Investigation Of Mycorrhizal Infection In Cowpea [*Vigna unguiculata* (L.) walp] In Calabar.

Anozie, Henry. I* and Orluchukwu, J. A

Department of Crop and Soil Science, Faculty of Agriculture, University of Port Harcourt, Nigeria.
* henifez@yahoo.com

Abstract: Mycorrhizal (fungus-root) infection on cowpea [*Vigna unguiculata* (L) walp] was investigated. The samples – uprooted mature cowpea plants, the plant's rhizosphere soils and non-rhizosphere soils of the same area were collected randomly at 10 different locations in the Staff Village of the University of Calabar. The terminal feeder root samples were used for the determination of the fungal – root colonization by applying the method of root//gridline intersects. The recovery and enumeration of fungal spores in the rhizosphere soils was done through wet sieving, decantation and centrifuging in a sucrose density gradient column. Available Phosphorus was also determined from the rhizosphere soils. The leaf sample – for total Nitrogen, Phosphorus and Potassium. The total Nitrogen, available Phosphorus exchangeable Potassium, Organic Matter and the textural soil type were determined in the non-rhizosphere soils. The investigation revealed 40.7 % arbuscules in the feeder roots and 58 fungal spores from 5g of the rhizosphere soils both at high magnifications (X14) of the microscope. The analysis showed 4.06 %, 0.205 % and 6.1 % of N, P and K respectively from the plant leaf samples, 0.116 % N, 38.25 mg/g P, 0.25 meq K/100 g and 2.31 % organic matter from the sandy loam non-rhizosphere soils (74.3 % Sand, 9.7 % Silt and 16 % Clay). The Phosphorus of the rhizosphere soils was 37.1 ppm. The presence of arbuscles in cortical cells of the stained feeder roots of cowpea is vesicular – arbuscular endomycorrhizal. The particle size analysis showed a well-drained fertile soils, and the correlation of the Phosphorus in non – rhizosphere soils with the plants P as well as the rhizosphere P with plants P, the t – test showed no significant difference ($P < 0.05$) indicating that the plants in a fertile soil like this, depended less on mycorrhizae for its uptake. Thus, there was no need for inoculation of the soil with mycorrhizal fungi unless in a later depleted state of the soil, since it is observed that fertile soils are not responsive to mycorrhizal inoculation.

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Introduction

Cowpea (*Vigna unguiculata* (L) walp) is a tropical grain legume widely grown in sub Saharan Africa (SSA), Asia, parts of the United States and Southern Europe (Singh *et al.,* 1997). Most cowpea does well in Africa, with Niger and Nigeria accounting for 66% of the world cowpea production. Between 2010 and 2014, Nigeria produced an average of 3.5 tonnes of cowpea followed by Niger with approximately 1.6 tonnes (faostart.org; updated in Aug, 2018). Saidi and Itulya (2010) have noted that it is one of the highly appreciated species of African leafy vegetable. It is of great importance to livelihoods of millions of people in West and Central Africa's semi-arid regions. Cowpea being a protein-rich grain is a suitable complement to starchy tuber crops and staple cereals. It serves as livestock fodder, improves the soil via nitrogen fixation, and benefits households by bringing in cash and diversifying income sources. Despite the great significance that cowpea has in regions such as the sub-Saharan Africa, the yields are significantly low. Plant growth is affected by low phosphorous availability in many soils as a result of P fixation by Fe, Ca and Al, which leads to formation of inorganic phosphates that are insoluble in soil (Ibijbijen *et al*., 1996). Additionally, cowpea growth and productivity are lowered by prevalence of various diseases and insect attacks. There is at least one major insect pest at every stage of cowpea's life cycle, which could cause serious damage and negatively impact the yield. Several plants possess different and specific inducible defence mechanisms for protection against insect attacks, allowing them to acquire nutrients more efficiently under stress conditions. Despite having such mechanisms, the major cowpea insect pests such as the legume pod borer, are still uncontrolled. Most farmers rely on costly commercial farm inputs such as insecticides and phosphate-rich fertilizers to address the mentioned constraints.

There is need to come up with alternatives for minimizing over-reliance on such commercial farm inputs. Host-plant resistance is the most economical and environmentally friendly way of controlling insect pests (Sharma and Ortiz, 2002). According to Cardoso and Kuyper (2006), biotic processes like symbioses have the potential of improving agricultural sustainability with less dependence on non-renewable inputs such as artificial fertilizers. Such symbiotic

processes include arbuscular mycorrhizal fungi (AMF), a group of beneficial soil microbiota that forms symbiotic associations with roots of higher plants enhancing plant nutritional uptake and resistance to biotic and abiotic stresses (Smith and Read, 1997). Although AMF are considered essential primarily for phosphorous uptake, they can also increase plant acquisition of other minerals such as Zn, N, Cu and Fe (Cavagnaro, 2008). In all legume plants, cowpea is able to form symbiotic association with microorganisms such as rhizobia and arbuscular mycorrhizal fungi (AMF). This can improve plant growth under low soil fertility condition, confer tolerance to some pathogens, improve the water balance of plants and can influence soil aggregation (Dalpé, 2005; Marulanda *et al*., 2006; Rillig and Mummey, 2006).

