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Original article

Flammulina velutipes: An option for "alperujo" use

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ABSTRACT

Background: Two-phase olive-mill wastes (or "alperujo") exhibit highly phytotoxic properties, mainly due to phenols. A valuable option for alperujo is its agricultural use, provided that no phytotoxic effects occur.
Aims: The present investigation was aimed at evaluating the efficacy of two strains of the lignin-degrading fungus *Flammulina velutipes* to colonize alperujo in order to produce edible mushrooms and to achieve its detoxification.
Methods: Some important cultural characters related to mushroom production (earliness, biological efficiency and quality of basidiomes) were estimated. The production of lignocellulolytic enzymes, phenol removal and detoxification of the substrate was evaluated.
Results: High biological efficiencies (70.8%) were obtained at 12 °C with *F. velutipes* strain BAFC 670/06 in a substrate containing poplar wood shavings and 90% of alperujo. The nature of the substrate did not seem to exert an important influence on pileus and stem morphology; nevertheless shortest stems were observed at higher temperatures. Endo-β-1,4-glucanase, endo-β-1,4-xylanase, laccase and Mn-peroxidase activities were detected in the extracts recovered from the solid-state cultures. Both *F. velutipes* strains were effective in removing the phenolic compounds. The initial concentration in the substrate with 90% alperujo was reduced in the case of *F. velutipes* BAFC 1763 by 84.31%, and 40.15% by *F. velutipes* BAFC 670/06. Germinability experiments on *Raphanus sativus*, showed that alperujo phytotoxicity was significantly reduced by *F. velutipes* cultures.
Conclusions: The experimented changes by the spent mushroom substrate resulting from *F. velutipes* cultivation with high amount of alperujo would allow its reuse for agricultural purposes.

Flammulina velutipes: una opción para el aprovechamiento del alperujo

Palabras clave:
Flammulina velutipes
Alperujo
Producción de basidiomas
Enzimas lignocelulolíticas

RESUMEN

Antecedentes: El alperujo, subproducto de las almazaras durante la extracción del aceite de oliva, tiene propiedades fitotóxicas importantes debido a su alto contenido fenólico. Su utilización en la agricultura es una opción válida, pero deben eliminarse sus efectos fitotóxicos.
Objetivos: El objetivo de este trabajo fue evaluar la eficacia de dos cepas del hongo ligninolítico *Flammulina velutipes* de crecer en sustratos con alto contenido de alperujo, detoxificarlo y producir basidiomas comestibles.
Métodos: Se estudiaron las principales características relacionadas con el cultivo para la producción de basidiomas: tiempo de aparición de primordios, eficiencia biológica y calidad. Se evaluó la producción de enzimas lignocelulolíticas, la remoción de compuestos fenólicos y la detoxificación del sustrato.
Resultados: Se obtuvieron altos valores de eficiencia biológica (70,8%) a 12 °C con la cepa BAFC 670/06 de *F. velutipes* en un sustrato compuesto de viruta de álamo y 90% de alperujo. La naturaleza del sustrato al parecer no ejerció influencias importantes en la morfología de los basidiomas, aunque a altas temperaturas los estípites presentaron una menor longitud. Se detectó actividad endo-β-1,4-glucanasa, endo-β-1,4-xilanasa, lacasa y Mn-peroxidasa en extractos recuperados de cultivos en estado sólido.

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Ambas cepas fueron efectivas en la reducción del contenido de fenoles del sustrato, reducción que alcanzó el 84,31% con *F. velutipes* BAFC 1763 y el 40,15% con *F. velutipes* BAFC 670/06. Los ensayos de germinación de semillas de *Raphanus sativus* mostraron una significativa reducción de la fitotoxicidad del alperujo.
Conclusiones: Los cambios experimentados por el sustrato remanente del cultivo de *F. velutipes* con altas concentraciones de alperujo podrían permitir su reutilización con fines agrícolas.

The white-rot fungus *Flammulina velutipes* is a xylophagous fungus belonging to the Physalacriaceae family, with health and medicinal benefits, widely cultivated in East Asia.[37] *F. velutipes* is one of the six most cultivated mushroom species in the world; over 300,000 t of this mushroom are produced per year.[28] It is particularly known for its taste, and preventive as well as curative properties for liver diseases and gastro enteric ulcers. In addition, it has also been reported to contain immunomodulatory, antitumor and antibiotic substances.[37] Its distribution is limited to temperate zones of the world because a cold period is required for fruiting.[37] Its genome has been recently sequenced and reveals a high capacity for lignocellulose degradation.[25] Moreover *F. velutipes* strain Fv-1 demonstrated its potential for the conversion of lignocellulosic biomass to ethanol by consolidated bioprocessing, a methodology that combines enzyme production, enzymatic saccharification and ethanol fermentation in one step, thus reducing the total cost of bioethanol production.[16]

Since *F. velutipes* grows on wood in nature, mixtures of lignocellulosic materials have been utilized as substrates in the commercial production of the mushroom. A common substrate used for its production is sawdust with agricultural residues, such as corncobs, cottonseed husk, sugarcane bagasse, etc.[37]

The olive oil extraction industry produces high amounts of olive wastes. One emerging technology for olive-oil extraction consists of a continuous centrifugation two-phase process that generates a liquid phase (olive-oil) and a semi-solid organic waste (alperujo), which are then dried and extracted with solvents to obtain an extra yield of oil and a dry olive mill residue (DOR).[34] Alperujo is a fibrous lignocellulosic paste which has a water content of about 65%, a slightly acidic pH and a very high content of organic matter (90.66%), mainly composed of lignin, hemicellulose and cellulose (38.82%, 29.70% and 23.47%, w/w, of total organic matter, respectively). It has also a considerable proportion of *N* [TN ($g \cdot kg^{-1}$) 11.99] and phenolic compounds (1.36%, w/w, of total organic matter).[21]

To preserve the ecology of olive oil producer's countries, degradation of these wastes have been studied using thermal, physico-chemical processes and biological treatments.[21] Several studies evaluated its detoxification through solid state fermentation and the addition of appropriate co-composting materials to deal with the particular structure of this effluent, using aerobic or anaerobic microorganisms. Apart from the exploitation of mixed microbial communities present throughout the composting process, the inoculation with white-rot basidiomycetes has also been used to decrease the contents of phenolics and phytotoxicity of the alperujo.[2,13,34] White-rot fungi are able to secrete specific ligninolytic enzymes causing significant phenolic removal.[31] Moreover, if edible mushrooms were utilized, a double goal could be achieved, decreasing the toxicity of this waste by means of the ligninolytic enzymes secreted by the fungus, while producing appreciated basidiomes.[42]

Up to now, the use of by-products from the olive-oil industry for mushrooms production has been scarcely investigated.[43] Olive-mill wastewater and olive-press cake were used as substrates for *Pleurotus* cultivation,[10,42] olive-mill by-products were used for *Agaricus bisporus* commercial exploitation,[1] and alperujo composted with wheat straw was evaluated as substrate for basidiome production of the edible mushrooms *Agrocybe cylindracea*, *Pleurotus cystidiosus*, *Pleurotus eryngii*, *Pleurotus ostreatus* and *Pleurotus pulmonarius*.[30,43]

The aims of this work were: (i) to study the capacity of two *F. velutipes* strains to grow with different alperujo concentrations and temperatures while producing lignocellulolytic enzymes, (ii) to evaluate if alperujo concentration influences yield values and morphological properties of the basidiomes obtained, and (iii) to assess the ability of *F. velutipes* for phenol removal and detoxification in this substrate.

Materials and methods

Fungal strains and culture conditions

F. velutipes strain BAFC 1763 (Fungal Collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) and BAFC 670/06 (IIB-INTECH Collection of Fungal Cultures) were used. Both strains were maintained in Petri dishes at 4 °C on ME agar (malt extract 1.2%, glucose 1%, and agar 2%).

Spawn production

Spawn production was prepared in 350 ml glass jars filled with boiled wheat grains (*Triticum durum*) and 1% (w/w) calcium carbonate ($CaCO_3$). Jars were sterilized for 1.5 h at 121 °C, cooled and inoculated with a 1 cm diameter plug of mycelium grown on Nobles' medium,[23] and then they were incubated at 25 °C, in the dark, with periodical shaking. Time required for spawn production was recorded.

