

Reusing ethyl acetate and aqueous exhausted fractions of dry olive mill residue by saprobe fungi

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Abstract

Some saprobe fungi (*Phlebia radiata*, *Trametes versicolor*, *Corioloropsis rigida*, *Pycnoporus cinnabarinus*, *Fomes sclerodermus* or *Pleurotus pulmonarius*) were able to bioconvert the ethyl acetate fraction (DEAF) and the corresponding aqueous exhausted fraction (EAF) of dry olive mill residue (DOR), reducing their phytotoxicity on *Lepidium sativum* seeds. Large amount of hydroxytyrosol together with other eight monomeric phenols were found in the native DEAF fraction, which represents a good source of antioxidants. *P. radiata*, *T. versicolor* and *F. sclerodermus* caused an effective phytotoxicity reduction of EAF in the concentration range of 25–3 g l⁻¹. In particular, in the range between 12.5 and 3 g l⁻¹, the EAF samples inoculated with *P. radiata* and *F. sclerodermus* surprisingly stimulated the germinability of *L. sativum*, suggesting their use as a potential biofertilizer. This is the first report which showed the bioconversion of the above fractions in shorter time with respect to the previous findings concerning DOR. The possible implications of laccase in the decrease of DEAF and EAF phytotoxicity was also discussed.

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1. Introduction

Olive production is a significant agricultural activity in the southern European Union Member States with important environmental, social and economic implications. The areas of greatest olive oil production are situated in Spain, Italy, Greece and Portugal (Barranco et al., 1997).

The latest technology for olive-oil extraction consists of a continuous two-phase centrifugation process (Vlyssides

et al., 1998) that generates a liquid phase (olive-oil) and a solid organic waste (alpeorujo or olive mill residue). The olive mill residue is usually sent for further chemical and thermal treatment in order to obtain a second-extraction olive-oil, with the production of a final solid waste named extracted alpeorujo or dry olive mill residue (DOR). DOR is a lignocellulosic by-product with a high C/N ratio. This residue is generally stored in piles close to olive-cake oil processing plants which created a problem for industries and a loss of market values for these sub-products (Madjón et al., 1998; Rosa et al., 2001).

Several studies have been performed in order to provide alternative solutions for the use of these solid residues. Because DOR contains >94% organic matter, its addition to agricultural soils has been proposed as a disposal strategy, which also serve to enrich soils poor in organic matter (Abu-Zreig and Al-Widyan, 2002). However, DOR

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contains toxic components capable of inhibiting the growth of microorganisms (Ramos-Cormenzana et al., 1996) and plants (Martín et al., 2002). Phenolic compounds seem to be mainly responsible for the phytotoxic effect of olive residues (D'Annibale et al., 2004). Nevertheless, DOR could be potentially transformed in a fertilizer because it can be detoxified through bioremediation by breaking down the toxic phenolic compounds (Giannoutsou et al., 2004; Brunetti et al., 2005).

The removal of phenols, by conventional waste-water treatment method (chemical coagulants, hydrogen peroxide and filtration), presents technical and economical difficulties (Flouri et al., 1996), therefore a biological process would be more appropriate. Despite the complex structure of phenols, there are fungi capable of degrading such compounds (Kirk and Farrell, 1987; Cullen, 1997). The white rot fungi seem to be associated with the release of extracellular enzymes namely lignin-peroxidases, Mn-dependent peroxidases and laccase capable of degrading a wide variety of pollutants (Sayadi and Ellouz, 1993). It has been described that the white rot fungi are particularly efficient for bioremediation of DOR (Sampedro et al., 2004b), however this is a time consuming process making it little useful to use on large scale.

On the other hand, it is known that some phenolic compounds possess also strong antioxidant properties (Galli and Visioli, 1999), which may turn the olive oil residues into a cheap source of natural antioxidants. In order to optimise the use of DOR, a study of the recycling of this residue by means of selected saprobe fungi in a short period of time with respect to previous findings (Sayadi and Ellouz, 1993) is highly desirable to be able to better understand its value as source of natural antioxidant and as a potential biofertilizer. In this light, we have performed an integrated physical–microbiological treatment of this material by extraction with ethyl acetate and its incubation of both the extracted ethyl acetate fraction (DEAF) and the corresponding DOR exhausted aqueous fraction (EAF) with the fungi *Phlebia radiata*, *Trametes versicolor*, *Coriolopsis rigida*, *Pycnoporus cinnabarinus*, *Fomes sclerodermus* and *Pleurotus pulmonarius*. The residual phytotoxicity of these extracts was evaluated using germination test with *Lepidium sativum* L. seeds.

