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Anatomical Studies of Four *Pleurotus* Species Cultivated on *Mansonia altisima* Sawdust

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Abstract

Pleurotus (Fr.) Quel is a genus of gilled mushrooms, and are one of the most commonly cultivated edible mushrooms in the world. This work was aimed at cultivating and identifying/determining the anatomical features of *Pleurotus* species at different developmental stages. Four species of *Pleurotus*, namely *Pleurotus florida, P. ostreatus, P.ostreatus* var. F. and *P. pulmonarius* were cultivated on *Mansonia altisima* sawdust and supplemented with rice bran. The sawdust was sterilized in an autoclave at 121^{o}C at 15 ibs pressure for 20 minutes. On cooling, spawn of each of the species were inoculated on the sterilized substrate. The inoculated bottles were incubated in a dark at controlled temperature of 15^{o}c. Pure cultures of each of the mushroom were grown on PDA to obtain the mycelia stage. After mycelial colonization of the substrate, primordia stage and mature fruit body stage were studied morphologically and samples were harvested in vial bottles. The pinhead formation took 26 and 27 days for *Pleurotus florida, P. ostreatus,* respectively; and 28 days for *P.ostreatus* var. F. and *P. pulmonarius.* Harvesting of the mature fruiting bodies was done after 30 days for *Pleurotus florida,* 31 days for *P. ostreatus* and *P.ostreatus* var. F, and 33 days for *P. pulmonarius*. The mature fruit body comprise of the stipe, gills and pileus. Anatomical sections were then taken at 25 micron thickness, stained and mounted on glass slide. Hyphae arrangement, cell polarity, cell density, cell shape, stainability, fruit bodies initials, presence of cuticle, hymenium length, hymenophore structure, cell wall structure, presence of nodulus, presence of copious granules, cell wall structure and cell size were diagnostic and varies among the species. Dendogram of characters produced *Pleurotus florida, P. Ostreatus* and *Pleurotus ostreatus* var. F. in a cluster and *Pleurotus pulmonarius* as an outlier. Classification based on cluster analysis confirmed the original identification of the *Pleurotus* species studied. *Pleurotus pulmonarius* was well separated from *Pleurotus ostreatus, Pleurotus florida and Pleurotus ostreatus* var. f with a taxonomic distance (D=0.90). The study has shown that *Mansonia altisima* sawdust is a good substrate for the cultivation of *Pleurotus* species. *Pleurotus ostreatus, Pleurotus florida and Pleurotus ostreatus* var. f in a cluster demonstrated closer zymographic relationships. Variations in the characters used are useful in the identification of the four species studied.

Key words: *Pleurotus, ostreatus,* Identification, Anatomical, Development

1. Introduction

Pleurotus (Fr.) Quel. belongs to the kingdom Fungi, Phylum Basidiomycota, Class Agaricomycetes, Order Agaricales, and Family Plurotaceae. It is a genus of the gilled mushrooms which comprise some the most widely eaten mushrooms. Species of *Pleurotus* are often called oyster, abalone, or tree mushrooms, and remains some of the most cultivated edible mushrooms in the world (Chang *et al*., 2004).

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The fungi have been used in mycoremediation of pollutants such as petroleum and polycyclic aromatic hydrocarbons (Stamets, 2005). *Pleurotus. florida*, *P. pulmonarius, P. ostreatus* and *P. tuber-regium* are some of the edible species reported in literature (Fasidi *et al.,* 2008). Adenipekun and Omolaso (2015) reported that wheat-bran supplement aids the development of better mushroom size, total yield, biological efficiency and proximate composition compared to rice straw supplemented on banana leaves.

Apart from the mushroom stem and cap, there is an entire network of mycelium beneath the mushroom. Mycelium is a tight network of cells under the ground; this underground mycelium is actually the plant of which the mushrooms are the fruits. During its life course, the mycelium has only one goal, the existence of the species. The mycelium does so by growing mushrooms (Vagi *et al*., 2013).

The combination of apical growth and branching/forking leads to the development of a mycelium, which is an interconnected network of hyphae. Mycelial tissues form the fungal fruiting bodies called the sporocarps (Vagi *et al*., 2013). The main function of the sporocarps is the support, protection and development of spore producing structures, to hold and protect them (Vagi *et al*., 2013). Sporocarps are sexual fruiting bodies that occur in the Ascomycota and are termed ascocarp or ascoma, and those in the Basidiomycota are called basidiocarp or basidioma (Vagi *et al*., 2013). The *Pleurotus* fruiting body is symmetrical in the early stages of development, such that their volumetric proportions is the same as the cross-sectional area proportions in the longitudinal sections of these medium (Sanchez *et al.,* 2006). A special pore anatomy, the dolipore, is exclusive to the phylum Basidiomycota. The border of this pore is swollen like a donut, and different structures (e.g. membrane-like bands) could occur in the tube of the pore (Vagi *et al*., 2013). The dolipore can be covered by the parenthesome, a membrane organization continuous with the wall endoplasmic reticulum (ER). The parenthesome can be sacculate, continuous or porate (Vagi *et al.,* 2013). The cultivation of the mushroom is currently gaining global attention owing to their importance as an alternative source of protein in food and their use in agro allied companies, pharmaceutical industries and medicines. Mushrooms have a lot of nutritional, economical and medicinal values as well as benefit. Notwithstanding of the fungi usefulness, literature on its microscopic studies are still relatively scanty.

2. Materials and Methods

Source of Materials

Spawn: Mother spawn of *Pleurotus ostreatus* var. *florida* was obtained from Zero Emission Research Initiative Unit (ZERI), Department of Biological Sciences, University of Namibia, *Pleurotus ostreatus* var. F was obtained from Forestry Research Institute of Nigeria while that of *Pleurotus ostreatus*, and *Pleurotus pulmonarius* were obtained from the Department of Botany, University of Ibadan, Nigeria.

Substrate: The substrate used in this research was saw-dust of *Mansonia altisima.* The sawdust waste was collected from Bodija plank market in Ibadan, Oyo State, Nigeria. The sorghum grain on which the spawn was sub-cultured was obtained from Bodija market, Ibadan.

Additives: Rice bran was used as additive and it was obtained from African Rice unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State.

Spawn Multiplication: The spawn was multiplied according to the method of Jonathan and Fasidi (2001). Sorghum grains are thoroughly washed in sufficient water to remove soil debris and undesirable seed of grasses. Washed grains were then soaked in sufficient water for 20-30 minutes and boiled in a pressure pot for 15-20 minutes. Excess water from the boiled grains was removed by spreading on sieve made of muslin cloth. The sieved grains were mixed with calcium carbonate so that they do not form clumps. The grains are then filled in previously washed spawn bottles and its opening was covered with aluminium foil. The spawn bottles with the grain content were then put in an autoclave and were sterilized at 22 ib pressure for 15-20 minutes. The autoclaved bottles were vortexed well before inoculation so that the water droplets in the bottle bags were absorbed by the grains. The sterilized bottles were kept on the laminar air flow under Ultraviolet tube for 20-30 minutes before they are inoculated with spawn from the mother spawn bottle. The bottles were then incubated at 28 ± 2^{o}C for mycelium spread.

Substrate Preparation: The substrate used for this study is *Mansonia altisima* sawdust. The substrate was prepared according to the method of Lawal *et al.*, (2011). The sawdust was soaked in water for 15 minutes and excess water was squeezed out with a muslin cloth. It was then supplemented with rice bran and thoroughly mixed and placed in 250 ml bottles, covered

with aluminium foil and sterilized in an autoclave at 121^{o}C at 15 Ibs pressure for 15 minutes and was allowed to cool.

Spawning, Incubation and Cropping: On cooling of the substrates, 5 gram of grain spawn of *Pleurotus ostreatus, P. ostreatus* var. f, *P. florida* and *P. pulmonarius* were inoculated in each substrate in the bottles respectively. The inoculated bottles were incubated in the dark at controlled temperature between 15-18^{o}C, relative humidity 80% and light intensity of 200lux. The bottles were incubated at 28 ± 2^{o}C and are inspected regularly to ensure proper fructification of mushrooms and to avoid contamination of substrates.

After full colonization of the substrates, the bottles were transferred to the mushroom house (for cropping) and their cover was opened. The bottles were adequately watered on a weekly interval to enhance fructification.

Mushroom Harvesting: Fruiting began 30 days after inoculation. Subsequently, harvesting of mushrooms commenced three days after fruiting. Harvesting was done by removing the fungus completely from the substrate.

Mycelial Production: 39g of synthetic Potato Dextrose Agar was dissolved in 1 litre of distilled water. Streptomycin was added to the dissolved PDA to avoid bacterial growth. The mixture was sterilized in autoclave at 1.051gcm^{2} for 15 minutes and was poured into sterile petri dishes. Matured stipe of the first flush was used for the tissue culture. Tissue culture of each mushroom was inoculated on the plates and labeled accordingly.

Mushroom Sectioning: The anatomical procedure was carried out in the Plant Anatomy laboratory, University of Ibadan. Thin sections (5-10mm) of the transverse section (T/S) of the mushroom primordial was obtained and matured fungus was used to obtain the mycelium, stipe, gills and the pileus of the samples using the Spencer "820" microtome. The finely sectioned specimens were carefully transferred into specimen bottle containing 50% ethanol and were labeled accordingly (Sanchez and Moore, 1999, Sanchez, 2000).

Staining of Mushroom Samples: The sections were stained with Safranin O, a red cationic dye for 3-4 minutes and later counter stained with Lactophenol blue for 3-4 minutes.

Mounting of Mushroom Sections: The stained sections were mounted on clean glass microscopic slides with a drop of 25% glycerol and covered gently with the cover slip. The edges of the cover slip were sealed with nail varnish and observed under the microscope. (Sanchez and Moore, 1999, Sanchez, 2000).

Photomicrograph of the Section: Light micrographs were recorded with a Sony digital camera attached to Fischer light microscope at different objectives (×10, ×40, ×100).
Statistical Analysis: Dendogram was constructed to establish the relationship among the four sampled *Pleurotus* spp.

3. Result and Discussion

The formation of primordial (pinhead) took 26, 27, 28 and 28 days for *Pleurotus florida, Pleurotus ostreatus, Pleurotus ostreatus* var. F and *Pleurotus pulmonarius*, respectively to initiate. The mature fruit bodies were developed within 30, 31, 31 and 33 days for *Pleurotus florida, Pleurotus ostreatus, Pleurotus ostreatus* var. F and *Pleurotus pulmonarius* respectively. The mycelial colonization on the Potato dextrose agar took the average of 8 days throughout the four species treated. The sorghum grain spawn ramification was at the range of 15 days for the species. Photomicrograph of the mycelia shows the presence of hyphae in mass. *Pleurotus florida* and *Pleurotus ostreatus* var. *F*. have an interwoven hyphae while *P. ostreatus* and *P. pulmonarius* have a branched hyphae. Irregularity was observed in the cells of all the species except that of *P. pulmonarius* which shows regular cell arrangement. The absorption of stain was high in all the species as they all took up the lactophenol cotton blue. Copious granules of cellular substances were exhibited by *Pleurotus ostreatus* among other species.

Initiation of fruiting bodies initials with polarity in different directions was observed at the primordial stage (Figure 1). The arrangement of the hyphae changed when compared with the mycelia stage with the exception of *Pleurotus pulmonarius* which maintained the same hyphae arrangement, thus having interwoven hyphae only in *Pleurotus ostreatus* var B; while those of *Pleurotus ostreatus* var F and *Pleurotus pulmonarius* were unbranched and *Pleurotus florida* hyphae not very visible. The cell shape is globular throughout the species and they possess a varying fruit body initials which appears to be ovoid in shape. Cell differentiation was noticed in all the species except in *Pleurotus ostreatus* var F. There is variation in both the cell density and cell elongation. All the species except *Pleurotus ostreatus* var. B were densely packed;

elongation was present in *Pleurotus florida* and *Pleurotus pulmonarius*, but were not observed in *Pleurotus ostreatus* var. B and *Pleurotus ostreatus* var. F. Also changes occurred in their ability to take up stain as *Pleurotus ostreatus* var. F and *Pleurotus pulmonarius* took up lactophenol cotton blue and safranin O respectively while *Pleurotus ostreatus* and *Pleurotus florida* took up less stain.

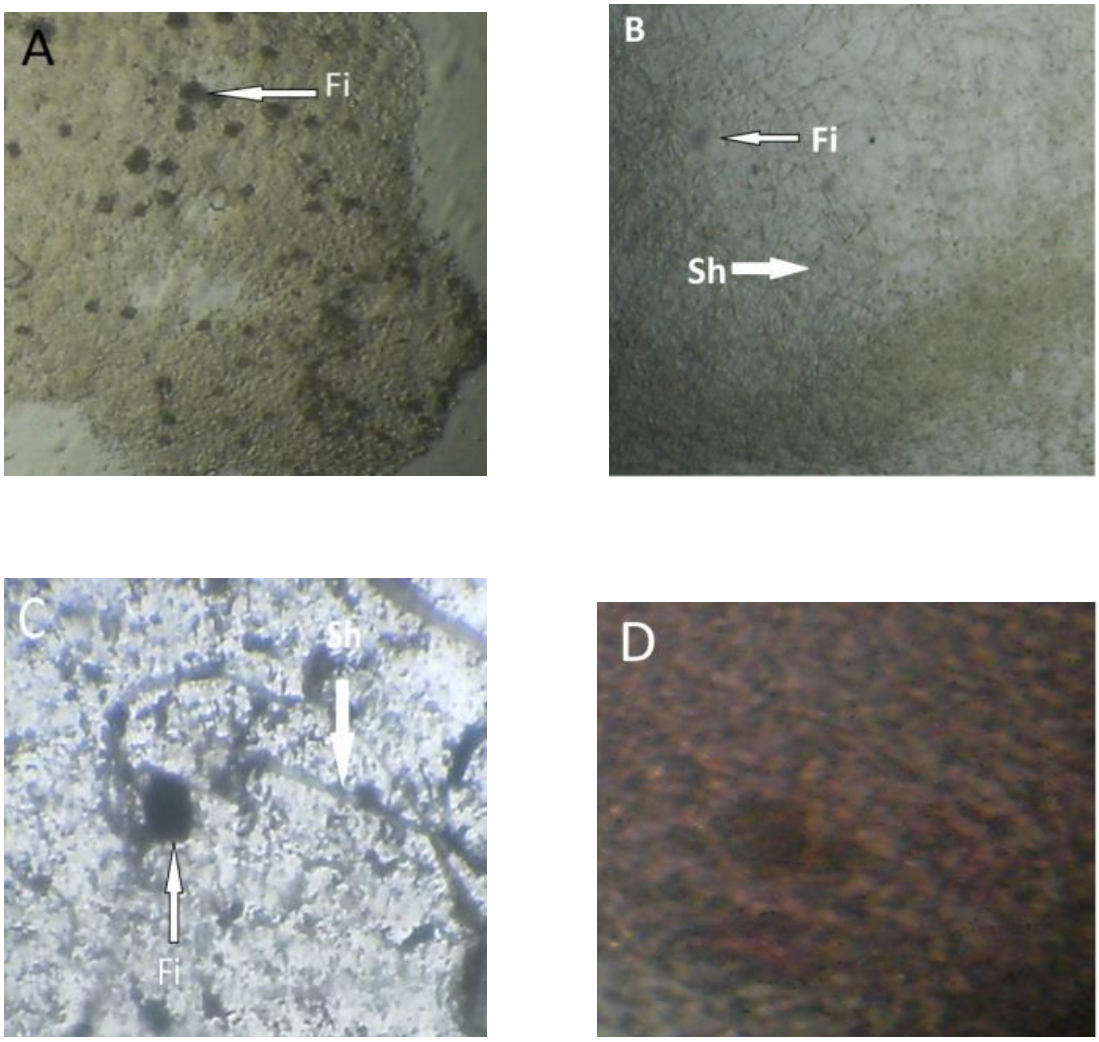

Figure 1. Photomicrographs of transverse sections of primordia of *Pleurotus* species.
A. *P. florida,* B. *P.ostreatus,* C. *P. ostreatus* var. F, D. *P.pulmonarius.*
Key: Fi: Fruit bodies initials Sh: Strands of hyphae

In the stipe of the species, uniformity in the cell surface was observed, as they all looks rough and densely packed (Figure 2). Spores-like particle was present throughout the species, even though their stainability varies. *Pleurotus florida* and *P.ostreatus* absorbed less stain while *P. ostreatus* var. F. and *Pleurotus pulmonarius* absorbed the stains well. The stipe in all the species

exhibit radial symmetry; with a meristemoids in *Pleurotus ostreatus* var. *F*. and *Pleurotus pulmonarius.*