Substrate preparation

Polypropylene bags of 20 cm × 40 cm were filled with 100 g (dry weight) of substrate [*Populus* wood shavings, alperujo (0%, 30%, 60% or 90%, w/w), and 3% $CaCO_3$]. Humidity was adjusted (w/w) to 75%. Bags were stopped with cotton plugs held by PVC (polyvinyl chloride) cylinders and autoclaved at 121 °C for 2 h. After cooling, bags were inoculated with 5% (wet weight) of spawn, and were incubated at 25 °C in the dark until total substrate colonization (21–25 days).

Basidiomes induction

After total colonization by the mycelium, polypropylene bags with the upper surface opened (with a neck of 10 cm approx. created from the same bag) were transferred to the basidiome production rooms. Cropping conditions to induce basidiome formation were 12 h light/12 h dark photoperiod (2000 lx of fluorescent light), 75–85% humidity levels and watering by automatic spray provided for 2 min, 3 times every day. Three different temperatures were assayed for basidiome production: 6 ± 1 °C, 12 ± 1 °C and 22 ± 1 °C.

Cropping period, crop yield and morphological traits assessment

Up to three flushes were harvested during the cropping period (80 days between the induction day and the last harvest) in the different substrates. Mature basidiomes (80% of expanded pileus) were collected manually. The parameters to test the suitability of the substrates under study for the cultivation of *F. velutipes* were (a) earliness, defined as the time elapsed between the day of inoculation and the day of primordial formation, (b) biological efficiency (BE), calculated as the percentage ratio of fresh mushrooms weight over the dry weight of the substrate, and (c) morphological quality traits: (i) pileus width and length and (ii) stem length and diameter.

Analytical determinations

Enzyme activities

Laccase activity (E.C.:1.10.3.2) was measured using 2,6-dimetoxiphenol (DMP) 5 mM in 0.1 M sodium acetate buffer (pH 3.6) at 50 °C. Oxidation of DMP was determined by the increase in A_{469} ($\varepsilon_{469} = 27\ mM \cdot cm^{-1}$).[26] Manganese peroxidase activity (MnP) (E.C.:1.11.1.13) was measured using phenol red as the substrate in 0.1 M sodium dimethyl succinate buffer (pH 4.5) ($\varepsilon_{610} = 22\ mM \cdot cm^{-1}$) at 50 °C.[6] Endo-β-1,4-glucanase (E.C.:3.2.1.4) and endo-β-1,4-xylanase (E.C.:3.2.1.8) activities were determined by measuring the reducing sugars released from carboxymethylcellulose or oat xylan, respectively, as substrates, in 50 mM sodium acetate buffer, pH 4.8. Liberated reducing sugars were quantified by the Somogyi–Nelson method,[22] using either glucose or xylose as standards.[40] Cellulolytic and xylanolytic activities were determined at 50 °C. Enzyme activity was expressed in International Units (U) as the amount of enzyme required to release 1 μmol of product in 1 min. In terms of production, the activity was defined as $U \cdot g^{-1}$ dry residue (substrate plus mycelium). Samples of substrates colonized by mycelium were collected at different stages of the solid-state fermentation: T1, after complete colonization of the substrate; T2, after the end of first flush; T3, after the end of the second flush (if present); T4, spent substrate after the last mushroom harvest. Crude extracts were obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w), stirring for 20 min, followed by filtration and centrifugation. The supernatants were stored at 20 °C until needed.

Total phenolic compounds quantification

Dried solid samples of non-inoculated and spent substrates were extracted with methanol:water 80:20 (v/v) (5:1, w/w), stirring for 2 h at 120 rpm, followed by filtration and centrifugation. The quantification of the total phenolic compounds was based on the Folin–Ciocalteu reaction, according to the method of Singleton et al.,[38] measuring the absorbance at 765 nm. Gallic acid was used as standard for the quantification.

Phytotoxicity bioassay

The phytotoxicity of the extract obtained from *Flammulina*'s spent mushroom was determined according to the method described by Zucconi et al.[44] using *Raphanus sativus* seeds incubated for 48 h at 28 °C with either (a) control:water; (b) the extract obtained from the non-inoculated substrate and (c) the extract obtained from the spent substrate after the last mushroom harvest. The germination index (GI) was calculated according to the expression: $GI = [(G/Go)/(L/Lo)] \times 100$, where Go and Lo are, respectively, the germination percentage and radicle growth of the control. Crude extracts were obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w), stirring for 20 min, and followed by filtration and centrifugation.