The present research was performed to achieve three objectives: (i) identification of potentially useful low molecular weight phenols in DEAF; (ii) assessment of the role of DEAF and EAF in the phytotoxicity of DOR and (iii) the selection of saprobe fungi able to detoxify EAF for the potential use of this material as a biofertilizer.

2. Material and methods

2.1. Sample preparation

DOR was collected from an “orujo” manufacturer (Aceites Sierra Sur, Granada, Spain). Total organic carbon (OC),

N, P, K, Mg, Ca, Na, Fe, Cu, Zn and Mn content of DOR was determined by Sampedro (personal communication). These were: C 215.7 g kg⁻¹, N 18.72 g kg⁻¹, P 2.11 g kg⁻¹, K 30.54 g kg⁻¹, Mg 3.82 g kg⁻¹, Ca 13.61 g kg⁻¹, Na 0.17 g kg⁻¹, Fe 1.12 g kg⁻¹, Cu 68.97 mg kg⁻¹, Zn 65.24 mg kg⁻¹, Mn 46.50 mg kg⁻¹. DOR was physically fractionated by solid–liquid extraction with ethyl acetate into DEAF and the corresponding EAF. DEAF was obtained by mixing and stirring DOR with ethyl acetate in a proportion of 1:2 (w/v) for 8 h at room temperature. The ethyl acetate extract was evaporated to dryness by rotary evaporation and the residue was resuspended in water with the same initial proportion. After this extraction, the remaining exhausted DOR was resuspended in water with the same solid–liquid proportion to obtain EAF.

These fractions were used as a growth medium for the saprobe fungi *P. radiata* IJFM A588 (CBS 184.83), *T. versicolor* IJFM A136, *C. rigida* (CECT 20449), *P. cinnabarinus* IJFM A720 (CECT 20448), *F. sclerodermus* (2752) and *P. pulmonarius* IJFM A578 (507.85). Inoculation of each fraction was carried out by growing the fungi under orbital shaking at 125 rpm and 28 °C on potato dextrose broth in the presence of 5% of aqueous DOR extracts for 5 d. The mycelia were collected by filtration, extensively washed with distilled water and homogenized. The fungi were grown in Erlenmeyer flask (250 ml) containing 70 ml of each fraction for 15 d at 26 °C with orbital-shaking at 125 rpm. The DOR ethyl acetate extract was added with nutrients from Czapek medium and adjust to pH 5.5. Each flask was inoculated with 0.45 g l⁻¹ of each inoculum. The culture liquid was separated from the mycelium by centrifugation (8000 rpm for 15 min) and after filtration through a disk of filter paper, the supernatants were analyzed. The mycelia were washed twice with distilled water, dried at 70 °C overnight and weighted. Appropriate controls were prepared by incubating each fraction without fungi. Experiments were performed in quadruplicate.

2.2. Chemical analysis

The total phenol content aqueous DOR extract (ADOR) of DEAF and EAF was estimated according to Ribereau-Gayon (1968), using tannic acid as standard and was expressed as g kg⁻¹ of DOR. Laccase activities were assayed according to the method of Saparrat et al. (2000) and the activity was expressed as IU (g⁻¹ DOR). One IU (International Unit) was defined as the amount of enzyme producing 1 μmol product min⁻¹ under the assay conditions.

2.3. HPLC and MS analyses

2.3.1. Materials

HPLC grade methanol and acetic acid were purchased from Carlo Erba (Milan, Italy). HPLC grade water

(18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

2.3.2. HPLC-UV analysis

The ethyl acetate extract was analyzed by HPLC-UV using a SURVEYOR MS micro HPLC (Thermo Finnigan, San José, CA, USA). Individual phenols were separated on a Hypersil BDS C18 column (250 mm × 4.6 mm, 5 μm) (Thermo, Bellefonte, PA, USA) at a flow rate of 1 ml min⁻¹; solvent A was 0.05% acetic acid and solvent B was methanol. After a 2 min hold at 5% solvent B, elution was performed according to the following conditions: from 5% (B) to 10% (B) in 3 min; 20% (B) in 13 min; 30% (B) in 25 min; 50% (B) in 10 min; and 95% (B) in 17 min, followed by 5 min of isocratic elution. The column effluent was monitored at 280 nm.