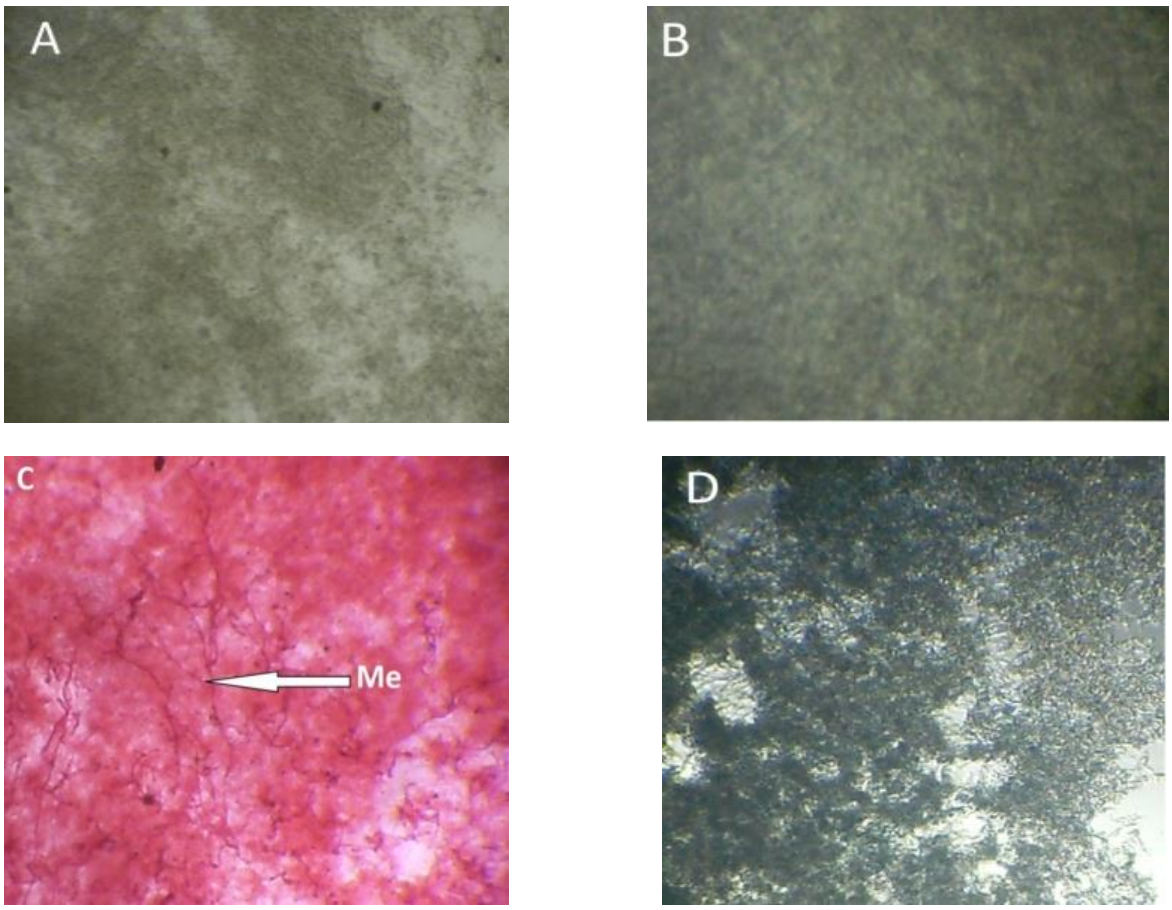

Figure 2. Photomicrographs of transverse sections of stipe of *Pleurotus* species.
A. *P. florida,* B. *P.ostreatus,* C. *P. ostreatus* var. F, D. *P.pulmonarius.*
Key: Me: Meristemoids.

Cell differentiation and extension was shown by the gills (Figure 3). The sectioned gill shows a rough surface just as it was observed in the stipe. Extension of cell was observed in all the species. There is a ground tissue from which the gills extended from i.e the hymeniophore. The hymenium was distant and vary in size and length throughout the species observed. There is a thick wall that binds all the gills with numerous spores' deposition. The ground tissue from which the gills extended from (hymeniophore) looks sculptural in both *Pleurotus ostreatus* var B and *Pleurotus ostreatus* var F; while the surface of *Pleurotus florida* and *Pleurotus pulmonarius* appears to be plain. The edge of the gills looks like a shell with no specialized shape though it is rough. There is variation in the length of the hymenium throughout the four species considered. The hymenium of *Pleurotus pulmonarius* were in folds, unlike in the other three species which are elongated with a pointing end. All the cells were differentiated and they

all absorb stain well. *Pleurotus ostreatus* and *Pleurotus ostreatus* var. F absorb safranin O; *Pleurotus pulmonarius* absorbed lactophenol cotton blue while *Pleurotus florida* absorbed both safranin and Lactophenol cotton blue. The gills throughout the species were spaced.