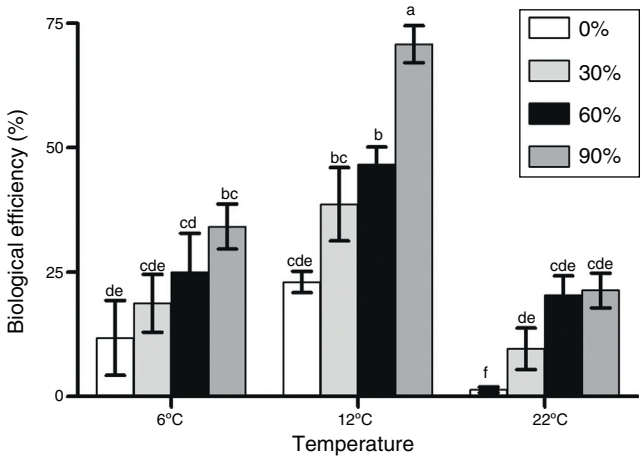

Fig. 1. Biological efficiencies attained by *F. velutipes* strain 670/06 with different alperujo concentrations and temperatures. Values with the same letter are not statistically different according to Tukey's test.

Experimental design and statistical treatments

For basidiome production each treatment consisted of ten replicates. Data of analytical determinations are the average of the results of at least three replications with a standard error of less than 5%. Analysis of variance (ANOVA) and repeated measures analysis were tested by the software Statistica v.7.0. The assumptions of normality and homogeneity of variances were checked by means of Kolmogorov–Smirnov and Bartlett tests, respectively, for the validity of ANOVA method. The significant differences between treatments were compared by Tukey's test at 5% level of probability.

Results

Effect of alperujo addition and temperature of incubation on F. velutipes *basidiome production*

Both strains of *F. velutipes* took 21–25 days to complete the colonization of all the substrates assayed. Growth was assessed visually, and while a dense mycelial mat could be observed in the substrates supplemented with alperujo, only scatter hyphae were seen on the control with solely poplar wood shavings. Lignocellulosic materials are generally low in protein content, insufficient for mushroom cultivation. Since the C:N ratio plays an important role in spawn running and the growth of basidiomes, nitrogen supplementation is an important factor for the growth and yield of mushrooms. The high content of organic matter provided by the alperujo might support better mycelial growth.

F. velutipes strain BAFC 670/06 demonstrated an exceptionally good adaptation to olive mill waste containing substrates since most of the alperujo media provided significantly higher BEs values in comparison to the control. Moreover, with alperujo addition mushroom primordia were formed within a shorter time period after substrate inoculation; the first flush was observed between 13 and 33 days after initial induction (Table 1). The highest BEs were obtained at 12 °C with *F. velutipes* strain BAFC 670/06, and BEs improved with increasing alperujo concentrations up to a maximum of 70.8 with 90% of this olive waste (Fig. 1). No significant differences were observed in BEs obtained with substrates containing 60 and 30% alperujo (46.60% and 38.68%, respectively) at 12 °C. More than 60, 75 and 90% of the total production was cropped during the first flush with 90%, 60% and 30% alperujo content,

Table 1
Effect of alperujo addition and temperature of induction on *F. velutipes* strain 670/06 basidiome production.

Temp.	Alperujo concentration	Earliness (days)	Time between induction and first flush	First flush (g)	Second flush (g)	Pileus		Stem	
						Width (cm)	Length (cm)	Width (cm)	Length (cm)
6 °C	0%	50.50 ± 2.38^{a}	30.25 ± 1.89^{a}	11.80 ± 7.54	–	2.53 ± 0.73^{a}	2.38 ± 0.67^{a}	0.33 ± 0.09^{bcde}	10.67 ± 2.50^{ab}
6 °C	30%	45.50 ± 1.00^{a}	25.50 ± 1.00^{a}	16.80 ± 6.72	2.50 ± 1.73	1.43 ± 0.51^{e}	1.30 ± 0.46^{f}	0.25 ± 0.06^{cdef}	9.46 ± 2.99^{a}
6 °C	60%	41.80 ± 1.09^{c}	21.80 ± 1.10^{c}	20.60 ± 5.94	5.50 ± 3.42	1.53 ± 0.56^{e}	1.41 ± 0.54^{f}	0.32 ± 0.17^{fg}	8.81 ± 2.84^{bcd}
6 °C	90%	50.20 ± 1.09^{b}	30.20 ± 1.10^{b}	34.17 ± 4.54	–	2.00 ± 0.40^{bcd}	1.81 ± 0.37^{bcd}	0.36 ± 0.10^{abc}	11.42 ± 1.37^{a}
12 °C	0%	46.5 ± 1.00^{b}	26.50 ± 1.00^{b}	22.00 ± 1.41	1.40 ± 0.36	2.00 ± 0.37^{cd}	1.84 ± 0.38^{cde}	0.26 ± 0.06^{defg}	10.76 ± 2.10^{ab}
12 °C	30%	34.80 ± 1.09^{c}	14.80 ± 1.10^{c}	36.50 ± 6.45	3.57 ± 1.69	2.59 ± 0.57^{ab}	2.42 ± 0.55^{ab}	0.38 ± 0.11^{ab}	12.27 ± 2.61^{ab}
12 °C	60%	42.40 ± 1.34^{c}	22.40 ± 1.34^{c}	34.67 ± 3.72	11.60 ± 2.30 (third flush: 3.40 ± 0.34)	2.26 ± 0.72^{abc}	2.12 ± 0.66^{abc}	0.34 ± 0.11^{bcdef}	10.34 ± 2.46^{abc}
12 °C	90%	40.33 ± 0.82^{c}	20.33 ± 0.82^{c}	44.60 ± 2.70	26.20 ± 2.77	2.19 ± 0.54^{cd}	2.00 ± 0.50^{cde}	0.39 ± 0.14^{bcdef}	9.97 ± 2.41^{cde}
22 °C	0%	35.2 ± 1.09^{c}	15.20 ± 1.10^{c}	1.40 ± 0.55	–	1.67 ± 0.39^{de}	1.42 ± 0.36^{ef}	0.23 ± 0.05^{g}	7.16 ± 1.73^{fg}
22 °C	30%	45.83 ± 0.75^{d}	25.83 ± 0.75^{d}	9.60 ± 4.16	–	2.08 ± 0.58^{cde}	1.79 ± 0.51^{def}	0.33 ± 0.11^{efg}	7.41 ± 1.55^{g}
22 °C	60%	40.86 ± 1.07^{d}	20.86 ± 1.07^{d}	20.40 ± 3.85	–	2.26 ± 0.61^{bcd}	1.96 ± 0.52^{cd}	0.40 ± 0.12^{abc}	8.52 ± 1.63^{efg}
22 °C	90%	35.33 ± 1.03^{d}	14.33 ± 1.03^{d}	21.33 ± 3.50	–	1.90 ± 0.63^{de}	1.64 ± 0.46^{def}	0.48 ± 0.16^{a}	9.73 ± 1.96^{def}

Data are the mean ± standard deviation of three determinations. Means followed by the same letter are not significantly different according to Tukey's test.

respectively, at 12 °C. Lower BEs were attained at 6 °C and 22 °C: at 6 °C BEs varied from 11.8 to 34.17%, while at 22 °C BEs ranged from 1.4 up to 21.33% with different amounts of alperujo; the highest values were obtained with 90% (Fig. 1).

The lowest BEs gotten at 22 °C might be related with various aborted primordia observed. Strain 1763 only produced basidiomes on 60% of alperujo at 12 °C and 22 °C, and the BEs were less than 10% (data not shown).

Pileus width and length, and stem length and diameter, were not significantly different among most of the treatments for the same strain, and hence the nature of the substrate did not seem to exert any important influence (exception *i.e.* in stem morphology with 90% of alperujo). On the contrary, the temperature of incubation held sway over morphology of *F. velutipes* basidiomes, resulting in mushrooms with shorter stems at 22 °C (Table 1).

Enzyme activities

Cellulolytic, xylanolytic and ligninolytic activities were detected in the extracts recovered from the solid-state cultures. Table 2 summarizes the highest titers of the different enzyme activities attained by both strains (and the day and medium in which these values were achieved). Time course of enzyme production in the conditions that rendered the highest BE, *F. velutipes* strain BAFC 670/06 grown at 12 °C with 90% of alperujo, is depicted in Fig. 2. Hydrolytic activities showed their highest values at initial stages of cultivation: endo-β-1,4-glucanase utmost titers detected were 5.46 ± 0.03 U·g^{-1} (30%, 12 °C) and 5.27 ± 0.01 U·g^{-1} (60%, 12 °C) for 670/06 strain, and 7.14 ± 0.20 (0%, 22 °C, and 30%, 22 °C) for 1763 strain, while endo-β-1,4-xylanase peaks were 10.16 ± 0.06 U·g^{-1} (60%, 12 °C) and 6.09 ± 0.01 U·g^{-1} (90%, 12 °C) for 670/06 strain. On the contrary, laccase activity showed its highest activity at final stages, at second flush or at the end of the culture period (80 days). *F. velutipes* BAFC 1763 displayed a higher activity of laccase [102.66 ± 6.02 U·g^{-1} (60%, 22 °C)], and MnP activity was only detected in this strain (1.67 ± 0.03 U·g^{-1} with 90% alperujo at 22 °C). Highest laccase activity detected in strain BAFC 670/06 was 0.98 ± 0.14 U·g^{-1} with 30% alperujo at 12 °C at the end of incubation. Laccase activity was influenced by the concentration of alperujo added to the substrate. Highest laccase activities recorded for strain BAFC 1763 at 12 °C with 0, 30, 60 and 90% alperujo were respectively: 0.64 ± 0.03; 5.46 ± 0.23; 21.74 ± 1.05 and 27.11 ± 2.90 U·g^{-1}. Strain BAFC 670/06 produced the highest laccase activity with 30% alperujo (0.98 ± 0.14 U·g^{-1} at 12 °C); laccase activity was 0.68 ± 0.11 U·g^{-1} with 90% alperujo at the same temperature while it not exceeded 0.35 ± 0.07 U·g^{-1} in the control without alperujo in the same conditions.

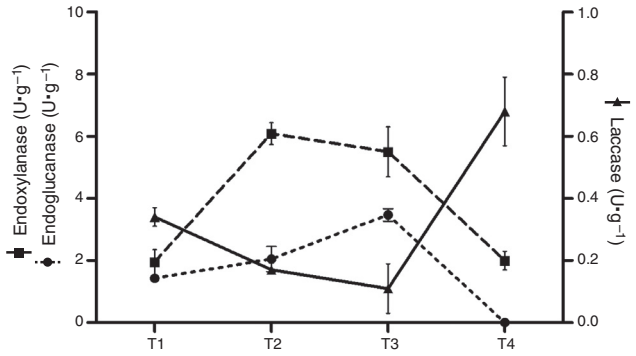

Fig. 2. Kinetics of enzyme production in the conditions that rendered the highest BE, *F. velutipes* strain BAFC 670/06 grown at 12 °C with 90% of alperujo. Each point represents the mean of three replicate experiments. S.E.M. (errors not shown were within the heights of the symbols).

Removal of phenolic compounds and detoxification of the substrate by F. velutipes

Both *F. velutipes* strains were effective in removing the phenolic compounds. Initial phenolic concentration in substrates with 30%, 60 and 90% of alperujo were respectively 2.61, 3.14 and 3.76 mg·g^{-1} (expressed as gallic acid equivalents). Phenol concentration at the end of the incubation in the substrate with 90% alperujo was reduced by 84.31% at 12 °C by *F. velutipes* BAFC 1763, and 42.28% by *F. velutipes* BAFC 670/06. Phenol removal by *F. velutipes* BAFC 1763 reached values of 93.06% and 87.26% with 30% and 60% of alperujo content, while those attained by *F. velutipes* BAFC 670/06 with 30% and 60% of alperujo at 12 °C were respectively 33.71% and 46.49%. GI of the treated substrate (with 90% of alperujo) was similar, or even higher than the control value (100%): 127% and 97% for strains BAFC 670/06 and 1763, respectively. GI value in the non-treated substrate was 26.67%.

Discussion

This work evaluated for the first time the potential use of olive mill wastes as substrate for *F. velutipes* cultivation. *F. velutipes* strain BAFC 670/06 formed primordia in a shorter time on substrates with alperujo. On the contrary, in previous works, always olive waste

Table 2
Extracellular lignocellulolytic activities displayed by *F. velutipes* strains on poplar wood shavings with different alperujo concentrations.

Enzyme activity	Strain BAFC 670/06 ($U \cdot g^{-1}$ substrate)[a]	Strain BAFC 1763 ($U \cdot g^{-1}$ substrate)
Endo-β-1,4-glucanase	5.46 ± 0.03 (30%, 12 °C, after 1st flush)[b] 5.27 ± 0.01 (60%, 12 °C, after 1st flush)	7.14 ± 0.20 (30%, 22 °C, initial post-incubation)
Endo-β-1,4-xylanase	10.16 ± 0.06 (60%, 12 °C, after 1st flush) 6.09 ± 0.01 (90%, 12 °C, after 1st flush)	3.46 ± 0.57 (30%, 22 °C, initial post-incubation)
Laccase	0.98 ± 0.14 (30%, 12 °C, end of incubation)	102.66 ± 6.02 (60%, 22 °C after 1st flush)
Mn-peroxidase	ND[c]	1.67 ± 0.03 (90%, 22 °C, 90 days)

[a] Data are the mean ± standard deviation of three determinations.
[b] The values shown correspond to the peak of enzyme production. Data between parentheses indicate the conditions under which these values were achieved.
[c] ND, not detected.

amendment had a distinct adverse effect on earliness.[30,42,43] Taking into account the nature of the residue used to cultivate the mushroom, BEs obtained in this work are acceptable and comparable to those obtained in other publications when growing *F. velutipes* using different formulation substrates: *i.e.* 50.9% using paddy straw with additives,[14] 90–106% on paddy straw,[39] 73% with maize straw,[7] 56% on coffee husks[12] and 78% while using coffee spent grounds.[12]

Pileus width and length, and stem length and diameter, were not significantly different among most of the treatments for the same strain when varying the amount of alperujo. Likewise, in the cases of *P. ostreatus* and *P. pulmonarius* no significant differences were observed in the size of the mushrooms obtained when assaying substrates with different alperujo concentrations.[43] Seven oyster mushroom strains were cultivated in wheat straw bags supplemented with up to 90% alperujo, and most of them showed no significant differences on cultivation parameters and basidiomes quality (except for color) between the control and the substrates supplemented with up to 50% alperujo, although high alperujo concentrations resulted in a significant yield, biological efficiency and productivity decrease, retarding of pinning and flushing.[30] Alperujo additions affected the yield, enzyme secretion, color and texture of the *Pleurotus* specimens obtained.[27] In both strains of *F. velutipes* assayed in this work, alperujo addition did not cause a decrease in mushroom size.

In order to obtain the nutrients required for growth and fruiting, *F. velutipes* is assumed to secrete the hydrolytic/oxidative enzymes that catalyze the degradation of the major macromolecular components (cellulose, hemicellulose and lignin) of its growth substrate. However, compared with other cultivated mushrooms, very little is known about the nature of the lignocellulolytic enzymes produced by *F. velutipes*, the parameters affecting their production, and enzyme activity profiles during different stages of the developmental cycle.[17,36] The production of these enzymes is important in substrate colonization, as well as decisive in basidiome production.[24] Moreover the production of ligninolytic enzymes by the fungus might be involved in its capacity to grow and detoxify substrates with high contents of phenolic compounds like alperujo.

