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EFFECTS OF TWO TYPES OF AMF ON GROWTH OF COCOA SEEDLINGS (*THEOBROMA CACAO* L.) IN GREENHOUSES

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ABSTRACT

Arbuscularmycorrhizae fungi (AMF) benefit to plants growth is not still to demonstrate. However, little is known about the effects of exogenous AMF (from temperate soil) on cocoa (*Theobroma cacao*) that plays an important role in the economic prosperity of Côte d'Ivoire. A greenhouse experiment was conducted to assess the effectiveness of AMF from temperate soil (*Rhizophagusirregularis* named exogenous inoculum) and Ivoirian soil (complex of strain found in soil named natives inoculums) on growth of cocoa plants during ten months. The experiment was a single factor experiment arranged in a completely randomized block design, with type of inoculum as a factor with twenty replications. Roots of all inoculated seedlings were colonized and the best frequency was observed with exogenous AMF. Analysis of growth parameters showed a significant difference between treatments for variables "Area", "Diameter", "Height", and fresh and dry biomass production. The best results were obtained with natives inoculum. However, exogenous inoculum showed much better results compared to the control. Indeed, Maximum plant diameter, height and leaf area of 14.49 mm, 1083.7 mm and 94.24 cm^2 respectively were obtained from seedlings inoculated with native's inoculum. The highest total fresh and dry biomass production (202.1 g and 53.86 g) was also recorded from seedlings inoculated with natives inoculum.

Keywords

Arbuscularmycorrhizal fungi (AMF), *Theobroma cacao*, Côte d'Ivoire, exogenous inoculum, growth, seedling, soil, inoculums, *Rhizophagusirregularis*.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the most important cash crops in Côte d'Ivoire and other countries in Central and Western Africa. Cocoa growth plays an important role in Ivoirian economic prosperity. It represents 40% of export incomes and contributes to 15% to the formation of the gross domestic product (GDP). At the social level, approximately 600,000 heads of farms feed about 6 million people [1] or more than 15% of the rural population deal with the cocoa sector. Côte d'Ivoire has held since the end of the 1970s, the Honourable rank of world's leading producer of cocoa [2] with an average production of 1,200,000 tons per year [3]. The ivorian production is estimated at 1,720,000 tonnes for the marketing year 2014/2015 [4] and corresponds to more than 40% of world production. This result is the combination of several factors, including a remunerative and attractive pricing policy that encouraged production, and a migration policy implemented since the colonial period, and encouraged by the ivorian authorities after the political independence of the country. Land availability that favoured the extension of orchards, also offered the opportunity for labourers (workers cocoa plantations) to be autonomous and have their own plantation. Despite this performance, levels of cocoa field productivity remain low and cultivated areas are still increasing. The current observed yields vary from 300 to 450 kg/ha against 2,000 to 2,500 kg/ha as estimated by research previsions [5]. Several reasons could explain this low productivity. They include the aging of the existing cocoa orchard, the depletion of forest reserves, the failure of spontaneous attempts of replanting on precedents non-foresters, the low rate of adoption of selected plant material and technical routes, the decline in the fertility of the soil and the emergence of new diseases and pests [5] as the swollen shoot. Various research programs have been developed to improve the productivity of ivorian cocoa. One of the possibilities for agriculture that is not yet very developed in Côte d'Ivoire is the use of arbuscularmycorrhizal fungi (AMF) potentialities in order to develop some sustainable technologies of production. Indeed, different studies have already demonstrated effectiveness of AMF's potentialities for plants growth [6], [7]. AMF are organisms, which form symbioses with the roots of most plant species [8]. In exchange for carbon from plant hosts, these fungi can help increase uptake of nutrients [9], [10], enhance resistance to disease [11], and increase drought tolerance [12]. It means that their use in agriculture should allow reducing intensive utilization of pesticides and fertilizers [13], [14].

Although AMF are non-host-specific in their ability to infect a wide range of hosts, the degree of benefit to each partner in any given AMF – host plant interaction can depend on the particular species involved. Such differential effects between individual AMF–host partners, may influence both host and AMF community structures. The composition of the AMF community may be strongly influenced by the host species through differential effects on hyphae growth and sporulation [15]–[19]. In return, the plant community structure may be strongly influenced by the specific composition of the associated AMF and the effectiveness of each of the fungal species in promoting growth of each host [20], [21].

