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Full Length Research Paper

Arbuscular mycorrizal fungi associated with *Theobroma cacao* L. in the region of Yamoussoukro (Cote d'Ivoire)

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Arbuscular mycorrhizae are the most widespread plant symbiosis on earth. This symbiosis is well-known for its positive impact on cultivated plant yields. Cocoa (*Theobroma cacao*) plays an important role in the economic prosperity of Côte d'Ivoire. However, cocoa yields remain low due to a loss of soil fertility and pest and disease damages. The myccorhizal symbiosis could be a way of resolving these constraints. However, its use requires the knowledge of the fungi symbionts. This study aimed at evaluating the diversity of arbuscular myccorhizal fungi (AMF) associated with cocoa in the Yamoussoukro region. Soil samples were collected from four cocoa fields. Spores from arbuscular myccorhizal fungi were extracted directly and after trapping by the wet-sieving method, then identified morphologically. Moreover, soil physical and chemical characteristics were determined and correlated with spore densities. Nine species of AMF belonging to the genera *Glomus*, *Acaulospora* and *Gigaspora* were found to be associated with cocoa. *Glomus* was the dominant genera. AMF spore densities were negatively correlated with phosphorus, magnesium and available potassium but positively with ammonium.

Key words: Arbuscular mycorrhizae, *Theobroma cacao*, Côte d'Ivoire, diversity.

INTRODUCTION

Côte d'Ivoire has held since the late 70's, the honorable rank of the world's largest producer of cocoa (Deheuvels, 2003) with an average production of 12 000 000 tons per year (UNCTAD, 2006). The cocoa sector contributes for 45% of export earnings and over 10% of gross domestic product (GDP). This sector occupies about 15% of the rural active population. Consequently, cocoa plays an important role in the economic prosperity of Côte d'Ivoire.

Despite this performance, levels of farm productivity remain low. The observed yields vary on average from 300 to 450 kg/ha against 2000 to 2500 kg/ha as indicated through research previsions (CNRA, 2008). There are numerous causes of this bad performance. These include the aging of the existing orchards, depletion of forest reserves, the failure of spontaneous replanting and the high cost of inputs (Janny et al., 2003). To this is added, the low rate of adoption of selected plant material and crop management, as well as the decline in soil fertility and the emergence of new diseases and pests (CNRA, 2008).

In order to solve these constraints, various research programs aiming at the development of alternative

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intensive and competitive systems of sustainable cocoa farming that have limited environmental impacts have been undertaken. These include the improvement and selection of high yielding and resistant cocoa plants, the development of techniques for regeneration and rehabilitation as well as cocoa cultivation techniques combining tree species and soil fertility management (CNRA, 2008). Another approach not very developed in Côte d'Ivoire is the use of soil microbial potential, especially arbuscular mycorrhizal fungi. These soil fungi are associated to roots of most land plants to form a body called mycorrhiza. Mycorrhizae are the seat of a two-way exchange of carbon compounds from the plant to the fungal symbiont and inorganic compounds and water of the fungus to the plant. This symbiosis improves water and mineral absorption, and plant resistance to biotic and abiotic stress (Smith and Read, 1997). It means that their use in agriculture should allow reducing intensive utilization of pesticides and fertilizers (Atkinson et al., 2002). Moreover, various studies of diversity, performance of selected strains and inoculation were conducted by researchers around the world.

This work led to the development of AMF inocula. However, the integration of this technology in agriculture in general and particularly in Ivorian cocoa agrosystems requires that endogenous strains of these fungi be identified. The initiation of such program is important in order to lay the foundations for sustainable agriculture in Côte d'Ivoire. This article aimed at identifying endogenous arbuscular endomycorrhizal fungi associated to cocoa fields in the region of Yamoussoukro. It also aimed at evaluating the correlation between a community of these fungi and the physico-chemical properties of agricultural soils.

MATERIALS AND METHODS

Presentation of the study area

This study was conducted in the region of Yamoussoukro, specifically in the sites of Konankro, Yobouékro and Toumbokro. These sites are located between latitudes 6° 49' and 6° 59' North and longitudes 5° 17' and 5° 43' West. The climate is equatorial, hot and humid with an average annual temperature ranging from 24.6 to 27.7°C and an average rainfall of 1100 to 1600 mm. It is divided into four seasons.

