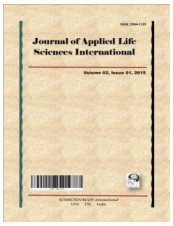

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Use of Agro-Wastes for Tissue Culture Process and Spawn Production of Oyster Mushroom (*Pleurotus florida*)

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Authors' contributions

This work was carried out in collaboration between both authors. Author FAB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AOS managed the analyses of the study. Author FAB managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

This study investigated the potentials of different growth media for the tissue culture process of Oyster mushroom as well as evaluated the response of different agro-wastes in Oyster mushroom spawn production. Agro-waste Powder Agar was prepared by mixing powdered agro-wastes which are corncobs and sugarcane bagasse, with agar-agar in the ratio 10:15. Twenty-five grams each of the media was suspended in 500ml of distilled water and autoclaved appropriately. Potato Dextrose Agar (PDA) was used as control growth medium. Oyster mushroom's pileus was surface sterilized, drained and transferred unto the growth media in a laminar flow. Corncob (CC), sugarcane bagasse (SB) were used as the main substrates while rice bran (RB) and groundnut shell (GS) were used as additives for spawn production. The different agro-wastes combination was inoculated with the tissue culture on growth media. Prior to inoculation, the agro-wastes were pasteurized, sieved and treated with limestone and gypsum in ratio (1:1; 1:2; 1:3; 1:4). Sixty grams of each CC: GS, CC: RB, SB: RB, SB: GS agro-wastes combinations were mixed together properly in a container in

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different percentages which are 80:20, 70:30, 60:40 that were later transferred into mayonnaise cream bottles, sterilized at 121°C for 30 minutes, allowed to cool and then inoculated. Data were collected on radial mycelia growth diameter (measured in the Petri dish), number of days for complete ramification and were subjected to analysis of variance and significant means were then separated using LSD at 5% level of probability. *Pleurotus florida* grown on agro-wastes growth media with 5% of sucrose had radial growth diameter which was significantly higher than the agro-wastes growth media without sucrose. These were found not to be significantly different from the conventional PDA growth medium. Spawn production on SB based substrates ramified faster than CC based substrates. *Pleurotus florida* grown on the combination of SBRB ramified faster than the other substrates. The combination of ratio 70:30 (70% main substrates: 30% additives) and 4% (1% $CaCO_3$ and 3% $CaSO_4$) of the calcium additives had the shortest days of ramification for spawn production. The study concluded that, agro-wastes possess great potentials as growth media for tissue culture of *P. florida* and also, provided a profitable means of producing viable spawns.

Keywords: Tissue culture; spawn; agro-wastes.

1. INTRODUCTION

Mushrooms are fleshy, spore-producing fruiting bodies with a distinctive fruiting body (basidiocarp) which can either be epigeous or hypogenous. The macro fungi have fruiting bodies large enough to be seen with the naked eye and to be picked up by hand [1]. They consist of two main parts; the mycelium and the fruiting body (sporocarp). The mycelium consists of a tree-like structure called “Hyphae” hidden in the soil. The mycelium absorbs food nutrients while hyphae forms into mycelia which in turn develop into the fruiting body (sporocarp) on the surface when atmospheric conditions particularly humidity is favorable. They are produced above ground on soil (hypogenous) [1]. They lack chlorophyll, they cannot, like green plants get their energy from the sun through photosynthesis [2]. The cultivation of mushroom has two phases, viz; spawn running phase (this is the period of mycelia growth) and fructification phase (this is the growth of the fruiting bodies) which are both dependent on temperature and humidity.

Mushroom substrate has been defined as a lignocellulosic material which supports the growth, development and fruiting of mushroom [3]. The growths of diverse type of mushrooms require different types of substrates and availability of various types of materials may dictate which type is used [4]. The lignocellulosic materials (agro-wastes) are rich in sugar and starch (carbon compounds), which are readily available carbohydrates sources. This speed up colonization and the consequent degradation of the substrate, thereby reducing the time of fruiting since the mycelium easily converts these carbohydrates in reserve for the fructification, increasing productivity [5]. Generally, some carbon rich agro-wastes are low in protein content and are thus, insufficient for the cultivation of mushrooms. They therefore require additional nitrogen, phosphate and potassium for proper use as growth medium for mushroom cultivation [6]. The supplements contain a mixture of protein, carbohydrate and fat, where the protein is the main source of nitrogen.

