

MONITORING OF MYCELIAL GROWTH AND FRUCTIFICATION OF *LENTINULA EDODES* ON SEVERAL AGRICULTURAL RESIDUES

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ABSTRACT

Four lignocellulosic substrates (oak-wood sawdust (OS), wheat straw (WS), corn-cobs (CC) and cotton wastes (CW), were comparatively evaluated for the cultivation of the shiitake mushroom Lentinula edodes. All five commercial strains (AMRL 118, 119, 120, 121, and 122), examined in 'race-tubes' experiments, demonstrated significantly higher mycelium extension rates on OS and WS than on CC and CW. Strains 121, 118 and 122 proved to be the fastest overall colonizers, but 120 performed notably better on CC and OS. Measurements of pH and electrical conductivity throughout the entire colonization process of substrates with strains 121 and 118 revealed a steady decrease of pH; electrical conductivity values increased until mycelium colonized 60 to 75% of the substrate, and then it slightly declined or remained constant until the end of incubation. Significant differences in electrical conductivity measurements were detected among fully colonized substrates: OS and CC showed the lowest values (0.79-0.93 mS/cm), whereas CW presented almost double salt content. Subjecting tubes fully colonized by strain 118 to cold-shock treatment resulted in fruiting 58-65 days after inoculation in all media tested; WS and CC substrates promoted earlier sporophore initiation than OS, while CW appeared to be the worst performing substrate. Monitoring of CO₂ concentrations in pilot-scale cultivation of strain 118 on synthetic



blocks, revealed higher respiration rates on OS and CC than on WS (peaks on 17^{th} , 20^{th} and 24^{th} day respectively), which are further correlated with substrate colonization rates. Results were evaluated for potential use of OS in combination with WS and CC as substrates for *L. edodes* cultivation.

INTRODUCTION

Lentinula. edodes (Berk.) Pegler, commonly known as the shiitake mushroom, is one of the most widely grown species of mushrooms and a very efficient biodegrader of wood. Although the mushroom is traditionally grown on wood logs (Harris 1986), this procedure has been largely replaced by artificial log cultivation that utilizes heat-treated supplemented substrates enclosed in plastic bags. The shorter time required to complete a crop cycle and the higher yields, resulting in consistent market supply, are the main advantages of this procedure (Miller and Jong 1986, Przybylowicz and Donoghue 1990).

Substrate formulation, strain genotype and length of the incubation period have already been identified as important variables in the efficient production of shiitake on synthetic substrates used in bag-log cultivation (Royse and Bahler 1986, Zadrazil 1973, Kalberer 1995). Worldwide, the most popular basal ingredient used in synthetic formulations of substrate for the commercial production of *L. edodes* is hardwood (preferably oakwood) sawdust, supplemented at a rate of 20-30% d.w. by wheat or rice bran, millet etc. (Miller and Jong 1986, Royse 1996, Pire *et al.* 2001). Wheat straw and other agricultural by-products resulting from processing maize, cotton, sunflower, flax, grape, coffee, cacao etc. have been examined as alternative substrates for their cultivation (Przybylowicz and Donoghue 1990, Levanon *et al.* 1993, Bis'ko and Bilay 1996, Philippoussis *et al.* 2001b).

Earlier works reported that mycelial growth, earliness, crop quality and productivity vary among strains on sawdust-based substrates (Diehle and Royse 1986, Royse and Bahler 1986). Furthermore, the close relationship between the rate of mycelial development during substrate colonization, CO₂ production and crop yield and the role of sufficient aeration have been demonstrated in sawdust based experiments (Leatham and Stahmann 1987, Kalberer, 1995, Donogue and Deninson 1995). Nevertheless, little is known concerning the performance of selected strains on different substrates composed of various lignocellulosic residues, especially regarding mycelial growth and the parameters affecting it.



Understanding the impact of substrate on mycelial growth and shiitake yield will be invaluable for the effective bioconversion of locally available agricultural wastes and for mushroom crop diversification in Mediterranean countries, where the respective mushroom market is largely dominated by *Agaricus* spp and *Pleurotus* species (Philippoussis and Zervakis 2000a,b, Zervakis and Venturella 2000). Our earlier works (Philippoussis *et al.* 2000, 2001a, Zervakis *et al.* 2001), reported on valorization of several low-value agro-industrial wastes through the cultivation of exotic mushrooms. In an attempt to introduce *L. edodes* cultivation into our region with no prior history of production of such mushroom species, experimental work is currently directed towards exploitation of locally available lignocellulosic residues as substrate components. The present study examines the influence of WS, CC and CW (in comparison to standard OS substrate) on the growth of mycelium and respiration rates of five *L. edodes* strains. The results of cultivation experiments are interpreted and discussed to determine whether the rate of colonization by the mycelium can be related to mushroom yield in different substrates.