2.3.3. Electrospray mass spectrometry

Electrospray Ionization/Mass Spectrometry (ESI/MS) in negative ion mode was performed by using a Finnigan LCQ DECA XP Plus ion trap instrument from Thermo Finnigan (San José, CA, USA) equipped with Xcalibur software. Fractions from HPLC analysis were infused in the ESI source by using a syringe pump; the flow rate was 3 ml min⁻¹. The capillary voltage was at -10 V, the spray voltage was at 3 kV and the tube lens offset was at

-10 V. The capillary temperature was 270 °C. Data were acquired in MS1 and MS/MS scanning mode.

The identity of the phenols was assessed by comparing the mass spectra of the peaks separated by liquid chromatography (LC) with those described elsewhere for the phenols naturally occurring in olive oil waste waters (Capasso, 1999; Della Greca et al., 2001).

2.4. Phytotoxicity bioassay

In order to assess whether DEAF and EAF were involved in the DOR phytotoxicity, and potentially utilize the corresponding bioconverted fractions, both the fractions were tested for their effects on *L. sativum* germination in raw form and after the treatment with the selected fungi.

The phytotoxicity bioassay was a slight modification of the method described by Zucconi et al. (1981). EAF and DEAF fractions inoculated or not with the fungi were incubated (25 °C) in the dark for 48 h with *L. sativum* seeds as follows. Twenty seeds were placed in 9 cm diameter Petri dishes lined with filter paper containing 1 ml of each extract undiluted and variably diluted (500, 400, 200, 100, 50, 25, 12.5, 6 and 3 g l⁻¹). Germinability tests conducted in the presence of distilled water (control) were also run in parallel. Incubations were carried out in quadruplicate. The

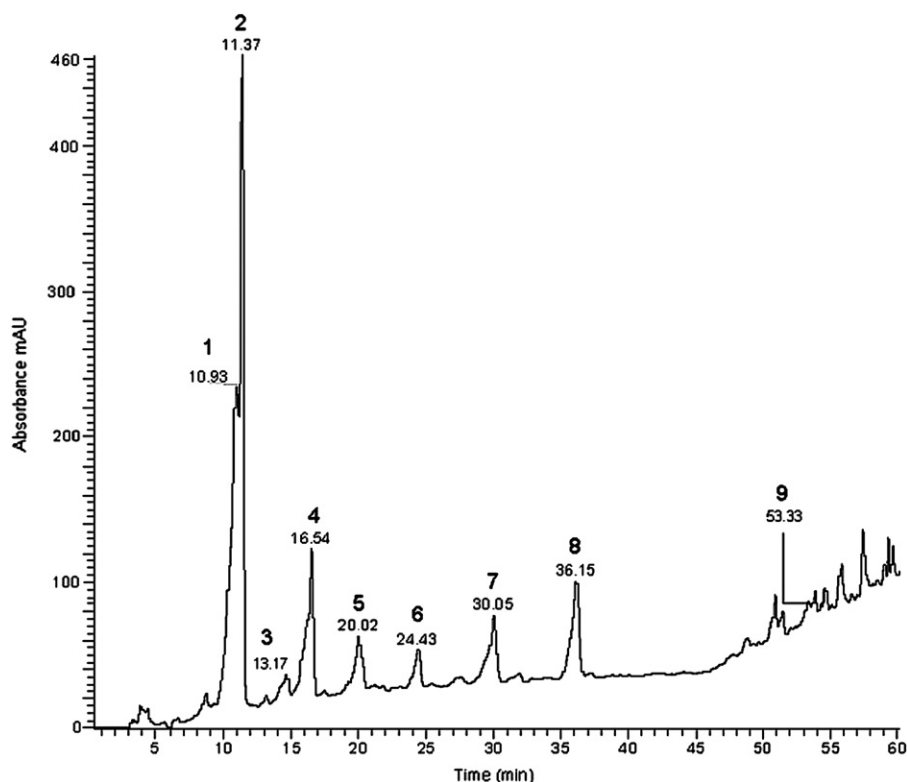
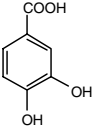
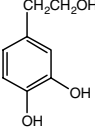

