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Removal of Monomeric Phenols in Dry Mill Olive Residue by Saprobic Fungi

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The dry olive residue (DOR) obtained from the olive oil extraction process has toxic components against plants and microorganism growth, particularly monomeric phenols. In this investigation nine saprobic fungi were found to be capable of completely removing these phenols from the solid after 20 weeks of growth, although the rate depended on the type of fungi and phenol. Results showed that most of the fungi tested first eliminated *o*-diphenols and then non-*o*-diphenols. However, some fungi did not follow this trend. *Phanerochaete chrysosporium* first removed hydroxytyrosol and tyrosol and later their glucosides and, in contrast, *Paecylomyces farinosus* hydrolyzed hydroxytyrosol and tyrosol glucosides at the first stage, 2 weeks of growth, and then eliminated all monomeric phenols. The behavior of this fungus seems of great interest for recovering phenolic antioxidants from the DOR. Similarly, differences in DOR decolorization capacity among the fungi tested were also observed. *Coriolopsis rigida* showed the highest capacity, followed by *Phebia radiata*, *Pycnoporus cinnabarinus*, and *Pha. chrysosporium*. Therefore, both decolorization and monomeric phenol elimination pointed out that saprobic fungi could be used to detoxify the DOR obtained from the two-phase system of the olive oil extraction process.

KEYWORDS: Dry olive oil residue; phenolic removal; saprobic fungi; decolorization

INTRODUCTION

At present two processes are used for the extraction of olive oil: the two-phase and the three-phase systems. The latter is applied in Italy, Greece, and other Mediterranean countries, whereas the two-phase is widely used in Spain, which is the main olive oil producing country.

Industries working under the three-phase system generate two main residues: a solid waste (olive pomace) and an aqueous liquid known as olive-mill wastewater (OMW), which is a highly pollutant matrix. To reduce this pollution, the new two-phase system was developed during the past decade, and it produces only a solid byproduct called "alpeorujo" (AL). It contains a higher proportion of water than the olive pomace and a great amount of lignin, cellulose, hemicellulose, and phenolic compounds (*1*, *2*). AL can be stored and treated with a second centrifugation by adding fresh water to extract the residual oil, but it produces a new contaminated wastewater as well as a solid residue. Alternatively, AL can be dried and subjected to chemical extraction with hexane, which has been a common practice for the olive pomace of the old two-phase system. This new dry olive mill residue (DOR) can be used for cogeneration of electric power. However, some problems have been raised lately such as the low residual level of oil in the unextracted solid cake, changes in cogeneration subsidies, and the discovery of polycyclic aromatic hydrocarbons in olive oil pomace, which make the study of alternative uses for this solid byproduct necessary.

Several methods have been proposed for olive-mill wastewaters and olive pomace disposal, based on evaporation ponds, thermal concentration, physicochemical and biological treatments, as well as their application to agricultural soils as an organic fertilizer either directly or after a composting process (*3*, *4*). However, olive-mill wastewater and olive pomace contain phytotoxic components capable of inhibiting microbial growth (*5*, *6*) and germination and vegetative growth in plants (*7*). There is a controversy over what the phytotoxic components of the olive residues are. Most researchers have reported a high phytotoxicity against plant and microbial growth by low molecular mass phenols (*8*, *9*), but high molecular mass polyphenols or lignin-like polymers have also shown toxic activity (*10*−*12*) and must also be considered (*7*, *13*). Furthermore, some researchers have not found a relationship between detoxification and removal of monomeric phenols from olive residues (*14*, *15*).

The lignin-degrading ability of white rot fungi seems to be associated with the release of extracellular enzymes, which

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mainly include lignin-peroxidases, Mn-dependent peroxidases, and laccase, and these enzymes could participate both in the removal of monomeric phenols and in the decolorization of olive residues. Pérez et al. (*16*) first described decolorization of OMW by *Phanerochaete chrysosporium*, and they suggested that the decolorization occurred through the breakdown of colored phenolic polymers into monomers, which were subsequently mineralized, but this is not a well-explained process.

Many other researches have studied the depolymerization and dephenolization of the olive residues by saprobic fungi (*17*, *18*), and a highly significant correlation was found between decolorization and laccase production (*19*); however, the same authors did not find a similar correlation in other experiments (*20*). Laccases alone are able to remove monomeric phenols from olive-mill wastewater (*21*), but the decolorization and dephenolization of OMW by fungi seems to be a sequential process: monomeric phenols are first oxidized and polymerized and then the depolymerization and, therefore, decolorization occurred (*22*, *23*).

Soil saprobic fungi are important and common components of the rhizosphere soil from which they obtain nutritional benefits in the form of inorganic compounds and exudates from the root (*24*). These soil fungi are important because they take part in the mobilization of nutrients and degradation of phytotoxic substances, they produce substances that promote or inhibit the growth of other rhizosphere microorganisms, they add great amounts of microbial biomass to the soil, and they also contribute to the optimum use of nutrients by the plant (*24*, *25*).

The objective of this research was to investigate the removal of the monomeric phenols of DOR during its biotreatment with nine saprobic fungi for the first time. Monomeric phenols of olive fruits and derived products have been studied extensively, and researchers have reported hydroxytyrosol and tyrosol as the main polyphenols in olive-mill wastewaters and olive pomace (*26*, *27*). However, the use of ethyl acetate as extraction solution and the difficulties in chromatographic analysis ignored the presence in olive pomace of two important phenolic compounds, hydroxytyrosol and tyrosol glucosides, recently characterized in this byproduct (*28*).

Finally, the effect of saprobic fungi on the color of DOR was also examined.

MATERIALS AND METHODS

Sample Preparation. Dry olive mill residue was collected from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain). The initial moisture content of the solid residue was 10−15%, and it was increased with added water up to 25% prior to the incubation assays.

The saprobic fungi used were *Paecilomyces farinosus* (*29*), *Fusarium oxysporum*-738, *Fusarium lateritum* (*30*), *Coriolopsis rigida* (CECT 20449), *Pleurotus pulmonarius* IJFM A578 (CBS 507.85), *Pycnoporus cinnabarinus* IJFM A720 (CECT 20448), *Phlebia radiata* IJFM A588 (CBS 184.83), *Phanerochaete chrysosporium* IJFM A547 (ATCC 24725), and *Poria subvermispora* IJFM A161. An aqueous suspension of fungal strains in sterile distilled water, containing $\sim 7.5 \times 10^3$ spores/mL of each saprobic fungus, was prepared from cultures grown in potato dextrose agar (PDA: Oxoid) for 1 week at 28 °C. The incubation process was carried out in glass jars containing 500 g of DOR steam-sterilized three times, inoculated or not with 3 mL of the saprobic fungal spore suspension. Each saprobic fungus was inoculated separately. Static incubation was performed at 28 °C for 0, 2, and 20 weeks.

Analysis of Phenolic Compounds. This process was based on the method described by Romero and co-workers (*28*). The phenolic compounds were extracted from the DOR samples (2 g) with a solution of methanol/water (80:20) at 0 °C (30 mL, six times). Methanol was evaporated under vacuum, and the residue was made up to a 25 mL solution with distilled water. Subsequently, a C_{18} cartridge was used to purify the phenolic extract; 1.5 mL of the solution was deposited in the cartridge, and monomeric phenols were eluted with methanol, which were eliminated by vacuum evaporation, and the residue was dissolved in 1.5 mL of a mixture methanol/water (1:1). