IMPROVEMENT OF SHIITAKE SPAWN FOR CULTURING ON PASTEURIZED WHEAT STRAW

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ABSTRACT

Results of a study relevant to the growth capacity of 15 strains of *Lentinula edodes* on a wheat straw substrate are presented. Four different types of spawn were prepared: F1 (sorghum seed), F2 (millet seed), F3 (peat moss, 1.3%; gypsum 1.3%; powdered coffee pulp, 8.8%; and sorghum seed, 88.5 %) and F4 (same formula as F3, but substituting millet seeds). Ten grams of pasteurized straw per petri dish were inoculated with 0.5 g of formula, and five petri dishes per formula were prepared. Controls were derived from mycelial implants that had been previously cultivated in an agar-water (AW) medium. Formula samples incubated for seven days at 26°C were used in estimating mycelial growth (i.e. total area). In the second part of this study, fungal biomass corresponding to metabolic activity for each formula was estimated for ten strains of *L. edodes*. Fluorescein diacetate hydrolysis (FDA), the technique used to carry out this estimation, was undertaken on formula samples that had been collected once a week for six weeks. All strains showed their greatest mycelial area on wheat straw, starting from F3 formula. Mycelial growth for the F1, F2 and F4 formulas were not significantly different. In all cases, the smallest mycelial area was associated with the AW medium. In contrast, FDA hydrolysis showed that the highest metabolic activity was found for the F3 formula. FDA hydrolysis also allowed spawn maturation to be determined, an event that usually occurs during the fifth week. The applied results of this study have permitted the selection of shiitake strains that can probably adapt rapidly to cultivation on pasteurized wheat straw.

INTRODUCTION

The traditional method for culturing shiitake mushrooms, which relied upon wood logs, has been partially replaced by a newer, more intensive cultivation system using substrates of sawdust or wood shavings. Because nutritional compounds are generally added to these materials, sterilization pretreatments are generally required in order to be able to inoculate under controlled conditions (Savoie *et al*. 2000). This system, also known as "cultivation on synthetic logs", requires less time to complete the cultivation cycle, and also has notably higher biological efficiencies. The principal disadvantages of this system are high costs of installation, greater consumption of energy during operations, and the tendency of substrates to become infected with antagonistic molds (Royse and Bahler 1989, Levanon *et al*. 1993, Kalberer 1998). Recently, the cultivation of shiitake on wheat straw has been proposed. In this case, the substrate receives a thermal treatment at 65°C (pasteurization) (Delpech and Olivier 1991). European cultivators have adopted this method and, little by little, it is gaining acceptance in the United States and the Americas (Savoie *et al*. 2000). Due to chemical and structural differences in the cultivation substrates, as well as in thermal treatments, the selection of genotypes adapted to these conditions is critically important to ensure a good production of fruiting bodies in the shortest time possible.

The capacity of a mushroom to grow on a lignocellulosic substrate is related to the vigor of its mycelium, as well as to its capacity to activate physiological mechanisms necessary to adequately exploit the medium (Buswell *et al.* 1993). If fructification traits are among the criteria for determining strain selection, particular emphasis must be placed on the strain's capacity to invade a given substrate. Initial colonization speed is even more important when a non-sterile substrate is utilized because of the presence of antagonistic microorganisms.

Shiitake cultivation on pasteurized wheat straw is not exempt from the problems produced by antagonistic fungi, and the number of shiitake strains that are well adapted to this system is relatively low (Mata and Savoie 1998). The preparation of a nutritionally supplemented spawn, as well as the preadaptation of the mycelium to the final components of the culture substrate, have allowed for a considerable reduction in contamination during the first growth stages, particularly with respect to molds of the genus *Trichoderma* (Mata *et al*. 1998, Savoie *et al*. 2000). Based upon the principle of nutritional preadaptation, this study has been undertaken to improve shiitake spawn among strains that grow rapidly on wheat straw substrates, as well as to propose a method for evaluating spawn maturation.