The present study is intended to test the efficiency of different AMF from temperate soil (*Rhizophagusirregularis*) and Ivoirian soil (complex of AMF found in soil) on growth of cocoa plants in tropical condition in greenhouse.

2. MATERIAL AND METHODS

2.1 Plant material

Plant material consisted in cocoa seedlings with four leaves. The seedlings were obtained from seeds sown, in terrines containing a sterile substrate consisted in a mixture of peat, clay, wood fiber and compost, four (4) days after harvest of the pods. Before sowing seeds, they were cleaned by washing in tap water and friction in sand in order to avoid problems related to contamination of the plantlets by parasites present on the sweet and tangy mucilage. Seeds were then washed again with tap water and a few drops of sodium hypochlorite and cleaned thoroughly with paper towels.

2.2 Inoculum

Two types of inoculum were used. The first type of inoculum consists of soil collected from the rhizosphere of ivoriancocoa trees, in three regions: Nawa, San-Pedro and Goh. Six soil samples were collected per region with a drill in the stratum of 0-20 cm depth. From one region to another, the drill was thoroughly sterilized with sodium hypochlorite diluted to 10%. Soil samples of each region were merged in order to obtain three composite samples: Nawa (SBE), San-Pedro (SPO), and Goh (GNA). The three soil samples contain species of AMF identified according to a high throughput sequencing method [22], especially of the genus *Glomus* (or *Rhizophagus*) considered as the most abundant AMF in tropical soils [23]. These three soil samples containing local species of AMF were termed native inoculum. The second type of inoculum, named exogenous inoculum, was composed of spores of *Rhizophagusirregularis* that was isolated and propagated *in vitro* on transformed carrot roots by the Sanders group (Department of Ecology and Evolution - University of Lausanne).

2.3 Growing conditions of cocoa seedlings

The study was performed in a greenhouse of the Department of Ecology and Evolution (DEE) of University of Lausanne in Switzerland for a period of ten (10) months. The greenhouse was set to the conditions of the tropics characterized by a temperature of 25±4 °C, 80±5% humidity rate. Cocoa seedlings were planted in plastic pots containing a substrate similar to the soil of Ivoirian cocoa orchard. 50% of the substrate consisted in a mixture of peat, clay, wood fibber and compost, characterized by a pH = 6.0, water retention capacity = 65% and conductivity = 30 mS/m. The other half of the substrate was composed of 33% crushed clay and 17% perlite. This mixed substrate was autoclaved at 120 °C for 30 minutes and then cooled for at least 24 hours at room temperature. Each

pot (18 cm x 16 cm) was filled with approximately 2.5 litters of substrate. The seedlings were inoculated with 65 g of each of the three native inoculums and 300 spores/ml of the exogenous inoculum when transplanting to the substrate (≈ 15 cm of height). During the two weeks following transplantation, seedlings were covered with a translucent plastic film in order to create growing conditions of a cocoa nursery by keeping moisture and avoiding direct exposure to sunlight. The plants were watered up to field capacity (150 ml) at regular intervals of three days.

2.4 Experimental design

Plants were arranged in a completely randomized block design (**Figure 1**) with eight treatments and twenty replications [24]. Treatments were divided in four (4) groups as follows: Control: CTL (without AMF); native inoculum (soil): GNA, SBE and SPO; exogenous inoculum: SC3 (AMF spores suspension); and mixed inoculum: MS1 (SBE + SC3), MS2 (SPO + SC3) and MS3 (GNA + SC3).

Plants were randomly rearranged two times (45 days after transplanting, and then one month and half later) for spacing in order to avoid a steric crowding that could result in the death of some and also to facilitate watering.

