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Studies on mycorrhizae development and anatomical confirmation of mycorrhizae formed on *Pinus caribaea* Mor. var. *hondurensis* Barr. and Golf. Seedlings

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The effects of three soil collections from old pine plantations in Nigeria: Enugu, Ugwuoba and Nsukka and a control soil on mycorrhizae development as well as morphology of the mycorrhizal rootlets, anatomical confirmation of mycorrhizae development were investigated. Enugu soil induced the greatest mycorrhizal infection (44%) and growth of seedlings. It is, therefore, recommended for nursery inoculations of pine seedlings. Bifurcate mycorrhizae characteristic of pines were observed in all treatments. Coherent rhizomorphs were found to be associated with mycorrhizae in Enugu, Ugwuoba and Nsukka soils. Anatomy of the mycorrhizae showed the presence of a fungal sheath and the cortical layer with associated Hartig net. The presence of clamp connections in the fungal sheath and rhizomorphs places the fungi involved in the class Basidiomycetes.

Key words: Mycorrhizae, *Pinus caribaea*, seedlings.

INTRODUCTION

By 1978, Nigeria already had two existing pulp and paper mills and there were plans of a third one being established at Iwopin in Ogun State. According to EC-FAO (2003), the Federal Government of Nigeria later became concerned with the huge amount of money spent on importation of timber for the pulp and paper industries and decided to embark on the establishment of plantations. So, in 1978, the Industrial Pulpwood Programme was launched with the aim of providing raw materials to the pulp and paper mills. The mills were projected to depend mainly on *Gmelina arborea* and tropical pines as sources of raw materials, the former providing 80% (short fibre) and the latter 20% (long fibre) of the requirements (Okoro, 1979).

The suggested 20 year plantation programme included 640,000 ha of pine plantations (Okoro, 1981). Furthermore, according to Okoro (1981), *Pinus caribaea* was favoured for the plantation programmes since *P. oocarpa* was found to have a natural requirement for

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very high altitude which occurs only in restricted areas in Nigeria. Unfortunately, insufficient supply of seeds cut-short the planting programme. The difficulty in seed supply arose as a result of high cost of imported seeds and poor seed setting of older plantations (Okoro and Okali, 1987). Consequently, Nigeria is yet to achieve self-sufficiency in production of industrial wood (National Planning Commission, 1993).

Aruofor (2000) reported that the pulp and paper industry has been largely constrained by inadequate long fibre availability among other things. The same author in 2001 noted that the total domestic production of paper which was 43,498 MT in 1990 declined to barely 5,314 tons in 1993 due to long fibre shortages. We, therefore, decided to investigate the possibility of by-passing the seed supply problem by using the technique of Plant Tissue Culture for mass propagation. Some of the results are reported in Akaneme and Ene-Obong (2001) and Akaneme and Ene-Obong (2005).

According to Odeyinde and Ekwebelam (1974), one of the major obstacles to wide-scale planting of pines in Nigeria was the lack of correct mycorrhizal fungus component. Reports of Madu (1967) showed that initial introductions of pines into Nigeria were unsuccessful

because suitable pine mycorrhizal symbionts were absent from Nigerian soils. However, the first successful establishment of *P. oocarpa* Schiede was at Vom in 1954. The success was due to mycorrhizae in imported soil samples collected from under pine stands in Zambia (Ekwebelam 1972).

Forest trees were first observed to require mycorrhizal associations when experimental plantations of exotic pines in different parts of the world failed until suitable mycorrhizal fungi were imported one way or another (Mikola, 1973; Marx, 1991). Ectomycorrhizal symbiosis is of mutual benefit to the symbionts: carbon from the plant flows to the fungus which absorbs and translocates nutrients from the soil to the host plant (Sylvia et al., 1997). Sylvia (2009) observed that the benefit is due to the fact that mycorrhizal fungi form a critical linkage between plant roots and the soil since they usually proliferate in both systems.

Hyphal strands in the soil absorb nutrients from regions of the soil not yet colonized by the feeder roots and transport them to the roots, thus increasing the absorptive surface area of the plant. Mycorrhizal plants are, therefore, more competitive and stress tolerant than non-mycorrhizal plants (Sylvia, 2009).

According to Gross et al. (2004), it is now the rule to inoculate nursery seedlings in reaforestation programmes. Different types of natural and pure mycelial inocula and different application methods have been used by many investigators (Marx et al., 2002). Marx (1991) reported that the most widely used natural inoculum, especially in developing countries is soil or humus collected from established pine plantations. However, the most biologically sound material for inoculation is the pure mycelia or vegetative inoculum but unfortunately, its large scale use in nurseries have been limited by the inability to produce large quantities of viable and economical inoculum (Marx et al., 1992). Thus Marx et al., 2002, noted that the use of soil inoculum is in agreement with the idea that any ectomycorrhizae are better than none.

This paper, therefore, is a report of the effects of three soil collections from old pine plantations in South Eastern Nigeria and a control soil on mycorrhizal development and subsequent growth of seedlings. Isolation of the ectomycorrhizal fungi in pure cultures would be undertaken subsequently. Anatomical confirmation of mycorrhizae development was also performed.

MATERIALS AND METHODS

The seeds of *P. caribaea* were obtained from old pine plantations of the Ministry of Agriculture Enugu and the new botanical garden of the University of Nigeria, Nsukka also in Enugu State.

Three wooden flat boxes were filled with river sand which was watered thoroughly. One hundred seeds were broadcast on each. Out of the 300 seeds sown, only 50 germinated by the 14th day. These were subsequently transferred to polyethene bags which were previously filled with river sand, top soil and manure (chicken droppings) in the ratio of 2:3:1 respectively. After three weeks of transplanting, only 26 seedlings survived.

Fresh seeds were collected and broadcast in three wooden flat boxes again. Equal numbers of seeds were sown (100 in each box). Out of the 300 seeds sown, 247 germinated by the 14th day. Transplanting was done as described previously. Three weeks after only 100 seedlings had survived. These were combined with the 26 seedlings initially obtained to set up a completely randomized experiment. Thus the main experiment was set up after two months and two weeks from the date of initial seed broadcast.

