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Organic matter evolution and partial detoxification in two-phase olive mill waste colonized by white-rot fungi

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Abstract

Dry olive mill residue (DOR) is a solid waste arising from the olive oil two-phase extraction system. The objective of the present study was to investigate the impact of an aerobic treatment of DOR with selected lignin-degrading fungi on both organic matter evolution and residual phytotoxicity of the waste. To this aim, several white-rot fungi, including *Phlebia radiata*, *Coriolopsis rigida*, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, *Poria subvermispora* and *Pleurotus pulmonarius* were inoculated under axenic conditions for 2 and 20 weeks. The chemical composition and phytotoxicity of DOR were scarcely affected by fungi after 2 weeks incubation. By contrast, both a significant depletion of phenolic compounds and a partial removal of phytoxicity towards *Lycopersicum esculentum* plants were generally obtained after 20 weeks. The most effective fungus in degrading lignin, total phenols and in removing phytotoxicity was *C. rigida*. A high correlation was observed between phytotoxicity and phenols added to soil with the waste.

Keywords: White-rot fungi; Phenols; Dry olive residue; Humification; Phytotoxicity

1. Introduction

Olive oil production is one of the most relevant agro-industrial activities in the Mediterranean area and generates huge amount of both solid and liquid wastes, the uncontrolled disposal of which might lead to serious environmental problems. Such problems that were formerly confined to historical oil-producing countries are now rapidly diffusing to other countries such as USA, Australia, New Zealand and Chile.

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 (alpeorujo), which are then dried and extracted with solvents to obtain an extra yield of oil and a dry olive residue (DOR) (Vlyssides et al., 1998). About 0.8 ton of solid waste is generated per ton of processed olives by the two-phase extraction system (TPES). It has been calculated that the annual production of DOR in Spain, where TPES is rather widespread, amounts to about four million tons (MAPA, 2002). Thus, new technological procedures allowing a profitable and environmentally sound use of the waste are required to minimize the environmental risks associated with the production of huge amounts of DOR.

Several studies have been performed in order to provide valuable solutions for the use of the solid waste arising from TPES. Two main alternatives have been proposed so far, the first one employing the waste combustion for energy production and the second one involving its composting (Madejón et al., 1998; Vitolo et al., 1999; Filippi et al., 2002). Due to its content in organic matter and mineral nutrients, DOR might be employed for agronomic purposes (Paredes et al., 1999; Bonanomi et al., 2006). However, the main technical constraint to the biological upgrading of DOR is due to the presence of a relevant phenolic fraction, the concentration of which may easily range from 12 to 26 $\mathrm{g\,kg^{-1}}$ (Sampedro et al., 2004). Both phytotoxicity (Martín et al., 2002; Casa et al., 2003;

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Sampedro et al., 2005; Bonanomi et al., 2006) and antimicrobial properties (Moreno et al., 1987; Kotsou et al., 2004) of DOR and olive mill wastes have been ascribed to the phenolic fraction. In addition to phenols, the high contents in lignin of the waste (Madejón et al., 1998; Felizón et al., 2000) might negatively affect the performances of a composting process even though indigenous microorganisms have been shown to be capable of performing lignin breakdown (Tuomela et al., 2000). Therefore, biodegradation of both lignin and phenols are key processes in DOR upgrading. In addition, molecular rearrangement and degradation of lignin are considered to be the main phenomena involved in the formation of humic-like substances in soil.

White-rot fungi (WRF) are widely recognized as the most effective lignin degraders in nature. Their capability of degrading lignin is mainly associated with the release of extra-cellular oxidases characterized by low substrate specificity and acting *via* radical mechanisms (Dehorter and Blondeau, 1992). For this reason, these fungi can be considered suitable candidates in DOR upgrading. The efficiency of lignin degradation by WRF depends on species employed and can largely vary from one substrate to another. In particular, WRF can be divided into either selective or simultaneous lignin degraders on the basis of the amounts of carbohydrates required to accomplish the removal of lignin (Otjen et al., 1987).

The objective of the present study was to assess whether an aerobic treatment of DOR with selected lignin-degrading fungi might be a valuable alternative to composting. To this aim, the impact of fungal colonization on both detoxification and chemical composition of the waste was studied in view of potential utilization of DOR for agronomic purposes.

2. Materials and methods

2.1. Fungi and preparation of inocula

Six WRF were employed in this study: *Phanerochaete chrysosporium* IJFM A547 (ATCC 24725), *Coriolopsis rigida* (CECT 20449), *Poria subvermispora* (CBS 34763), *Pycnoporus cinnabarinus* IJFM A720 (CECT 20448), *Pleurotus pulmonarius* IJFM A578 (CBS 50785) and *Phlebia radiata* (CBS 18483). Strains were maintained at 4 °C and periodically subcultured on potato dextrose agar (PDA) added with 2% malt extract (w/v). Barley seeds were inoculated with a 1 cm^2 disk of PDA withdrawn from 14-day-old fungal cultures grown at 28 °C.

2.2. Sample preparation and fungal treatment

Dry olive mill residue was collected from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain). The main characteristics of DOR were as follows: total organic carbon (TOC), 58.5%; total nitrogen (TN), 1.87%; total phosphorus, 0.21%; lignin, 24.7%; cellulose, 18%; hemicellulose, 11.4%; total phenols, 2.65%; total lipids, 0.2%; ashes, 9.2%. The most abundant elements, the concentration of which is reported in $g\,kg^{-1}$ DOR were: potassium, 30.5; calcium, 13.6; magnesium, 3.8; iron, 1.1; sodium, 0.17; copper, 0.07; zinc, 0.06 and manganese, 0.04. The pH of the aqueous extract of DOR was 5.13.