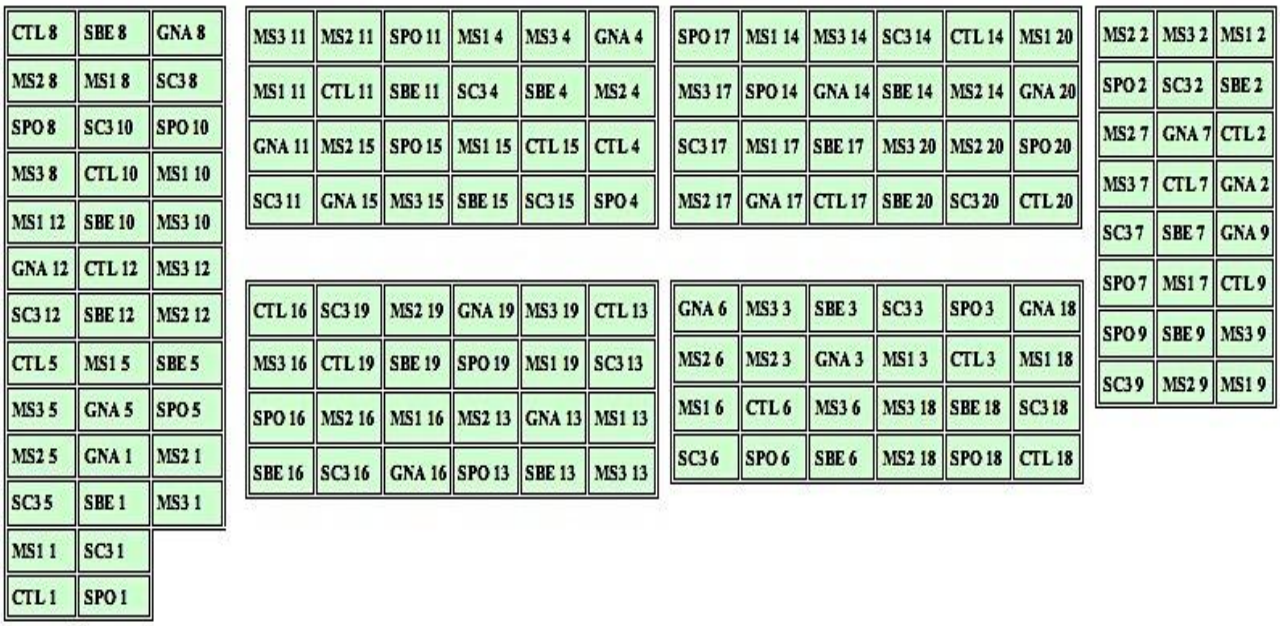

Figure 1: Experimental design (Randomized Block Design) with 8 treatments and 20 replications.

Legend: Control: CTL (without AMF); native inoculum (soil): GNA, SBE and SPO; exogenous inoculum: SC3 (AMF spores suspension); and mixed inoculum: MS1 (SBE + SC3), MS2 (SPO + SC3) and MS3 (GNA + SC3)

2.5 Assessment of the mycorrhizal colonization status

Ten months after inoculation, roots of cocoa plants from six replicates of each treatmentwere carefully extracted, dried and freeze-dried for DNA extraction. This six replicates were chosen randomly. Mycorrhizal colonization status of inoculated plants was accessed by PCR amplification of DNA of Arbuscular Mycorrhizal Fungi using primers AML1 and AML2 [25]

2.6 Data scoring

Ten months after transplanting, stem diameter (Diameter), height (Height), number of leaves (Leaf), and chlorophyll rate of cocoa seedlings were recorded. Leaves, stem and roots were cleaned and separated.Roots and shoots (stem + leaves) fresh weights were determined. Roots and shoots were then dried at 65 °C for 3 days and roots and shoots (stem + leave) dry weights were determined. The leaf area (Area) was done by photography and area was calculated on the software "Image.j". Mycorrhizal frequency is determined by counting of bands emerged agar's gel electrophoresis after PCR.

2.7 Statistical Analysis

All the data obtained were subjected to the analysis of variance (ANOVA) with the software RStudio version 3.1.1. Treatment means were further separated by SNK (Student-Newman-Keuls) for test of significance at significant level alpha = 0.05 [26].

3. RESULTS

3.1 Mycorrhization frequency

The cocoa seedlings (shoot) responded positively to all AMF inoculation with natives inoculums and strain of *Rhizophagusirregularis* in a series of substrate pots. But, they exhibited various degrees of mycorrhizal colonization (Figure 2). The highest colonization was 66.7% and it was observed in cocoa seedlings inoculated with SC3. Plants inoculated with mixed inoculum (MS1, MS2 and MS3) had 50, 55.6 and 55.6% respectively of their roots infected. The lower colonization frequency was observed in the root of plants inoculated with native inoculum. None of the roots from control (CTL) plants were colonized by AMF.