Generally, the cultivation of edible fungi often follows two distinct steps which are fundamental to the preparation of the matrix: obtaining pure inoculum of the fungus and the preparation of the "spawn" or matrix itself. Spawn is the vegetative mycelium from a selected mushroom grown on a convenient medium like wheat, pearl millet, sorghum, etc for raising mushroom. It essentially involves preparation of pure culture of mushroom from tissues/ spores that is generally maintained on any agar medium, followed by culturing on sterilized grains and further multiplied on grains. The spawn thus comprises of mycelium of the mushroom and a supporting medium which provides nutrition to the fungus during its growth. Obtaining the primary matrix of mushroom can be performed both by sexual or by asexual process [6]. It is relative to the mycelial or vegetative phase of the fungus colonizing a previously sterilized nutritional substrate (growth medium). Its production starts by the isolation of a fungus using tiny fragments of a mushroom, placed in sterile culture medium under aseptic conditions. Tissue culture is recommended for mother culture production because genetic characteristics of the mushroom are preserved in the isolated mycelia. Basically, a medium for microbial cultivation contains various organic and inorganic constituents which the microbe degrades and utilizes with the aid of enzymes it

secretes so as to carry out its metabolic activities [7].

Potato dextrose agar and Sabouraud dextrose agar have been widely used conventionally for culturing fungi and have been recommended by the American public health association for Plate count of fungi [8]. It is also used for the stimulation of sporulation (slide preparation), maintenance of stock cultures of certain fungi [9]. However, agro-wastes though unpalatable for human consumption support the growth of fungi thus serving as a cheaper and readily available laboratory media that can compare favourably with the internationally accepted microbiological growth media for fungi [7]. Although the most used media to obtain the primary matrix are potato-dextrose-agar and malt extract [10], the sawdust-dextrose-agar (SDA) medium is the most indicated by avoiding the physiological adaptation that can occur when the used culture medium has very different characteristics of production substrate [11]. The current trend is to produce inoculum from the cultivation substrate. [6] reported the use of sawdust to produce the inoculum ("seed") or grain mixed with sawdust. "Spawn" or "seed" is the substrate colonized by mushroom mycelium, with the goal of facilitating the distribution of the inoculum in different points of cultivation, thereby contributing to a more uniform and rapid colonization of the substrate, reducing the possibility of contamination.

The rate at which agro-wastes turn out often surpasses the rate of their utilization. This leads to wastage of cellular carbon rather than been incorporated into the carbon cycle within the biomass present at various trophic levels. This study investigated the potential of different growth media for the tissue culture process of Oyster mushroom and produced spawn on augmented agro-wastes without the incorporation of edible food crops like sorghum, wheat, maize and other cereal crops thus, increasing the use of agro-wastes in a positive way (Bio-recycling), as a means to spawn production.

2. MATERIALS AND METHODS

2.1 Location of the Study

The study was carried out at the Mycology Laboratory, Department of Crop Production and Protection, Faculty of Agriculture, Obafemi Awolowo University, Ile-Ife.

Cultured mycelia of Oyster Mushroom for this experiment was obtained from the mushroom collection culture centre of the same Mycology unit. Two centimeters of the mushroom's cap was washed in 1% parazone for five minutes, these little portions from the parazone solution were then transferred on to a pair of filter paper to drain the water droplets from the mushroom. Aseptically in the laminar flow, these tissues on the filter were transferred unto Potato Dextrose Agar (PDA) Plate and incubated for 3 days at 28°C and stored in the slant bottles for future use. Corn cobs, sugarcane bagasse, rice bran and groundnut shell were the agro-wastes used in this study (where Corn cobs, sugarcane bagasse only were used for the tissue culture). A (4 x 3 x 4) factorial experiment laid out in a randomized complete block design was used in this study.

2.2 Media Preparation

Two agro-wastes, corn cobs (CC) and sugarcane bagasse (SB) were air dried. Each of these substrates was grounded into powder in a dry milling machine. The powders were separately kept in clean specimen bottles for use as fungi growth medium for the tissue culture process [7].