The initial moisture content of the solid residue was 10–15% (w/w) and it was adjusted to 25% by adding tap water and sterilized twice at 120 °C prior to inoculation. The incubation process was carried out in 1l Erlenmeyer flasks containing 80 g of steam-sterilized DOR, inoculated with four barleys seeds previously colonized by the mycelium for 7 days. Flasks were covered with sterile cotton plugs and incubated under stationary conditions at 28 °C for 2 and 20 weeks. Non-inoculated DOR samples were prepared and incubated as above and will be referred to as incubation controls from here onwards.

2.3. Analytical assays

Samples were ashed at 600 °C for 12 h and organic matter (OM) obtained by subtracting ash content from the whole sample weight (López et al., 2002). Total N, P, K, Fe, Mn, Cu and Zn content in DOR were determined by the method described by Mingorance (2002). TOC was determined by dichromate oxidation (Springer and Klee, 1954). Pyrophosphate-extractable-C (PEC) was extracted with $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1:20 solid–liquid ratio by mechanical shaking at 37 °C for 24 h. The suspension was then centrifuged (8000*g*, 20 min) and filtered through a 0.45 μm membrane (Millipore, USA). Humic acids (HA) were precipitated from the filtrate by adding H_2SO_4 up to pH 2.0 and then centrifuged as above. The carbon contents in PEC and the fulvic acid fraction (C_{FA}) in the surnatant after precipitation of HA were determined as above. The humic acid carbon (C_{HA}) was calculated by subtracting C_{FA} from PEC (Paredes et al., 2002). Humification index (HI) and humification ratio (HR) were calculated from the C_{HA}/TOC and C_{HA}/C_{FA} ratios, respectively, as previously reported (López et al., 2002; Paredes et al., 2002). Nitrate was colorimetrically determined after extraction in 0.5 M K_2SO_4 (Cataldo et al., 1975). Ammonium was extracted in 1 M KCl and determined according to Anderson and Ingram (1993). To extract phenols, 2 g DOR was incubated for 24 h in 100 ml distilled water/acetone mixture (50:50 v/v) under orbital shaking (200 rpm). Total phenolic contents (TP) of extracts were determined according to Linares et al. (2003) using syringic acid as the standard and expressed as $mg\,g^{-1}$ of DOR. Lignin, cellulose and hemicelluloses were determined as previously reported (Giovannozzi Sermanni et al., 1994). Losses in OM, nitrogen and lignin were calculated according to Paredes et al. (2002). The extent of fungal biomass was indirectly determined by the chemical determination of the chitin content in the solid substrate (Ride and Drysdale, 1972).

2.4. Phytotoxicity experiments

The soil used was a grey loam obtained from the field of the Estación Experimental del Zaidín (Granada, Spain). The soil had a pH of 8.1 in a 1:1 soil:water ratio. Both fungal-treated and incubation controls of DOR were sterilized and added to soil pots at concentrations of 25 $g\,kg^{-1}$ soil. The same soil in the absence of DOR was used as the control.

Tomato (*Lycopersicum esculentum* L.) was used as the test plant. Experiments were carried out in 0.3l pots of soil which were steam-sterilized and mixed with sterilized quartz sand (1:1 v/v). Plant seeds were pre-germinated and selected for uniformity prior to planting. Plants were grown in a greenhouse with natural light supplemented by Sylvania incandescent and cool-white lamps giving 400 $nmol\,m^{-2}\,s^{-1}$ in the wavelength range of 400–700 nm; there was a 16–8 h light–dark cycle at 25–19 °C and 50% relative humidity. Plants were watered from below, and fed with a nutrient solution at 10 ml per week (Hewitt, 1952). Plants were harvested after 4 weeks and the dry weight of epiphytic biomass was measured. Percent growth inhibition (GI%) was calculated from the following expression:

$$GI\% = \left(1 - \frac{B}{B_{dwc}}\right)100,$$

where B is the amount of biomass obtained in the presence of fungal-treated or DOR incubation controls and B_{dwc} that obtained in control soil.

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2.5. Statistical treatment of data

Data obtained were subjected to ANOVA, and multiple pair-wise comparisons were performed by the Tukey test using a confidence level of 95%.

3. Results

3.1. Mycelial growth on DOR

Fungal colonization of DOR, as assessed by visual inspection, did not appear to be complete within the end of the second week of incubation for the majority of fungi. To obtain a quantitative, albeit indirect estimation of fungal biomass, the chitin content of the solid substrate was determined and showed that after 2 weeks incubation the lowest and highest contents of this parameter were observed for *Pl. pulmonarius* and *Po. subvermispora* amounting to 1.6 and 2.7 μg glucosamine mg^{-1} DOR, respectively (Fig. 1A). By contrast, at the same incubation time, the chitin contents in the remaining fungal cultures on DOR did not significantly differ from each other. The extension of the incubation time from 2 to 20 weeks generally resulted in an increase in fungal biomass content, as expected. The highest increases throughout incubation were observed for *Ph. radiata* and *Po. subvermispora* (from 2.3 to 8.0 and from 2.7 to 4.2 μg glucosamine mg^{-1} DOR, respectively).