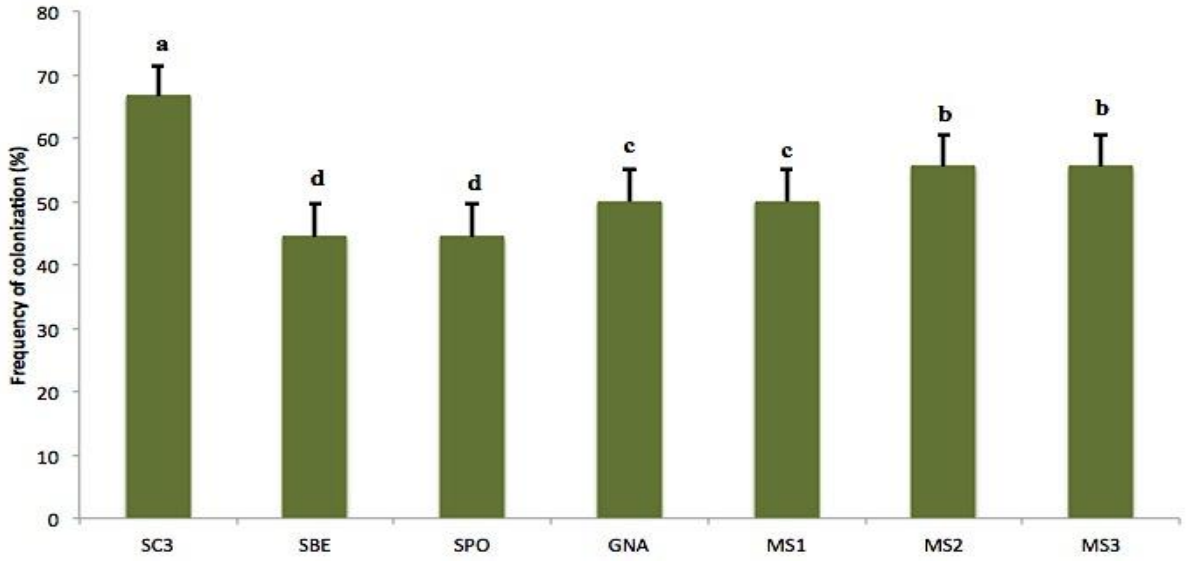

Figure 2: Frequency of root colonization of plants inoculated with native or exogenous inoculum. Different letters above indicate a significant difference among means of treatments Legend: **native inoculum (soil): GNA, SBE and SPO; exogenous inoculum: SC3 (AMF spores suspension); and mixed inoculum: MS1 (SBE + SC3), MS2 (SPO + SC3) and MS3 (GNA + SC3).**

3.2 Non-destructive growth parameters (Diameter, Height, Leaf)

Data collected ten months after the start of the experiment were analysed. It shows there is a significant difference between treatments for non-destructive growth parameters that are the diameter and height of plants (Table 1). Indeed, seedlings inoculated with native inoculums showed a larger diameter (14.49, 14.39 and 14.14, respectively, for SBE GNA and SPO) compared to control seedlings (CTL = 12.43) and other treatments (mixed inoculum). Although the inoculated seedlings with exogenous inoculum SC3 seems better than the control plants (CTL), the SC3-inoculated seedlings have produced a smaller diameter than the inoculated seedlings with native inoculums. The average height of the cocoa plant ("Height") are 1083.70 mm, 1064.82 mm and 1004 mm for seedlings inoculated respectively with GNA, SBE and SPO against 1002.17 mm with SC3 and 875.41 mm for non-inoculated seedlings (CTL). These values are significantly different at alpha significant level of 5% selected (Table 1). No significant difference was observed between treatments for the variable "Leaf".