A long dry season from mid-November to mid-March, characterized by the presence, in December and January of the harmattan, a dry and powerful wind from the Sahara, which considerably lowers the temperature; a long rainy season from mid-March to mid-July; a short dry season from mid-July to late August; a short rainy season from late August to mid-November. The relative humidity of this region is 75%. However, it is higher during the rainy season (60 to 85%). The minima are in the range of 60 to 65% with 40% falling from December to March.

Sample collection

Sampling was carried out at four different cocoa fields: Konankro, Yobouékro1, Yobouekro2 and Toumbokro. In each cocoa field, two points distant from 200 m were considered. At each point, soils closer to cocoa plant rhizosphere were sampled at twelve subpoints in circles of 3 and 6 m. The sampling was done using an auger of 20 cm in diameter. Before moving from one field to another, the auger is disinfected with bleach.

Soils from each point was recovered in a polyethylene bag, and then mixed. Soils from both two points distant from 200 m are then mixed to form a composite sample. Only the horizon of the topsoil (0 to 20 cm) was sampled because it was shown that AMF colonize young and thin roots (Burle, 1961). Also, 95% of root hairs are concentrated in this horizon (Jadin and Vaast, 1990). The soil sample from Yobouékro1 was taken at the beginning of November (end of the short rainy season). The other three sites, samples were taken at the beginning of June (long rainy season).

Soil physico-chemical characteristic measurement

Of each composite soil sample collected in each cocoa field, was taken 1 kg of soil for physicochemical characterization. The samples were dried in open air laboratory for 4 days and clods were crushed by hand and gravels removed. Subsequently, the soil was slightly crushed and passed through a 2 mm calibrated sieve. Analyses were performed on soil fractions smaller than 2 mm. Physical characteristics (fraction of clay, sand and silt) and soil chemical parameters were analyzed. pH was measured in a water-soil (2/5) solution with a pH-meter. The methods of Walkley and Black (1934) and Kjeldahl (1883) were used for the determination of organic carbon, total nitrogen and organic ammonium (Thomas, 1982). Exchangeable cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) were extracted by the ammonium acetate method (Thomas, 1982). The total phosphorus (Pt) and cation exchange capacity (CEC) were extracted by perchloric acid. All these parameters were correlated with the density of spores obtained in cocoa fields. Saturation (V) of the elements Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} was calculated with the following formula:

$$V\,x = ([X] / CEC) \times 100$$

where x: ion and [X]: concentration of the ion.

Direct extraction of spores

This consisted of extracting spores directly from field soils. For each sample, 25 g of soil were analyzed in triplicate. Spores were extracted following the wet sieving technique described by Gerdemann and Nicolson (1963). In a beaker of 2 L, 25 g of soil were dissolved in 1 L of water. The mixture was stirred and mixed. After one minute, the supernatant of the beaker was passed through a series of sieves of mesh 500, 125, 90 and 45 microns. These operations were repeated four times with the same soil. Deposition in the sieve of 500 microns mesh generally made up of debris was discarded. The contents of the other three screens were transferred in a beaker containing water using a spatula.

Extraction of spores after trapping using cowpea (*Vigna unguiculata*)

In field soils, it can sometimes be difficult to find spores. It is therefore necessary to use the soil as inoculum to allow possible spore germination. Cowpea (*V. unguiculata)* was used as host plant. Pre-germinated seeds of cowpea were sown in plastic pots containing 700 g of a sandy soil previously sterilized in an oven at 120°C for 2 h 30 min. To this soil was added in triplicate 100 g of soil from each sample in pot cultures. The pots were kept in a growth chamber where plants were exposed to light. Plants were

Table 1. Clay, silt and sand content of soils collected from different cocoa fields.

Proportion of soil physical components			
Site	**Clay (%)**	**Silt (%)**	**Sand (%)**
Konankro	15.75	26.66	56.82
Toumbokro	37.9	37.98	23.08
Yobouekro 1	14.35	14.03	70.78
Yobouekro 2	39.67	37.89	21.47

Table 2. Chemical characteristics of soils.