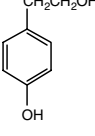
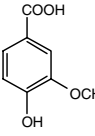
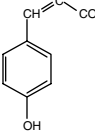
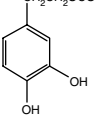
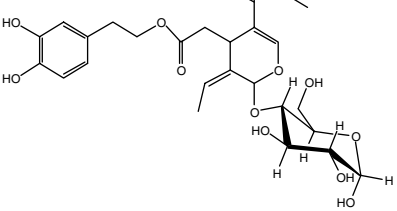


Fig. 1. HPLC-UV chromatogram of phenolic compounds of DEAF (detection at $\lambda = 280$ nm). Peaks: (1) protocatechuic acid, (2) hydroxytyrosol, (3) protocatechol, (4) *p*-tyrosol, (5) vanillic acid, (6) not identified compound (under investigation) (7) *p*-coumaric acid, (8) monoacetylhydroxytyrosol, (9) oleuropein.

Table 1
Common name, chemical structure, mass and retention time (t_r) of the phenols identified in DEAF

| Number | Common name | Chemical structure | Mass | t_r |
|--------|--------------------------|--|--------|-------|
| 1 | Protocatechuic acid |  | 154.12 | 10.93 |
| 2 | Hydroxytyrosol |  | 154.16 | 11.37 |
| 3 | Protocatechol |  | 110.11 | 13.17 |
| 4 | Tyrosol |  | 138.16 | 16.54 |
| 5 | Vanillic acid |  | 168.15 | 20.02 |
| 6 | – | – | 216.14 | 24.43 |
| 7 | <i>p</i> -Coumaric acid |  | 164.16 | 30.05 |
| 8 | Monoacetylhydroxytyrosol |  | 196.16 | 36.15 |
| 9 | Oleuropein |  | 540.51 | 53.33 |

number of germinated seeds was counted and radicle growth measured. The germination index (GI) was calcu-

lated according to the formula $GI = (G/G_0) \times (L/L_0) \times 100$, where G and G_0 are the number of grown

seeds in sample and control and L and L_0 are the average sum of root lengths in sample and control, respectively.

2.5. Statistical treatment of data

The data obtained were subjected to ANOVA. The mean values of four replicates were compared using Tukey test.

3. Results and discussion

The chromatographic separation of the DEAF phenols determined by HPLC and their ESI/MS/MS analyses revealed DEAF to be mainly composed of hydroxytyrosol (2) and, to a lower extent, protocatechuic acid (1), tyrosol (4), monoacetylhydroxytyrosol (8), *p*-coumaric acid (7), vanillic acid (5), (6) not identified compound (under investigation), oleuropein (9) and protocatechol (3) in the order of decreasing quantity (Fig. 1 and Table 1).

The phenolic compounds detected have also been reported as the main phenols in virgin olive oil (Amiot et al., 1986; Romani et al., 2001), olive oil waste-water (Visioli et al., 1999) and DOR (Sampedro et al., 2004a). Hydroxytyrosol naturally occurring in DOR can be regarded as a compound of high added value due to its high antioxidant properties (Visioli et al., 2000; Casalino et al., 2002; Ranalli et al., 2003). Although, diverse synthesis procedures for the production of hydroxytyrosol have been developed (Bai et al., 1998; Espin et al., 2001), the production methods so far proposed are expensive and/or produce low yields. The ethyl acetate extraction of DOR could open a new possible methodology to obtain hydroxytyrosol from two-phase olive waste.

The fungi were able to grow in both the fractions, but it was necessary to add external organic supplements for DEAF (Table 2).