3.3 Destructible growth parameters (Area, Fresh and dry weight)

Leaf chlorophyll ("Chlo") production occurred regardless of treatment, this means that no significant differences were found. The average values vary between 308.53 and 330.29. Leaf area ("Area") for its part, varies depending on the treatment. Analyses highlight two homogeneous groups of treatments. The largest average area of 94.24 cm^2 leaves was observed in plants inoculated with GNA while non-inoculated plants (CTL) have the lowest average is 80.17 cm^2. Furthermore, the fresh biomass and dry biomass were measured ten months after inoculation of the plants. Results for biomass production were shown in Figure 2. As compared to control (non-inoculated plants) which have an average of 122.89 g and 35 g of fresh and dry biomass respectively, the biomass production is important in all plants inoculated. This production is greater in seedlings inoculated with natives inoculums. The inoculated seedlings with SBE, SPO and GNA were presented respectively 163.05 g, 155.86 g and 151.71 g of fresh biomass of plant aboveground port. The same took place in root biomass (Hypogeum of the plant) fresh and dry roots. The values range from 26.69 g to 39.05 g of fresh biomass, respectively non-inoculated seedling and seedling inoculated with SBE. It was noted 5.8 g of dry biomass for the control seedlings (CTL) while inoculated seedlings with GNA were 8.51 g dry biomass. Figure 3 shows the total fresh biomass and total dry biomass. For these two variables, it was observed a significant difference between treatments.

4. DISCUSSION

To assess the effectiveness of AMF on young plants of cocoa, the presence of fungal structures has been sought in plant roots by pcr using specific primers (AML1/AML2). The mycorrhizal frequencies ranging from 44% to 66.7% in this study are substantially the

same as those of Ndiaye [27], which are from 37.02% to 55.6%. But they seem low compared to studies of Zougari-Elwedi [28] which shows 95.8% colonization in roots of leeks and a maximum of 100% on palm *Phoenix dactylifera* L. as well as [29] with avocado. The difference could be justified by the fact that in this study, inoculums were soil but also by the host plant and substrate of culture that seems less poor nutrient [30].

Table 1: Growth parameters of cocoa plants non-inoculated and inoculated with different complexes of AMF.

Treatments	Area	Chlo	Leaf	Diameter	Height
CTL	80.17 ± 2.10 b	319.58±6.40 ns	42.70±2.58 ns	12.43±0.14 c	875.41±14.11 cd
GNA	94.24±2.34 a	329.47±7.47 ns	37.64±2.52 ns	14.39±0.18 a	1083.70±22.70 a
MS1	88.16±2.61 ab	313.82±8.86 ns	38.64±1.83 ns	12.50±0.12 c	910.11±22.19 cd
MS2	89.59±4.05 ab	308.53±11.14 ns	38.35±2.83 ns	12.33±0.22 c	840.05±35.66 d
MS3	91.49±2.83 ab	310.00±7.69 ns	36.70±1.70 ns	12.47±0.23 c	924.17±23.98 c
SBE	84.79±3.42 ab	330.29±6.23 ns	42.35±2.93 ns	14.49±0.12 a	1064.82±14.98 ab
SC3	83.74±3.41 ab	321.59±8.40 ns	40.88±3.23 ns	13.31±0.22 b	1002.17±11.73 b
SPO	90.47±2.50 ab	328.41±5.78 ns	42.00±2.56 ns	14.14±0.20 a	1004.00±14.12 b
P value	0.01906 *	0.22076	0.4904	2 x 10^{-16} ***	1.681 x 10^{-14} ***

Means in columns followed by the same letter are not significantly different at 5% level. *: Significant difference, ns: Non-significant difference