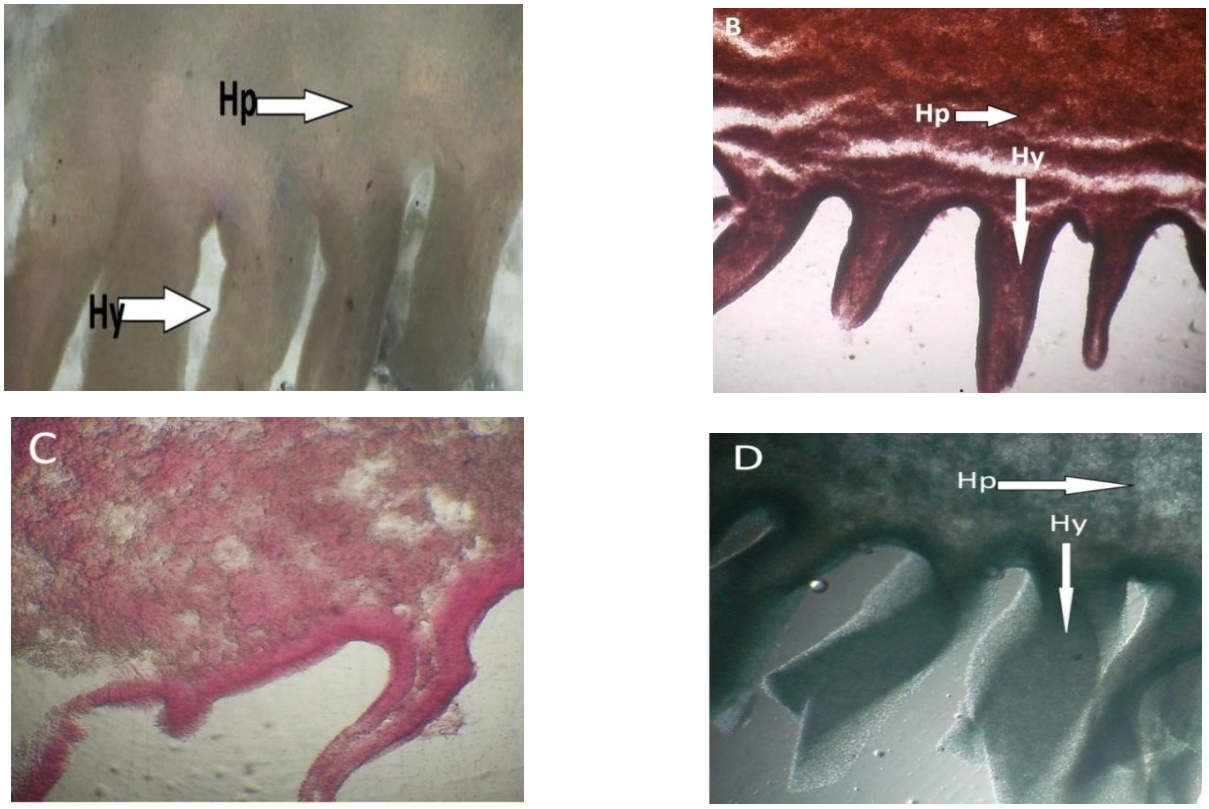

Figure 3. Photomicrographs of transverse sections of gills of *Pleurotus* species.
A. *P. florida,* B. *P.ostreatus,* C. *P. ostreatus* var. F, D. *P. pulmonarius.*
Key: Hy: Hymenium, Hp: Hymenophore

Dispersed fragment of hyphae was observed on all the cell surfaces of the four species in the pileus section (Figure 4). Table five describes the characters observed in the photomicrograph of the four species. Differentiation of cell was observed only in *Pleurotus florida*, and trichoderm type of pileipellis was found in the species as well. The traits were absent in *Pleurotus ostreatus, Pleurotus ostreatus* var. *F.*, and *Pleurotus pulmonarius*. Their cells were purportedly dense throughout the species. A centrally located nodule was observed at the center of *Pleurotus florida* and *Pleurotus ostreatus* cells. The cells did not absorb stains in all the species except *Pleurotus ostreatus* var. F which absorb safranin