The initial 26 seedlings were randomly shared into four equal parts – there were 6 seedlings in each part. The 100 seedlings were also randomly shared into four equal parts (25 seedlings per part). Finally, there were 31 seedlings in each part. Three different mycorrhizal soils were obtained from old pine planations at Ugwuoba (Enugu State), Enugu-Ngwo (Enugu State), Nsukka (Enugu State) respectively. Top soil obtained from the old botanical garden of the University of Nigeria, Nsukka served as the control. Each soil collection was applied to 31 seedlings. The weight of each soil applied per seedling was 442.83 g.

However, before soil inoculation, chemical analyses of samples of the 4 soil collections were performed in the Department of Soil Science, University of Nigeria, Nsukka. Soil pH was measured with a glass electrode in a 1:2:5 soil to water suspension after mechanical shaking for 30 minutes. Particle size analysis followed the Bouyoucos (1951) hydrometer method while potassium was determined by neutral (ammonium acetate) extraction method and analysed using flame photometer. Finally with the aid of Bray 11 extracting solution (0.03N NH4F - ammonium fluoride in 0.1 N hydrochloric acid) available phosphorus was determined.

Growth attributes studied were: Plant heights (cm)/plant, number of needles/5 plants/soil collection, dry weights of the root and shoot systems (4 plants/soil collection). Mycorrhizae development was scored based on 4 plants/soil collection. Data were taken monthly on the first two attributes for eleven months. Four months after soil inoculation, the first set of data on mycorrhizae development was taken and four months later, the second set of data was collected. The dry weights of the root and shoot systems were obtained at the end of the experiment. The shoots systems were cut off from the root systems at soil level and the root systems subsequently dug up. The dry weights were obtained after being oven-dried for 24 h at 70°C.

Comparisons of relative mycorrhizae development were based on “the number of mycorrhizae expressed as a percentage of the total number of short roots per seedling (“mycorrhiza percent”). The criterion of mycorrhizal infection was the presence of dichotomous branching or forking of the short roots (Richards and Wilson, 1963; Molina, 1980). The estimate of mycorrhiza percent in this experiment was based on a complete enumeration with the aid of a hand lens of the root systems of four seedlings per soil collection selected at random. The results were later averaged for each soil collection (Richards and Wilson, 1963) . Analyses of variances were performed for all the attributes studied.

Studies on the morphology and anatomy of mycorrhizal rootlets

Two seedlings per soil collection were uprooted and the root systems washed thoroughly in tap water and subsequently they were cut into manageable sizes and transferred to petri dishes. With the aid of a hand lens the root tips were viewed to determine colour and forms of the ectomycorrhizas.

For the study of anatomical details, mycorrhizal root tips for the respective soil treatments were fixed in FAA (formaldehyde 10 mls; glacial acetic acid 5 mls; ethyl alcohol 50 mls; water 35 mls) and dehydrated for one day each in the following: 90% ethanol, absolute ethanol, propanol-tertiary butyl alcohol mixture (50:50

Table 1. Chemical analyses of the four soil collections.

	Mechanical analyses (%)					pH value	
Soil collection	**Clay**	**Silt**	**FS**	**CS**	**Total sand**	**H_20**	**KCl**
Control	10.3	2.0	7.1	80.6	87.7	4.87	4.81
Enugu	12.3	6.0	11.7	70.0	81.7	4.90	4.15
Ugwuoba	6.3	3.0	22.4	68.3	90.7	5.11	4.79
Nsukka	16.3	6.0	17.7	60.0	77.7	4.05	3.88

		Organic matter (%)		Potassium	Phosphorus (ppm)
Soil collection	**Carbon**	**Organic matter**	**Nitrogen %**	**(MEQ)**	
Control	0.47	0.82	0.028	0.014	5.6
Enugu	1.21	2.08	0.056	0.011	6.5
Ugwuoba	0.97	1.67	0.042	0.014	12.1
Nsukka	1.03	1.78	0.056	0.136	7.9

pH 4.5 and below = extremely acidic, 4.5 – 5 = very strongly acidic, 5.1 – 6.0 = strongly acidic; ORGANIC MATTER: 0.4% and below = very low, 1 - 1.5 = moderate, 1.5 - 2.0 = high, 2% and above = very high; PHOSPHORUS: 3 ppm = very low, 3 - 7 ppm = low, 7 - 20 ppm = moderate, 20 - above = high; POTASSIUM: 1.0 - 2% = very high, 0.6 - 1.2% = high, 0.3 - 0.6 = moderate, 0.2 - 0.3 = low, 0.2 and below = very low; NITROGEN: Less than 0.05% = very low, 0.05% = low, 0.1 - 0.15% = moderately low, 0.15 - 0.2% = medium, 0.2 - 0.25% = moderately high, 0.25 - 0.3% = high, 0.3 and above = very high.

vol/vol) and tertiary butyl alcohol. The processes of wax infiltration and dewaxing of specimens are as reported earlier in Akaneme and EneObong (2008).
Sections were cut at 10 - 12 m thickness and stained as follows:

(a) 1% safranin for 5 min.
(b) Safranin was drained off and the sections washed three times with distilled water.
(c) They were washed again with 97% and absolute alcohol (two times each).
(d) The sections were counter stained with 1% fast-green for 5 minutes and washed with absolute alcohol three times.
(e) They were transferred into blocks containing 50% alcohol: 50% xylene until fairly cleared.
(f) Finally they were transferred into pure xylene for more clearing and then mounted in D.P.X mountant (Anon, 1968).

The sections were examined under a light microscope to:

(a) Determine the presence of Hartig net and Fungal mantle
(b) Measure the mantle thickness, the diameter of mantle hyphae and also the diameter of hyphae of the hartig net in order to find out the differences among the four soil collections (Adapted from Chilvers, 1968).
(c) Obtain line drawings of transverse or longitudinal sections.

Photomicrographs of good sections were obtained at ×100 and x400 magnifications. Mantle thickness and the various diameters were measured with the aid of ocular or eyepiece micrometer. The number of divisions of the eyepiece graticle that corresponded with the mantle, its hyphae and hyphae of the Hartig net were recorded.

The stage micrometer was used in calibrating the actual value of the measurement made with the micrometer eyepiece in milimetres. By superimposing the eyepiece micrometer calibrations on that of the stage at x40 magnification, it was found that 100 divisions of the stage (equivalent to 1 mm or 1000 m) coincided with 39 divisions of the eyepiece. Hence 1 division of the eyepiece micrometer was calculated to be equal to 0.0256 mm or 25.641 m. The values on mantle thickness and diameters of the various hyphae obtained with the aid of the eyepiece micrometer were converted to mm/ m by multiplying with 0.025 mm or 25.641 m.