MATERIALS AND METHODS

Strains, culture media and spawn

Five commercial strains of *L. edodes* (Berk.) Pegler deposited at the fungal culture collection of Edible Fungi Laboratory of Athens of NAGREF (National Agricultural Research Foundation) with the accession numbers AMRL 118, 119, 120, 121 and 122 were used in this study. The culture media used for routine culture and storage purposes were complete yeast medium (CYM) and potato dextrose agar (PDA, Difco). Grain spawn of individual species was prepared in 500 ml Erlenmayer flasks filled with 200 g of boiled millet grains (*Panicum miliaceum* L.) supplemented with 3% calcium carbonate and 1.5% gypsum (w/w, in terms of dry weight). The ingredients of each flask were thoroughly mixed, autoclaved for 1.5 h at 121 °C, allowed to cool for 24 h and inoculated with 5 agar plugs cut from a growing colony in a PDA petri dish. Flasks were incubated at 25 °C for 3 weeks, in the dark, with periodical shaking to prevent mycelial knitting of the grain.

Substrate preparation and analysis

Four mushroom cultivation substrates were prepared as follows: 60% of the total weight consisted of oak-wood sawdust (OS), or wheat straw (WS), or corn-cobs (CC), or cotton wastes (CW) which were used as the basal ingredient, plus ca. 15% wheat straw to

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regulate the substrate porosity level at 35-45% (Philippoussis *et al.* 2001b). Then, substrates were left to soak in water for 12-24h, and after the surplus water had been drained off, they were supplemented with 10% millet, 10% wheat bran, 4% soybean flour and 1% calcium carbonate (all ingredients based on oven dry substrate weight).

For substrate analysis, samples were dried to a constant weight in a 60 °C oven, milled and sieved. The moisture content, porosity, pH, carbon/nitrogen (C/N) ratios and electric conductivity of each substrate were determined. The carbon content was estimated from the ash content of the substrate and the nitrogen content was determined by the Kjehldal method. The moisture content of the sterilized substrates was measured to be 65-70% and porosity 35-50%, pH 5.0-6.5, Ec 0.6-0.7 (measured in the substrate extracts) and the C/N ratio in the range of 25-50/1. Ten replicates per substrate and strain were used. For bag-log cultivation, polypropylene-autoclavable bags were filled with 2 kg of substrate, closed with a cotton plug and sterilized twice for 1 h at 121 °C. After cooling, inoculation was carried out by pouring about 60 g of grain spawn (3% w/w on fresh weight basis) along the central vertical axis of the block.

Colonisation studies, mycelial growth rate determination and sporophore induction in tubes

Linear growth rate tests were performed in glass 'race' tubes (200×30 mm) uniformly filled to 100 ml volume with the substrates as previously described (Philippoussis et al. 2001a) and sterilised for 1.5 h at 121 °C. Ten replicates per strain and substrate were inoculated with grain spawn at a rate of 2% (w/w) and incubated at 25 °C in the dark. Fungal growth was recorded daily by measuring the visible penetration of mycelia into the substrate in two perpendicular directions and the extension rate K_r (mm/day) was calculated after the mycelium front had reached more than 30 mm. At 0, 50, 75 and 100% colonisation, (expressed as height of the colonised substrate column in proportion to the total height of the substrate column), three replicate tubes per substrate and strain were analysed (dry matter, substrate extract) pH and electrical conductivity (Ec) values were recorded. Associations between extension rate and Ec levels of different substrates were determined by correlation analyses. Finally, to induce sporophore primordia, a set of three replicate tubes from each of the substrates, fully colonized by strain 118, were subjected to a low temperature shock at 5 °C for three days, later were grown in a 24h/ day illuminated fruiting room (200-300 lux) at 17.5 ±1 °C and the number of fruit bodies produced was scored.

Fungal respiration during substrate colonization in bag-log cultivation



The influence of OS, WS and CC lignocellulosic residues on fungal growth, respiration and substrate properties during spawn run was examined using ten replicate bags per substrate inoculated with strain 118. Colonisation of the substrates took place in growth chambers, at 25±1 °C. Twelve days after spawning 140 holes were punched in the headspace of the bags, in two opposite directions above the substrate, with a set of needles 1.1 mm in diameter. From the 13th to the 35th day of incubation and at intervals of 3-4 days, the percentage of substrate colonization and the CO₂ and O₂ concentrations were monitored by gas chromatography (Perkin Elmer 8700) in the headspace of four bags from each substrate. Gas samples were taken by a 1 ml syringe (Hamilton Co.) through a 15 mm thick silicone patch glued to the bag that served as a septum to prevent gas leakage during sampling. During colonization, progress of the mycelium through the medium was recorded, along with indications of fungal development, such as browning, lumping and development of primordia in the bag. After complete colonization of the substrate, polypropylene bags were removed and three blocks per treatment were weighed and substrate extracts analyzed to determine changes in moisture content, dry weight, pH and electric conductivity.