MATERIALS AND METHODS

Strains

Fifteen strains of *Lentinula edodes* (Berk.) Pegler were studied. They are deposited in the Fungus Strain Collection of the Instituto de Ecologia. That strain collection has been registered as IE in the World Data Centre of Microorganisms and catalogued as number WDCM 782. Among these strains are certain commercially cultivated lines used in industrial production as well as others that were obtained by laboratory crossing (Table 1). All strains were maintained on a culture medium derived from agar and malt extract (MEA).

Table 1. Strains studied and their origins.

Strain	Original number	Country
IE 40	MSCN - 433	Hong Kong
IE 105	CS.2 Fungi Perfecti	United States
IE 112	Mori P - 5	Japan
IE 122	IBUG - 16	Mexico
IE 123	IBUG - 17	Mexico
IE 124	IBUG – 18	Mexico
IE 149	JCT	United States
IE 171	CBS (390.89)	Netherlands
IE 207	DSH 92 – 147 PNG	New Guinea
IE 242	A9	France
IE 243	M115	United States
IE 244	4055	France
IE 245	V084	France
IE 246	S600	France
IE 247	S610	France

Spawn formulations

Spawn were prepared from sorghum (*Sorghum vulgare* Pers.) and millet (*Panicum miliaceum* L.) seeds to which were added powdered peat moss, gypsum, and powdered coffee pulp. In order to observe the effect of different spawn compositions on mycelial growth, four spawn formulations were prepared: F1: 100% sorghum seeds; F2: 100% millet seeds; F3: 88.5% sorghum seeds, 1.3% peat moss, 1.3% gypsum, and 8.8% coffee pulp; and F4: 88.5% millet seeds, 1.3% peat moss, 1.3%
gypsum and 8.8% coffee pulp. In all cases, seeds were hydrated separately by soaking them for 12 hours. Later, they were drained of excess moisture and then the remaining

ingredients were added. Ingredients were mixed and the moisture content had stabilized at 75%. Formulas were placed in plastic bags and sterilized for 90 min at 121°C. Once samples had cooled, 30 g of each formula were placed in petri dishes (90 mm diameter) and inoculated with mycelial implants (8 mm diameter) from each of the strains that had been pre-cultured in MEA for 15 days. Five samples of each strain-formula combination were prepared and incubated in darkness at 26°C for 16 days.

Mycelial growth on wheat straw

In order to determine mycelial growth for the strains, wheat straw (*Tritucum aestivum*) was cut into small pieces (< 3 cm) and hydrated by soaking in water for 12 h. Once substrate had been drained of excess water, it was placed in permeable plastic bags and pasteurized at 95°C with steam for 1 h. Later, 10 g of straw were placed in petri dishes, and then each dish was individually inoculated with 0.5 g of one of the previously prepared spawn formulas. Control samples were inoculated with mycelial implants that had been cultured for 16 days in a water and agar medium (WA). Because implants in control samples lacked nutrient supplements, it was considered that shiitake strains might express their maximum growth capacity by directly feeding upon the elements present in the substrate. Five replications were prepared for each strain-formula combination, and then these were incubated in darkness at 26°C for 7 days. Mycelial growth was determined at the end of the incubation period by tracing the area occupied by the mycelium upon the petri dish of each sample. Total surface areas for each sample were calculated using a gauge for measuring foliar area (LI-COR Mod. 3100). To determine significant differences among samples, The data were submitted to analyses of variance and Tukey multiple range tests (α = 0.05).

Metabolic Activity

Fungal biomass associated with mycelial metabolic activity for the different spawn formulas was determined through fluorescein diacetate hydrolysis (FDA) on fresh spawn samples. To carry out this test, ten strains were chosen that had shown their greatest mycelial growth on wheat straw using the four previously mentioned formulas. These formulas were prepared according to the methods cited earlier. After formulas had been sterilized, they were placed in glass flasks (40 g per flask) and inoculated with two mycelial implants (0.8 mm diameter) taken from strains that had been pre-cultured for 10 days on MEA. These flasks were incubated at 26°C for 42 days. After 14 days, flasks were agitated to redistribute their contents uniformly, and then left undisturbed until the end of the experiment.