Site	pH_{H2O}	C%	N%	NH_4^+	Pt	CEC	Ca^{2+}	Mg^{2+}	Na^+	K^+
Konankro	5.9	2.05	0.22	0.29	167	8.80	3.16	0.38	0.07	0.08
Toumbokro	5.9	3.82	0.35	0.07	333	28.20	7.24	1.84	0.06	0.30
Yobouekro 1	5.4	1.56	0.16	0.21	222	4.60	2.18	0.39	0.06	0.11
Yobouekro2	6.5	3.31	0.29	0.02	344	27.2	8.95	1.93	0.04	0.29

watered every morning for 15 days and then every two days. No amendment was made to the plants. After 60 days of culture for samples from Yobouékro 1 and 27 days for the other samples, 25 g of soil from each sample was used to extract spores using the wet-sieving method described by Gerdemann and Nicolson (1963).

Spore identification

Spores extracted directly from soil or after trapping were placed on Petri dishes and observed under a binocular microscope. Based on morphological characteristics, the spores were separated and counted. Also, spores were placed between plates and strips in polyvinyl-lacto-glycerol (PVLG) for morphological identification. The spore morphological features were compared with original descriptions of AMF species (Schenck and Perez, 1990) and with the online reference culture database published at http: invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm. Then, names were assigned to each spore type. Spore density and relative abundance (RA) of different types were determined. Spore density was defined as the number of spores in 25 g of soil and relative abundance (RA) of each type was determined by the formula used by Johnson et al. (1991):

AR = [(total number of spores observed of one genus in all sites / total number of spores observed in all sites) × 100]

Analysis and data processing

Data analysis was performed with XL STAT SOFTWARE 2010.2.01. Analyses of variance (ANOVA) of spore density were carried out. Tests of significance between these densities were performed in agreement with the Fisher test. When significant differences were observed between treatments, the Student Newman Keuls test (SNK) at 5% was used for comparison of mean. Also, the Pearson correlation test at 5% level of significance was performed to study the correlation between spore density of AMF and soil physico-chemical characteristics.

RESULTS

Physical and chemical characteristics of soils

The results of soil physico-chemical analyses are indicated in Tables 1 and 2. It was shown that the soil physical characteristics (texture) vary from field to field. Depending on the content of sand, clay and silt, the soil was sandy loam (Konankro), silty clay (Toumbokro, Yobouékro 2), and sandy (Yobouékro 1). The different soils were acidic with a pH (H_2O) ranging from 5.4 (Yobouékro 1) to 6.5 (Yobouékro 2). The C/N ratio in soils were in agreement with generally expected values ranging from 7 to 16%.

The Pt content of the soil sampled in Konankro is 167 ppm. This value is lower than the relative value that range from 200 to 400 ppm. The other three sites had a normal Pt content. Soils from the four fields had low organic NH_4^+ contents that ranged from 0.022‰ (Yobouékro 2) to 0.294‰ (Konankro). However, it was shown that the soil from Toumbokro had a higher capacity of cation exchange (CEC) (28.2 méq/100 g) than the other sites. Moreover, the soils from the four fields showed low levels of base saturation as compared to suitable values for better cocoa growth that range from 60 to 70%.

Occurrence of AMF spores in different cocoa field soils and in the traps

The total amounts of spores obtained directly from each field and after trapping are indicated in Table 3. It was

Table 3. Comparison of spore densities occurring in field soils SD(F) and obtained after trapping (SD(T)). Spore densities with the same letters lane are not significantly different.

Site	SD (F)	SD (T)
Konankro	6292.3[a]	2333.7[a]
Toumbokro	1094[b]	1897.5[a]
Yobouekro 1	162.1[b]	2039.8[a]
Yobouekro 2	1167[a]	5237[a]

