally, it appears from our observations that the number of spores in the substrate varies with the mesh size of the different sieves; the sieve with large meshes made it possible to detect a greater number and great variability of the spore structures.

Table 1: Number and morphotypes of spores identified from the substrate

Mesh size (µm)	Morphotypes	Color	Genus	Number of spores/100g of substrate
250	M1	Brown		111.04
	M8	Translucent	*Glomus*	111.04
	M6	Brown		83.25
	M7	Red		55.52
	M3	Black	*Gigaspora*	83.25
	M2	Black		111.04
125	M4	Black		83.35
	M5	White	*Entrosphora*	27.76
	M1	Black		222.08
45	M1	Black	*Gigaspora*	277.6

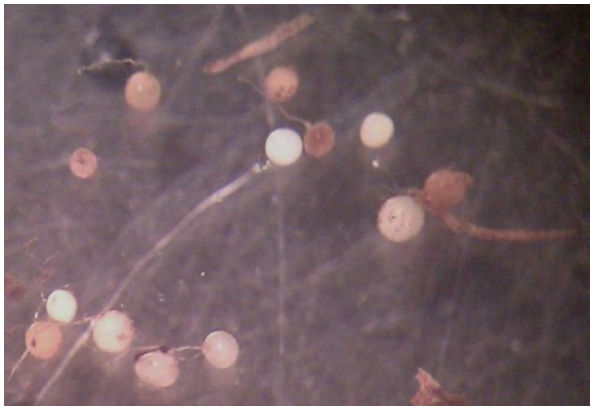

Figure 2: Microscopic view of some AMF spores in the substrate

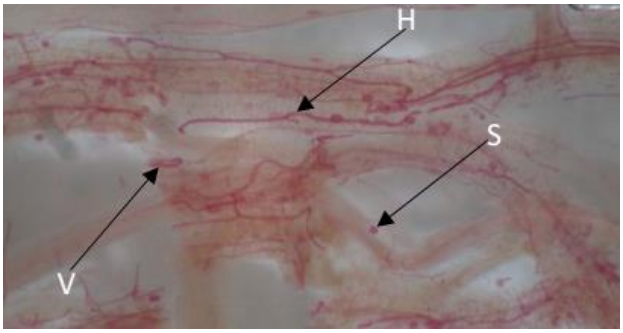

Figure 3: Some AMF structures (H: hyphae, S: spore; V: vesicle)

Fuchsine staining of the roots of cocoa plants

The results recorded in Table 2 below show the distribution of the AMF structures in the roots of the different substrates from different experimental pots. From this table, it appears that only the roots of young cocoa plants from the sterilized substrate are free from any mycorrhizal structure to the detriment of the others. The mycorrhizal structures identified in the roots of plants are hyphae, spores, and vesicles (Figure 3). Furthermore, the proportion of these root mycorrhizal structures is variable and is thus presented in order of importance: hyphae (72%), vesicles (14.67%) and spores (3.33%).

Table 2: Distribution of AMF structures from roots of different treatments

	AMF structures			
Pots	Arbuscular	Hyphae	Vesicles	Spores
T0	-	-	-	-
Autres	-	+	+	+

Legend: + = present; - = absent

Effect of biofertilizers on the growth of cocoa plants in the nursery

The results contained in Table 3 show the influence of the different treatments applied to the development parameters of young cocoa plants in the nursery. It appears from this table that after three months, the height of the young plants ($F_{3, 32} = 15.41$; $P < 0.05$), the diameter at the collar ($F_{3, 32} = 9.33$; $P < 0.05$), the average fresh mass ($F_{3, 32} = 11.87$; $P < 0.05$), the dry average mass ($F_{3, 32} = 5.93$; $P < 0.05$), the chlorophyll rate ($F_{3, 32} = 13.14$; $P < 0.05$) as well as the rate of root colonization of mycorrhizal structures ($F_{3, 32} = 32.11$; $P < 0.05$) differ significantly between the different treatments. Overall, these parameters are more important in the T_3 treatment, the pots of which benefited from the AMF inoculum compared to the T_2, T_1, and T_0 treatments. This means that the inoculation of AMF in the nursery results in the most vigorous cocoa plants.

Table 3: Variation of growth parameters of cacao seedlings in nursery according to treatments

T	Height (cm)	Diameter at the collar (cm)	Fresh biomass (g)	Dry biomass (g)	Chlorophyll rate (%)	Root colonisation rate (%)
T_3	27.23 ± 1.18 a	7.04 ± 1.01 a	8.10 ± 1.25 a	2.77 ± 0.18 a	35.87 ± 5.35 a	70.03 a
T_2	23.33 ± 0.75 b	5.02 ± 1.06 b	4.53± 0.25 b	1.91 ± 0.43 ab	33.62 ± 1.38 b	26.33 b
T_1	22.78 ± 1.47 b	3.66 ± 0.58 b	7.09 ± 0.74 a	2.43 ± 0.75 a	26.77 ± 0.88 c	3.33 c
T_0	10.83 ±9.45 d	2.13 ± 1.76 c	2.22 ± 1.93 c	0.82 ± 0.72 d	19.87 ± 17.35 d	0.00 c
$F_{3, 32}$	15.41	9.33	11.87	5.93	13.14	32.11