Metabolic activity was monitored in spawn samples of the four formulas, taking weekly samples of each strain. FDA hydrolysis was carried out according to the methods of Inbar *et al*. (1991) by placing 0.7 g of spawn from each formula in test tubes containing 10 ml of previously sterilized buffer solution [1M potassium phosphate (8.7 g K_2HPO_4 + 1.3 g KH_2PO_4 / l distilled H_2O) at pH 7.6]. To initiate the enzymatic reaction, 0.2 ml of FDA (400 µg) were added. For each treatment, three replicates and a control (without FDA) were prepared. Samples were incubated for 30 min at 30°C; manually agitating them for 10 sec every 5 min. To stop the reactions, 10 ml of acetone were added to each sample. Samples were filtered with a filter paper (Wathman No.1), and then the optical density of the recovered filtrate was determined with a spectrophotometer set at 490 nm and read against the standard curve for FDA. A unit of metabolic activity was defined as the equivalent of 1 µmol of hydrolyzed FDA min^{-1} g^{-1} (dry weight) for a given formula. Results were the averages of three replications.

RESULTS

Mycelial growth

Mycelial growth results for 15 strains of *L. edodes* are shown in Table 2. Greatest growth was obtained for samples inoculated in F3 and F2, whereas the control treatment (WA) showed the least growth. On average, growth obtained with samples of F3 was greater than, and significantly different from, the other treatments. Maximum growth was observed for F3 using strains IE 243 (47.6 cm^2) and IE 112 and IE 244 (the latter two each measuring 46.4 cm^2). Significant differences in growth within each of the treatments were found among the strains. However, in the WA treatment the strains were associated in smaller numbers of groups (Table 2).

To select the strains that would be studied in the next phase of work, growth values for each strain in all treatments were taken into consideration. On the basis of similarities obtained from the statistical analysis of the data, a value of 1 was assigned to strain-treatment combinations taken from the two groups showing greatest growth. The sum of these values was called the selection value (SV). There were differences in SV among many strains. Specifically, strains IE 124, IE 105 and IE 112 had an SV of 5 and IE 122 and IE 246 had values of 0 (Table 2). To determine metabolic activity, strains with SV between 3 and 5 were selected.

Table 2. Mycelial area (cm^2) for shiitake strains cultivated in wheat straw after seven days of incubation and using different spawn formulas.

	WA (control)	F1	F2	F3	F4	Selection value
IE 40	**5.2 abc ***	**35.8 fg**	**39.2 cde**	37.8 bc	**39.0 fg**	4
IE 105	**10.2cd**	**35.2 fg**	**44.2 e**	**39.8 bcd**	**37.2 defg**	5
IE 112	**6.4 abc**	**37.0 g**	**44.8 e**	**46.4 de**	**42.0 g**	5
IE 122	2.4 a	19.0 ab	20.6 a	36.6 b	22.4 b	0
IE 123	**7.0 abcd**	**31.0 defg**	**41.6 de**	37.6 bc	**37.6 efg**	4
IE 124	**12.8 d**	**33.4 efg**	**37.8 cde**	**42.4 bcde**	**37.2 defg**	5
IE 149	3.0 ab	27.2 cde	28.6 abc	**43.8 bcde**	30.2 cd	1
IE 171	**6.0 abc**	**30.0 def**	29.0 abc	**39.2 bcd**	**32.0 cdef**	4
IE 207	**6.8 abcd**	25.4 bcd	**32.0 bcd**	20.2 a	30.6 cde	2
IE 242	3.8 ab	22.6 bc	**39.0 cde**	**41.6 bcde**	31.6 cde	2
IE 243	**4.4 abc**	**31.2 efg**	**35.6 cde**	**47.6 e**	29.2 bc	4
IE 244	**4.0 abc**	26.2 cd	**31.0 abcd**	**46.4 de**	**39.4 g**	4
IE 245	**5.2 abc**	**33.8 efg**	**43.4 e**	13.8 a	9.0 a	3
IE 246	2.4 a	15.0 a	21.4 ab	13.4 a	14.6 a	0
IE 247	**9.2bcd**	**31.2defg**	29.8 abc	**44.4 cde**	**35.6 cdefg**	4
Mean	**5.9 A ****	**28.9 B**	**34.5 CD**	**36.7 D**	**31.2 BC**	

Results are averages from five replicates.