Fig. 1B shows that in 2-week-old fungal cultures on DOR the lowest extents of OM losses were observed with *Py. cinnabarinus* and *Pl. pulmonarius* (4.77% and 8.24%, respectively). At the same incubation period, *C. rigida* growth on DOR resulted in the highest value of OM losses (around 17.3%) (Fig. 1B). The significant increase that was observed in ash concentration as the incubation time was prolonged from 2 to 20 weeks clearly showed that effective organic matter degradation occurred throughout the bioconversions (Fig. 1C). In fact, the extension of incubation time to 20 weeks resulted in a marked decrease in the OM content and the highest losses were observed with *Ph. radiata*, *Po. subvermispora* and *C. rigida* (47.3%, 37.6% and 37.4%, respectively) (Fig. 1B). By contrast, the lowest OM losses were observed for these species, *i.e. Py. cinnabarinus* and *Pl. pulmonarius* that had shown a scarce initial growth.

The pH values in aqueous extracts of DOR which had been colonized with fungi for 2 weeks did not appear to significantly differ from the incubation control with the exceptions of those of *Pha. chrysosporium* and *Ph. radiata* cultures where pH values were shifted to opposite directions with respect to the incubation control (2.6 and 6.87 *vs.* 5.33, respectively) (Fig. 1D). An alkalinization of the substrate was observed in both *Po. subvermispora* and *Ph. radiata* 20-week-old cultures on DOR, where pH values amounted to 9.03 and 8.47, respectively (Fig. 1D). By contrast, in the remaining fungal cultures, pH values did not differ from those of the incubation control.

3.2. Effect of fungal growth on chemical composition and organic matter stabilization in DOR

Total carbon losses in solid substrate cultures are usually associated with a decrease in OM due to mineralization (which is directly related to respiration). Table 1 shows that the highest carbon losses were observed in 20-week-old *Ph. radiata* and *Po. subvermispora* cultures on DOR where they both amounted to around 13%. Table 1 also shows that the large majority of TN content in DOR, accounting for 18.7 g kg^{-1}, was organic nitrogen. In fact, low levels of ammonium and nitrate were detected in the waste in agreement with other studies (Cayuela et al., 2006). After 2 weeks colonization, TN contents in biotreated DOR did not significantly differ from those of the incubation control, irrespective of the fungus employed. By contrast, after 20-weeks incubation marked TN losses (around 34%) were observed in *Po. subvermispora* cultures where a significant alkalinization of the substrate had been observed. After 2 weeks incubation, C/N ratio in *C. rigida* and *Pha. chrysosporium* cultures was significantly lower than the control (24 and 21.9, respectively, *vs.* 31). This ratio was found to significantly increase in *Po. subvermispora* and *Pha. chrysosporium* cultures as the incubation time was increased from 2 to 20 weeks incubation (Table 1). By contrast, after 20-weeks incubation, the C/N ratios in *Ph. radiata* and *Pl. pulmonarius* cultures on DOR were significantly lower than those observed in relative incubation controls (25 and 25 *vs.* 31, respectively). The most abundant biopolymer in DOR was lignin, the content of which was around 24.7 ± 0.6%, while hemicellulose and cellulose contents ranged from 11.4 ± 1.5% to 18 ± 1%, respectively. Fig. 2A shows that fungal incubation did not substantially affect the contents of these biopolymers in 2-week-old cultures on DOR with respect to the relative incubation control. By contrast, hemicellulose contents were mostly affected in DOR that had been incubated for 20 weeks with fungi. In particular, hemicellulose degradation ranged from 34.5% to 62% as in the cases of *Ph. radiata* and *C. rigida*, respectively (Fig. 2B). Lignin content in DOR was not significantly affected by fungi, with the exceptions of 20-week-old *Ph. radiata*, *Py. cynnabarinus* and *C. rigida* cultures on DOR where lignin was degraded by 26%, 21.2% and 25.4%, respectively (Fig. 2B).

In order to assess a possible effect of fungal treatment on the stability of the carbon pools in the waste, some humification parameters were determined. Fig. 3 shows that C_{HA} contents in DOR which had been incubated for 2 weeks with fungi were lower than or equal to those observed in incubation controls. A remarkably higher content in C_{HA} than the relative incubation control was observed in 20-week-old DOR incubated with *Ph. radiata*, *Po. subvermispora* and *Py. cinnabarinus* (42.3, 39.6 and 43.5, respectively, *vs.* 25.5 g kg^{-1}). The previously mentioned tendency of C_{HA} to accumulate a long time in cultures of *Ph. radiata*, *Py. cinnabarinus* and *Po. subvermispora* in DOR was also confirmed by the increase in