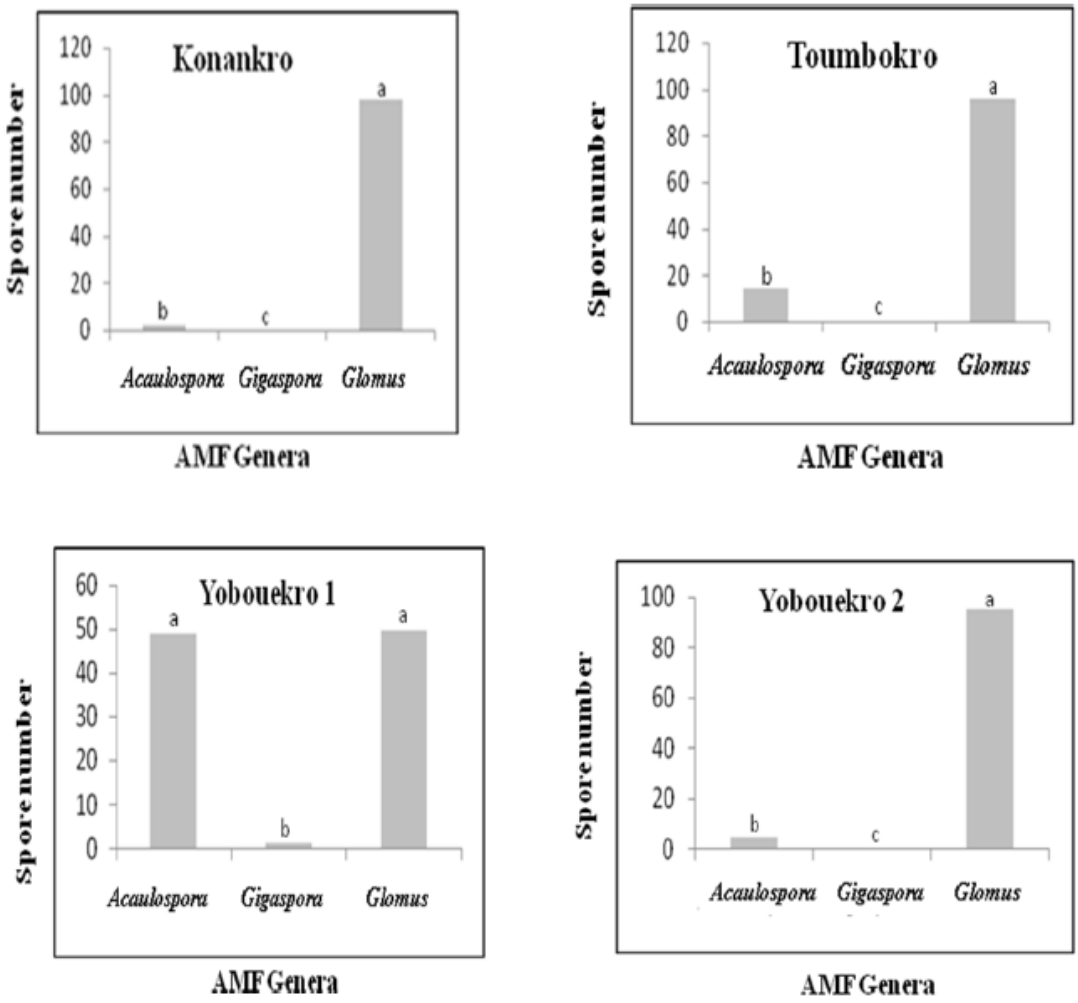

Figure 1. Occurrence of AMF genera identified in different cocoa field soils. Different letters indicate significant differences between the means ($p < 0.05$).

shown that the cocoa field located in Konankro (6292 spores/25 g of soil) had an amount of spores significantly higher than the spores in the other fields. The field in Yobouékro1 (162 spores/25g) had the lowest spore amount. Konankro and Yobouékro2 showed no significant difference between densities of spores extracted before and after trapping. On the other hand, in Toumbokro and Yobouékro 1, densities of spores extracted by trapping were significantly higher than the densities of spores obtained directly from the fields.

AMF spores densities in the cocoa fields

Identification based on spore morphology allowed the detection of three main AMF genera (*Acaulospora*,