* Values in the data columns with equivalent small letters indicate no significant differences were found with the Tukey test ($\alpha = 0.05$).

** Equivalent capital letters for values in rows indicate that no significant differences were found with the Tukey test ($\alpha = 0.05$).

Shaded values were obtained from the group with greatest mycelial growth for each treatment.

Underlined values were obtained from the group with the second greatest mycelial growth for each treatment.

Metabolic activity

Metabolic activity for 10 strains studied for the four spawn formulas is seen in Figure 1. Mycelial growth in the formulas was generally dense. However, in F3 and F4 rapid colonization and apparently greater density were observed. In a general way and in spite of quantitative differences, strains displayed similar behavior and showed lower

activity during the first few weeks of incubation. Peak activity was observed after 35 days, with a descent noted after 42 days of incubation. Maximum values were obtained with strains IE 247 (188 U g^{-1}), IE 105 (128 U g^{-1}), and IE 123 (58 U g^{-1}) in F3, and with strains IE 123 (143 U g^{-1}) and IE 245 (77 U g^{-1}) in F4. Some strains had high values, but these were expressed in a delayed fashion. This was noted especially for strain IE 124 (77 U g^{-1}) in F3 and IE 112 (90 U g^{-1}) in F4. Results indicated greater metabolic activity for strains in F3 and F4. This might be associated with greater mycelial biomass and ultimately with spawn maturity.

DISCUSSION

An adequate spawn is one possessing a mycelium that is capable of rapid growth when invading a particular substrate. In the particular case of pasteurized substrates, it must also have a competitive advantage over other colonizers that might potentially utilize the same space and nutrients (Leatham and Griffin 1984). The utilization of an unconventional substrate in shiitake culturing implies the preparation of spawn that will develop adequately on that substrate (Donoghue *et al*. 1996). Providing nutritional supplements to the spawn has, as its principal objective, the production of a vigorous mycelium. It is also important to count on a large number of points from which the mycelium might invade and colonize the substrate. Both substrate composition and spawn composition play very important roles in reducing the incidence of antagonistic organisms and contaminants that might affect the shiitake mushroom (Mata *et al*. 1998, Ohmasa and Cheong 1999, Savoie *et al*. 2000). Results obtained in this study have allowed for the classification of strains according to their growth capacity on a wheat straw substrate; but even more, the use of different spawn formulas has demonstrated that the strains under investigation have differential adaptive possibilities on this substrate. The growth test based on agar implants (WA) proved to be a useful tool for determining growth potentials among strains. In this case, the mycelial implant lacked nutrients to a degree that growth on this substrate was the product of the capacity of each given strain to immediately utilize the nutrients that were present. Eleven strains showed their greatest growth on the WA treatment; and of these, ten also showed their maximum selection values (SV) with this treatment.

On the other hand, results for metabolic activity demonstrated that the supplements used in F3 and F4 increased this activity in the strains after about 35 days of incubation. Reductions in levels of hydrolyzed FDA after 42 days might have been associated with changes in metabolism that would permit the mycelium to prepare for fructification. This phenomenon has been observed in the enzymatic profiles of laccase and peroxidase

manganese dependent, which are produced by certain shiitake strains (Mata and Savoie 1998). Although adequate mycelial growth is not sufficient for obtaining acceptable biological efficiencies, it is fundamental in ensuring rapid initial mycelial growth on substrates. Although there are many factors that affect the initiation of mycelial growth from the spawn, temperature and incubation time are critical (Terashita *et al.* 1997). The results of this study suggest that incubating in F3 for 35 days might yield a spawn with high metabolic activity, and thus permit vigorous initiation of growth on the wheat straw substrate. However, other experiments must still be undertaken in order to confirm the production capacity of the strains studied in this investigation. This additional work might begin with the strains IE 247, IE 105, IE 123, IE 124, IE 112 and IE 245, all of which showed good growth and high metabolic activity on wheat straw.

