

Edible Mushrooms from Olive Oil Mill Wastes

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The biological remediation of olive oil mill wastes has been attempted several times in the past through the use of different types of microbes. Among them, a relatively large array of fungi were studied for neutralizing the heavy pollutant effects and/or for converting these wastes into new value-added products. The present investigation was aiming at examining whether olive oil mill wastes could be exploited for the cultivation of mushrooms of the genus *Pleurotus*. At a preliminary stage, two *Pleurotus* species, i.e. *P. eryngii* and *P. pulmonarius*, were tested for their ability to colonize an olive press-cake (OPC) substrate supplemented with various dilutions of raw olive mill wastewater (OWW). Some important cultural characters related to mushroom production (earliness, yield, biological efficiencies and quality of basidiomata) were estimated. The outcome revealed different cultural responses for each *Pleurotus* species examined; the *P. pulmonarius* strain showed better earliness values and *P. eryngii*, although it was a slow growing fungus, produced basidiomata in high yields and of a very good quality. On the other hand, the OPC substrate supplemented with low concentrations of OWW (12.5% v/w) behaved satisfactorily as regards the fungal colonization rates and mushroom yield, but when the addition of higher rates of raw, untreated OWW (75–100% v/w) was attempted then the *Pleurotus* strains were completely unable to grow. The optimal concentration of OWW for *Pleurotus* mycelial growth was assessed through measurements of the biomass produced in liquid nutrient media and was found to lie within the 25–50% range, depending on the *Pleurotus* species and on the properties of the substrates examined. Furthermore, the phytotoxic effects that the spent liquid medium possessed were examined in comparison with the phytotoxicity of the raw liquid waste. The prospects of exploiting olive oil mills wastes for mushroom cultivation is discussed. © 1997 Elsevier Science Limited. All rights reserved

INTRODUCTION

Olive cultivation maintains an essential position in the economy, ecology and social life of the Mediterranean countries, where almost 98% of the world olive tree growing is concentrated. Because of the size of this agricultural activity in the Mediterranean Basin, the environmental problems associated with the olive oil extraction process have a crucial importance. Commonly, olive mills produce residual solids, the olive press-cake or OPC which make up a fibrous lignocellulosic waste generally used as fuel; and a black liquid effluent, the olive oil wastewater or OWW, which is a heavy pollutant mainly due to its high phenolic content and organic load. Thus, remediation of OWW presents severe difficulties

because phenolics and certain aromatic compounds are very phytotoxic and are held responsible for the strong antimicrobial properties and the recalcitrant black colour of the waste (Pérez *et al.*, 1986; Paredes *et al.*, 1986; Rodriguez *et al.*, 1988).

In the past, several investigations were carried out to select microorganisms (notably fungi) capable of eliminating the inhibitory effects of those substances and of converting the initial waste either directly into a useful end-product or by making it susceptible to further physiochemical and biological treatments (Fiestas Ros de Ursinos, 1961; Ercoli & Ertola, 1983; Hamdi *et al.*, 1991; Martinez-Nieto *et al.*, 1992). Among Basidiomycotina in particular, white-rot fungi belonging to the genus *Pleurotus* (Fr.) Kummer are selective decomposers of lignin and are among the most efficient producers of lignocellulosic enzymes (Ander & Eriksson, 1978; Platt *et al.*, 1983). In addition, they can be cultivated

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on a large array of substrates transforming them into human and/or animal food. Previous works reported the use of *Pleurotus* strains for the production of biomass (=mycelium) and basidiomata (=mushrooms) on OWW, by achieving in parallel a significant decrease in the phenolics concentration (Sanjust *et al.*, 1991; Tomati *et al.*, 1991).

The present work describes the preliminary results of a study on the biodegradation of olive oil extraction by-products through the use of selected mushroom fungi. OPC (untreated or supplemented with various concentrations of raw OWW) was tested for its suitability to serve as a substrate for the production of *Pleurotus* basidiomata. In addition, *Pleurotus* was cultured in liquid media with OWW to assess the levels of biomass production and the extent of elimination of the waste phytotoxicity. Future plans are discussed in conjunction with the results obtained herein and by taking into consideration other related works from the literature.