Therefore, these symbiotic associations are increasingly gaining popularity as a significant factor of sustainable agro-ecosystems. AMF are especially important for plant nutrition in organic and low input farming systems because these systems do not utilize synthetic fertilizers and fungicides, which can tremendously reduce levels of root colonization by AMF (Cavagnaro *et al.,* 2011). However, in Nigeria particularly in Calabar Municipality, Cross River State; little is known about the influence of ecological site conditions on AMF community composition. Therefore, the aim of this study was to analyse the AMF communities composition in cowpea cultivated in Calabar Municipality, Cross Rivers State, Nigeria.

Materials and Methods

The study area

Samples of roots and rhizosphere soil were collected within Calabar in the Cross River Basin area located between latitude 4.96°N and longitude 8.31°E at an elevation of 700 km above the sea level (Tageo.com 2013)

Sample Collection

Matured cowpea plants of about two months old were uprooted at random from the different cowpea farm plots (a total of ten plants, one from each plot). Rhizosphere soil samples were collected by shaking the roots of the uprooted cowpea plants to release attached soil. The collected soil samples was bulked, packed into polythene bags, labelled and taken to the laboratory for analysis.

Root samples used for quantifying mycorrhizal infection were collected by severing-off the terminal feeder roots (unsuberized) from the uprooted cowpea plant's roots using a pair of Scissors. The roots were packed in polythene bags and taken to the laboratory for analysis. Leaves samples were collected from the uprooted cowpea plants using a sharp knife. The leaves collected were packed in polythene bags and taken to the laboratory.

Non-rhizosphere soil samples were collected at random from ten different maize plots at depth of 0 – 15 cm using spades, bulked and carried to the laboratory using polythene bags (well labelled).

The bulked rhizosphere and non-rhizosphere soil samples were air-dried, ground and passed through a 2 mm sieve, labelled and stored for laboratory analysis. The leaves samples were washed, chopped into smaller fragments, packed in brown envelopes, dried in an oven for two days at 160^0 C, ground and stored for laboratory analysis.

Laboratory Analysis

Laboratory analysis carried out in this study were both soil chemical and physical analysis. Particles size distribution was determined using the hydrometer method as described by Bouyoucos (1965). Organic carbon was determined by the wet oxidation method (Walkley and Black, 1934). Total Nitrogen was by the micro-Kjeldhal digestion method. Available phosphorus was determined by Bray 1 method (Bray and Kurtz, 1945). Exchangeable bases (Ca, Mg, K and Na) were extracted in 1N NH_4OAC at pH 7. Sodium and K were determined with a flame photometer while Ca and Mg were determined with the atomic absorption spectrophotometer (AAS). Effective Cation Exchange Capacity was by summation of exchangeable bases (Ca, Mg, K and Na).

Arbuscular Mycorrhizal Fungi Colonization Assessment

Freshly harvested feeder roots were thoroughly washed in tap water and fixed in 50% ethanol. They were then cleared and stained after Koske and Gemma (1989) method. Colonization was determined by viewing under a dissecting microscope at × 45 magnifications using a grid intersection method of Giovannetti and Mosse (1980).

All data were subjected to a combined analysis of variance using the Windows version of Statistical Analysis System (1996) and Duncan's Multiple Range Test (DMRT) was used to separate the means at 0.05 level of probability when the F-ratio was significant.

Isolation and Estimation of Arbuscular Mycorrhizal (AM) Spore Population

The rhizosphere soil samples in three replicates were used to determine AM spore population from 50 grams portion of soil. The AM fungal spores were isolated from composite soil samples by employing "wet sieving and decanting technique" as described by Gerdemann and Nicolson (1963) and were quantified by using the "grid-line intersect method" as described by Adoleya and Gaur (1994). Results were ex- pressed as mean of three replicates for each sample. The abundance of spores determined for each sample was

expressed as the number of AM fungal spores per 10 grams of soil for all the samples studied.

Identification of Arbuscular Mycorrhizal Fungal Species

Intact spores and sporocarps were mounted in lacto- phenol and identified according to their spore morphology by using taxonomic key [Schenck and Perez (1990) and Morton and Redecker (2001). The qualitative estimation was expressed as percentage frequency occurrence of AM fungal species.