Anatomy of the rhizomorphs

Rhizomorphs attached to mycorrhizal root tips which developed with the Enugu, Ugwuoba and Nsukka soils were fixed in FAA. They were later boiled in two changes of potassium hydroxide, cooled and were subsequently rinsed in two to three changes of distilled water and transferred to a 30% bleach (containing 5.25% sodium hypochlorite) acidified with a few drops of concentrated Hydrochloric acid for 3 - 10 min. Rhizomorphs were cleared to a pale straw colour and were washed in distilled water and later stained by boiling in alcoholic lactophenol. The materials were left in the stain for 2 h for stain intensification. Fungal structures were stained dense blue (Adapted from Dhingra and Sinclair, 1985).

RESULTS

Soil analyses

The characteristics of the four soil collections before soil inoculation of seedlings could be seen in Table 1. With respect to soil texture, the control and Ugwuoba were found to be sandy with 88% sand, 10% clay and 91% sand, 6% clay respectively. Nsukka soil, on the other hand, was a sandy-loam with 78 sand, 16% clay while Enugu soil was loamy sand with 82 sand, 12% clay. In terms of the pH value, water determinations showed that the control and Enugu soils were very strongly acidic while Nsukka soil was extremely acidic and Ugwuoba strongly acidic.

Organic matter content was very high for Enugu soil, high for Nsukka and Ugwuoba soils and very low for the

Table 2. Values of mean squares (MS) and variance ratios (VR) obtained from ANOVA for the attributes studied.

Attributes		MS	VR
Plant Heights	Soil type	89.130	22.361**
	Error	3.986	
Number of needles	Soil type	58013.214	11.880**
	Error	4883.144	
Shoot dry weight	Soil type	22.1563	5.441*
	Error	4.0721	
Root dry weight	Soil type	0.5053	5.125*
	Error	0.0986	
Total plant dry weight	Soil type	29.3002	5.594*
	Error	5.2382	
Root/shoot ratio	Soil type	8.0794	1.0057^{NS}
	Error	8.0334	
Mycorrhizae development	Soil type	743.4192	6.017*
	Observation period	90.5828	0.733^{NS}
	Error	123.5451	

Probability Levels used: ** = P < 1%; * = P < 5%; NS = P > 5%.

Table 3. Soil Type Means (X ± SE) and F-LSD at 5% Probability for attributes studied.

Attributes	Control	Nsukka	Ugwuoba	Enugu	F-LSD
Plant heights	21.372^{b} ± 2.13	21.58^{b} ± 2.01	18.56^{c} ± 1.84	25.49^{a} ± 3.14	1.738
Number of needles	214.89^{b} ± 38.50	259.18^{b} ± 60.55	271.69^{b} ± 42.174	385.42^{a} ± 75.84	60.845
Shoot dry weight	1.54^{b} ± 0.280	3.718^{b} ± 1.012	3.895^{b} ± 0.921	7.243^{a} ± 1.457	3.1092
Root dry weight	0.263^{b} ± 0.102	0.545^{b} ± 0.135	0.515^{b} ± 0.200	1.105^{a} ± 0.173	0.4838
Total plant dry weight	1.8025^{b} ± 0.361	4.2625^{b} ± 1.147	4.410^{b} ± 1.110	8.3475^{a} ± 1.60	3.5264
Mycorrhizae development	14.739^{b} ± 3.672	16.291^{b} ± 3.065	20.413^{b} ± 4.495	43.990^{a} ± 8.816	17.299

Note: Values with the same lower case letters are not significantly different at 5% level of probability.

Control soil. The nitrogen content was very low for the Control and Ugwuoba soils and also low for Enugu and Nsukka soils. The C/N (Carbon: Nitrogen) ratios, however, indicated moderately balanced contents of carbon and nitrogen for the soils. The standard ratio is 18 - 20% asagainst 16.8% for the Control soil, 18.4% for Nsukka soil, 23.6% for Ugwuoba soil and 21.6% for Enugu soil. Exchangeable potassium was very low for all the soils while available phosphorus was low for the Control and Enugu soils and moderate for Nsukka and Ugwuoba soils.

Mycorrhizae development and growth of seedlings

Analyses of variance (Table 2) showed that there were significant differences ($p < 0.05$) among the soil types in the development of mycorrhiza on the seedlings. Periods of observation were not significantly different. Enugu soil influenced the greatest mycorrhizae infection (Table 3) and it differed significantly from the others. The Control soil produced the least effect on mycorrhizae development on seedlings but it did not differ significantly from Nsukka and Ugwuoba soils.

There were highly significant differences in plant heights and number of needles among the soil types (Table 2). Seedlings treated with soil from Enugu grew higher than the others and also produced the highest number of needles (Table 3). The least plant height was produced by seedlings treated with Ugwuoba soil and the number of needles produced by these seedlings did not differ significantly from those produced by Nsukka and the control soils.

From Table 2, it could be observed that there were significant differences ($P < 0.05$) among the soil types with respect to shoot dry weight, root dry weight, and total plant dry weight. Enugu soil had the greatest influence on these characteristics and its effect was significantly different from others (Table 3). All treated plants produced less root/shoot ratio than the Control. However, the analysis of variance (Table 2) showed that there were no significant difference among the soil types in terms of the

Table 4. Estimates of linear correlation (r), linear regression (b) and coefficient of determination (R^2) between attributes studied and percent mycorrhiza.

Attributes	Mycorrhiza %			
	r	R^2	b	VR
Plant heights	0.325	0.1056	0.1737	1.6525^{NS}
No. of needles	0.316	0.0999	4.005	1.5579^{NS}
Shoot dry weight	0.309	0.0955	0.0717	2.7435^{NS}
Root dry weight	0.495	0.2448	0.0134	4.5364^{NS}
Total plant dry weight	0.420	0.1764	0.0851	3.003^{NS}

Note: NS = not significant.