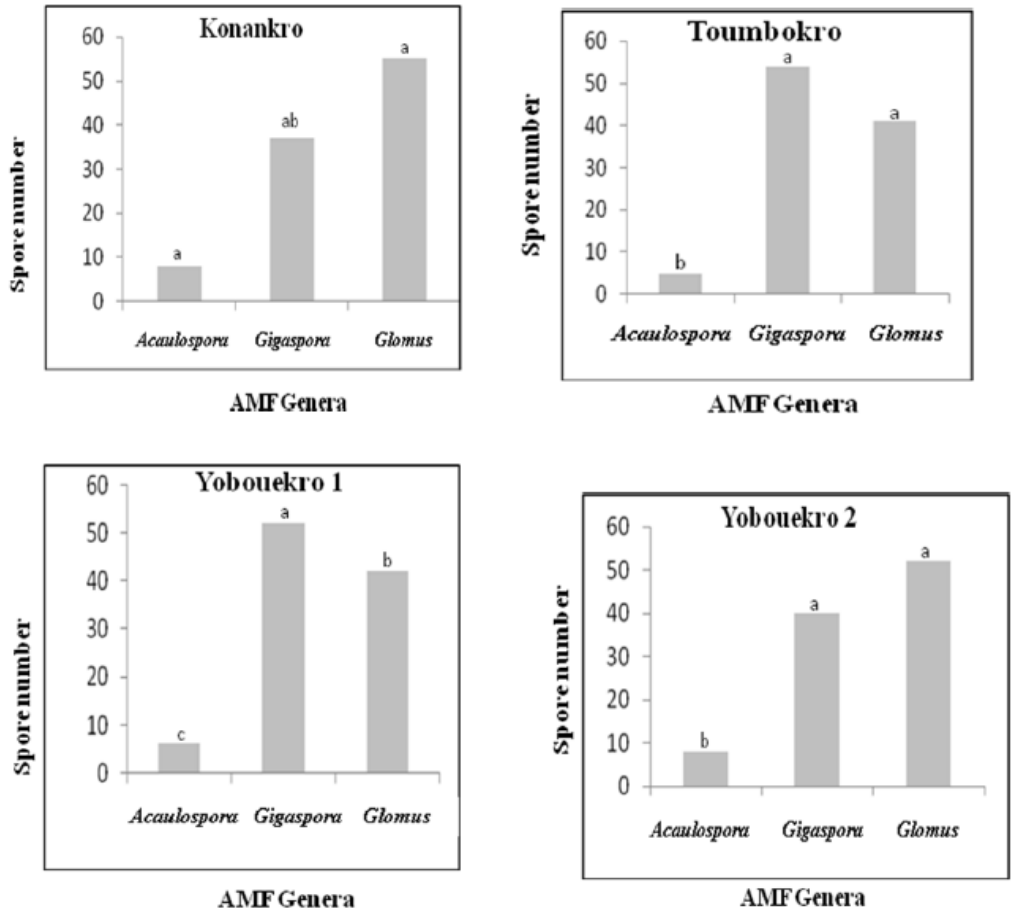

Figure 2. Occurrence of AMF genera obtained from soils after trapping with Vigna unguiculata. Different letters indicate significant differences between the means ($p < 0.05$).

Gigaspora and *Glomus*) in the four cocoa fields. These three genera occurred in the fields at different densities (Figure 1). Overall, the genus *Glomus* appeared to be the most abundant in the four cocoa fields except in Yobouekro 1 where *Acaulospora* and *Glomus* were both abundant. *Gigaspora* was the rarest genus. After trapping, all the same genera were detected in the four fields (Figure 2). However, in this case the rarest species was *Acaulospora* while *Gigaspora* and *Glomus* were the most abundant in Toumbokro and Yobouekro 2. However, in Yobouekro 1, *Gigaspora* was the most abundant while in Konankro *Glomus* was the most abundant.

Composition of AMF species associated to cocoa fields

The observation of morphological characteristics of spores allowed the identification of 8 spore types (2 types of *Acaulospora*, 1 type of *Gigaspora* and 4 types of *Glomus*) in the four fields. There was no difference in AMF diversity from one cocoa field to another. However, the densities of spore types were variable (Figure 3). The spore type *Glomus* sp.2 was the most abundant in cocoa fields from Konankro, Toumbokro and Yobouekro2 while there was no significant difference from one field to another for the other 7 types of spores. The cocoa field from Yobouekro1 had the lowest spore densities no matter the spore type. After trapping, 9 types of spores (1 type of *Acualospora*, 3 types of *Gigaspora* and 5 types of *Glomus*) were identified (Figure 4). The trapping increased both *Gigaspora* and *Glomus* spore types while the *Acaulospora* type decreases to only 1 type. Again there was no difference in species composition from one field to another. Moreover, there was no significant difference between fields in terms of spore densities.