2.3 Formulation of Mushroom Growth Medium Agar

Each labeled powder, growth media were formulated. Each Agro-waste powder (AP) was treated 1% Calcium carbonate and 1% Calcium sulphate (gypsum) and mixed with agar - agar in the ratio 5:7 to form Substrate Powder Agar (SPA). The various treatments are; CC: Agar-agar powder with 5% sucrose, CC: Agar-agar powder without sucrose, SB: Agar-agar powder with 5% sucrose, SB: Agar-agar powder without sucrose.

2.4 Sample Analysis

Twenty-five grams of each of the formulated growth medium was mixed with 500 ml distilled water in 1000 ml conical flask, boiled to melt the agar and then sterilized at 121°C for 15 minutes. The media were allowed to cool to 45°C before they were aseptically poured into sterile petri-dishes for them to set. Commercially prepared Potato Dextrose Agar (PDA) (Lab M) prepared by suspending 39 g of powdered agar in 1000 ml of distilled water in a conical flask and then sterilized at 121°C for 15 minutes and used as control. The inoculated growth media were

incubated at 28°C for 5 days and the radial mycelia growth diameter (cm) was measured with the metre rule in the petri dish.

2.5 Spawn Production Using Agro-Wastes

Corn cob (CC) and sugarcane bagasse (SB) were used as the main agro-waste while rice bran (RB) and groundnut shell (GS) were used as additives. These agro-wastes used for this study were combined in different ratio such as: (CC: GS, CC: RB), (SB: RB, SB: GS). They were soaked in hot water at 80°C for 30 minutes, and pressed to expel excess water. Sixty grams of each CC: GS, CC: RB, SB: RB, SB: GS agro-wastes combinations were mixed together properly in a container in different percentages which are 80%: 20%, 70%: 30%, 60%: 40% that were later transferred into mayonnaise cream bottles and sterilized at 121°C for 30 minutes. These bottles containing the aforementioned combination were allowed to cool before inoculating with a 5 days old culture of mushroom mycelia and incubated at room temperature. To keep the pH of the growing media in check, calculated percentages of $CaSO_4$ and $CaCO_3$ were added. These include:

1% CaSO4 + 1% $CaCO_3$; 2% CaSO4 + 1% $CaCO_3$; 3% CaSO4 + 1% $CaCO_3$; 4% CaSO4 + 1% $CaCO_3$.

(N.B: percentages were determined based on the dry weight of the agro-waste combination. i.e. 1% of 1000 g of $CaCO_3$ were calculated by the formula 1/100 *1000 g = 10 g of $CaCO_3$). The quantity of the above calculated percentages was added to the agro-waste combination and thoroughly mixed for equal distribution. The tissue content of the prepared Plates was used to inoculate the combined agro-wastes for spawn production. The inoculated agro-wastes combinations were incubated in dark cupboards and monitored for growth until full ramification of the substrates was attained.

3. RESULTS

3.1 Tissue Culture Process of *Pleurotus florida*

Pleurotus florida tissue was cultured in axenic condition on both conventional growth medium and growth media produced from agro-wastes' powder (Plates 1A, 1B and 1C). *Pleurotus florida* grown on media made from agro-waste and 5% of sucrose had a radial mycelia growth diameter which is significantly higher than the agro-waste media without sucrose but not significantly different from the conventional Potato dextrose agar (PDA) growth media (Fig. 1). *Pleurotus florida* spread with a fluffy white mycelium radially on the media (potato dextrose agar, corncobs with sucrose agar, corncobs agar without sucrose, sugarcane bagasse sucrose agar and sugarcane bagasse agar without sucrose) used in this study (Plate 1). Although, *P. florida*'s growth (using the radial mycelia diameter) was faster at first on PDA but it was not significantly different from the observed mycelia diameter on corncobs sucrose agar and sugarcane bagasse sucrose agar. *Pleurotus florida* grown on corn cobs agar without sucrose (CCA2) and sugarcane bagasse agar (SCBA2) without sucrose had reduced growth rate which was significantly lower than the other media used in this study (Fig. 1). After 5 days of inoculation, SCBA2 had the least radial growth diameter followed by CCA2 with 4.3 cm and 4.5 cm values respectively which were not significantly different from each other and the *P. florida* on both media (CCA2 and SCBA2) did not fill the Plate after 5 days (Fig. 1). A different pattern was observed on CCA1, SCBA1 and PDA. It was observed that *P. florida* grown on CCA1 and PDA growth media had a radial mycelia growth diameter of 9 cm and filled the Plates after 5 days while *P. florida* on SCBA1 growth media had a radial growth diameter of 8.05 cm which did not fill the Plate, but was found not to be significantly different from PDA and CCA1 (Plates 1A, 1B, 1C and Fig. 1).