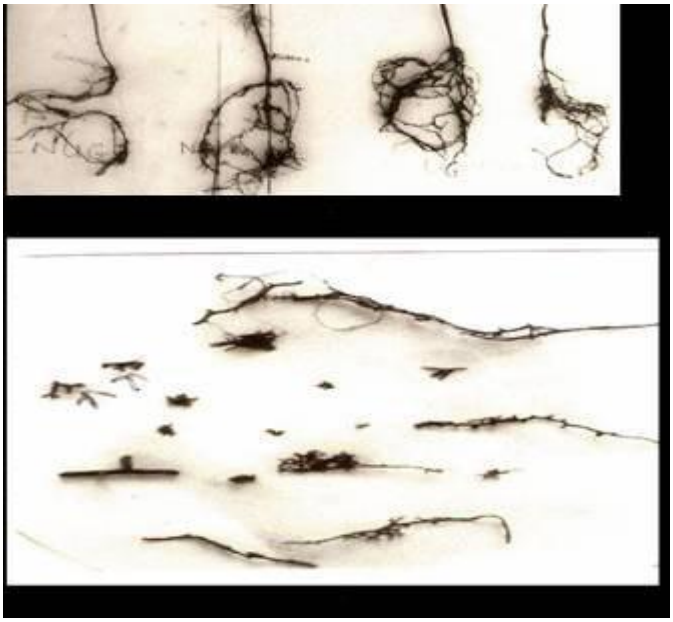

Figure 1. a) Root systems of some *P. caribaea* seedlings
b) Arrows indicate bifurcate mycorrhizae.

ratio between the roots and shoots.

Tables 4 showed that plant heights/plant, number of needles/plant, shoot dry weight, root dry weight and Total plant dry weight were positively but not significantly correlated with ectomycorrhizae percent. The coefficient of determination (R^2) indicated that mycorrhizae development did not contribute much to the variations observed in the attributes (Table 4). Furthermore, the linear regression coefficient shows that mycorrhizae development could be a predictor of number of needles and plant heights of treated seedlings, although the values were not significant at 5% level of probability.

Forms and anatomy of mycorrhizal rootlets

Appearance under the hand lens

Bifurcate mycorrhizae characteristic of pines were observed in all treatments (Figure 1). Mycorrhizae from Enugu soil were found to be glistening white at the apex and maturing to a golden brown colour some distance further back. Those from Ugwuoba soil appeared pale translucent yellow at the tips while the tips of mycorrhiza from Nsukka and the Control soils were glossy black in colour. In the three soils, Enugu, Ugwuoba and Nsukka, coherent rhizomorphs were found to be associated with these mycorrhizae.

Anatomy of the rootlets

All observed mycorrhizae were ectotrophic.

Enugu soil treated seedlings: The mycorrhizal short roots had a well-developed mantle typically 0.429 mm thick and consisting of moderately loose felt prosenchyma throughout (Figures 2 and 3). The mantle hyphae were narrow, about 0.061 mm in diameter and bearing prominent clamp connections. Below the mantle is a layer of heavily tanninized cells followed by enlarged cells in the cortex. The fungal hyphae penetrated in between the cortical cells forming a small- celled hartig net which consists of thin hyphae, 0.057mm in diameter. The hyphae were very irregular in appearance. Rhizomorphs (Figure 8) were not very common and exhibited no tissue differentiation and consisted of fairly straight septate hyphae bearing frequent clamp connections.

Ugwuoba soil treated seedlings: Mantle structure consisted of a single more or less homogenous layer of irregular synenchyma tissue, 0.211 mm thick and woven from very large and small diameter hyphae, 0.060mm. Occasionally a brief net prosenchyma overlies this. Next to the mantle layer is a small layer of tanninized cells. The hartig net consisted of hyphae, 0.054mm in diameter, discontinuous at the inner cortical layer and often irregular in appearance (Figure 4). Rhizomorphs showed no tissue differentiation and consisted of wide hyphae with frequent clamp connections and the hyphae were also frequently wavy or twisted.