Estimation of Arbuscular Mycorrhizal Root Colonization

Roots of the test plant species after thorough washing with tap water were cut into approximately 1-cm pieces and processed by employing "rapid clearing and staining technique" as described by Phillips and Hayman (1970). 50-stained root bits were examined under microscope for mycorrhizal root colonization. The observations were recorded from each sample containing 50 root segments according to the method suggested by Bierman and Linderman (1981). The relative abundance of mycelium, vesicles and arbuscules of mycorrhizal fungi in the host tissues were recorded as abundant, moderate and scanty. Per cent root colonization was quantified based on the number of root segments colonized by AM fungi using the formula:

$$\text{Percentage root colonization} = \frac{\text{Number of root segments colonized}}{\text{Number of root segments observed}} \times \frac{100}{1}$$

Statistical Analysis

Statistical Packages for Social Sciences (SPSS) (Systat Co., http://www.spss.com) 16.0 version package were used for the statistical analysis of the data. Pearson Correlation analysis was performed to assess the relationship between AM fungal spore density of rhizosphere soils, percent root colonization by mycorrhizal fungi, fungal population of rhizosphere and non-rhizosphere soils and soil edaphic factors at 0.01% and 0.05% levels of significance and found a good association between them.

Result

Physicochemical analysis of the soil revealed that it is characteristically sandy loam consisting of 75.6% sand, 7.7% silt and16.7% clay. The chemical composition of the soil was 0.10% total N; 35.5mg/g P; 0.30 cmol kg^{-1} K; 1.18% organic carbon and 2.04% organic matter.

Fungi – Root Infection

The result of the observation of the stained root of cowpea (*vignaunguiculata*) as seen in Table 3 under microscope (X14) showed the presence of some arbuscles in the cortical cells of the roots, indicating infection of the roots by vesicular-arbusclar endomycorrhizas.

Table 1: fungi-roots colonization and fungi Spore Count in the Rhizosphere Soils

Fungi-Root Infection (%)	No. of fungi Spores in the Rhizosphere Soil/g soil
40.7	58

One of the such factors include soil fertility and nutrient concentration, considering Table 4, there was a high level of the concentration of macro nutrients elements that was tested for (NPK) in the soil, this coupled with the high level of organic matter in the soil, Table 4 indicated a high fertility level of the soil.

Table 2: Nutrient Concentrations in the Plant (Cowpea) and Soils

PLANT			SOIL				
N (%)	P (%)	K (%)	N (%)	P (mg/g) Rhi	Non-Rhi	K Meq/100	OC (%)
4.06	0.205	6.1	0.116	37.1	38.25	0.25	2.311

Rhi = Rhizosphere, Meq = milliequivalent

Marx (1975) and Zhihui *et al.,* 2016) observed that high Nitrogen and P concentration in the soil could enhance root growth and protein synthesis causing a decrease in carbohydrate in feeder root, this he observed can cause a dramatic reduction in their susceptibility to mycorrhizal infection especially ectomycorrhizas. This agrees with Ibrahim Ortas (2012) who conducted that mycorrhizal infection decreases in nutrients rich soils and after fertilizers applications.

They showed that increasing application of KH_2PO_4 can reduce the root length of a plant which is infested by VAM, Slankis (1974), also showed that the application of Nitrogenous fertilizer like ammonium nitrate and Cadmium nitrate to boost soil fertility could reduce VAM infection.

Another possible cause of low infection level is the pH of the soil. Though the soils' pH was not tested for, the abundance of the major macro nutrient elements in the soil (Table 2) can encourage a speculation that the pH of the soil as at the time of sampling was within the range of 5.5 and above, since that is the range where most macro nutrient elements are in available form to plant, below this range is acidic soil where most macro elements are in fixed form. Mycorrhiza infection is considered to be more in an acidic soil. This agree with Slankis (1974) who stated that mycorrhizal formation in rots is greater under acidic condition. This observation may be due to low nutrient availability rather than soil reaction. He also reported that alkaline soil conditions in the rhizosphere severely inhibit the growth of some type of mycorrhizal fungi, whereas most macro nutrients (N, P, K, Ca and S) are in available form at pH of 6.6 – 7.8 which is mildly alkaline to moderately alkaline. This agrees in part with Asghari and Cavagnaro (2012) who ascribed the reduced mycorrhizal infection in alkaline soil to an increased Nitrogen availability due to enhanced nitrification rather than the high pH per se. other factors like aeration and moisture might not have had much impact on the level of infection since the mechanical analysis shows a well-drained sandy loam soil type.

It is however worthy to mention that fumigants applied to soils may linger in the soil or years in the course of this research, one does not overlook the possibility of the soil to have fumigated years back and some of these systemic fungi-toxicant like Bordeaux mixtures, triphenyltin acetate, chloroneb (Demosan) can persist in the soil for years after application and Max (1981) observed that this can suppress fungal invasion into roots.