3.2 Spawn Production Using Agro-Wastes

The substrates (agro-wastes) used for this experiment were found to support the full growth of *P. florida* in form of ramification of the substrates in the production of viable spawns (as shown in Plates 2a and 2b). Eight days after the inoculation of the substrates with the mycelia of *P. florida* grown on the agar growth media, complete growth of *P. florida* in form of ramification of the substrates was observed on the sugarcane bagasse substrates (Table 1). Whereas, *P. florida* inoculated on corncobs substrates was found to complete the full growth in form of ramification of the substrates after 16 days (Table 1). This was significantly different from the previous observation on the sugarcane bagasse substrates (Table 1). Increased growth

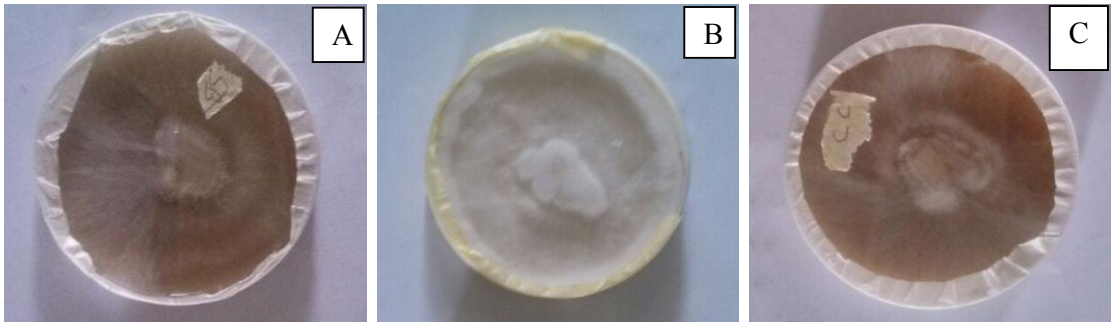

Plate 1. Mycelia of growth of Pleurotus florida on different growth media 5 days after inoculation

Plate 1A: Mycelia growth of P. florida on sugarcane bagasse agar (SCBA); Plate 1B: Mycelia growth of P. florida on corncobs agar (CCA); Plate 1C: Mycelia growth of P. florida on potato dextrose agar (PDA)

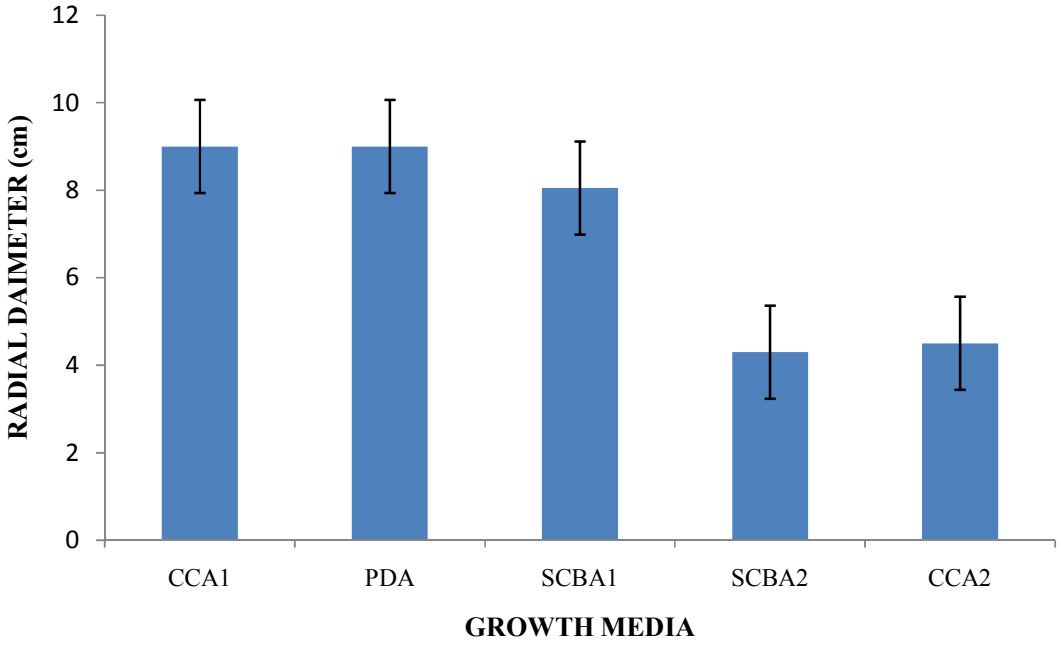

Fig. 1. The radial diameter (cm) of the mycelia of *P. florida* on different growth media 5 days after inoculation

