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Base Substrate Sorghum Supplied with Nitrogen Additive Enhanced the Proliferation of Oyster (*Pleurotus ostreatus* (Jacq.: Fr.) Kummer) Mushroom Spawn Mycelium

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Abstract: ***The experiment was conducted to evaluate the performance of Pleurotus ostreatus (Jacq.: Fr.) Kummer mycelium on spawn media prepared from base substrate sorghum supplemented with combination of additives namely wheat bran, $CaCO_3$, $CaSO_4$, urea, sucrose and citric acid. Five spawn substrate combinations in 3 replications had been tested and data on fresh mycelial weight change 5, 10, 15, and 20 days after inoculation (DAI) and percentage mycelium coverage (PMC) at 20 DAI were collected. Completely randomized design was employed and the data was analyzed using SAS version 9.0. The result indicated differences in mycelial fresh weight ($p < 0.05$) after inoculation with the highest substrate weight reduction recorded at 20 DAI. Consequently, spawn substrate media combined from sorghum, wheat bran, $CaCO_3$, $CaSO_4$, and urea was found to be the most favorable media mix while 20 DAI was incubation period for better mycelial growth. Similarly, the qualitative data was found to be in support of the above result and there was significant difference in PMC at 20 DAI. Therefore, this study indicated substrate media combined from sorghum (87.2%), wheat bran (10%), $CaCO_3$ (1.5%), $CaSO_4$ (0.5%), and urea (0.8%) as the best alternative media for the production of P. ostreatus mushroom spawn.***

Keywords: Pleurotus ostreatus, substrate additives, spawn, mycelia

1. Introduction

Mushrooms are fruiting bodies of fungi. They are classified in Kingdom of Fungi, which is also called Kingdom of Mycetae [1]. The use of mushrooms as food is an old age practice [2,3] and there is cultivation record in 60 A.D. [4]. Mushrooms are considered as a source of high quality protein and crops possessing high medicinal and economical values [4-6]. Mushroom farming has two inseparable phases; spawn production and fruiting body cultivation. Spawn is the planting seed of mushroom [7] and technically, it is an expanding mushroom mycelium colonizing a given substrate media. Currently, albeit mushrooms are cultivated in more than 100 countries [8], the main constraints facing the majority of mushroom producers in developing countries including Ethiopia is the lack of good quality spawn that meets consumers preference [9,10]. Production of good quality spawn requires a strict laboratory procedure in which maintaining sanitation and purity of the spawn are critical importance [11]. The quality of spawn affects both yield and quality of cultivated mushrooms [12,13].

On the other hand, the production of good quality spawn is directly associated with the type and quality of substrate used [4]. It has long been known that, for most mushroom types, cooked or soaked cereal grains and sawdust are commonly used substrates for spawn production [4,7]. Essentially, the substrate should supply nutrient and provide stability for expanding mushroom mycelium [14]. Among cereal grains, rye, wheat, sorghum and millet are the common substrates. Accordingly, Jiskant et al. [15] have tested two different varieties of sorghum for better and early spawn growth of *P. ostreatus*. Similarly, Stanley [13] has tested the mycelial growth response of two oyster mushroom species on substrates made of wheat, yellow maize, Guinea corn, millet, red sorghum and white maize.

Empirical studies indicated that supplementation of the grain substrate with small amounts of component additives such as wheat bran [16] can furnish nitrogen, vitamins and minerals and ultimately improve the suitability of the substrate for mycelium proliferation. Additives like vermiculite are known to prevent the grain from getting sticky, $CaCO_3$ and $CaSO_4$. $2H_2O$ have a positive effect on the structure of the substrate and stabilize the pH [7]. Environmental conditions of the production milieu and the media including temperature, O_2 and CO_2 balance, humidity, light and pH have also been reported to affect mycelial growth in the process of spawn preparation [17-20]. Generally, the type of base substrate and proportion of additives could affect carbon to nitrogen ratio (C:N), pH, moisture content, compaction, O_2 and CO_2 concentrations and the temperature of the media [21,22].

In their experiment for commercial spawn production of shiitake mushroom (*Lentinus edodes*), Ramkumar et al. [23] observed the substrate Ill-filled paddy with $CaCO_3$ additive significantly enhanced the mycelial growth and basidiocarp formation. Yang et al. [24], cultivated *P. ostreatus* on rice, wheat, cotton seed hull basal substrates alone and supplemented with different proportions of rice and wheat straw to find a cost effective substrate. Tajudeen et al. [16] evaluated the effect of supplementing fermented pine sawdust substrate with different levels of wheat bran on the yield of oyster mushroom (*P. ostreatus*). In addition to these, various spawn grains, culture media, oil types and rates reported to show a highly significant effect on the dimensions and weights of *L. squarrosulus* [18].

In conclusion, there are a wide range of base substrate materials and component mixtures which can be prepared in various proportions for spawn production. In turn, cultivated mushrooms respond differently for different substrate materials, component mixtures and additive proportions [12,25]. Consequently, the vigor, proliferation and quality of spawn vary with the type of substrate media onto which it is growing [4]. However, research work on the effect of multiple additives on the vigor of *P. ostreatus* mycelium growing on cereal grain has not been tested at this proportion. Thus, as spawn is often located at the center of the value chain of mushroom industry [10,26], the quest for better substrate media calls the attention of several workers. In view of this, this research was conducted to identify best alternative spawn substrate media for oyster (*P. ostreatus*) mushroom production.

2. Material and Methods

2.1 Experimental Location

The experiment was conducted between September 2013 and February 2014 at Bahir Dar University, Department of Food and Nutrition Engineering Laboratory, Bahir Dar, Ethiopia at environment controlled condition.

2.2 Research design

The experiment was laid out in completely randomized design (CRD) in five treatments replicated three times. Base substrate, supplemental and additives and their corresponding proportions used in the final test substrate component for this experiment are listed in Table 1.

Table 1: Base substrate and proportion of additives combination in dry weight bases

Base substrate, supplemental and additives	*Treatment (Trt) dry weight substrate combination in kg*				
	Trt1	Trt2	Trt3	Trt4	Trt5
Sorghum	0.860	0.862	0.870	0.872	0.880
Wheat bran	0.10	0.10	0.10	0.10	0.10
$CaCO_3$	0.015	0.015	0.015	0.015	0.015
$CaSO_4$	0.005	0.005	0.005	0.005	0.005
Urea	0.008	0.008	0.008	0.008	0.00
Sucrose	0.01	0.01	0.00	0.00	0.00
Citric acid	0.002	0.00	0.002	0.00	0.00
Total	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**

2.3 Material, media and culturing

Source of material and preparation of potato dextrose agar culture medium

The mushroom species (*P. ostreatus*) that was used for this work was supplied by Kiflemariam Horticulture Development Enterprise, Bahir Dar, Ethiopia. The white sorghum grains and additives namely wheat bran, urea, chalk ($CaCO_3$), gypsum ($CaSO_4$), sucrose, and citric acid were all purchased in Bahir Dar and Addis Ababa, Ethiopia. Commercial Potato Dextrose Agar (PDA) was employed as medium of growth for culturing *P. ostreatus*. The culture medium from commercial PDA was prepared using previously described guidelines (http://wiki.bugwood.org/Potato_dextrose_agar).

Culture of fungi

Fresh cultures of *P. ostreatus* were used for this experiment. Small pieces of fresh tissue from young and well grown mushroom were taken as follows. The selected fresh mushrooms were thoroughly surface sterilized with 75% alcohol and then each mushroom stipe (stem) was split in to two with a sterilized surgical blade under laminar flow cabinet and small rectangular tissue (about 4 mm^2) was taken from the inner upper most part of the stipe and placed on autoclaved (121 ^{0}C at 15 bar pressure for 20 min) and cooled PDA medium in Petri-dishes. In this way, 10 inoculated dishes were incubated at 25 ^{0}C for 12 days in incubator until the mycelium branches were stretched out of the agar.

Grain substrate media and spawn preparation

White sorghum grain (4.34 kg) was washed three times and boiled for 50 min in 6 liters of water and excess water was drained at the end of the boiling time. Then, the boiled sorghum grain was weighed and the moisture content was calculated to be 41% as per the formula indicated in Oei [7] as:

Percent moisture content = [(wet weight – dry weight)/wet weight] *100

Following this, the wet sorghum was divided for each treatment based on their calculated share. Afterwards, wheat bran, $CaCO_3$, $CaSO_4$, urea, sucrose, and citric acid were separately prepared for each treatment and thoroughly mixed with the base substrate sorghum as per the treatment combination indicated in Table 1. For each treatment (when applicable) urea, sucrose and citric acid was applied in solution form after being dissolved by 16 ml, 20 ml, and 4 ml water, respectively and wheat bran was wetted with water on the bases of 0.5 ml/1g and mixed with the base substrate sorghum. The substrate mixes (base + additives) was then filled into spawn bottles of 500 g capacity up to 3/4th of the bottles' capacity and autoclaved at 121 ^{0}C (at a pressure of 1kg/cm^2) for 1 h. Following this, each bottle was shaken well when taking it out of the autoclave to avoid subsequent caking. Ten g of sample from each treatment combination was taken and the pH of each substrate mix was determined following appropriate laboratory procedures. In the following day, when the substrate cools down, each treatment bottle was exposed into laminar flow cabinet gamma radiation for 30 min and inoculated with two squares (10x10 mm^2) of PDA grown pure culture mycelia and shaken very well to mix the mycelia evenly and finally incubated at 25 ^{0}C for 20 days.

2.4 Data collected

Data collection included weight of empty bottle, weight of bottle and substrate in the bottle upon inoculation, mycelial fresh weight at 5, 10, 15, 20 DAI, and percent mycelial coverage of each replicate in each treatment 20 DAI.

Mycelial fresh weight was determined using the methods described by Stanley [13] as follows: Weight of bottle = X g; Weight of bottle + spawn grains = (X+Y) g; Weight of

bottle + spawn grains + weight of fresh mycelia= (X+Y+Z) g; and fresh mycelial weight = (X+Y+Z)-(X+Y) g = Z g.

Percent mycelium coverage 20 DAI was scored visually by three data collectors and the average of the three was taken as final score for each of treatment replicates.

2.5 Data analysis

CRD factorial data analyses had been employed and the data analysis was performed using SAS version 9 statistical software package. Mean comparison among treatment groups was also conducted using least significant difference (LSD) method and differences between means at p value ≤ 0.05 were considered as significant.

3. Results

Based on the previous 5 years experience of the lead investigator in spawn making and available literature references, five spawn substrate media combinations had been tested to study the effect of the media mixes on fresh mycelial weight change of *P. ostreatus* 5, 10, 15, and 20 DAI and the percent mycelium coverage (PMC) at the end of incubation period; 20 DAI.

Urea substrate additive increased mycelial fresh weight gain

The result of the study indicated that the sorghum base substrate supplemented with wheat bran, $CaCO_3$, $CaSO_4$ and urea significantly ($P < 0.05$) increased the mean mycelial fresh weight gain (-2.375 ± 0.506 g) compared to other treatment groups (Table 2) and thus, this spawn substrate was found to be the most favorable media for mycelial weight gain and expansion of *P. ostreatus*. However, further supplementation of this substrate media with sucrose and citric acid did not affect the mean mycelium weight gain of *P. ostreatus.*

Table 2: Effects of substrates on *P. ostreatus* spawn mycelial weight gain

Trt	pH	Mean ± SE
1	5.63	0.450 ± 0.506^{a}
2	6.19	-0.800 ± 0.506^{a}
3	5.39	-0.383 ± 0.506^{a}
4	6.21	-2.375 ± 0.506^{b}
5	6.09	0.008 ± 0.506^{a}

Means with different superscripts are significantly ($P < 0.05$) different.

Substrate weight loss across incubation period explains mycelial weight gain

To understand whether the incubation period influences the mycelial establishment or not, the mycelia fresh weight change among treatments was evaluated at 5, 10, 15 and 20 DAI. The result has shown that with the exception of treatment 4 (sorghum + wheat bran + $CaCO_3$ + $CaSO_4$ + urea), all treatments showed weight gain during the first 10 days of incubation. The highest mean weight gain was observed during the initial period of mycelial establishment; 5 DAI in treatment 5 (sorghum + wheat bran + $CaCO_3$ + $CaSO_4$) followed by treatment 1 (sorghum + wheat bran + $CaCO_3$ + $CaSO_4$ + urea + sucrose + citric acid) and 3 (sorghum + wheat bran + $CaCO_3$ + $CaSO_4$ + urea + citric acid). As the time of incubation period increases, decrease in weight of colonized substrate media became evident and mycelial growth further continued with a negative weight gain 15 & 20 DAI across treatments. At 15 DAI, the highest weight loss was observed in treatment 4 followed by treatment 2 and 5 (Fig. 1). Furthermore, the weight loss increasingly continued across treatments and at the end of the incubation period (20 DAI) the highest weight loss was also observed in treatment 4. This significant weight loss difference may indirectly explain the use of citric acid in treatment 1 and 3 and the addition of sucrose in treatment 1 and 2 had no significant effect on mycelial proliferation during the incubation periods. On the other hand, the use of urea as nitrogen source additive in treatment 4 (a treatment without citric acid and sucrose) showed significant difference at $P < 0.05$ when compared with the change observed in the remaining treatments.