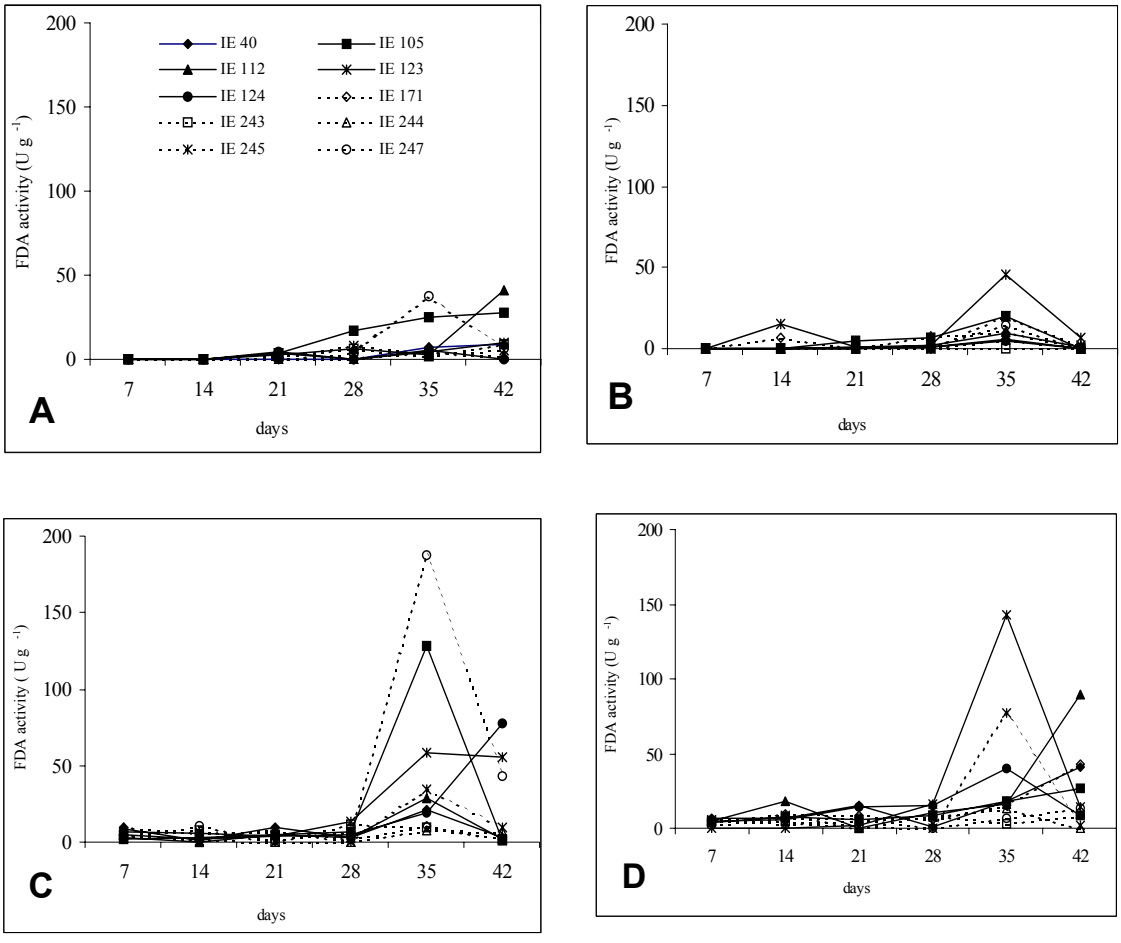

Figure 1. Metabolic activity for shiitake strains (U g^{-1}) in four spawn formulas after 42 days of incubation. A: metabolic activity in F1, B: metabolic activity in F2, C: metabolic activity in F3, D: metabolic activity in F4.

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