Correlation between spore density and soil physico-chemical characteristics

The matrix of Pearson correlation (r) which defines the correlation between spore densities and soil physico-chemical characteristics are given in Tables 4 and 5. It

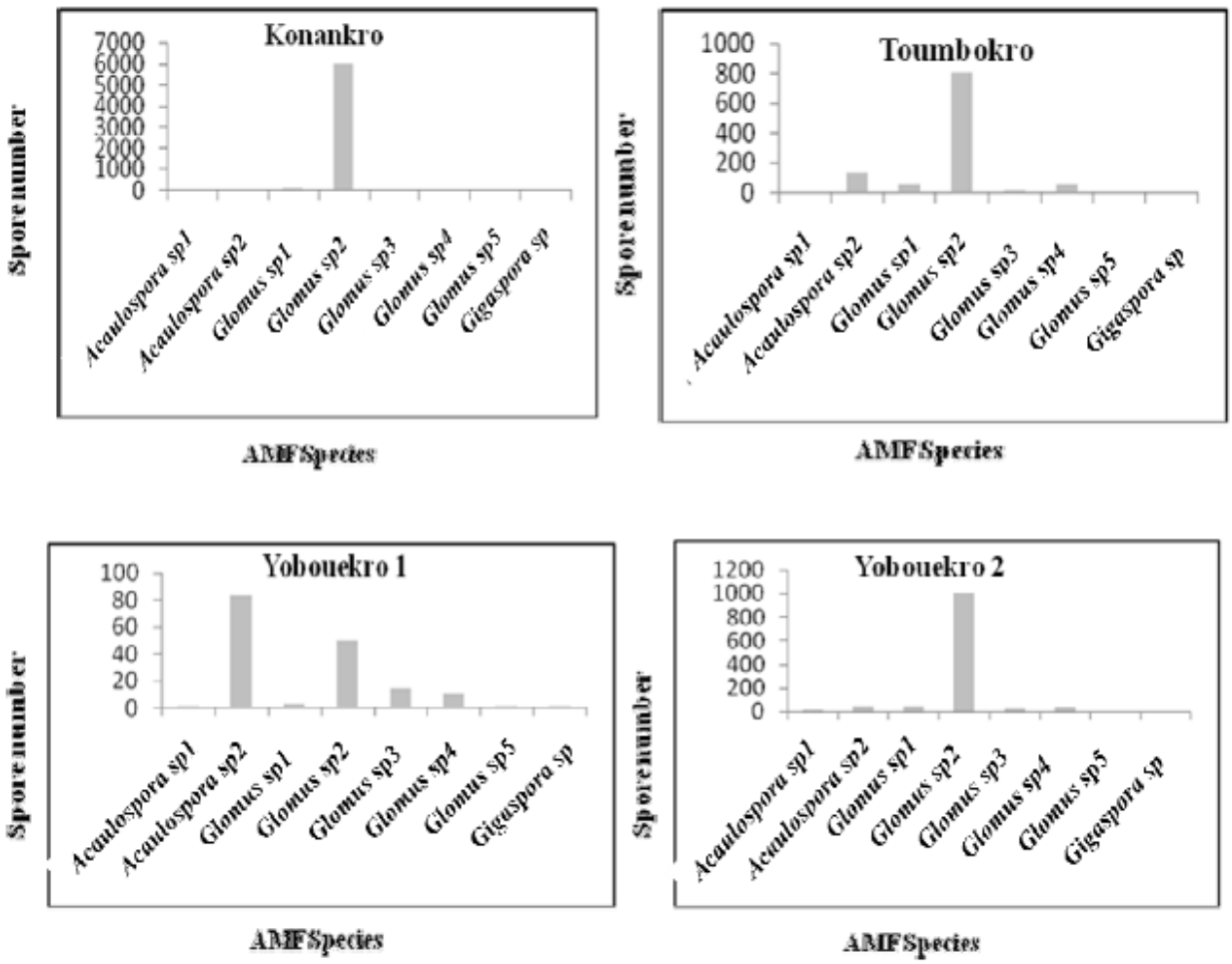

Figure 3. Composition of AMF communities in different cocoa field soils.

was shown that Mg2+, K+ and total phosphorus (Pt) having Pearson correlation (r) coefficients respectively -0.95, -0.58 and -0.65 were significantly negatively correlated with spore density ($P<0.05$). However, with a value of r=0.65, the organic ammonium form (NH4+) was significantly positively correlated with spore density ($P<0.05$).