Where PDA: Potato Dextrose Agar; SCBA1: Sugarcane Bagasse Agar + 5 % Sucrose; CCA1: Corncobs Agar + 5 % Sucrose; SCBA2: Sugarcane Bagasse Agar without Sucrose; CCA2: Corncobs Agar without Sucrose

rate of *P. florida* was observed when the supplements/ additives were added to the main substrates (corncobs and sugarcane bagasse). This also decreased the number of days spent by *P. florida* to complete the full ramification of the substrates combination for spawn production. *Pleurotus florida* grown on the combination of corncobs with groundnut shell (CCGS) and corncorbs with ricebran was found to complete its full growth in form of ramification of the substrates after 13 days (Table 1). This recorded number of days for complete ramification of the combined substrate with additives/ supplements was significantly different from the recorded number of days (16 days) when concorbs without additives was used (Table 1). Futhermore, this same trend was observed for the *P. florida* grown on sugarcane bagasse substrates for spawn production. The addition of supplements/ additives to the main substrate (sugarcane bagasse), was found to significantly decreased the number of days spent by *P. florida* to completely ramify of the substrates for spawn production from 8 days (SB) to 7 days (SBGS)

and 6 days (SBRB) respectively at p< 0.05 (Table 1).

Table 1. Effect of different combinations of Agro-wastes on days to complete ramification of substrates for spawn production

Combination	DTCR (days)
CCGS	13.00
CCRB	13.00
SBGS	7.00
SBRB	6.00
CC	16.00
SB	8.00
$LSD_{0.05}$	0.09

(Means separation at p<0.05); DTCR: Days to complete Ramification; Where CCGS: corncobs + groundnut shell; CCRB:corncobs + rice bran SBGS: sugarcane bagasse+ground nut shell; SBRB: sugarcane bagasse+ rice bran; CC: Corn cobs; SB: Sugar cane bagasse

The effect of different ratio of combinations (80:20, 70:30, and 60:40) of the main substrates to the additives on days to complete ramification of the combined substrates (CCGS, CCRB, SBGS, SBRB) for spawn production is presented in Table 2. The different ratio of combination of the main substrates and the additives (all agro-wastes) was found to be highly significant on the number of days to complete growth of *P. florida* in form of ramification of the substrates for spawn production. *P. florida* grown on substrates with 70% main substrates: 30% additives for spawn production was found to completely ramify the substrates combination after 8 days (Table 2). This number of days recorded for *P. florida* grown on substrates combination with 70% main substrates: 30% additives was found to be significatly different from the other (80:20 and 60:40) ratio of combinations (Table 2). *P. florida* was found to completely ramify the substrates combination with ratio 80:20 (80% main substrates to 20% additives) after 11 days of inoculation for spawn production while full growth of *P. florida* in form of ramification of the substrates with combination ratio 60:40 (60 % main substrates to 40% additives) occurred 9 days after inoculation (Table 2). The effect of different percentages of calcium derivatives (1% $CaCO_3$: 1% $CaSO_4$, 1% $CaCO_3$: 2 % $CaSO_4$, 1% $CaCO_3$: 3 % $CaSO_4$, 1 % $CaCO_3$: 4 % $CaSO_4$) added to the agro-wastes on days to complete ramification of substrates for spawn production was presented in Table 3. *Pleurotus florida* grown on substrates with calcium augumentation ratio 1:3 (1% $CaCO_3$: 3% $CaSO_4$), was found to completely ramify the substrates after 8 days of inoculation (Table 3). Whereas, the *P. florida* inoculated on substrates with calcium augumentation ratio ratio 1:1 (1% $CaCO_3$: 1% $CaSO_4$) and 1:4 (1% $CaCO_3$: 4% $CaSO_4$) was found to have the longest days (10 days) for complete ramification of the substrates for spawn production (Table 3).