Experimental design and data analysis

In all experiments a completely randomized design with 6-10 replicates per treatment was followed. Variance analysis was performed using the Least Significant Difference (LSD) test at 5 % level of probability to compare mean values.

RESULTS

Mycelium growth kinetics on different media in race tubes

In order to determine the air-porous space formulation of each individual substrate that supports fast growth of *L. edodes* mycelia, various formulations of OS, WS, CC and CW giving different porosity levels in the range of 30–50% were prepared and colonization rate of strain 121 was assessed in 'race-tubes'. Although in OS and WS faster colonization was recorded at 33% and 47% porosity levels the effect of substrate texture on mycelium extension rate was not remarkable. This effect was more pronounced on CC and CW where significantly better growth results were obtained in 35% and 39% porosity levels respectively (Figure 1) and therefore they were used in all subsequent experiments.





Figure 1: The influence of substrate type and porosity level on colonization rate of *Lentinula edodes* strain 121.

Mycelial growth rates (mean of four replicates) in two selected porosity levels of each media are presented

In a following experiment, the mycelium growth characteristics (lag time after inoculation, colonization period, extension rate) of five *L. edodes* strains were comparatively evaluated in 'race-tubes' filled with OS, WS, CC and CW. Strain 121 followed by 122 presented low lag time values t (r=30 mm) and proved to be the fastest colonizers among all strains tested. The former exhibited growth rates in the range of 4.92 – 3.38 mm/day and needed only 30, 37, 46 and 52 days to colonize OS, WS, CC and CW respectively, while for the latter the respective colonization period was 34, 38, 49 and 55 days. Strains 118 and 119 presented inferior growth rates, while 120 proved to be the slowest strain in all media. Among different substrates, OS and WS supported faster colonization of all *L. edodes* strains examined (Figure 2). In general, CC and CW presented inferior growth results. Particularly CW proved to be unsuitable for fast growing of *L. edodes* mycelia, as all strains needed 3 weeks more to colonize this substrate than OS.





Figure 2: Linear extension rates of *Lentinula edodes* strains on different lignocellulosic substrates



Figures 3 a, b: pH and Ec values measured on OS, WS, CC and CW substrates during their incubation with *Lentinula edodes* strain 118 (a) and strain 121 (b).Black marks are for pH, white marks are for Ec values.

Measurements of pH and electrical conductivity of different substrates throughout the colonization process by strains 118 and 121 revealed similar results for both strains (Figure 3a,b). A steady decrease of pH in all media tested was observed; the initial pH values, ranging 6.37 - 7.31, fell to 4.49 - 5.01 at the end of incubation. In contrast, the Ec values increased until mycelium colonized 60 to 75% of the substrate, and then they slightly declined or remained constant until the end of incubation. Significant differences in Ec measurements were detected among fully colonized substrates: OS showed the lowest values (0.79 and 0.82 mS/cm in strains 121 and 181 respectively), whereas CW presented almost double salt content (1,54 mS/cm) in both strains (Figure



3 a,b). Moreover, mycelial growth rate was found significantly and negatively correlated (P=0.05) with salinity levels of different substrates at 50% ($R^2 = 0.7645$) and 100% ($R^2 = 0.7235$) of the substrate column colonization.



Figure 4. Lentinula edodes fructification in glass tubes in the laboratory

Sporophore primordia appeared 4-5 days after the cold-shock treatment, first in tubes containing WS and CC, in 58 and 62 days respectively after inoculation (Figure 4). Although OS presented fast

Days after	CO ₂ (%)		
	OS	WS	CC
12	0.5200	1 7225	0.9242
15	0.5300	1.7325	0.8242
1/	4.2/48	2.7309	3.3/92
20	2.5645	3.3404	4.4588
24	3.8796	3.8495	3.8423
27	2.0770	2.0614	1.8963
32	3.3884	3.7208	3.2767

Table 1. Levels of CO₂ (%) in the perforated headspace of bags during colonization of OS, WS and CC substrates by *Lentinula edodes* strain 118.

colonisation, basidiomata formation achieved in the 65th day while for CW fructification process started considerably later, 75 days after inoculation of the substrate. As far as primordia and fruit bodies number is concerned, WS appeared to yield better than OS and CC (5, 4 and 3 fruit bodies respectively), while CW was the least efficient producing only one basidiocarp initial.