T = Treatments; Values followed with the same latter are not significantly different by Tuckey Kramer test

The present study clearly shows that the growth of young cocoa plants in the nursery is improved by the presence of AMF, with the corollary being a very high rate of mycorrhizal root colonization. Furthermore, as demonstrated, the mycorrhizal structures listed in the pots that have not received the AMF inoculum came from the progressive contamination of the roots of the corresponding young plants by the spores contained in the substrate used. Finally, sterilization of the substrate, which causes the absence of mycorrhizal structures within it, results in cocoa plants being stunted after three months.

Discussion

Our results on spore extraction and counting are almost analogous to those obtained in the North-West and South-West regions of Cameroon [49] concerning the genus number of AMF spores. Indeed, studying the substrates from the Cameroon, Oku, and Manenguba mountains, this author also obtained three kinds of spores from AMF; however, unlike the

genus *Entrophospora*, the latter author found in addition to the genera *Glomus* and *Gigaspora* the genus *Acaulospora*. Furthermore, if these results show four AMF morphotypes, two of which belong to the genus *Glomus*, one to the genus *Gigaspora* and one to the genus *Acaulospora*, our results revealed eight sporal morphotypes of AMF including six belonging to the genus *Glomus*, one to the genus *Gigaspora* and one to the genus *Entrophospora*. These compared results confirm the fact that the genus *Glomus* represents the major component of the mycorrhizal flora in several rhizospheres as several other authors have already suggested [7, 17, 31].

Similarly, our results are different from those obtained in Ebolowa and Nkolbisson in the southern and central regions of Cameroon [32]. Indeed, working on the predominantly Sisongo soils sampled from indicated sites, this author detected 22 sporal morphotypes belonging to five genera classified according to their importance: *Gigaspora* (13 morphotypes), *Entrophospora* (5 morphotypes), *Scuttelospora* (2 morphotypes), *Acaulospora* (1 morphotype) and *Glomus* (1 morphotype).

Regarding root coloring, the absence of mycorrhizal structures in the roots of young cocoa plants from the sterilized substrate can be explained by the inhibition of the germination of the AMF spores present in this substrate. Furthermore, the results obtained by other authors have shown that the rate of germination and elongation of the hyphae are generally affected by physical or chemical factors such as the use of fungicides that prevent any mycorrhizal symbiosis with the roots of young seedlings of the cocoa tree [10, 54].

Compared to the NPK regularly used in the nursery by cocoa farmers in the locality, treatments based on AMF inoculum are more significant and allow good results to be obtained than those expected for this chemical fertilizer. Indeed, biofertilizers appear as natural sources of NPK for plants [12, 42]. Besides, NPK-based chemical fertilizers are long-term causes of soil depletion and contamination of groundwater [4].

Concerning the different growth parameters recorded in our trial, our results are in agreement with those obtained on *T. cacao* by other authors [47]. According to these authors, the agronomic parameters are improved by the inoculation of AMF in the substrate used. Several works have already been carried out on the symbiotic networks between certain cultivated plant species and the AMF. This is particularly the case for corn or *Zea mays* [46], bean or *Phaseolus vulgaris* [25], celery or *Apium graveolens* [45], and of course the cocoa tree *T. cacao* [13, 47, 52]. These various works made it possible to promote the use of biofertilizers in agronomy with a view either to improving the growth parameters of young plants in the nursery or to boost the fertility of the substrate while preserving the environment and the physical-mineral balance soil, or finally to reinforce the resistance of plants against the induced pathogenicity of certain microorganisms [13, 29, 30, 31, 32, 34, 35, 36, 37, 38, 40, 47, 48, 52].

Conclusion

Our study focused on the influence of the inoculation of Arbuscular Mycorrhizal Fungi on the growth of young cocoa plants in the nursery. It emerged that the substrate used in our test contained a variety of spore morphotypes. When conditions were favorable, these spores germinated and established symbiotic contact with the roots of young experimental plants. This interaction resulted in a constant supply of nutritive and protective substances for mycorrhizae whose role was to vitalize the host plants allowing them to grow usefully. Overall, the inoculation of mycorrhizal fertilizers in the experimental pots had a positive impact on the growth parameters of the young cocoa plants in the nursery compared to the plants from the other pots. The absence of mycorrhizal structures is detrimental to the good development of cocoa plants in the nursery; these are puny compared to the rest. Cocoa farmers would, therefore, benefit from integrating, for their nursery needs, the AMF instead of or in addition to the usual chemical fertilizers. This arrangement can allow them to better ensure the good growth of their cocoa plants in the nursery.

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