Table 2. Effect of different ratio of combinations of Agro-wastes on days to complete ramification for spawn production

Ratio	DTCR (days)
80:20	11.00
70:30	8.00
60:40	9.00
$LSD_{0.05}$	0.08

(Means separation at p<0.05); DTCR: Days to complete Ramification; Where 80:20: 80% Main substrates: 20% additive; 70:30: 70% Main substrates: 30% additive; 60:40: 60% Main substrates: 40% additive

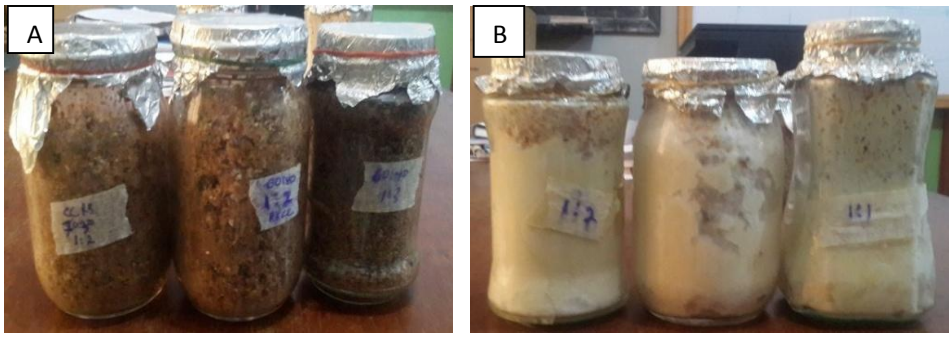

Plate 2. *Pleurotus florida* spawn production using different Agro-wastes
Plate 2a. Sterilized bottles containing agro-wastes combination; Plate 2b. Fully ramified bottles containing agro-waste combination

Table 3. Effect of different percentages of calcium added to the agro-wastes on days to complete ramification for spawn production

% Calcium	DTCR (days)
1:1	10.00
1:2	9.00
1:3	8.00
1:4	10.00
$LSD_{0.05}$	0.09

(Means separation at p<0.05) Where 1:1: 1 % $CaCO_3$: 1% $CaSO_4$; 1:2: 1% $CaCO_3$: 2 % $CaSO_4$; 1:3: 1% $CaCO_3$: 3% $CaSO_4$; 1:4: 1 % $CaCO_3$: 4% $CaSO_4$; DTCR: Days to complete Ramification

4. DISCUSSION

Agro-wastes were used in this study as a result of its manifold availability, its cheap sources of essential nutrients and the ability of the organism (*P. florida*) to easily breakdown the contained polysaccharides for its growth when compared to other substrates used for cultivation. The possibility of convertng the 'wastes' from the farm into potential raw materials for efficient use within the agro-system also informed the choice of agro-wastes for Oyster mushroom (*P. florida*) spawn production in this study. This conforms to the submission of Shah et al. [4] who reported that mushroom can be grown on agricultural and industrial wastes, and more than half of produce from the land remain unused as waste in the form of straws, leaves, stems, roots etc. These wastes can be recycled into food and the environment will be less harmful in form of pollution. The substrates used in this study supported the spawn production of *P. florida* significantly although at different levels. The observed differences can be alluded to the differences in the chemical and elemental composition of these agricultural waste [12].

[8] reported that Potato dextrose agar and Sabouraud dextrose agar have been widely used conventionally for culturing fungi and have been recommended by the American public health association for Plate count of fungi. *Pleurotus florida* cultured on media made with agro-waste (corn cobs and sugarcane bagasse) with 5 % sucrose performed excellently and has no significant difference with tissues cultured on the conventional PDA. The agro-waste growth media without the addition of sucrose also supported the growth of *P. florida* although not at the the same rate with the agro-waste growth media with sucrose. The fact remains that the growth media contained the needed substances that is required for the growth of the organism but a lag period will be needed for the breakdown of these components into absorbable nutrients which may likely require more time for the process to be completed. Thus, increasing the time it will take the organism to fill the Plates. This is similar to [7] who reported that waste cobs from variously processed maize (*Zea mays*) were used as raw materials to prepare growth agar media for fungi and all the formulated media supported the growth of the microorganisms tested. This further asserts the fact that *Pleurotus* species have extensive enzyme systems capable of utilizing complex organic compounds that occur as agricultural wastes and industrial by-products [13]. It was also discovered that *P. florida* tissues, cultured on agro-waste based media sustained and preserved the growth of the tissues longer than the conventional PDA. This can be attributed to the extraordinary metabolic activity of mushrooms growing on organic materials as reported by [6] and the gradual decomposition of the substrates which continually supports the growing mycelia. Hence, the media can be used for maintenance of stock cultures of certain fungi. This is in contrast to the report of [9] who used PDA as maintenance of stock cultures for certain fungi. [7] also reported that agro-wastes though unpalatable for human consumption support the growth of fungi thus, serving as a cheaper and readily available laboratory media that can compare favourably with the internationally accepted microbiological growth media for fungi.