Quantification of Fungi Spores

In the recovery and enumeration of fungi spores from the rhizosphere soils, 58 spores were recovered from 5g of the Rhizosphere soils. This was quite high but as enough to increase the level of infection into root above 40.7% all things being equal. Therefore, this underscores the speculation that most of the fungi were dormant existing as spores without infecting the roots, the high fertility level of the soil might have induced the dormancy of the fungi. Again, fumigation of the soil might have contributed to the dormancy of the fungi. This is in agreement with the findings of because Ceustermans *et al.* (2018) which reported that fumigation can cause a lengthy delay in mycorrhizal development, even in soils with large native fungi populations.

Phosphate Correlation

The result of the chemical analysis as shown in Table 3 indicates a high percentage of Phosphate level in the plant since it is mycorrhizal, thus higher growth. Though it fell slightly below the sufficiency range of 0.2 – 0.5 % which is naturally hardly attained by many plants. This is due to the fact that phosphate ions diffuse very slowly in soils thus making it not easily available to plants. The high level of Phosphate in the plant is well explained by Lizadeh (2011), who observed that mycorrhizas can exploit insoluble or sparingly soluble P sources which are not available to roots and cowpea being mycorrhizal enjoys this advantage.

Table 3: Phosphate – P in the plant, Rhizosphere and the Non-Rhizosphere Soils

Plant – P	Rhizosphere Soil – P (mg/g)	Non-Rhizosphere Soil – P (mg/g)
0.205	37.1	38.25

Table 4: Levels of Phosphate in Plants and Soils

PLANT	Non Rhizo Soil P mg/g X_2	Rhizo Soil P mg/g X_1	Plant P (%) Y
Telfairia	61.5	50.1	0.358
Calopogonum	35.5	39.8	0.071
Cocoyam	28.25	41.3	0.258
Cowpea	38.25	37.1	0.205
Pepper	12.87	23.1	0.252
Okro	62.87	45.3	0.375
Centrosema	36.5	46.6	0.190
Cassava	17.37	22.2	0.258
Water Leaf	44.13	42.7	0.241
Maize	78.62	33.9	0.143
Potato	30.5	18.8	0.238
Σx	446.36	400.9	2.589
Mean	40.58	36.45	0.24

Rhizo = Rhizophere, P = Phosphorus

The high fertility of the soil as reflected in Table 2 adversely affected the extent of mycorrhizal infection in the root, thus checking the absorption of P by the plant as reported by Ibrahim Ortas (2012). Also, the plant was sampled almost at the vigorous vegetative growth stage, this may also explain the reason behind the low P level of 0.205 %. This agrees with Araújo *et al.,* 2018) who observes that the absorption of P in cowpea occurs principally at the end of the growth period, during pod formation.

The t-test results of t-Cal (0.604), t-tab (2.09) indicated no significant difference ($P < 0.05$) in the mean level of % P in the non-rhizosphere soils and the rhizosphere soils of the different plants considered (Table 4). This can thus underscore the speculation that because of the low level of mycorrhizal infection (Table 1) in the root probably resulting from the high level of soil fertility (Table 2), the plant therefore did depend less on mycorrhizas for its P uptake. The correlation coefficient between the rhizosphere soils P/Plants' P and the non-rhizosphere Soils' P/Plants' P. both reveal a positive correlation of 0.92 and 0.867 respectively. The direct proportional relation should be expected since soil is the only source of phosphate to plant, it follows that the higher the available P in soils, the higher the Plants' P grown on it and vice versa.

Conclusion

Vigna unguiculata (L) walp is a mycorrhizal crop, thus has a beneficial dependency upon mycorrhizal fungi for normal root functioning including increase in the absorption rate of phosphorus and other micro element present in the soil.

The rhizosphere is influenced by the activities of the root, including the release of organic substances which would stimulate the growth of fungi leading to increased number of spore in the rhizosphere. The type of exudate by plant root can influence the level of infection by the fungi and even the colonization of the rhizosphere. Phosphorus is a major nutrient element to plant which help in fruit formation but the phosphate in the soil is not readily available to plants of phosphate from the soil.

The area under research (Calabar) is made up of depleted soils with a greater of phosphorus being in a fixed form, thus a greater need for mycorrhizal establishment to make available, the phosphate to plant. The area also depends on cowpea as one of the major stable foods. Proper management of soil including artificial inoculation of soil with mycorrhizal fungi and guarding against systems and practises that can encourage mycorrhizal deficiency like fumigation can help to increase the production of a healthier, drought and disease resistant; and high yield cowpea in the area.

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