The fungi tested were able to decrease the phenolic content of DEAF and EAF fractions, except for *P. cinnabari-*

mus, *F. sclerodermus* and *P. pulmonarius* in EAF specifically (Table 3) and the decreased phenol content appeared not to be growth associated (Table 2). It has been observed phenol content (total *o*-diphenols) reduction of all the inoculated samples of DEAF up to 0.24 g kg⁻¹ (10-fold for *T. versicolor*) of the original sample (Table 3). This abatement was confirmed by the disappearance of the peaks of the above mentioned phenols by LC-MS analysis (chromatograms not shown). With regard to the EAF content of phenols, results have shown that the phenols strongly decreased in the samples inoculated with *P. radiata*, *T. versicolor* and *C. rigida*, whereas, it remained substantially unchanged in those inoculated with *P. cinnabarinus*, *F. sclerodermus* and *P. pulmonarius* (Table 3).

The total phenol measured in the ADOR extract proved to be 19–20 g kg⁻¹. This value was very similar to that of EAF (Table 3), so the higher concentration of this latter than DEAF (sevenfold) untreated by the fungi was expected.

Several studies have reported the phytotoxic and antimicrobial effects of DOR due to the phenol, organic and fatty acid content (Riffaldi et al., 1993; Linares et al., 2001). In order to establish whether DEAF and EAF were involved in the phytotoxicity of this material and to what extent, they were inoculated with the selected fungi and tested for the effects on *L. sativum*. As shown in Table 4, the DEAF samples non-inoculated, and those inoculated with the selected fungi inhibited the germination of *L. sativum* in the range of concentration between 500 and 25 g l⁻¹, except when inoculated with *T. versicolor* which decreased its phytotoxicity in the range of concentration between 400 and 25 g l⁻¹.

As shown in Table 5, both the EAF non-inoculated and inoculated with the selected fungi inhibited the germination of *L. sativum* in the range between 500 and 200 g l⁻¹. The effective detoxification was shown by the fungi to be active on the sample diluted up to 50 g l⁻¹, except for *P. cinnabarinus* and *P. pulmonarius*, suggesting this concentration

Table 2
Mycelium dry weight expressed in g of the selected fungi growth on DEAF and EAF^a

| Fraction | <i>Phlebia radiata</i> | <i>Trametes versicolor</i> | <i>Corioloopsis rigida</i> | <i>Pycnoporus cinnabarinus</i> | <i>Fomes sclerodermus</i> | <i>Pleurotus pulmonarius</i> |
|----------|------------------------|----------------------------|----------------------------|--------------------------------|---------------------------|------------------------------|
| DEAF | 0.26 a | 0.69 b | 0.81 b | 0.60 b | 2.84 c | 2.80 c |
| EAF | 4.79 b | 2.22 a | 2.43 a | 5.99 b | 4.67 b | 6.57 c |

^a Data are the means of four determinations. Row values followed by the same letter are not significantly different as determined by one way ANOVA followed by the Tukey test ($P = 0.05$).

Table 3
Phenol content expressed in g (kg DOR)⁻¹ as tannic acids equivalents in DEAF and EAF inoculated or not (control) by saprobe fungi^a

| Fraction | Control | <i>Phlebia radiata</i> | <i>Trametes versicolor</i> | <i>Corioloopsis rigida</i> | <i>Pycnoporus cinnabarinus</i> | <i>Fomes sclerodermus</i> | <i>Pleurotus pulmonarius</i> |
|----------|---------|------------------------|----------------------------|----------------------------|--------------------------------|---------------------------|------------------------------|
| DEAF | 2.21 b | 0.34 a | 0.24 a | 0.52 a | 0.80 a | 0.33 a | 0.60 a |
| EAF | 15.14 b | 7.09 a | 7.66 a | 7.58 a | 16.10 b | 16.10 b | 15.12 b |

^a Data are the means of four determinations. Row values followed by the same letter are not significantly different as determined by one way ANOVA followed by the Tukey test ($P = 0.05$).

Table 4
Germination index (%) of *L. sativum* in presence of DEAF inoculated or not (control) with saprobe fungi^a