MATERIAL AND METHODS

Organisms

In the experiments performed, two *Pleurotus* dikaryons were used: *P. pulmonarius* LGAM P46 (isolated from central Greece, on *Fagus sylvatica*) and *P. eryngii* LGAM P63 (isolated from Crete, on *Eryngium* sp.). All strains are deposited in the Fungal Culture Collection of the Laboratory of General and Agricultural Microbiology, Agricultural University of Athens; and they are maintained in complete yeast medium slants at 4°C.

Culture media and conditions

Raw OWW and OPC, which originated from a three extraction phases/continuous olive oil mill plant (Pylos-Messinia, Greece), were stored at -20°C and used in the experiments exactly as they were supplied from the plant, unless otherwise stated. Accordingly, all OWW dilutions were performed in water, unless otherwise stated.

For the production of *Pleurotus* basidiomata, four OPC-based substrates were examined and the procedure adopted for their preparation was as follows. OPC (max. particle size: 5mm) was left to soak into: (i) plain tap water; (ii) 12.5% OWW; (iii) 25% OWW; and (iv) 50% OWW. After 24h, the surplus water was drained off and the

individual substrates were mixed with calcium carbonate (0.6% d.w.). Plastic cylindrical beakers (*ca* capacity: 1l) were then filled, sealed and sterilized twice (121°C/1.1atm, for 2h). The relative humidity of the sterilized substrate was measured to be around 50%, while the pH values ranged from 5.2 to 6.0 (depending on the OWW content). Five replicates for each particular substrate per strain were prepared.

Inoculation of each substrate followed by means of placing agar plugs with mycelium of *P. pulmonarius* and *P. eryngii* on the surface of the medium. The beakers were then incubated at 23°C in the dark. As soon as the substrates were densely colonized by the *Pleurotus* hyphae, the environmental conditions (temperature, relative humidity, aeration and light intensity) were altered to induce fruiting and were maintained at the appropriate levels during the entire length of the production cycle (Zervakis & Balis, 1992). The harvesting period lasted for three production flushes and fruitbodies were collected before they started to curl up.

Liquid cultures were carried out in 250ml Erlenmeyer flasks containing 100ml of various raw OWW dilutions (100%, 50%, 25% and 12.5%). Inoculation with the two *Pleurotus* species was performed with two mycelium agar plugs (6mm dia) for each flask. Cultures were then incubated at 23°C in the dark and were shaken twice daily. After a period of 4 weeks, the biomass was harvested on a Whatman filter paper by vacuum filtration; was dried in an oven at 80°C for 24h and weighted.

Cultural characters

In order to evaluate and compare the cultural performance of the *Pleurotus* strains, a number of characters were selected: earliness, yield and biological efficiency (BE). Earliness was defined as the time elapsed between the day of inoculation and the day of the first appearance of the basidiomata. After harvest was over, the mushrooms were weighted and the BE% (percentage ratio of the fresh weight of harvested mushrooms over the dry weight of the substrate) was calculated.

Phytotoxicity

The phytotoxicity of OWW serving as a culture medium for the two *Pleurotus* species was assessed

by measuring the germination rates (germinability) of the seeds of *Lattuga romana* plants. Twenty-five seeds per treatment were placed for germination into a Petri dish containing a pack of five filter papers (Whatman No. 1) which were previously soaked with 5ml of the spent OWW substrate to be examined (25%, 12.5% and 6.25% of OWW). Measurements were taken after 3 days and each assay was performed in triplicate.

Mycelium growth rates

Pleurotus linear growth rates were calculated on media composed of OWW solidified with agar. Mycelial growth took place in 90mm Petri dishes, containing 15ml of the OWW-Agar to be tested (100%, 75%, 50% and 25% of OWW). Inocula (4mm dia) taken from the periphery of 5-day-old colonies, were placed in the centre of the dishes and measurements (increase in mean colony diameter along two lines perpendicular to each other) were then taken every 24h over a period of 10 days at 23°C.

RESULTS

Four different OPC-based media (with plain water and supplemented with 12.5%, 25% or 50% OWW) were examined for their potential to serve as substrates for the cultivation of *P. eryngii* and *P. pulmonarius*.