DISCUSSION

This is the first study reporting the population dynamics of AMF associated to cocoa fields in Cote d'Ivoire. Due to the fact that AMF are widespread, this study reported the occurrence of AMF spores in all cocoa field soils. However, a variation could be observed in spore density and distribution within the four fields. The results showed that the soil from Konankro cocoa field contained the highest number of spores while the lowest was observed in Yobouekro1.

This variation could be due to environmental factors and soil physico-chemical characteristics that can affect the density, distribution and composition of AMF spores in fields as previously reported (Khadge, 1988, Bardwaj et al., 1997; Barrow et al., 1997; Li et al., 2007). According to the physical characteristics, the soils could be classified in three main types: 1) sandy, 2) sandy loam and 3) silty clay. Depending on the content of sand, clay and silt, the soil was sandy loam (Konankro), silty clay (Toumbokro, Yobouékro 2), and sandy (Yobouékro 1). The different soils were acidic with a pH (H_2O) ranging from 5.4 (Yobouékro 1) to 6.5 (Yobouékro 2). The fact that the soil in Konankro was sandy could explain the massive presence of AMF in this field. Sandy loam soils have been previously reported to contain a high number of AMF spores associated to tomato (Sreevani and Reddy, 2004). Moreover, spore densities were significantly ($P<0.05$) negatively correlated with P content in all the four fields. The negative correlation between spore density and the P content in the root zone obtained in this study corroborates the work of Nemec et al. (1981). The researchers found similar results in a study of the distribution and ecology of AMF in *Citrus* nurseries.

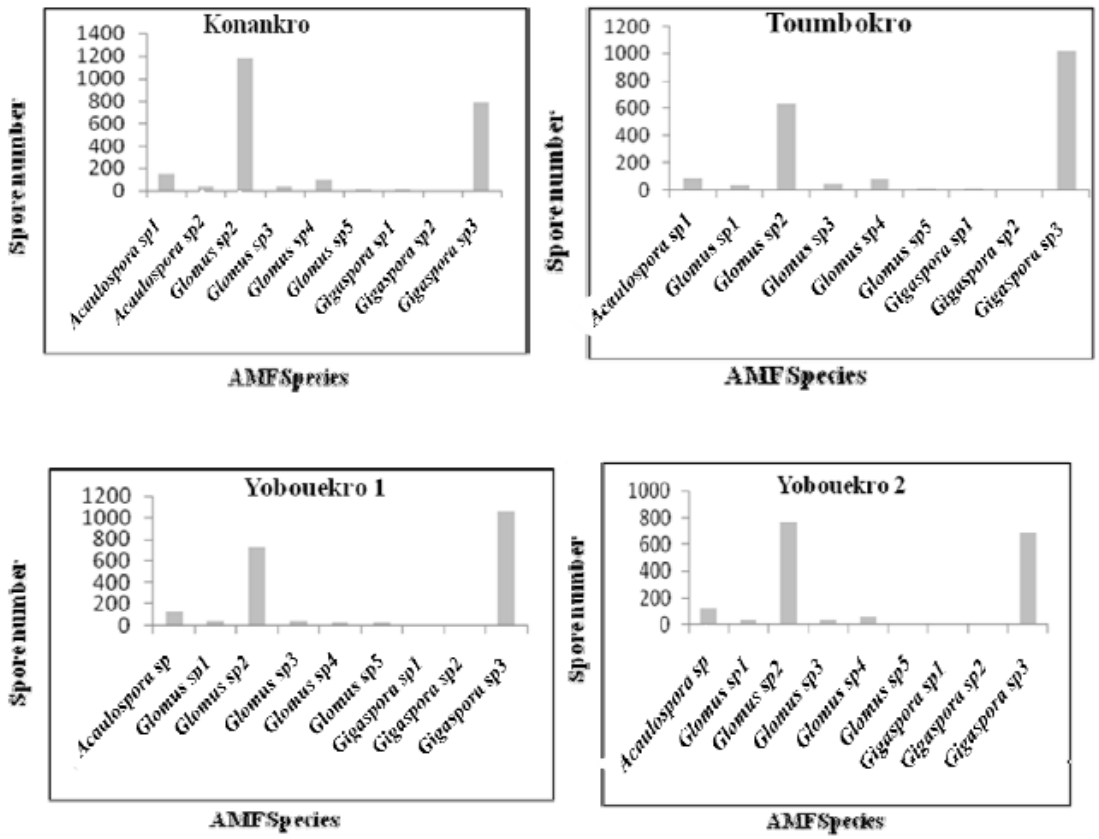

Figure 4. Composition of AMF communities after trapping using soils from different cocoa fields.