Spawn production had been conventionally produced with sorghum grains. The main function of the grain is to serve as means of dispersion of mycelium, since it is impossible to handle the mycelium without damaging the fragile structure of the hyphae walls [14]. In contrast, this study investigated the use of agro-wastes in the production of viable spawns. These spawns were later used to produce the fruiting bodies that was harvested. Agro-wastes used in this study provides an alternative and profitable means of producing viable spawns without the incorporation of edible food substances/ constituents in the production cycle of edible mushroom. The spawn produced with agro-wastes then became the substrate colonized by mushroom mycelium, with the goal of facilitating the distribution of the inoculum in different points of cultivation, thereby contributing to a more uniform and rapid colonization of the substrate, reducing the possibility of contamination [6].

The combination of substrates further signifcantly reduced the number of days needed for the complete ramification of the substrates for spawn production. The number of days taken for the complete ramification of the substrates used for spawn production is very important as it determines the number of successful cycles of spawn that can be produced at a given space of time. Thus, determining the number of days that will be needed for the mushroom grower to proceed to the next phase of the production cycle. The combined substrates provided the essential nutrients for the fast and full ramification of the substrates by the organism. The constituents present in the combined substrates were available at required amount and the organism also efficiently breakdown the substrates to derive the necessary nutrient for its growth. *P. florida* grown on the combination of sugarcane bagasse and ricebran was able to ramify faster than the other substrates due to the fact that the combination gave a larger surface area for the spread of the organism since the size of these substrates were smaller compared to the other substrates. Hence, a faster spread of the orgamism's mycelia on the substrates. Although, rice bran alone used as a substrate for spawn production took a longer period of time. This may be due to the small particle size of the bran thus, providing room for compaction and a blockage to the spread of the mycelia. It is thus, best used as supplements/additives with other substrates. This is similar to [6] who suggested the ratio of supplement to main substrates for fruiting body production.

The ratio of main substrates to the supplements for spawn production used in this study proved that adding one-third of supplements to the main substrate is most appropriate for faster growth of the organism to ramify/colonize the substrates. Increasing the supplements beyond this proportion, the days to complete growth in form of ramification of the substrates for spawn production was delayed which is detrimental to the production cycle of edible mushroom. Calcium augumentation effect depends on the substrates used and the initial calcium content. Augumentation of the substrates with calcium based compound evaluated in this study revealed that 4% (1% $CaCO_3$ and 3% $CaSO_4$) of the calcium based substrates is most appropriate for rapid ramification of the substrates. This is similar to the report of [5,6,15] who recommended 3% calcium oxide.

5. CONCLUSION

Powdered agro-wastes of corncobs and sugarcane bagasse were effective as growth media for fungi most especially *P. florida* used in this study. The agro-waste growth media competed substantially with the conventional growth media (PDA) for fungi growth in the tissue culture process. This opens the way for the elimination of edible food products (potato) or other food source in the production of growth media and also offer a cheaper means for fungi growth media formulation with the use of agro-wastes. Also, agro-wastes have the potential to support the growth of *P. florida* from the tissue stage to the spawning stage. The combination of substrates significantly reduced the number of days needed for the complete ramification of the substrates for spawn production. *P. florida* grown on the combination of SBRB (sugarcane bagasse and ricebran) ramified faster than the other substrates. Ratio 70:30 (70% main substrates: 30% additives) and 4% (1% $CaCO_3$ and 3% $CaSO_4$) of the calcium additives were most appropriate for rapid ramification of the substrates for spawn production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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