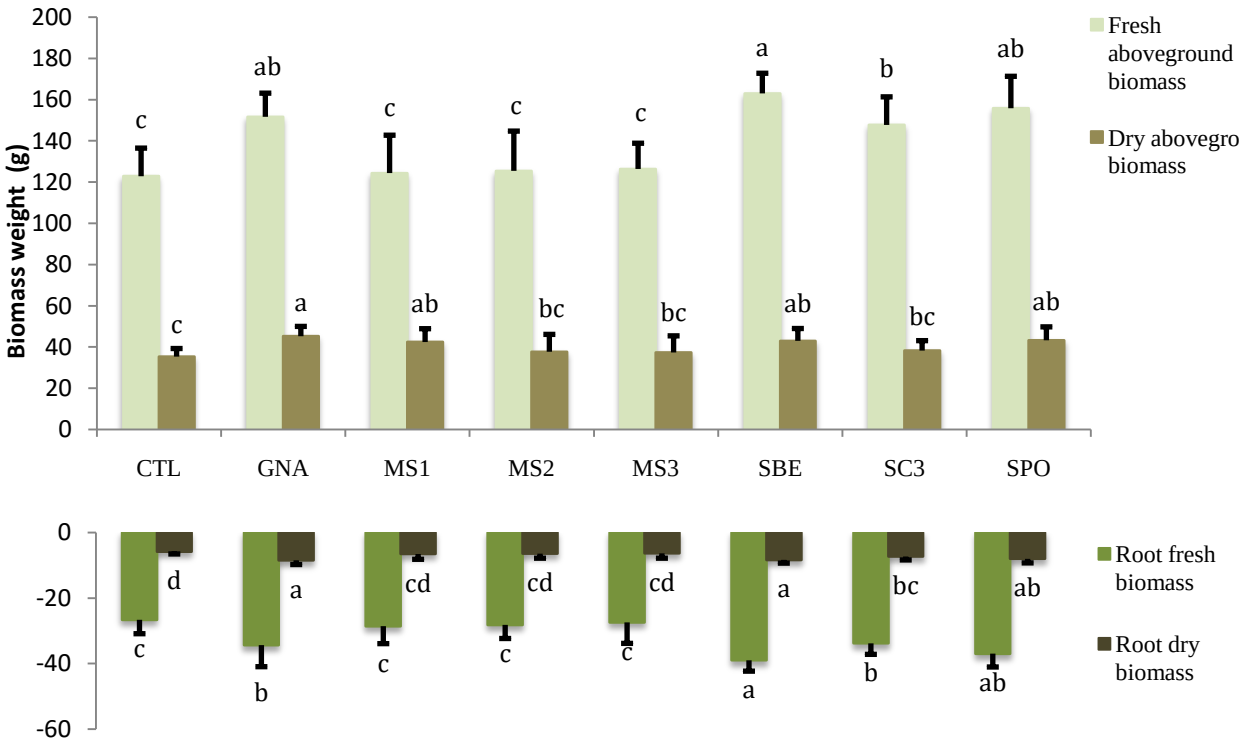

Figure 3: Effect of inoculation on stem and root weight of cocoa plants.

Fresh aboveground weightpvalue= 2.10^{-16}, Dry aboveground weightpvalue= 26.10^{-06}, Root fresh weightpvalue= 2.294.10^{-14}, Root dry weight =4.348.10^{-11}. Different letters above bars indicate a significant difference among means of treatments at 5% level. Control: CTL (without AMF); native inoculum (soil): GNA, SBE and SPO; exogenous inoculum: SC3 (AMF spores suspension); and mixed inoculum: MS1 (SBE + SC3), MS2 (SPO + SC3) and MS3 (GNA + SC3).