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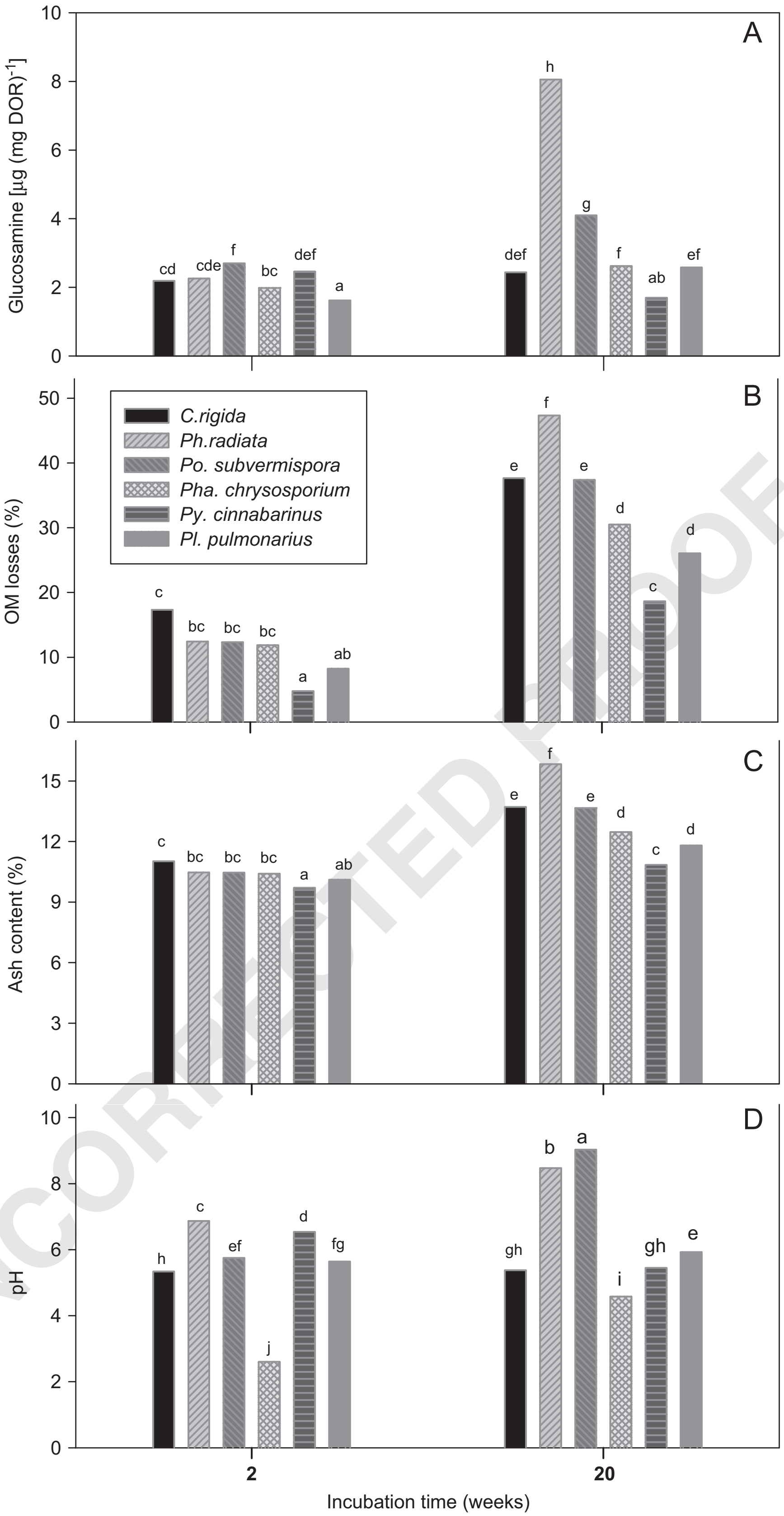

Fig. 1. Glucosamine content (A), organic matter losses (B), ash contents and pH of aqueous extracts (D) in dry olive residue (DOR) incubated with *C. rigida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus* and *Pl. pulmonarius* for 2 and 20 weeks at 28 °C under stationary conditions. Data are the mean of triplicate experiments. The significance of differences between fungal treatments of DOR was tested by the Tukey test and same letters above bars indicate lack of statistical significance ($P \leqslant 0.05$). Ash contents and pH in 2 and 20 weeks incubation controls were 9.0% and 9.2%, respectively, and 5.33 and 5.15, respectively.

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Table 1
Total organic carbon, total nitrogen, C/N ratio and ammonium and nitrate concentrations in dry olive residue ~~in~~ DOR colonized by *C. rígida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus*, *Pl. pulmonarius* and in related incubation controls

Sample	Total organic carbon (g kg^{-1})		Total nitrogen (g kg^{-1})		C/N		NH$_4$∗ N (mg kg^{-1})		NO$_3^-$ N (mg kg^{-1})	
	2 w	20 w	2 w	20 w	2 w	20 w	2 w	20 w	2 w	20 w
Incubation control	585^{ab}	577^{a}	18.7^{c}	18.7^{c}	[illegible]	31^{bc}	11^{ab}	16^{abc}	351^{cd}	374^{cd}
C. rígida	500^{ab}	525^{abc}	20.8^{b}	17.9^{cd}	24^{ef}	29^{cd}	4^{a}	131^{e}	219^{abc}	419^{d}
Ph. radiata	497^{a}	502^{ab}	18.7^{c}	20.0^{b}	26.5^{de}	25de	10^{ab}	22^{bcd}	312^{bcd}	135^{a}
Po. subvermispora	500^{ab}	501^{ab}	20.3^{b}	12.3^{f}	24.6^{de}	41^{e}	13^{abc}	20^{bcd}	321^{bcd}	150^{ab}
Pha. chrysosporium	510^{abc}	524^{abc}	23.2^{a}	15.8^{e}	21.9^{f}	33^{b}	12^{abc}	29^{d}	355^{cd}	334^{cd}
Py. cinnabarinus	540^{abc}	533^{abc}	18.3^{cd}	17.3^{d}	29.5^{cd}	31^{bc}	12^{abc}	21^{bcd}	316^{bcd}	608^{e}
Pl. pulmonarius	495^{ab}	521^{abc}	18.9^{cd}	19.8^{b}	26.2^{de}	26de	6^{a}	24^{cd}	258^{abcd}	292^{abcd}

Incubations were performed at 28 °C for 2 and 20 weeks under stationary conditions. Data are the mean of three replicates, and column means followed by the same superscript letter did not significantly differ as assessed by the Tukey test ($P \leqslant 0.05$).