The *P. pulmonarius* strain cultivated on both plain OPC and on OPC with 12.5% OWW was the first one to respond to environmental stimuli and started forming basidiomata after the 45th day. On the other hand, fungi grown on OPC with 25% OWW presented a different fructification cycle and higher yields (in comparison to the former two treatments), despite the delay in the appearance of basidiomata initials to 55 days from inoculation. In general, the total yield of *P. pulmonarius* mushrooms was 39g, 32g and 58g, while the BEs were 10%, 9% and 14% for plain OPC, OPC with 12.5% OWW and OPC with 25% OWW, respectively (Table 1).

The strain of *P. eryngii* was a late fruiter and formed basidiomata after 75 days, when *P. pulmonarius* had already completed its production. However, the yields and quality of *P. eryngii* mushrooms were significantly higher than those reported for *P. pulmonarius*, although the former failed to fructify when cultivated on OPC with

Table 1. Cultural Characters (Earliness, Total Mushroom Yield and BEs) Related with Mushroom Production of *P. pulmonarius* and *P. eryngii* Cultivated in Plain OPC or Supplemented with OWW Substrates

Substrate ^a	Species				
	<i>P. pulmonarius</i>			<i>P. eryngii</i>	
	A	B	C ^b	A	B
Earliness ^c	45 (75)	45 (75)	55 (80)	75 (115)	80 (120)
Yield	39±9	32±5	58±9	181±34	136±51
BE%	10	9	14	46	36

^aA, plain OPC; B, OPC supplemented with 12.5% OWW; C, OPC supplemented with 25% OWW.

^bValues were obtained from only two out of five replicates.

^cThe first value indicates the number of days between inoculation and first mushroom appearance, while the value in parentheses indicates the time period within which three production flushes were completed and harvested.

25% OWW. The distribution of mushroom quantities harvested in each one of these flushes was quite uneven and dissimilar for the two substrates examined. The total mushroom yield of *P. eryngii* reached 181g and 136g, while the BEs were 46% and 36% for plain OPC and OPC with 12.5% OWW, respectively (Table 1).

Liquid cultures of *Pleurotus* species were carried out in various concentrations of raw (12.5%, 25%, 50% or 100%) OWW. The *P. eryngii* strain performed better than *P. pulmonarius* in all OWW concentrations tested, except the 100% raw OWW medium in which minimal growth was obtained for both species (Table 2). The 25% OWW dilution produced the higher mycelium yields for all *Pleurotus* strains, whereas the 50% OWW concentration supported a very poor hyphal growth. In general, differences in biomass quantities became more evident among the two species in higher concentrations of OWW. The pH of the liquid media did not show any significant fluctuations before and after fungal culturing or among the individual experimental treatments.

The phytotoxic properties of the spent OWW

Table 2. Dry Weight (mg) of the Mycelium of Two *Pleurotus* Species after 4 weeks Growth in Liquid Cultures Composed of Different OWW Concentrations

OWW Concentration (%)	<i>P. eryngii</i> (pH: 5.2/5.3) ^a	<i>P. pulmonarius</i> (pH: 5.2/5.3)
12.5	100±10	60±10
25	150±10	70±20
50	80±10	20±10
100	No growth	No growth

^apH values of the liquid substrate before and after *Pleurotus* culturing.

substrate originating from *Pleurotus* liquid cultures were tested by measuring the germinability of plant seeds. At an initial stage, different concentrations of the raw OWW medium were assayed (6.25%, 12.5% and 25%). The germinability stayed at very high levels in all spent OWW dilutions ($>83\%$), and this was particularly obvious in the 25% concentration for which the germination rate of the control decreased sharply and almost halved the value attained in 12.5% (Table 3). Both *P. pulmonarius* and *P. eryngii* had superior germination rates in the 6.25% and 12.5% dilutions, but the rates decreased as the OWW concentration was increased. It is noteworthy that the substrate originating from *P. pulmonarius* cultures performed less well in all dilutions examined.

The mycelium growth rates of *Pleurotus* species were assessed on solidified nutrient media containing raw OWW at the following concentrations: 25%, 50%, 75% and 100% (Table 4). The strain of *P. pulmonarius* presented the faster growth rates in all dilutions but at the same time showed longer lag phases especially at 75% and 100%. The gradients for the 25% and 100% dilutions were very high (6.8 and 6.9 mm day⁻¹, respectively), while the lowest value noted was for the 75% dilution (5.0 mm day⁻¹). Accordingly, *P. eryngii* performed better in the 25% and worst in the 100% OWW concentration as far as growth rates were concerned (4.7 and 3.4 mm day⁻¹, respectively), whereas lag phases were almost identical among the different OWW dilutions examined.