Hydrolysis of cellulose and hemicellulose provides the nutrients required for vegetative growth as a prelude to basidiome production. Endoglucanase and endoxylanase activities increased with the incubation time and also during the formation of basidiomes for both strains. Matsumoto[19] also found that cellulase and xylanase activities increased during the development of *Lentinus edodes* basidiomes cultivated on eucalyptus sawdust, with highest levels during mushroom maturation. Luz et al.[15] registered similar results when incubating *P. ostreatus* in different agro industrial wastes. Kurt and Buyukalaca[9] detected the highest endoglucanase activities when incubating *P. ostreatus* and *Pleurotus sajor-caju* in different agro industrial wastes after first flush and on the 5th day of mycelial growth. The increase in the enzyme activities during basidiome production may be due to the fungus' need to mobilize large amounts of carbon for mushroom formation.[18]

High ligninolytic activities during growth phases in lignocellulosic substrates, with drastic reductions during the period of basidiome formation, were observed in the white-rotting basidiomycetes *P. ostreatus*,[9] *P. sajor-caju*,[9] *Lentinus tigrinus*,[11] *L. edodes*,[18,24] *A. bisporus*[41] and *Grifola frondosa*.[20] In contrast, during *Volvariella volvacea* solid state fermentation, laccase activity increased greatly after vegetative growth at basidiome initiation and stayed at high level until the basidiomes matured.[4] Laccase activity was influenced by the concentration of alperujo added to the substrate. Ruiz Rodriguez et al.[31] also found an increase in laccase and peroxidase levels secreted by *P. ostreatus* and *P. pulmonarius* on substrates supplemented with olive mill wastes than on control.

DOR and its components have been described as a source of phenols and stimulators of fungal growth that induce several enzymes such as MnP and Lac.[5,29,35]

Ko et al.[8] evaluated endoglucanase, endoxylanase and laccase activities recovered from spent mushroom compost of *F. velutipes* grown in sawdust; specific activities obtained were respectively 151, 110 and 48.5 $nkat \cdot g^{-1}$. Compared with these data, our results showed higher recovery of all the enzyme activities evaluated. Moreover, as far as we know this is the first report of MnP activity in *F. velutipes*.

Due to its content in organic matter and mineral nutrients, alperujo might be employed for agronomic purposes.[2] However, the main technical constraint to its biological use is the presence of a relevant phenolic fraction, the concentration of which may easily range from 12 to 26 $g \cdot kg^{-1}$, exhibiting significant phytotoxicity.[32] Both spent mushroom substrates under trial showed low phytotoxicity (GI > 60%) within the range of safety for agronomic use.[44] Thus, the spent mushroom compost derived from *F. velutipes* cultivation might be used as organic fertilizer.[1] *F. velutipes* BAFC 1763 removed these toxic compounds more efficiently probably by synthesizing and excreting higher titers of ligninolytic enzymes (laccase and MnP) than the strain BAFC 670/06 into the alperujo. The effect of laccase and MnP on DOR, as well as their aqueous and organic extractives, has to some extent been described *in vivo* and *in vitro*.[29] *Coriolopsis rigida*, another white-rot fungus, is capable of transforming certain phytotoxic monomeric phenols in DOR, into non-phytotoxic polymeric phenols.[3] The degree of polymerization of these phenols may limit the accessibility *via* plant cell membrane, decreasing phytotoxicity.[33] The incubation of DOR with *Bjerkandera adusta* also produced a sharp decrease in total phenolic content in 4 weeks, as well as a reduction in phytotoxicity.[29]

In conclusion, the results achieved in this investigation contribute to expand the knowledge on the lignocellulolytic enzyme system of *F. velutipes*, scarcely investigated up till now. Taking into account that *F. velutipes* successfully colonized this waste, this substrate formula might be considered for its growth. The inclusion of poplar wood shavings provides nutrients and enhances gas

exchange and water retention, thus facilitating the degradation of this toxic compound.[13] Both *F. velutipes* strains decreased the phenolic content whilst at the same time yielded a less phytotoxic substrate. *F. velutipes* BAFC 1763 cultures in alperujo/poplar wood shavings mixtures resulted in aqueous extracts containing about 80% less phenolic compounds, which inhibited seed germination to a much lesser extent. Experiments comparing abiotic control with the fungal treated substrate containing 90% of alperujo, showed that phytotoxicity was totally suppressed in the waste that underwent 100 days of fungal treatment. The experimented changes undergone by alperujo after degradation would allow its reuse (after the harvest of the mushrooms) for agricultural purposes as organic amendments or soil conditioner for growing plants.[32]

Conflict of interest

The authors declare that there are no conflicts of interest.

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