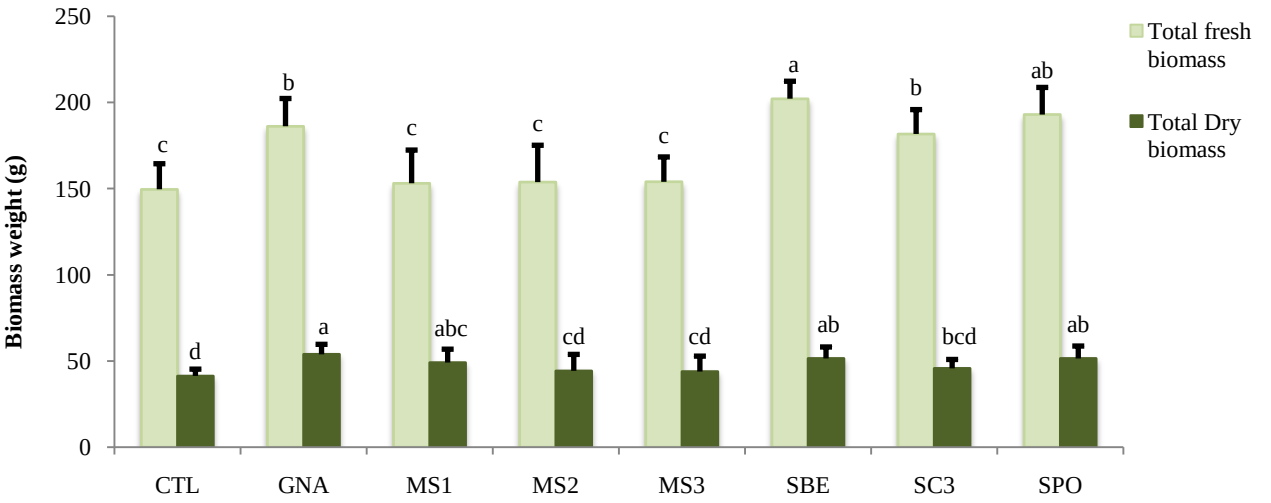

Figure 4: Effect of AMF inoculation on cocoa biomasses production.

Total fresh biomass ($p=2.10^{-16}$), Total dry biomass ($p=5.462.10^{-07}$). Different letters above bars indicate a significant difference among means of treatments at 5% level. Control: CTL (without AMF); native inoculum (soil): GNA, SBE and SPO; exogenous inoculum: SC3 (AMF spores suspension); and mixed inoculum: MS1 (SBE + SC3), MS2 (SPO + SC3) and MS3 (GNA + SC3).

The pure strain comes from temperate country [23] and would probably require a prior adaptation. So, low infectivity of strains can justified this result. Another important aspect showing rigour in the experiences and good sterilization of the substrate is that no roots of control seedlings (CTL) showed presence of AMF.

On the other hand, endomycorrhization of cocoa seedlings has improved vegetative growth parameters and therefore nutrition. It is well known that plant species have very different dependency levels with regard to mycorrhizal colonization[31]. Several studies have focused on AMF action in plants nutrition [32]–[34].This improved nutrition has resulted in biomass production with a gain of 35.1% and 30.3%, respectively, of total fresh and dry biomass observed in seedlings inoculated with GNA. Similarly, leaf area of inoculated plants increased to 17.6% with GNA then that Diouf[35] have obtained an increase of 45% with *Glomusintraradices* strain DAOM 197 198, in their work on sesame. The best performances were obtained in plants inoculated with GNA. However, these performance are low compared to those obtained by Bousselmane[36] on argan tree in greenhouses for six months. They got 83% of growth height with *Glomus* strains compared with the control and fresher biomass gain was 77% in stems and 80% in roots. In dry biomass, the gain was 120% in stems and 70% in the roots. These performance differences could be explained by rate of mycorrhizal colonization that is low in this study compared to 80% observed in only four months on argan tree roots. Strullu[31] attributed this performance to extrametrical hyphae of AMF that allow to explore a significant volume of substrate, in addition to intramatricalarbuscules that increase surface of exchanges and assimilation of minerals for the host plant. This can be vital for replanting of cocoa in natural environment, as the Ivorian orchard is aging. Transplanting plants with effective and adapted to their roots symbionts, can improve the nutrition water and mineral nutrition. Therefore, the plants develop tolerance to abiotic stress they face in developing countries.

The potential of controlled mycorrhization allowed significant improvements in seedlings survival rate, in unfavourable environments, of many forest species. This improvement was observed in chestnut [37], oak [38], pine and Hazel [31]. The contribution of fungal symbionts improves water and nutrients absorption by plants and, consequently, contributes to an improvement in their survival rate especially in the first months following their establishment in natural conditions.

5. CONCLUSION

This study allowed confirming possibility that AMF from temperate area (exogenous AMF) can colonize cocoa (a tropics plant). Inoculation with AMF to different origin has improved vegetative growth (Diameter, Height and Area) and cocoa seedlings nutrition resulted in biomass production. This potential of AMF could be useful for farmers in Côte d'Ivoire to reduce chemical fertilizer inputs, improve soil fertility and increase their yields. Certainly the superiority of infection is attributed to the exogenous strain (SC3: *Rhizophagusirregularis*), but native inoculums (GNA, SBE and SPO) hold superiority of the improvement of studied parameters. However, mixed inoculums (natives AMF and exogenous AMF) showed lower results than natives inoculums.

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