Table 3. Germination Rates (Expressed as Percentages of the Respective Values on Water) of *Lattuga romana* Seeds when Germinated for 3 days on Spent Liquid *Pleurotus* Substrates Composed of Different Raw OWW Concentrations

Concentration (%)	OWW	<i>P. eryngii</i>	<i>P. pulmonarius</i>
Water	100		
6.25	95.9	100	100
12.5	94.6	97.2	97.3
25	46.6	83.6	86.3

Table 4. Linear Growth Rates (mm day⁻¹) of *Pleurotus eryngii* and *P. pulmonarius*, Colonies on Solid Nutrient Media Containing Different OWW Concentrations

OWW concentration (%)	<i>P. eryngii</i>	<i>P. pulmonarius</i>
Control	4.4	8.9
25	4.7	6.8
50	4.3	5.9
75	3.5	5.0
100	3.4	6.9

DISCUSSION

The present study aimed at examining to what extent and how efficiently could olive oil mill wastes be transformed into a substrate suitable for the production of fungal biomass. Two *Pleurotus* species were employed to convert OPC supplemented with raw OWW into mushrooms and to remediate the phytotoxic effects of OWW through the growth of mycelial colonies.

This work demonstrated the suitability of the OPC alone or supplemented with low dilutions of OWW as a promising substrate for the cultivation of certain *Pleurotus* species. Hence, mycelial colonization of untreated OPC proved to be a fast process being completed within 1–2 months, depending on the species examined. This time period would have been even shorter if inoculation was performed with spawn in the appropriate dosage instead of placing just three agar-plugs of fungal mycelium on the substrate surface. Nevertheless, the earliness values recorded here (after 45 and 75 days for *P. pulmonarius* and *P. eryngii*, respectively, in the plain OPC medium) were comparable to those quoted from other *Pleurotus* strains already integrated in established mushroom production schemes or from similar experimental trials on conventional mushroom substrates (Imbernon *et al.*, 1983; Zervakis & Balis, 1992). As regards OPC supplemented with OWW, a delay of 3–8 days in the appearance of mushrooms (and consecutively at the completion of the three flushes cycle) was monitored, which was related to OWW concentration (greater dilutions resulted in longer cultivation periods). A similar effect was noted for yield and BEs: plain OPC substrate presented higher values for both parameters and *Pleurotus* species examined than OPC supplemented with 12.5% OWW. Previous cultivation studies on *P. pulmonarius*, growing on various agroindustrial wastes (corn cobs, supplemented wheat and paddy straw), gave BE values ranging from 63 to 125% (Bano & Rajarathnam, 1982; Imbernon *et al.*, 1983; Royse *et al.*, 1991; Zervakis & Balis, 1992). In another work, two different substrates (supplemented wheat straw and corn cobs) were evaluated through the use of the same *P. eryngii* strain and the BE results were slightly superior in all experimental treatments: 68–87% against 36–46% on OPC (Zervakis & Balis, 1992). The OPC substrate produced *Pleurotus* mushrooms of very good organoleptic qualities (shape, form, colour,

flavour and taste). In addition, it has previously been demonstrated that no accumulation of phenolics occurs on OWW-grown fruitbodies, whilst their protein content is higher than in *Pleurotus* mushrooms sold in markets (Sanjust *et al.*, 1991).

This is the first report on the ability of the OPC to serve for the production of mushrooms of any kind. Despite its present low economic significance, its future looks promising if more effort is focused into the adoption of suitable cultivation techniques, selection of the appropriate *Pleurotus* strains and the improvement of the OPC properties to support more efficiently mycelium and basidiomata development.

Only lately have there been reports on the treatment of OWW with edible mushrooms, notably of the genera *Lentinula* and *Pleurotus* (Grappelli *et al.*, 1991; Tomati *et al.*, 1991; Sanjust *et al.*, 1991). In previous works, higher fungi were rather scarcely used and only for decolourization purposes (Saiz-Jimenez & Comez-Alarcon, 1986; Pérez *et al.*, 1987; Sayadi & Ellouz, 1993); demonstrating that the lignin degrading system of these organisms, and notably of *Phanerochaete chrysosporium*, was involved in the colour removal of OWW (Sayadi & Ellouz, 1992).