HR that were significantly higher than those of the related incubation control (0.49, 0.36 and 0.34, respectively, *vs.* 0.11) (Fig. 3C). After 20 weeks incubation, HI in *Ph. radiata*, *Po. subvermispora* and *Py. cinnabarinus* cultures on DOR was significantly higher than the related incubation control (8.5%, 7.9% and 8.2%, respectively, *vs.* 4.4%) (Fig. 3D).

3.3. *Fungal dephenolization and detoxification*

Olive mill wastes have been shown to be phytotoxic and phenols have been suggested to be the main determinants for this effect (Casa et al., 2003). In the present study, the content in total phenols (*i.e.* 26 g kg^{-1} DOR) was not significantly reduced by fungi after 2 weeks incubation with the sole exception of the waste that had been colonized by *C. rigida* where TP were depleted by about 36% (Table 2). By contrast, with the sole exception of *Ph. chrysosporium* cultures, TP percent reduction was higher than 65% in DOR incubated for 20 weeks with all of the fungi, the highest removal extents being attained with *Ph. radiata*, *C. rigida* and *Py. cinnabarinus* (95.8%, 89.2% and 88.7%, respectively).

In the present study, toxicity was inferred by the amount of epiphytic biomass reduction in *L. esculentum* plants grown in soil in the presence of 2.5% of either non-inoculated or inoculated DOR with respect to plants grown in the absence of the waste (Table 3). In this respect, the waste proved to be highly phytotoxic. In fact, the shoots dry weight of plants grown in the presence of both 2-week and 20-weeks incubation controls of DOR were dramatically lower than those obtained in the absence of the waste (10 and 10.3 *vs.* 417.3 mg, respectively, corresponding to about 97% growth inhibition). Table 3 shows that in 2-week-old DOR samples where fungal incubation did not lead to substantial TP reduction, phytotoxicity was not reduced at all. By contrast, five out of six species which had resulted in high TP removal upon 20 weeks incubation, led to a significant detoxification with respect to the incubation control. However, regardless of the fungus employed, the extent of detoxification was only partial (Table 3). Biomass production was negatively correlated with the amount of phenols added to the soil, irrespective of the treatment, and the high value of the squared correlation coefficient adjusted by the degrees of freedom ($R^2_{\mathrm{adj}} = 0.76$) clearly indicated that residual phenol contents in DOR significantly contributed to the overall toxic effect (data not shown).

4. Discussion

This study was prompted by the increasing evidence that DOR, despite its potential fertilizer value, exerts both phytotoxic and anti-microbial activity when applied to soil (Sampedro et al., 2004; Baeta-Hall et al., 2005; Bonanomi et al., 2006). Phytotoxicity of DOR has been tested on a significant number of crops and its effects shown to be plant species-dependent (Sampedro et al., 2004; Bonanomi et al., 2006). With regard to its effect on soil microorganisms, a stimulating effect of undecomposed DOR on both radial growth and hyphal density of widespread plant pathogens, such as *Sclerotinia minor* (SM) and *Botrytis cinerea* (BC), was reported (Bonanomi et al., 2006). Disease severity by both pathogens was significantly increased in the presence of DOR (Bonanomi et al., 2006). The same investigators concluded that agronomic management criteria of DOR, dosages in particular, had to be reconsidered taking into account both the susceptibility of crop to the waste and its possible impact on the plant–pathogen interactions. In practical terms, the generally recommended dosage of 4 kg waste m^{-2}, equivalent to about 20 g kg^{-1} (Ocampo et al., 1975), had to be reduced in the case of highly susceptible crops such as lettuce (Bonanomi et al., 2006).

An alternative track to pursue a safe agronomic use of DOR should rely on its bioconversion aimed at both removing its toxic components and at stabilizing its organic matter, prior to its application into soil (Paredes et al.,

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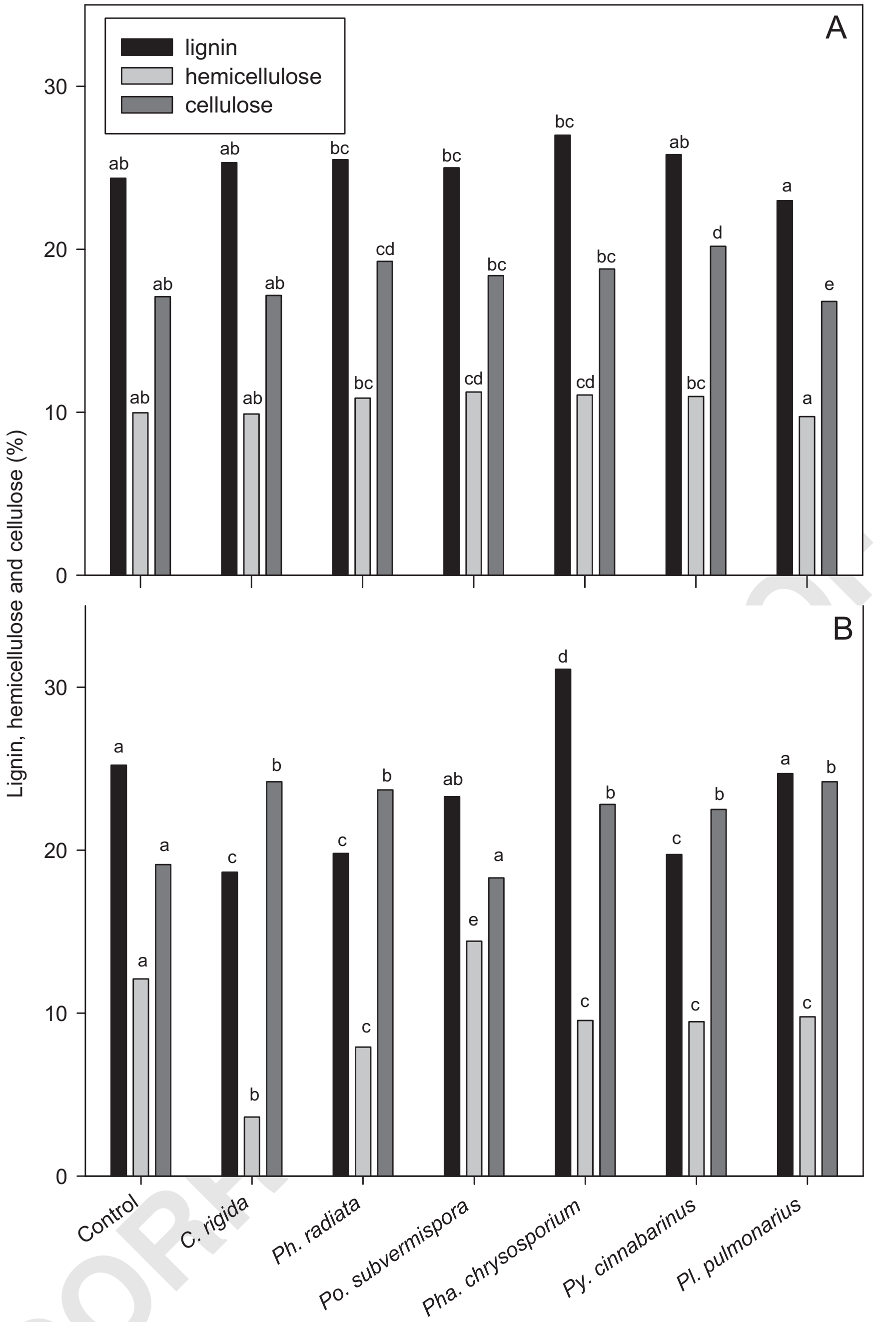

Fig. 2. Lignin, hemicellulose and cellulose contents in DOR incubation controls and in the waste that had been colonized at 28 °C under stationary conditions with *C. rigida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus* and *Pl. pulmonarius* after 2 weeks (Plot A) and 20 weeks (Plot B). Data are the mean of triplicate experiments. The significance of differences between fungal treatments of DOR was tested by the Tukey test, and same letters above bars indicate lack of statistical significance ($P \leq 0.05$).

1999). In this respect, both composting (Baeta-Hall et al., 2005; Cayuela et al., 2006) and co-composting with lignocellulosic wastes (Paredes et al., 2000, 2002) have been shown to be valuable approaches.

In the present study, the capability of six distinct species of WRF to both detoxify and upgrade DOR was thoroughly investigated. The selection criteria was based on the reported capability of these species to efficiently carry out the breakdown of lignin, that was the main component in DOR (around 25% on a mass basis) and to deplete phenols from a variety of both liquid and solid matrices. To provide evidence about the feasibility of using WRFs, DOR was not either added with nutrient supplements or with bulking agents, such as lignocellulosic wastes, and gaseous exchanges were allowed only by passive aeration. Under these conditions, all of the fungi employed showed the ability to grow on DOR albeit at slow rates as indicated by low glucosamine contents,

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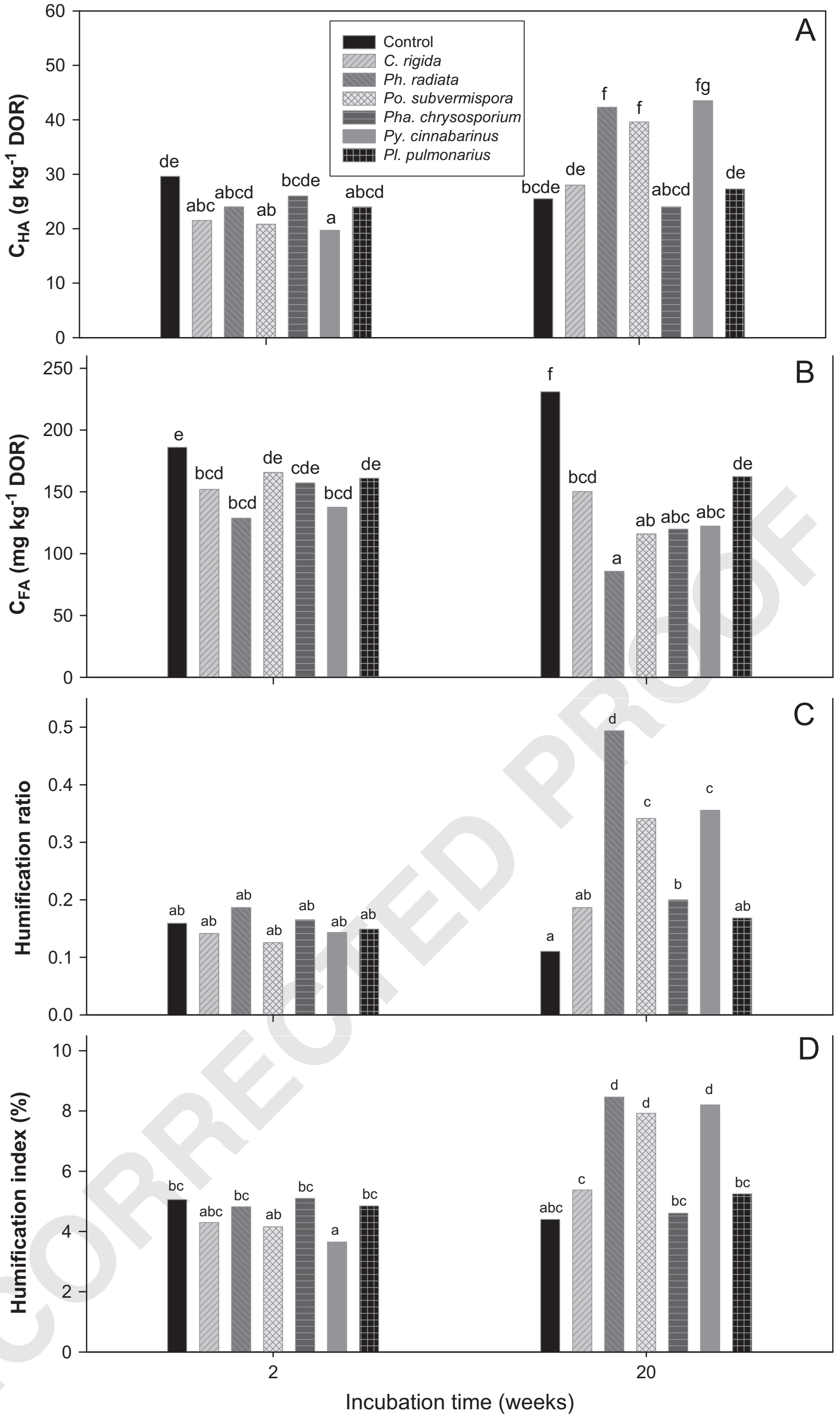

Fig. 3. Humic acid carbon (C_{HA}) (A), fulvic acid carbon (C_{FA}) (B), humification ratio (C) and humification index (D) in dry olive residue (DOR) incubated with *C. rigida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus* and *Pl. pulmonarius* for 2 and 20 weeks at 28 °C under stationary conditions. Data are the mean of triplicate experiments. The significance of differences between fungal treatments of DOR was tested by the Tukey test and same letters above bars indicate lack of statistical significance ($P \leqslant 0.05$).

limited organic matter losses and negligible impact on fiber components in DOR within the first two weeks incubation. Among them, the most actively growing species were *Ph. radiata* and *Po. subvermispora*, the colonization of which gave rise to OM losses of 47.3% and 37.6% after 20 weeks incubation. Lignin degradation took place only in *C. rigida*, *Py. cinnabarinus* and *Ph. radiata* but its removal did not exceed 26%. The limited lignin degradation observed in these cultures and the failure of the other fungi in depleting it from DOR might be presumably ascribed to

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Table 2
Total phenols content in DOR incubation control and the waste colonized by *C. rigida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus* and *Pl. pulmonarius* for 2 and 20 weeks

Sample	Total phenols (g kg^{-1} DOR)	
	2 weeks	20 weeks
Incubation control	26.3 ± 1.2^{a}	21.3 ± 0.5^{a}
Coriolopsis rigida	17.7 ± 0.8^{b}	2.3 ± 0.05^{b}
Phlebia radiata	22.8 ± 0.8^{cde}	0.9 ± 0.03^{c}
Poria subvermispora	25.0 ± 1.1^{ad}	5.9 ± 0.3^{d}
Phanerochaete chrysop~~s~~orium	25.7 ± 1.3^{adf}	18.2 ± 0.1^{e}
Pycnoporus cinnabarinus	26.4 ± 0.5^{ad}	2.4 ± 0.06^{b}
Pleurotus pulmonarius	25.4 ± 1.2^{ad}	7.2 ± 0.5^{f}

Data are the mean ± standard deviation of triplicate experiments. Column means followed by the same superscript letter were not significantly different as assessed by the Tukey test ($P \leqslant 0.05$).