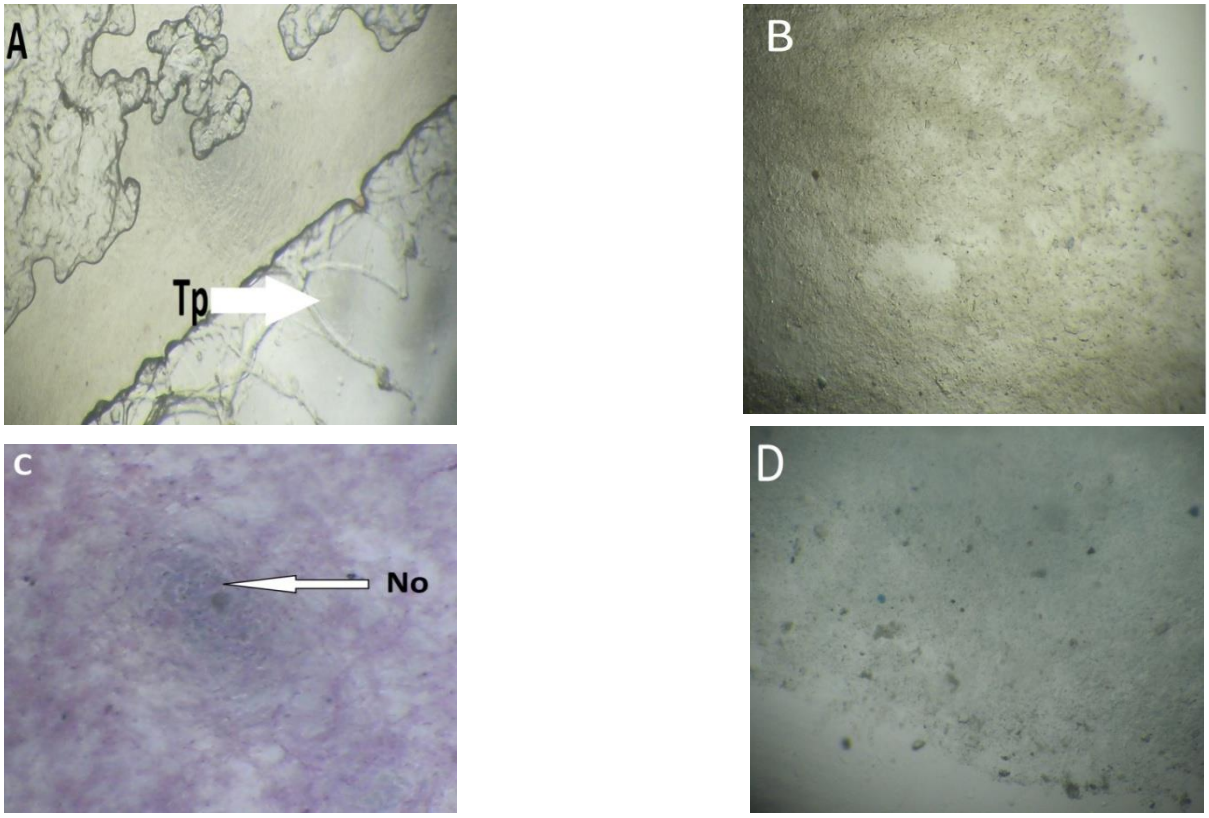

Figure 4. Photomicrographs of transverse sections of pileus of *Pleurotus* species.
A. *P. florida.* B. *P.ostreatus,* C. *P. ostreatus* var. F and D. *P.pulmonarius.*
Key: Tp: Trichoderm pilleipelis, No: Nodules

Classification based on cluster analysis confirmed the original identification of the *Pleurotus* species studied. *Pleurotus pulmonarius* was well separated from *Pleurotus ostreatus, Pleurotus florida and Pleurotus ostreatus* var. f with a taxonomic distance (D=0.90). A dendogram showed the relationships among the four species generated from the characters observed (Figure 5). Dendogram of characters produced *Pleurotus florida, P. Ostreatus* and *Pleurotus ostreatus* var. F. in a cluster and *Pleurotus pulmonarius* as separate.

The results obtained from this study showed that *Pleurotus ostreatus, Pleurotus florida, Pleurotus ostreatus* var. F and *Pleurotus pulmonarius* can be cultivated successfully on *Mansonia altisima* sawdust under favourable conditions. Physical observation of the experiment showed that the mycelia of *Pleurotus ostreatus, Pleurotus florida, Pleurotus ostreatus* var. F and *Pleurotus pulmonarius* ramified through the entire substrates at the level of additives added. The substrates used in this study supported significantly the growth of the four oyster mushrooms: *Pleurotus florida, Pleurotus ostreatus, Pleurotus ostreatus (F),* and *Pleurotus pulmonarius*.

This is in line with the findings of Okwujiako (1992) who reported that agricultural wastes are good growth media for *Pleurotus* species. This is in line with the conclusion of Lawal *et al.*, (2011) on their study of effect of additives on the cultivation of *Auricularia Auricula* (St.Amans) on *Mansonia altisima* (A.Chev) chev sawdust. They reported that *A. auricula* degraded *M. altisima* sawdust with different levels of additives. The addition of rice bran as additive to supplement the substrate used in this study enhanced the growth of the mushroom. This conforms to the findings of Gbolagade *et al.*, (2006) who reported the effect of different supplement on the yield of *Lentinus subnudus* in which it was observed that supplement such as rice bran contributed to the high yield of mushrooms and also aid sporophore emergence.

Variations observed in the number of fruiting bodies produced may be associated with the nature of the mushroom. This fall in line with Quimio *et al.*, (1990) who stated that there are variations in fruiting of mushroom and could be attributed to ventilation, light, moisture and humidity.