The *Pleurotus* biomass produced in liquid cultures differed among the species examined; *P. eryngii* gave higher mycelium yields in all OWW concentrations, and the 25% dilution of OWW presented the heavier biomass. These data provide evidence for the mode of enzyme function in this particular substrate. The decomposition of xenobiotic compounds has been shown to be dependent on the lignin-degrading system of white-rot fungi (Bumpus *et al.*, 1985; Bumpus & Aust, 1987) and laccase induction is primarily regulated by lignin concentration or by its transformation products, phenolics belonging to these categories (Keyser *et al.*, 1978; Ander *et al.*, 1983; Faison & Kirk, 1985). Due to the presence of such inducer-compounds in the OWW medium, *Pleurotus* laccase could act by both detoxifying compounds in the substrate and oxidizing phenolic groups, as an initial enzyme involved in the cleavage of side chains and aromatic rings of lignin phenolic moieties (Higuchi, 1990). Thus in this experiment, it appeared to operate best around the 25% raw OWW dilution, being activated just below the 12.5% level.

The mycelium growth rates recorded on solid nutrient media containing 25–100% raw OWW

were particularly high, especially for the *P. pulmonarius* strain. The values ($5.0\text{--}6.9\text{mm day}^{-1}$) attained by this isolate were superior than the mean values reported for the same species growing on other substrates, i.e. $4.8\text{--}5.7\text{mm day}^{-1}$ on PDA and $2.0\text{--}3.4\text{mm day}^{-1}$ on cellulose medium (Zervakis & Balis, 1991). Similarly, *P. eryngii* performed equally well or better on OWW based media than on the conventional media mentioned previously. As was the case with the liquid cultures, both *Pleurotus* species appeared to metabolize most effectively the organic compounds present in raw OWW at dilutions of about 25%. However, their growth could also be supported on much higher concentrations, e.g. 75–100%, without significant decrease in the rates measured. Furthermore, there are *Pleurotus* species, e.g. *P. dryinus*, which presented higher growth rates in the 100% raw OWW dilution than on malt extract based controls (Flouri *et al.*, 1996). In fact, what seems to be most influenced in this dilution range is the lag phase which was prolonged considerably in most cases as a result of the time needed by the organism to adapt its degradation mechanism for the particular type of OWW substrate examined. Other reasons for this extended lag period could be the high content of inhibitors (possibly quinones) obstructing enzyme operation in its early stages. A similar behaviour for *Pleurotus* strains adapted to grow on OWW was reported by Tomati *et al.* (1991) and Sanjust *et al.* (1991).

The degree of elevation of the OWW phytotoxic effect was the subject of the germination assays conducted with plant seeds. The germinability was found to be higher for the spent liquid substrates in comparison with the control. The differences detected for the 25% dilution of OWW were significant, confirming the results outlined above for the levels of OWW responsible for optimal mycelium yields and growth rates, and the conclusions derived concerning laccase activity and its correlation with substrate concentration. Both strains efficiently decomposed the substances responsible for OWW phytotoxicity. The germinability maintained high values even in relatively dense OWW concentrations (25–50%).

In general, it seems feasible to elaborate an OWW-application scheme for plant fertilization purposes (in order to take advantage of the rich organic and mineral load of this waste) as long as *Pleurotus* biomass production is performed on

treated OWW (<50% dilutions) and then the spent liquid substrate is used alone or further diluted into water. Of course, the selection of the suitable crops and the controlled spreading of OWW is very important, also taking into consideration the soil characteristics; this waste possesses the potential to serve as a natural herbicide too (Bonari *et al.*, 1993). In this way, two useful products might emerge out of one pollutant: fungal mycelium which could further serve for fodder enrichment and a biofertilizer with enhanced nutritive properties.

Hence, *Pleurotus* species could be suitable organisms to cope with the difficult task of remediating olive mill wastes, not only by elevating some toxic load and facilitating further biological treatment and exploitation of this pollutant, but also by yielding other products with important added value such as mushrooms, fodder and fertilizers. The findings of this preliminary work support further research towards the evaluation of each species potential at bioconverting olive mill wastes into the most appropriate and demanded product.

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