Nsukka soil treated seedlings: Mantle was 0.224 mm

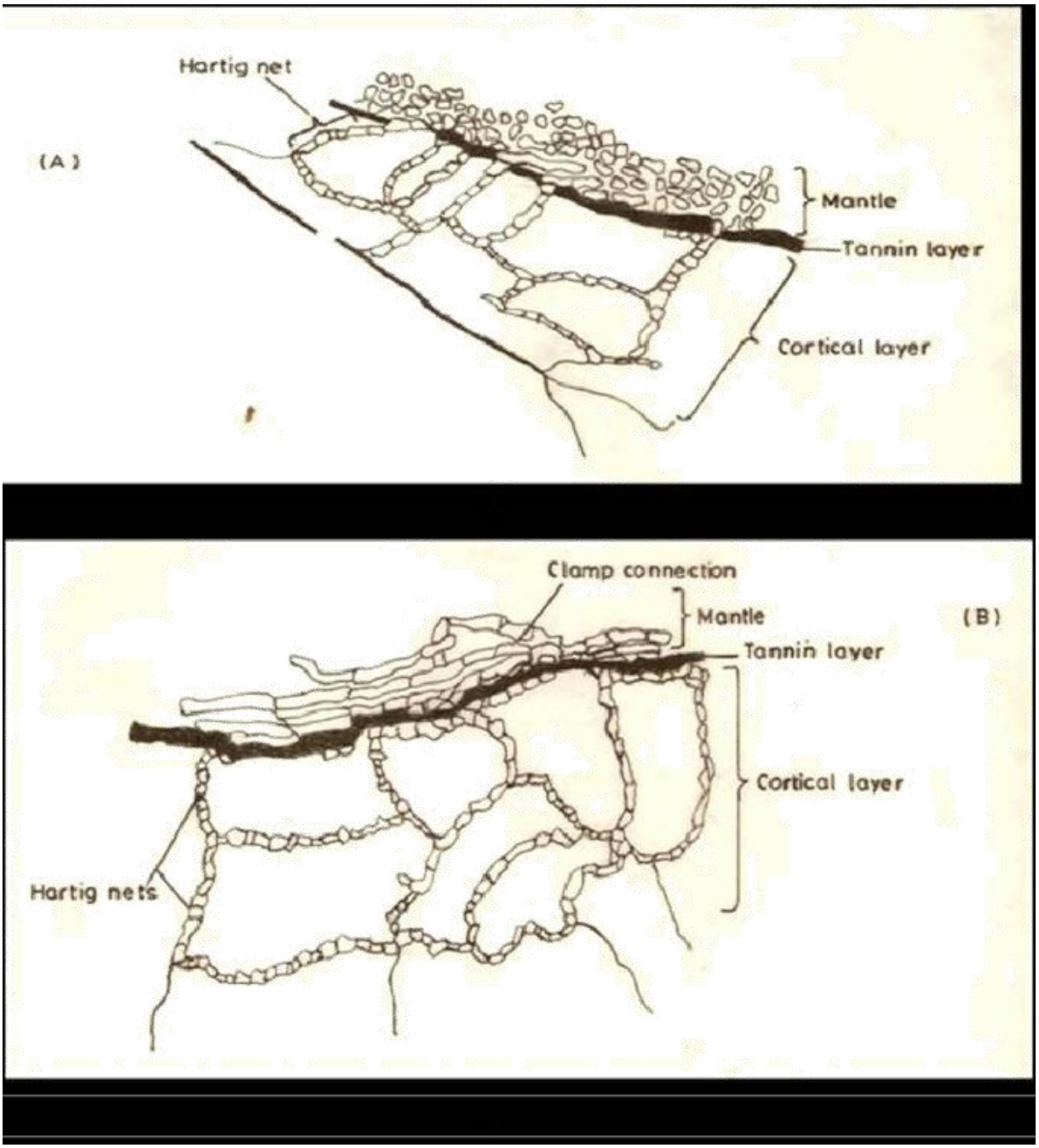

Figure 2. Enugu soil treated seedlings longitudinal (a) and transverse (b) sections through the mantle and adjacent tissues - line drawings of portions of the sections.

thick, consisting of irregular synenchyma tissue woven from very large diameter hyphae, 0.074 mm. There were many pockets of heavily tanninized layers underlying the mantle and close to the endodermal layer. Few clamp connections were observed. The hartig net consisted of very large diameter hyphae, 0.070 mm and were very discontinuous in the cortical layer and often irregular in appearance (Figure 5). The rhizomorphs showed no tissue differentiation and consisted of wide septate hyphae with frequent clamp connections. The hyphae were moderately twisted.

Control soil treated seedlings: The mantle consisted of a single more or less homogenous layer of irregular synenchyma tissue, 0.22 mm thick and woven from moderate diameter hyphae, 0.064 mm. A layer of tanninized cells underlied the mantle. The hyphae of the Hartig net were 0.060 mm in diameter and were very

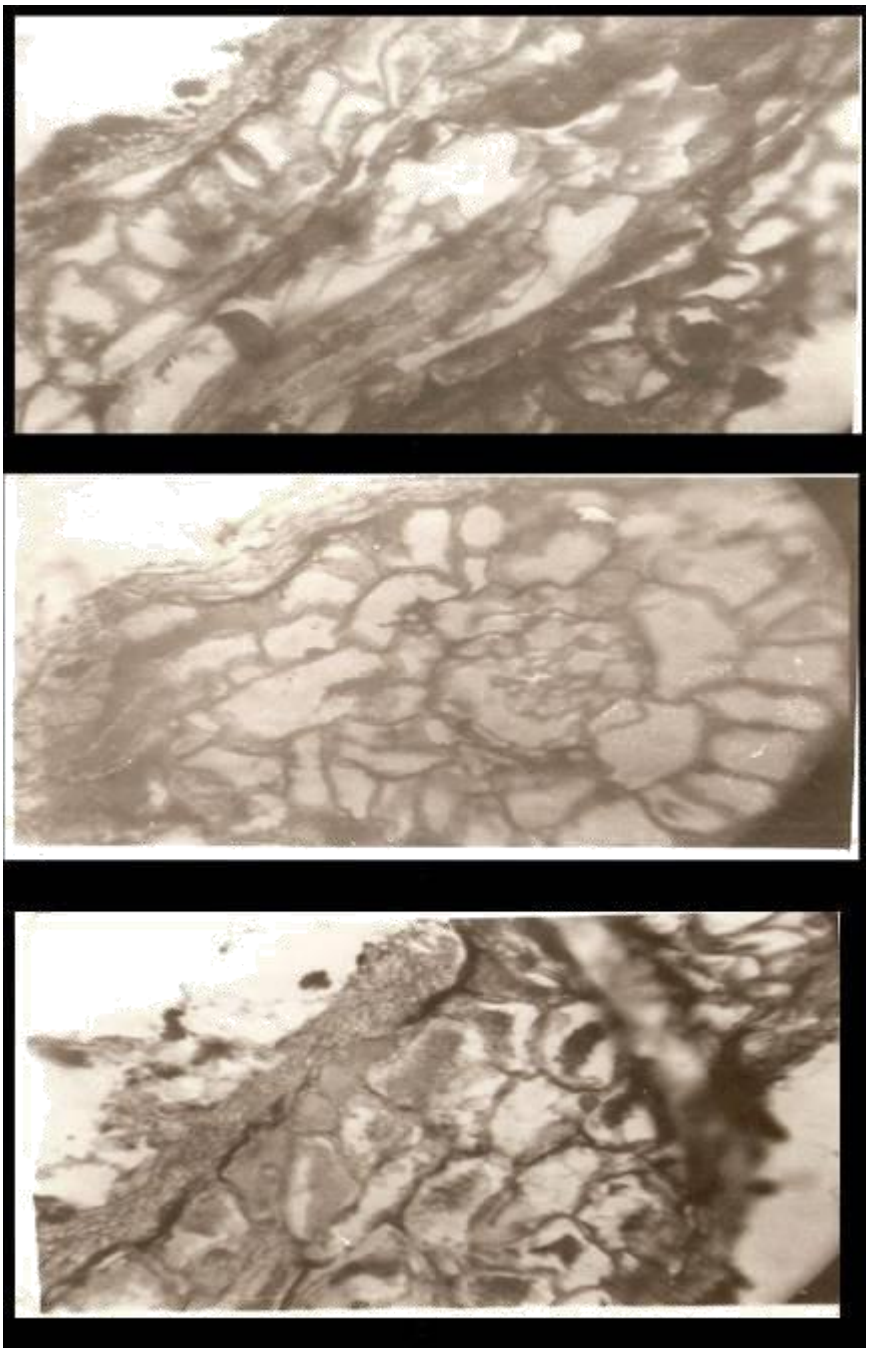

Figure 3. Pictures of the line drawings in Figure 2.

discontinuous in the cortical layer and also fairly smooth in appearance (Figure 6). Rhizomorphs were not observed.

Uninfected short roots: These were unbranched, usually dark brown in colour than the mycorrhiza and showed no hypertrophy of the cortical cells. They had neither a mycorrhizal mantle nor a hartig net (Figure 7).

It can be concluded that the presence of clamp connections places the fungi involved in the class Basidiomycetes.