| DEAF concentration (g l ⁻¹) | Control | <i>Phlebia radiata</i> | <i>Trametes versicolor</i> | <i>Corioloopsis rigida</i> | <i>Pycnoporus cinnabarinus</i> | <i>Fomes sclerodermus</i> | <i>Pleurotus pulmonarius</i> |
|---|----------|------------------------|----------------------------|----------------------------|--------------------------------|---------------------------|------------------------------|
| 500 | 1.2 b | 1.2 b | 1.6 bc | 0.5 a | 0.6 a | 1.4 bc | 1.4 bc |
| 400 | 1.2 ab | 1.7 b | 2.3 d | 0.9 a | 0.9 a | 1.6 b | 0.9 a |
| 200 | 2.1 a | 2.7 ab | 4.3 c | 2.9 ab | 2.2 a | 2.0 a | 2.1 a |
| 100 | 11.35 ab | 19.1 bc | 25.2 c | 6.4 a | 4.3 a | 6.7 a | 11.7 ab |
| 50 | 48.9 bc | 66.6 cd | 73.9 d | 30.7 ab | 16.1 a | 45.4 bc | 56.2 cd |
| 25 | 78.6 ab | 80.8 ab | 97.6 c | 84.0 ab | 59.0 a | 85.3 ab | 82.4 ab |

^a Data are the means of four determinations. Row values followed by the same letter are not significantly different as determined by one way ANOVA followed by the Tukey test ($P = 0.05$).

Table 5
Germination index (%) of *L. sativum* of EAF inoculated or not (control) with saprobe fungi^a

| EAF concentration (g l ⁻¹) | Control | <i>Phlebia radiata</i> | <i>Trametes versicolor</i> | <i>Corioloopsis rigida</i> | <i>Pycnoporus cinnabarinus</i> | <i>Fomes sclerodermus</i> | <i>Pleurotus pulmonarius</i> |
|--|----------|------------------------|----------------------------|----------------------------|--------------------------------|---------------------------|------------------------------|
| 500 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 200 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 0.4 a | 0.8 ab | 1.9 c | 2.1 c | 0.6 ab | 1.2 b | 1.8 c |
| 50 | 1.6 a | 16.7 bc | 19.1 c | 26.9 c | 6.9 ab | 23.8 c | 4.4 a |
| 25 | 32.4 ab | 78.5 c | 81.5 c | 51.9 b | 46.62 ab | 85.6 c | 31.5 ab |
| 12.5 | 72.91 ab | 126.4 c | 82.4 a | 79.1 ab | 86.8 b | 122.1 c | 56.9 ab |
| 6 | 72.6 a | 138.1 b | 116.5 ab | 76.8 a | 96.7 ab | 141.7 b | 71.8 a |
| 3 | 80.9 a | 147.1 bc | 115.7 abc | 93.2 ab | 99.4 ab | 165.6 c | 94.2 ab |

^a Data are the means of four determinations. Row values followed by the same letter are not significantly different as determined by one way ANOVA followed by the Tukey test ($P = 0.05$).

to be a critical one. The most effective detoxification activity of the fungi was evidenced starting from the concentration 25 g l⁻¹ mainly for *P. radiata*, *T. versicolor* and *F. sclerodermus*. This suggests that the incubation of EAF with these fungi provide a residue free of toxicity and rich in organic matter that may be as an organic amendment. In the range of concentration between 12.5 and 3 g l⁻¹, the EAF samples, inoculated with *P. radiata* and *F. sclerodermus* surprisingly stimulated the germinability of *L. sativum*. In particular, the germinability index of EAF, inoculated with these fungi, at the concentration of 3 g l⁻¹ increased to values greater or equal to 100%, compared to the non-inoculated (Table 5). These data suggest that EAF can be used as an organic amendment in the range of the concentration between 12.5 and 3 g l⁻¹, when inoculated by *P. radiata* or *F. sclerodermus*.

Phenols have been considered as being mainly responsible for the phytotoxicity of olive residues (Linares et al., 2003). It has been described that phenols like *p*-coumaric acid inhibit plant growth regardless of their concentration (Li et al., 1993). The same authors indicate that other phenols can inhibit plant growth according to their concentration. The occurrence of synergistic inhibitory effects between phenols has also been shown (Della Greca et al., 2001).

Our data indicate that there were no relationships between the decrease in the phenols content and the phytotoxicity reduction of DEAF and EAF by fungi. In fact, all of the fungi tested were able to decrease the phenol content of DEAF, but only *T. versicolor* induced the reduction of

its phytotoxicity (Tables 3 and 4). On the other hand, *P. radiata*, *T. versicolor* and *C. rigida* induced a strong decrease of the phenols content of EAF but only *P. radiata* caused the decrease of its phytotoxicity (Tables 3 and 5). Furthermore, EAF inoculated with *F. sclerodermus* was able to stimulate the germinability of *L. sativum* but its phenol content was similar to the non-inoculated.