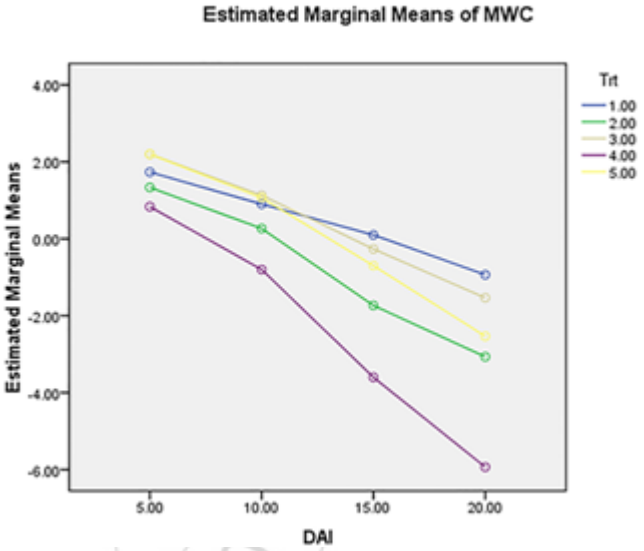

Figure 1: Mean mycelial weight change 5, 10, 15, and 20 DAI

The figure indicating weight change differences among treatments across the incubation period. In addition to analyzing the effect of incubation period on mycelial weight gain in each media combination, the data obtained in each incubation period was merged irrespective of the media used. The analysis has revealed the presence of significant difference in mycelia fresh weight during 5, 10, 15, and 20 DAI (Table 3). During the first 10 days of incubation, though the trend was decreasing, the mycelial weight gain was showed to be positive. However, during 15 and 20 DAI, the trend of weight gain was negative, with the highest weight reduction recorded -2.800 ± 0.452 g 20 DAI and -1.240 ± 0.452 g 15 DAI (Table 3).

Table 3: Effects of days after inoculation on *P. ostreatus* spawn mycelial weight gain

Days after inoculation (DAI)	Mean ± SE
5 DAI	1.660 ± 0.452^{a}
10 DAI	0.513 ± 0.452^{b}
15 DAI	-1.240 ± 0.452^{c}
20 DAI	-2.800 ± 0.452^{d}

Means with different superscripts are significantly ($P < 0.05$) different.

PMC is affected by substrate combination

Apart from analyzing the quantitative data on mycelial weight change, qualitative analysis indicating the live mycelial expansion had also been recorded. Likewise, percent colonization of the substrate with expanding mycelium was found to be in support of the quantitative data obtained through weight measurement during the experimental period (Fig. 2 and 3).

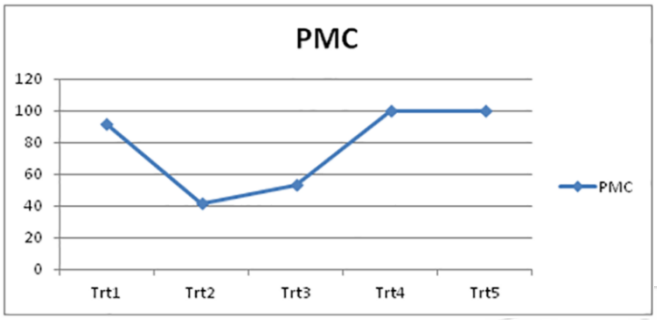

Figure 2: Line chart indicating mean percentage mycelial coverage 20 DAI

The line chart indicates, the highest coverage of mycelial colony, 100%, was observed in both treatment 4 & 5 followed by treatment 1 (91.67%). The lowest coverage was observed in treatment 2 (41.67%) followed by treatment 3 (53.33%), indicating sucrose and citric acid had no effect on mycelial expansion.