DISCUSSION

A fertile soil is one which has any or all of the following features:

(a) A sandy-loam and loamy soil texture giving good drainage and aeration, as well as an adequate water-

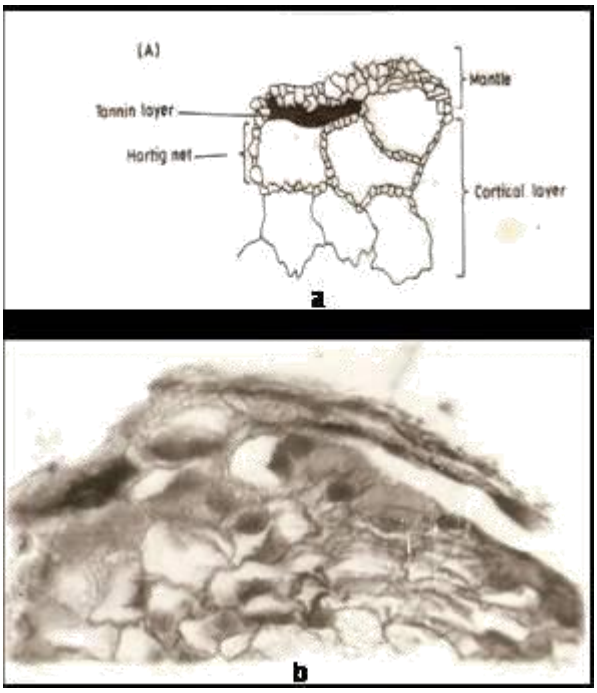

Figure 4. Ugwuoba soil treated seedlings. Transverse section through the mantle and adjacent tissues – Line drawing in (A) and the picture in (B).

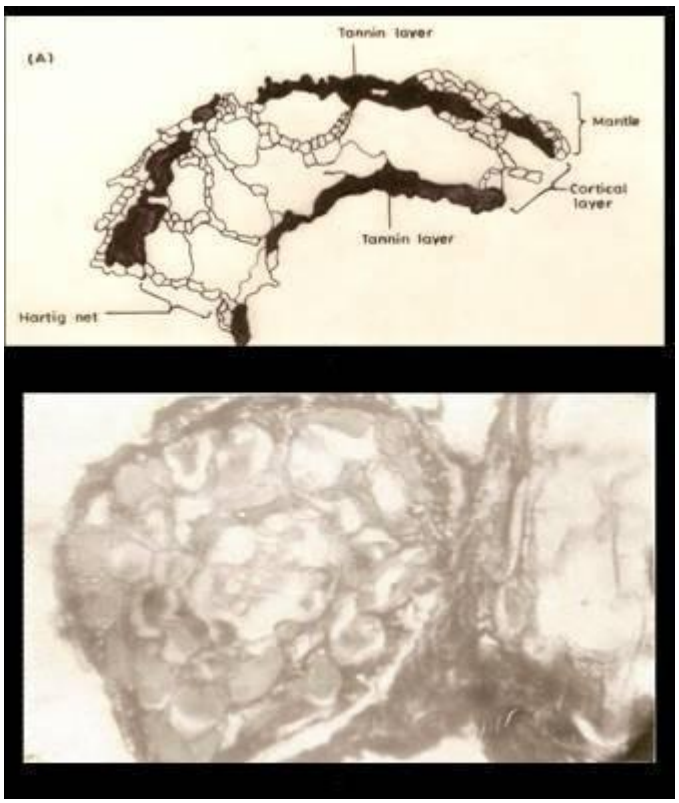

Figure 5. Nsukka soil treated seedlings. Transverse section through the mantle and adjacent tissues - Line drawing in (A) and Picture in (B).

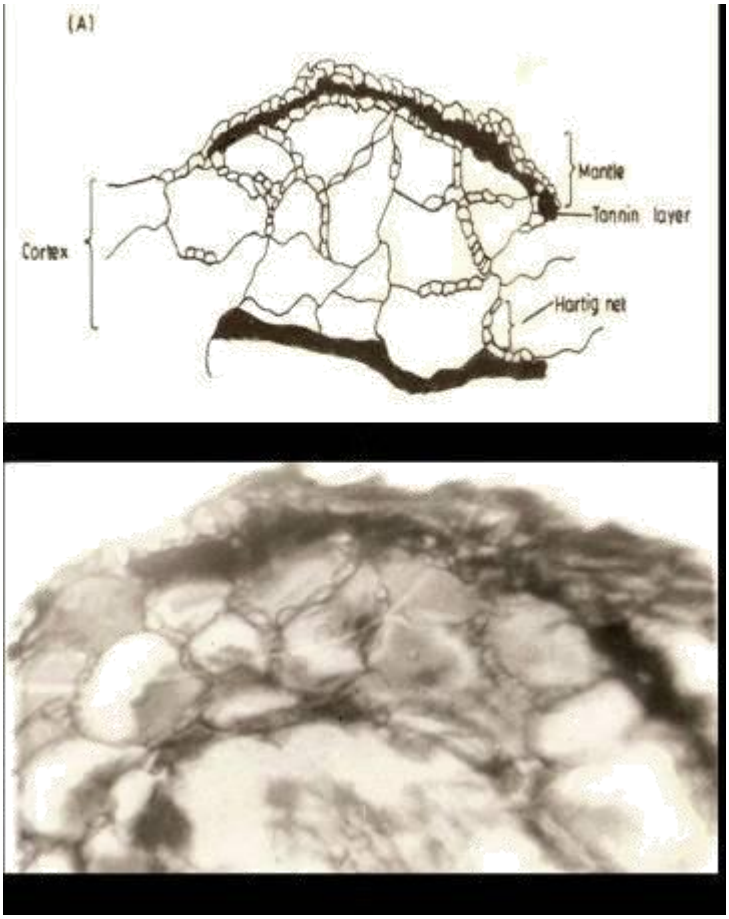

Figure 6. Control soil treated seedlings. Transverse section through the mantle and adjacent tissues - Line drawing in (A) and pictures in (B).