The different behaviour of fungi in the decrease of phytotoxicity of the DEAF and EAF extracts can be attributed to differences in the enzymatic machinery implicated in the degradation of phenolic compounds (Camarero et al., 1994; Giovannozzi-Sermanni et al., 1994). The decrease in monomeric phenols from DOR has been related to the phenoloxidases produced by fungi which transform the monomeric phenols in reactive products, which, in turn, undergo non-enzymatic transformation into polymeric phenols with different phytotoxicity levels (D'Annibale et al., 2004).

Therefore the possibility that the fungi such as *P. cinnabarinus* and *P. pulmonarius* induced the decreasing of the monomeric phenols of DEAF by their transformation into polymeric ones of similar phytotoxicity can not be excluded (Tsioulpas et al., 2002) and will be investigated further (see in correspondence Tables 3 and 4).

The laccase activity observed in DEAF inoculated with *P. radiata*, *T. versicolor* and *P. cinnabarinus* and in EAF inoculated with *P. radiata* and *C. rigida* (Fig. 2) was closely related with the decrease in the levels of phenols observed as a consequence of these treatments, indicating that laccase could be implicated in the degradation of phenols

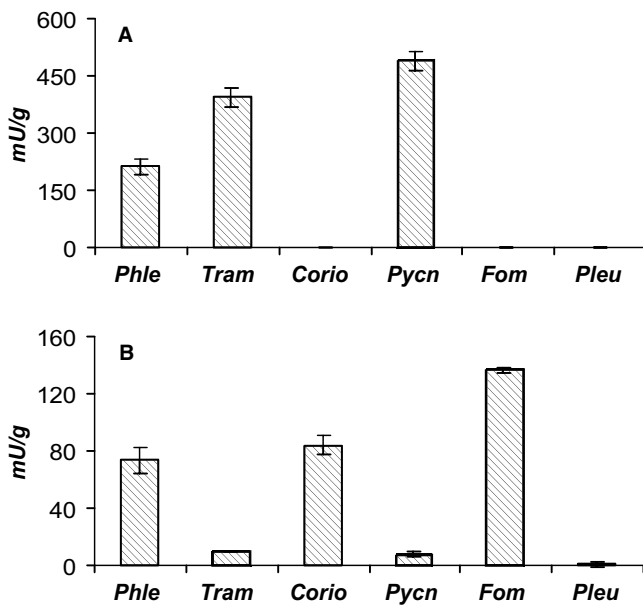


Fig. 2. Laccase activity produced by different saprophytic fungi grown on DEAF (A) and EAF (B) from DOR. Phle (*Phlebia radiata*), Tram (*Trametes versicolor*), Corio (*Coriolopsis rigida*), Pycn (*Pycnoporus cinnabarinus*), Fom (*Fomes sclerodermus*), Pleu (*Pleurotus ostreatus*). Bars show standard errors of means.

from DOR. However, the phenols level was decreased by *C. rigida*, *F. sclerodermus* and *P. pulmonarius* in DEAF and by *T. versicolor* in EAF in spite of the fact that no laccase activity was detected. These findings indicate that phenols removal is not only associated with the laccase activity, but it should be taken into account that other auxiliary degradative mechanisms involving enzymes, and small MW agents, such as hydrogen peroxide, oxalic acid and siderophores, are implicated in the breakdown of aromatic substances brought about by white-rot fungi (Tsioulpas et al., 2002; D'Annibale et al., 2004).

In conclusion, the raw DOR decreases its toxicity by the treatment with fungi as previously reported (Sayadi and Ellouz, 1993) and DEAF and EAF are involved in its toxicity. These fractions can be detoxified after treatment with selected fungi, which exert this process by a complex enzymatic activity.

The native DEAF fraction can be regarded as a source of commercially valuable products, with particular interest for hydroxytyrosol; in addition its recovery from DOR by extraction with ethyl acetate represents a preliminary physical detoxification process of this waste. Data obtained in this research suggest that the EAF fraction can be used as an organic fertilizer after a shortened incubation period with some saprobe fungi and further agronomical research can confirm this statement.

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