Furthermore, as indicated in Table 4, there was significant difference among treatment means in percent mycelia coverage 20 DAI at $P < 0.05$.

Table 4: Effect of various substrate combinations on PMC 20 DAI

Trt	Mean %age
1	91.67^{ba}
2	41.67^{b}
3	53.33^{ba}
4	100.00^{a}
5	100.00^{a}

Means with different superscript are significantly ($P < 0.05$) different.

Figure 3: Mycelial coverage 20 DAI

Figure 3 indicates from left to right in order of their arrangement the extent of mycelium coverage 20 DAI in treatment 1, 2, 3, 4, and 5. The picture for treatment 4 appears to be more vigorous and covered with white bright mass of mycelium suggesting the noticeable quality difference between treatment 4 and 5 regardless of the equivalent score they share in PMC.

4. Discussion

In spawn production, mycelia growth, rate and extent of mycelia expansion are affected by the type of base substrate, substrate combination and proportion of additives supplemented. This is supported by studies demonstrating the existing variation in mycelium proliferation among cereal grain types [12,13], mixed grain media[25], sawdust[16,27,28] and cereal grain[24] supplemented with various types of cereal bran, and the use of various additives to enrich the media with minerals, vitamins, and protein[18]. Moreover, $CaCO_3$ [20,23,29], $MgSO_4$ and $CaSO_4$ are added to improve the structure of the media and offset the pH. Apart from the media type, there is also response variation among mushroom species to the level of the pH of the media [17,29,30]. Values of pH are affected by differences in media, growth conditions, and strains or stocks used. Generally, the pH range for optimal mycelial growth of *P. ostreatus* is between 5.4 and 6.0[4].

Similarly, this study demonstrated treatment 4; substrate media combined from base substrate sorghum supplemented with wheat bran and with $CaCO_3$, $CaSO_4$ and urea at pH of 6.21 as the best media for the production of spawn for *P. ostreatus*. In this treatment, faster growth of mycelium was observed as evidenced by progressive weight loss beginning from 5 DAI and continuing until the end of the incubation period; 20 DAI (Fig. 1). Weight losses are associated with high rate of energy utilization by the invading mushroom mycelium resulting decrease in the overall weight of the substrate as the fungus feeds it. In the same way, Jiechi et al.[31] observed weight loss of substrates when *Auricularia auricula-judae* was cultured on sawdust substrates. Their result indicated that regularity in growth rate, weight loss and high rate of respiration correlates with the state of mycelial growth and substrate consumption. Thus, high rate of weight loss in the colonized substrate of treatment 4 may indicate that the proliferation of the mycelium and its aggressive utilization of the carbohydrate in the substrate mix and more importantly the relative favorability of the media for faster mycelial colonization and rapid nutrient utilization. The result of this study has also been supported with the qualitative data taken as PMC at 20 DAI (Fig. 2 and Table 4).

The insignificant differences observed in weight change of the remaining treatments suggest lower pH values in treatment 1 and 3 (Table 2) that was brought due to the addition of citric acid additive may have reduced the favorability of the medial for mycelial proliferation while the addition of sucrose in treatment 2 may have changed the C:N ratio of the media.

5. Conclusions

Spawn substrate media combined from sorghum, wheat bran, $CaCO_3$, $CaSO_4$, and urea was found to be significantly different from other media combinations in terms of mean fresh mycelia weight change and mean percent mycelia coverage and found to be the most favorable media mix in the production of spawn for *P. ostreatus* mushroom. Therefore, substrate combination of treatment 4, i.e., sorghum (87.2%) + wheat bran (10%) + $CaCO_3$ (1.5%) + $CaSO_4$ (0.5%) + urea (0.8%) is recommended as the best alternative spawn production media for *P. ostreatus* mushroom. Moreover, weight change in mycelial fresh weight showed significant difference at 5, 10, 15 and 20 DAI at $p<0.05$ with the highest weight reduction of -2.800 ± 0.452 g 20 DAI and -1.240 ± 0.452 g 15 DAI. Accordingly, 20 DAI was found to be favorable incubation period in this substrate media combination for better spawn mycelial growth of *P. ostreatus*.

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