Table 4. Correlation coefficients (r) between spore number and soil nutrient contents in different fields. Coefficient in bold are significantly correlated (P<0.05).

Nutrient factors	Sites				Correlation
	Konankro	Toumbokro	Yobouekro1	Yobouekro 2	
No AMF	6292.3	1094	162.1	1167	-
C (%)	2.05	3.82	1.56	3.31	-0.25
N (%)	0.22	0.35	0.16	0.29	-0.09
NH4	0.294	0.0672	0.2128	0.0224	**0.65**
Pt	167	333	222	344	**-0.65**

This showed evidence that soil P content negatively impact sporulation in field soils. The high spore density extracted in the site from Konankro may be related to low soil phosphorus. It seems that the plant is able to regulate its state of mycorrhization depending on the amount of available soil P, when the maintenance cost exceeds the benefit of mycorrhizae provided by the fungus (Marshner, 1995). Indeed, when the soluble phosphorus is present in high enough quantities, although the AMF always induces its accumulation in the plant, it would have more influence on plant growth (Al-Karaki, 2002). Thus, soils with low phosphorus contents as in Konankro exhibit a high spore density compared with soils rich in P. The soil from Konankro had the least K content. Potassium was shown to be negatively correlated to spore densities (r=-0.58; P<0.05). This result is similar to that found by Khade and Rodrigues (2009) in a study conducted in India on the diversity of AMF associated with varieties of papaya (*Carica papaya* L.) a significant negative correlation between potassium and spore density. Moreover, the soil from Konankro had a low Mg^{2+} content that was negatively correlated to spore density. All these factors may have contributed to a better AMF spore formation in the Konankro field. The

Table 5. Correlation coefficients (r) between spore number and soil properties in different fields. Coefficient in bold are significant (P<0.05).

Cation exchange capacity factors	Sites				Correlation
	Konankro	Toumbokro	Yobouekro1	Yobouekro 2	
No AMF	6292.3	1094	162.1	1167	
Ca^{2+}	3.16	7.24	2.17	8.95	-0.12
Mg^{2+}	0.38	1.84	0.39	1.93	**-0.94**
Na^{+}	0.07	0.06	0.06	0.04	0.08
K^{+}	0.08	0.30	0.11	0.29	**-0.58**
pH_{H2O}	5.9	5.9	5.4	6.5	0.11

lowest spores density was observed in the Yobouékro 1 cocoa field.

This could be due to the saturation level of salt (NaCl) in Yobouekro soils. Indeed, it was shown that high salt concentrations could inhibit mycorrhizae formation and restrict the activity of most mycorrhizal fungi (Juniper and Abbott, 1993). The spores of the AMF associated to these soils were classified in three genera (*Glomus*, *Acaulospora* and *Gigaspora*). *Glomus* was found to be the most abundant genus in the four cocoa fields. This result was not a surprise since it was previously shown that *Glomus* species are the most abundant glomeromycetes in tropical areas (Husband et al., 2002; Snoeck et al., 2010). It could also be attributed to the wide range of distribution (ubiquity) of this genus. Moreover, most studies have shown that *Glomus* was the most common AMF genus found in cultivated soil (Nemec et al., 1981; Khade and Rodrigues, 2008a, b).

Conclusion

This study is part of a research program aiming at improving cocoa production in Côte d'Ivoire by optimizing mineral nutrition as well as plant resistance to biotic and abiotic factors. Its main objective was the identification of various strains of arbuscular endomycorrhizal fungi associated with cocoa in the region of Yamoussoukro. The morphological characteristics of spores showed different types of arbuscular mycorrhizal fungi associated with cocoa fields.

However, morphological identification did not allow distinguishing the species associated with different types of spores obtained. The use of new identification tools such as molecular biology, in particular the amplification and sequencing and phylogenetic analyses using the18S rRNA could be considered.

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