Table 3
Epiphytic biomass production and percent growth inhibition of *Lycopersicum esculentum* L. plants grown in soil added with 2.5% (w/w) of either DOR incubation control or the waste colonized by *C. rigida*, *Ph. radiata*, *Po. subvermispora*, *Pha. chrysosporium*, *Py. cinnabarinus* and *Pl. pulmonarius* for 2 and 20 weeks

Amend~~ant~~/organism	Biomass production (mg)		Growth inhibition (%)	
	2 weeks	20 weeks	2 weeks	20 weeks
None/none	412 ± 12.1^{a}	417 ± 22.9^{a}	0	0
DOR/non-inoculated	10.0 ± 1.0^{b}	10.3 ± 1.5^{b}	97.6	97.5
DOR/*Coriolopsis rigida*	43 ± 9.2^{c}	249.7 ± 59.9^{c}	89.6	40.1
DOR/*Phlebia radiata*	7.6 ± 0.7^{d}	117.0 ± 7.0^{d}	98.1	71.9
DOR/*Poria subvermispora*	22.9 ± 4.1^{e}	262 ± 65.1^{c}	94.4	37.2
DOR/*Phanerochaete chrysop~~s~~orium*	30.0 ± 2.6^{c}	162 ± 11.8^{de}	92.7	61.1
DOR/*Pycnoporus cinnabarinus*	12.8 ± 3.5^{f}	114 ± 8.3^{d}	96.9	72.6
DOR/*Pleurotus pulmonarius*	22.7 ± 1.5^{e}	121.0 ± 10.5^{d}	94.5	70.9

Growth inhibition: Calculated by relating biomass production in the presence of DOR to that observed in the absence of the waste.
Data are the mean ± standard deviation of triplicate experiments. Column means followed by the same superscript were not significantly different as assessed by the Tukey test ($P \leqslant 0.05$).

the compact structure of DOR (Baeta-Hall et al., 2005) that did not allow an adequate aeration with subsequent negative impact on ligninolysis. In fact, the ligninolytic capacity of white-rot species, *Py. chrysosporium* in particular, is strongly dependent on oxygen supply (Faison and Kirk, 1983; López et al., 2002). The degradation activity of WRF may be promoted by either intermittent or continuous forced aeration (Breen and Singleton, 1999; López et al., 2002). The low macroporosity of DOR has been identified as one of the main technical constraints encountered in DOR bioconversions thus both hindering colonization of microorganisms and negatively affecting ligninolysis (Baeta-Hall et al., 2005). This has led, for instance, to the development of co-composting processes of DOR employing lignocellulosic wastes, such as wheat (Madejón et al., 1998) and poplar sawdust (Filippi et al., 2002) as bulking agents.

Lignin is mostly present in the endocarp of the drupe, that is often referred to as olive stone, rather than in mesocarp and epicarp (Felizón et al., 2000; Niaounakis and Halvadakis, 2004). Although the mesocarp (*i.e.* olive pulp) is scarcely lignified a lignin-like fraction has been found in this part of the drupe (Felizón et al., 2000). The low surface area of chopped stones has been suggested to be another determinant for the limited lignin biodegradation in olive mill wastes (Madejón et al., 1998; Baeta-Hall et al., 2005).

In contrast to lignin, hemicelluloses were found to be significantly degraded in 20-week-old cultures in DOR, their removal being particularly significant in *C. rigida.* The higher susceptibility to biodegradation of hemicelluloses than lignin and cellulose is not surprising since it has been shown that their amorphous structure makes them rather prone to microbial attack (Ward and Moo-Young, 1989). The biotransformation of lignocellulosic materials by WRFs is known to result in the formation of several OM fractions, some of which may be assigned to humic substances, having lignin as the main core (López et al., 2002; Yavmetdinov et al., 2003). In the present study, both C_{HA} and humification parameters were not necessarily highest in those cultures where lignin had been significantly degraded. In fact, the largest increase in C_{HA} was observed in 20-week-old *Py. cinnabarinus* cultures where lignin was reduced by 8%. In this respect, it was previously suggested that the balance between lignin depolymerization/polymerization reactions in solid-state cultures of WRFs variably affected the relative amounts of HA and FA (Reid, 1988). In addition, it has to be taken into account that WRF are able to depolymerise HAs (Dehorter and Blondeau, 1992).

HAs constitute the high molecular weight fraction of humic substances while FA represents the most labile carbon pool of humic fraction (Stevenson, 1994; Kostov et al., 1994). The value of the HR attained by both *Ph. radiata* and *Po. subvermispora* and *Py. cinnabarinus* on DOR might be considered promising on a comparative basis. In fact, on a lignocellulosic mixture with initial HR similar to DOR (*i.e.* 0.1), two effective WRFs, such as *Coriolus versicolor* and *Phanerochaete flavido-alba*, led to a maximal HR of 0.3 within 13 weeks colonization (López et al., 2002).

Besides increasing OM stabilization, 20-week-old *P. radiata* and *Po. subvermispora* gave rise to phenol removal yields higher than 70%. However, a scarce impact on OM stabilization was observed in *C. rigida* cultures where phenols had been significantly degraded. Dephenolization of DOR obtained in the present study with the aforementioned fungi was very similar to that reported in olive husk composting in aerated piles after 17 weeks incubation (Baeta-Hall et al., 2005).

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The high phytoxicity of DOR was significantly reduced in cultures where phenols had been removed by fungi (present study) as indicated by the increase in biomass obtained in the waste that underwent fungal colonization. In general, the suppression in plant biomass production was found to be highly correlated with the amount of phenols added to the soil thus indicating the significant contribution due to phenols to the overall toxic effect of the waste.

In conclusion, albeit being promising candidates to perform DOR treatment, lignin-degrading fungi required long colonization times to deplete phenols, to attain a partial stabilization of the organic matter and toxicity removal from the waste.

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