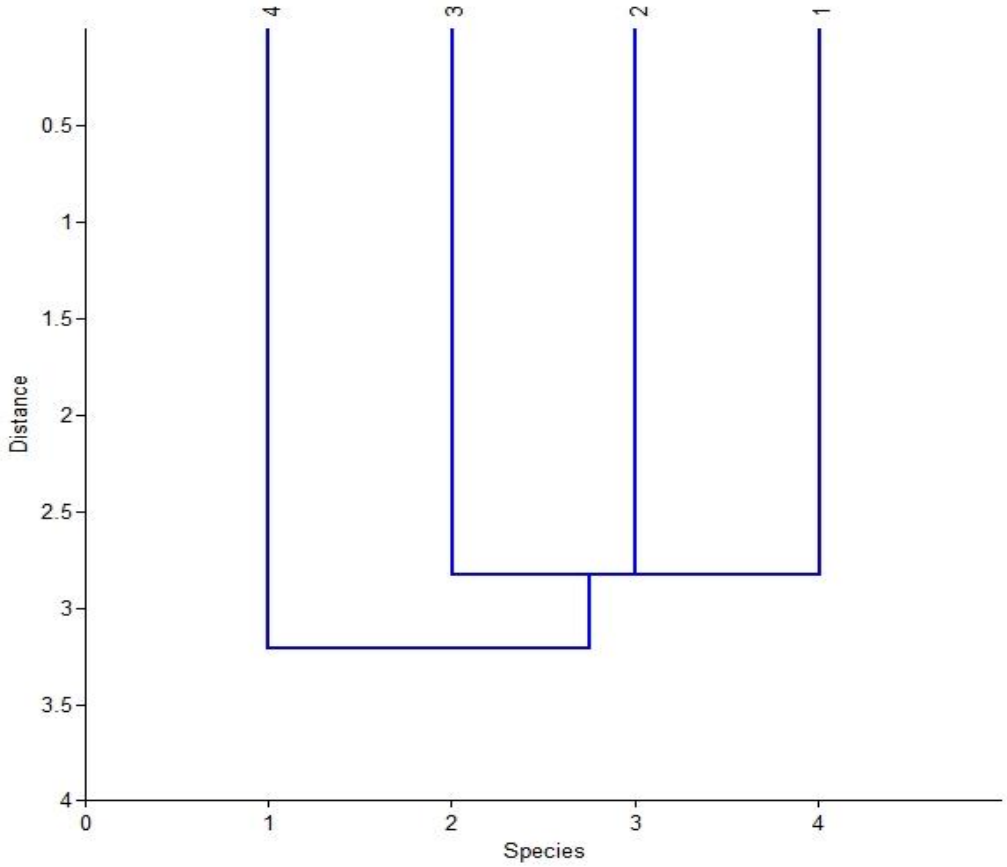

Figure 5. Relationships among the four *Pleurotus* species studied based on observed characters.
1: *Pleurotus florida*, 2: *Pleurotus ostreatus*, 3: *Pleurotus ostreatus (F)*, and 4: *Pleurotus pulmonarius*

The mycelia of *Pleurotus ostreatus* can be gloeoplerous since their high refractive index gives a granular appearance under the microscope. The presence of interwoven mass of hyphae in *Pleurotus florida* and *Pleurotus ostreatus* variety F. as against uninterwoven mass of hyphae found in *Pleurotus pulmonarius* and *Pleurotus ostreatus* is a developmental difference that has implication for their taxonomic status (Clemencon, 1997; 2004).

In this study, the observations of the fruiting body of *Pleurotus ostreatus* and *Pleurotus ostreatus* var. F reveals a mycelial cord with tiny spherules attached to it. This falls in line with Umar and Van Griesven (1999) observations on *Agaricus bisporus* fruit body. *Pleurotus florida* and *Pleurotus pulmonarius* reveals a clustered mycelium instead of mycelial cords. Umar and Van Griensven, (1995) stated that cluster mycelium, characteristically produces a massive, undifferenciated, pseudosclerotial tissue instead of mycelia cords. Further development is characterized by a polarized growth resulting in formation and elongation of the stipe and steadily enlarging cap. The globular pattern of primordia observed in this study is similar to that observed by Umar and Van Griensven (1999) in *Agaricus bisporus.*

In the central parts of *Pleurotus ostreatus* var. *F.* and *Pleurotus pulmonarius* stipe, hyphae are disposed in a longitudinal or parallel manner. This aligns with the observation made by Sanchez *et al.,* (2006) on the stipe of *Pleurotus pulmonarius* where they stated that the longitudinal hypha arranged in a parallel manner presumably due to the stretching tensions resulting from increasing vacuolation. The dark-stained area in the middle portion of *P. ostreatus* var. *F.* pileus could represent a distinct cellular region, denoting tissue specialization which might control some kind of morphogenetic signals as reported by Umar and Van Griensven (1999) on their study on *Agaricus bisporus* primordia. *Pleurotus ostreatus*, *Pleurotus florida and Pleurotus ostreatus* var. f in a cluster demonstrated closer zymographic relationships. This conform with the finding of Zerkavis and Labarere (1991) where they reported that the separation of *Pleurotus pulmonarius* from *Pleurotus ostreatus* isolates at their respective clusters were found to be distinct. Their outcome is also in accordance with the finding of preceding taxonomic study which applied morphological and physiological criteria to distinguish strains from those 2 species (Zervakis and Balis, 1991).

4. Conclusion

This study has shown that *Mansonia altisima* sawdust is a satisfactory substrate for the cultivation of *Pleurotus* species. It further reveals that rice bran as additives enhance better

performance of the substrate. It could thus be said that characters such as hyphae arrangement, cell polarity, cell density, cell shape, stainability, fruit bodies initials, presence of cuticle, hymenium length, hymenophore structure, cell wall structure, presence of nodulus, presence of copious granules, cell wall structure and cell size can be informative and relevant in identifying and characterizing the *Pleurotus* species. Variation in the characters generated could further be used for their identification.

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