Fungal respiration on different substrates in bag-logs

The CO_2 concentration courses in the perforated bag headspace during the 20 days of OS, WS and CC colonisation by L. edodes strain 118 are illustrated in Table 1. The type of substrate influenced CO_2 level and the timing that peak values were observed, especially in the first three weeks of spawn-run. In OS, a rapid increase of CO_2 occurred until the 17th day of incubation, where gas concentration was maximum, then declined sharply and presented a smaller peak after a week. These peaks corresponded approximately to 30% and 70% of full bag-log colonisation. However, CC and WS presented different time courses of the gas concentration as in the former CO_2 level peaked on 20th day (40% of substrate colonisation), while the latter presented the lowest CO_2 production rate and a smaller peak on day 24 (45% of substrate colonisation). Hereafter, CO_2 curvesof all substrates run identically, declining until 27th day, presented a third peak of decreased height on day 32 (85-95% of substrate colonisation) and declining slowly until the end of incubation.

DISCUSSION

The expansion of the cultivation of wood inhabiting edible and medicinal mushrooms such as shiitake is based on good crop yield produced in reduced time through utilization of locally available rural and agro-industrial residues. As the length of the incubation period relates to both the duration cycle and the reduction of potential contamination risks, it is of prime importance to minimize the duration of the colonization phase.

Nevertheless, little has been published regarding measurements of *L. edodes* extension rates during colonization of lignocellulosic substrates. Our growth assays, conducted to assess the effects of OS, WS, CC and CW lignocellulosic substrates on *L. edodes* colonization rates, confirm the fact that both substrate formula and the stain used affect mycelial growth rate and therefore the incubation and crop cycle duration (Przybylowicz and Donoghue 1990, Zadrazil 1993). Previous studies have demonstrated the influence of substrate wood-chip particle size on shiitake yield (Donoghue and Denison 1996, Royse and Sanchez-Vazquez 2000, 2001); in this study we recorded different growth responses when the same strain 121 colonized various substrates or porosity formulations of the same substrate.



Actually, OS and WS supported faster mycelium growth of all tested strains. The overall growth rate of all the strains on OS and WS was similar. In contrast, CC substrate did not perform adequately; the lower (relatively to the other media used) N content of CC (0.96 %) could be held responsible for the depression observed in colonization rates. Previous reports assess that nitrogen could be a growth-limiting factor in shiitake cultivation (Adno 1974, Boyle 1998, Kalberer 2000). Moreover, enhancement of ligninolytic activity in nitrogen enriched media has been indicated by Kaal et al. (1995). On the other hand, the low growth rate recorded in CW could be explained by the high moisture content of this substrate (72.5%), which exercised a negative effect on mycelial growth. Han et al. (1981) reported that L. edodes colonization rate declined significantly when the water content of the substrate increased over 60%. In addition, a linear negative correlation between moisture content and growth rate was found by Badham (1989). This could be partly attributed to the suppression of the oxidative nature of lignin degradation by deficient gaseous exchange and O₂ unavailability (Burla et al. 1992). Furthermore, results obtained in this investigation showed that there is a negative relation between mycelial growth rate and substrate salinity, supporting previous findings demonstrating that L. edodes mycelium is sensitive to salt-induced water unavailability (Badham 1989). Our results also indicate increase of the acidity in all substrates during incubation; low pH value of the substrate is probably a prerequisite for primodia formation (Kalberer 1995).

There are several works reporting successful fruiting of *L. edodes* on artificial media and amended hardwood sawdust in the laboratory approximately 3-4 months after substrate inoculation (Adno 1974, Tan and Moore 1992, Pacumbaba and Pacumbaba 1999). However, our fruiting process conducted in glass tubes, furnished a remarkable reduction in the time necessary for the first fructification (2 months for OS, WS and CC and 2,5 months for CW substrates). Furthermore, the obtained sporophore yields make it possible to estimate the production potential of individual substrates. This, along with the use of growth rate measurement for valorization of the substrate incubation efficacy, renders the 'race-tube' method a dependable technique for screening-selecting purposes.

Regarding cultivation in the bags, the CO_2 level reported here is lower (because of the perforation of the bag headspace) than the one recorded by Donoghue and Denison (1995) when standard 12 cm² filter patches were used. However it is consistent with the level registered by the same authors in bags with double size filter. In general, the results reported here are in agreement with the CO_2 profile obtained previously with supplemented hardwood sawdust as substrate (Kalberer 1995). Yet our results broaden the data concerning CO_2 production during incubation of different substrates and



indicate that the type of substrate affected both the CO_2 level and steepness of the gas peak in the bag headspace, since the CO_2 concentration peaked on 17^{th} , 20^{th} and 24^{th} day after inoculation in OS, CC and WS respectively. These studies support the potential of WS and CC for use as alternative substrates for *L. edodes* cultivation, alone or in mixtures with OS.

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