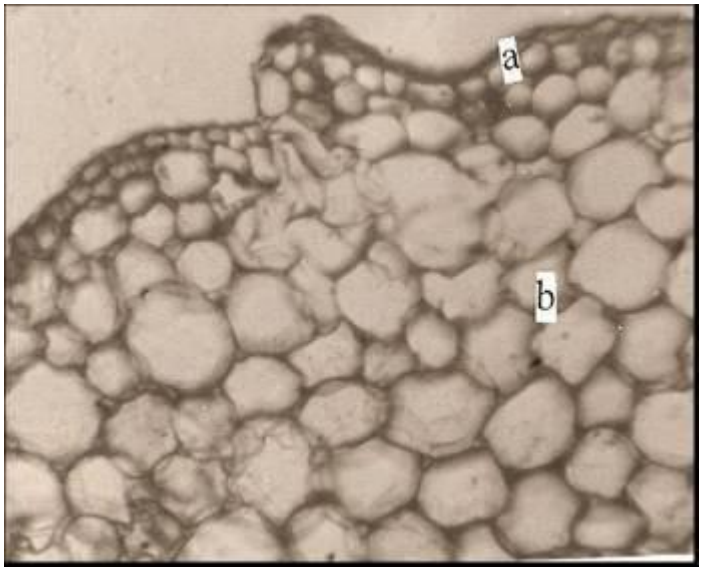

Figure 7. A transverse section of an uninfected rootlet showing the absence of fungal mantle at the epidermal layer (a) and in the cortical layer (b) ×400.

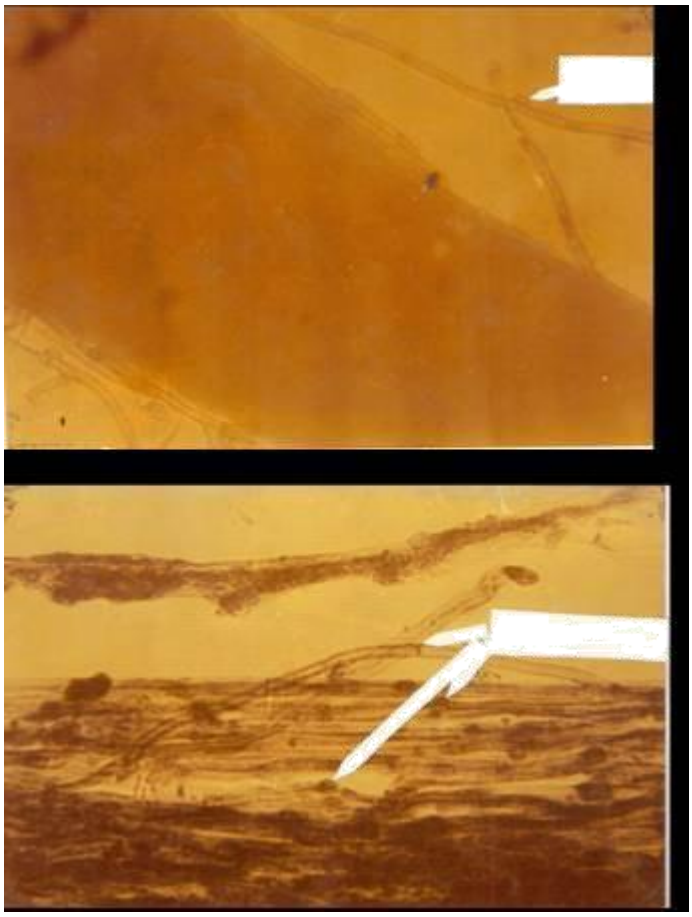

Figure 8. Whole mounts of Rhizomorphs (arrows indicate clamp connections) ×1000.

holding capacity.
(b) A well-developed crumb structure associated with adequate quantities of soil humus
(c) A neutral-to-slightly-acid pH etc. (O"Hare, 1988).

Based on the results of soil analysis, only Nsukka soil qualified for criterion (a) being a sandy-loam. However, Kimmins (1987) reported that the availability of nutrients for plant growth is determined partly by the pH because of its relationship to solubility and rates of decomposition of organic matter into humus.
Nsukka soil with (pH 4.05) was characterized by a type of humus called „Mor" which has pH values in the range of 3.5 - 4.5. According to O"Hare (1988), Mor humus is a black poorly decomposed acid type of humus, lacking in nutrients. It does not become intimately mixed with the underlying mineral matter, because there are very few soil animals in acid Mor soils. Instead the Mor forms a cap or mat over the mineral matter below. Consequently, Mor humus layers usually contain very small amounts of mineral particles. The scarcity of large soil animals is beneficial for the development of fungi which dominate the microflora.

The control, Ugwuoba and Enugu soils, on the other hand, were characterized by Moder humus which has pH values between 4 - 5. The Moder is a transitional type of humus with characteristics between Mull and Mor humus. [The Mull is a dark-brown to black, well decomposed, crumbly type of humus which is rich in nutrients and only slightly acidic (pH 5.5 - 6.5). The humus is thoroughly mixed with the underlying mineral matter by means of earthworms and other soil fauna and the active mixing of the organic material inhibits the development of fungal mycelium and consequently bacteria dominate the microflora] (O"Hare, 1988).

The Moder humus is characterized by a more rapid and complete decomposition of organic matter and a more gradual transition from the forest floor to the mineral soil than in Mor type. In addition there is less abundant fungal activity than in Mor forest floors because of the increased activity of soil animals. Moder forest floors are more acid than Mull forest floors and the humus is partially mixed with and grades into the underlying mineral soil. Thus, it can be concluded that all the experimental soils had low potentials for nutrient availability to pine plants.

According to Kimmins (1987), soil nitrogen is available

over a wide pH range but decreasingly so below pH 6 and above pH 8, an important reason being the adverse effect of pH extremes on soil microbes. The results of the present study conform to this because all the experimental soils had low nitrogen contents and pH values below pH 6. The author further noted that phosphorus is available over a narrower range of pH and availability declines above pH 7.7 and below 6.5. This explains the low phosphorus levels observed in the experimental soils. Low availability of potassium may be explained by the fact that sometimes, potassium become firmly fixed in clay lattices or immobilized in decomposition - resistant organic matter.

The reports of Sanchez and Salinas (1981) are in accordance with the above observations. They noted that "of the soil-related constraints on crop production, low chemical soil fertility is the most severe on more than half of the arable land in the tropics. Furthermore, they stated that „the infertile soils are acidic (pH < 5.3) and that a combination of stress situations frequently occur - on about 43% of the tropical area, deficiency of plant nutrients (nitrogen, phosphorus, potassium, calcium, zinc and boron), toxicities of others (aluminum and manganese) and a high phosphorus-fixing capacity (at pH < 5.3 and > 7.5) are the rule".

These results of the soil analyses are important because they show clearly that the four soil collections are the types normally colonized by ectomycorrhizal fungi (soils of moderate deficiency of nutrients). Harley (1969) had earlier reported that mycorrhizal intensity is greatest in soils of moderate deficiency of nutrients especially of nitrogen and phosphorus.

Adjustment of soil conditions is often an indispensible prerequisite for successful mycorrhiza inoculation (Mikola, 1973). He further noted that fertilization, acidification and addition of organic matter are the most common measures used for promoting mycorrhiza development in forest nurseries. However, according to him, the addition of organic matter alone in the form of forest humus has promoted the formation of mycorrhizae in treated seedlings. The results of the present study are thus confirmed since the addition of the four forest humus promoted some mycorrhiza development in the pine seedlings.

The differential formation of ectomycorrhiza by the different soil types and growth of *P. caribaea* seedlings could be explained by the report of Meyer (1973). He observed that although mycorrhizae are widespread in forests influenced by man, the component fungi may differ in effectiveness in terms of their ability to utilize and transmit to the host the organic "nitrogen" of humus compounds or phosphorus and potassium minerals that are difficult to dissolve. Theodorou and Bowen (1970) reported that the growth of pine seedlings was consistently promoted more by *Boletus granulatus* and *Rhizopogon luteolus* than by *Boletus luteus* and *Cenococcum graniforme.* Great differences have also been noticed between different strains of the same species, for example, *Pisolithus arhizus* (Lamhamedi et al., 1990).

Sources of infection in soils believed to be free from mycorrhiza forming species might be either vegetative mycelia or dormant spores which must have been present in the soil (Deacon and Fleming, 1992). This could explain the formation of mycorrhizae on seedlings treated with the control soil. The correlation coefficients between ectomycorrhiza percent and growth parameters were not significant. Several authors like Marx et al. (1982) and Dixon et al. (1987) have suggested that an ectomycorrhizal colonization of approximately 50% is necessary for an enhancement of seedling growth. From the results obtained only Enugu soil with its inherent mycorrhizal fungi approached this standard with 44% mycorrhiza development and this could be the reason for the better performance of Enugu- soil- treated -seedlings.

Attempts have been made to classify ectomycorrhiza (Marks, 1965; Chilvers, 1968) to enable investigators study their structures and better understand their many interrelationships with tree, fungus and environment. These classifications have used descriptions of the mycorrhizal roots. The descriptions were made on the bases of visible, morphological characters such as colour of mycorrhiza, degree of branching and thickness of the mantle (Bledsoe, 1992). Chilvers (1968) further made use of microscopic examination of the actual mycorrhiza. This revealed differences in tissue organization of the mantle, the hartig net, associated mycelium and rhizomorphs. He was able to describe successfully eight distinctive types of Eucalypt mycorrhiza. Zak (1973) noted that the classification system derived from these descriptions would make it possible to test laboratory findings and later apply them in the forest and nursery in order to grow more vigorous and healthier trees.

In the present study, attempts were made to describe the observed mycorrhizae on the bases of colour, attached rhizomorphs and their microscopic features, mantle thickness, structure and diameter of the component hyphae, presence and character of the hartig net and diameter of its component hyphae. Some differences were observed among the four sets of treated seedlings and these perhaps correspond to differences in the mycorrhizal fungi which colonized the seedling roots.

The fungal sheath has been reported to have a number of functions. Read (1992) postulated that it provides a mechanical barrier to pathogenic fungi. It also has an important role in nutrient storage (Isaac, 1992; Read, 1992). Fogel and Hunt (1979) estimated that the sheaths of ectomycorrhizal roots contained up to 50% of the below ground "nitrogen" in a Douglas fir forest. Baath and Söderström (1979) from their work in pine-heaths of Sweden estimated that the fungal biomass contained up to 20% of total soil nitrogen. They concluded that the proteolytic capabilities of the mycorrhizal mycelium will provide the potential for direct recycling of this "nitrogen"

fraction. Based on these, it is speculated that the better growth shown by Enugu-soil-treated-seedlings might be due to the amount of nutrients made available to the seedlings through the much thicker fungal mantle observed in the mycorrhizal roots.

According to Isaac (1992), individual hyphae also extend from the mantle surface to the soil particles and they provide extensive surface area for absorption. They scavenge nutrients from the surrounding soil not yet colonized by the plant roots and therefore, act as an extension to the root system. She also reported that networks of fungal hyphae become anastomosed to form an extensive and integrated mycelial system with the hyphae often grouped together in „strands" or „rhizomorphs". She noted that the main connection between the fungal mantle and the mycelium in the soil is the rhizomorph emanating from the base of the root. In such circumstances according to her, the mycorrhizal root is not itself an absorbing structure. Rather it forms a base on which the fungus is dependent for carbon with which to sustain its foraging activities. It also provides a facility for storage of products, absorbed and translocated over considerable distances by the fungi.

Marks and Foster (1973) reported that a well-developed Hartig net is the distinguishing feature of a true ectomycorrhiza and that association without intercellular penetration are asymbiotic because there is insufficient common surface area of close contact for nutrient exchange to occur between the two symbionts. Well developed hartig nets were observed in the sections of the mycorrhizae obtained in this study, so it can be concluded that true ectomycorrhizae were formed.

Below the mantle, a layer of tanninized cells were also observed in the sections. The secretion of tannins by the host plant is considered to be a defense mechanism and it is documented that ectomycorrhizae never penetrate as far as the endodermis which is sometimes filled with tannins (Marks and Foster, 1973).

Conclusion

Mycorrhizal inoculations are indispensible part of nursery practices in pine nurseries and soil from Enugu-Ngwo pine plantation is recommended for such nursery inoculations. The presence of clamp connections in the sections of rhizomorphs places the fungi involved in the class Basidiomycetes.

The result obtained with Enugu soil can perhaps be correlated with the report of Okoro and Okali (1985). They conducted an investigation across the planting range of *Pinus caribaea* in Nigeria with a view to identify areas that could be designated seed cone production areas. They discovered that seed cone production was greatest at Enugu-Ngwo, followed by Afaka in Kaduna State and lowest in Miango (Plateau State) and Awi (Kwara State). Perhaps the high seed cone production in Enugu-Ngwo might be due to improved growth of the trees as mediated by the mycorrhizal fungi in the soil.

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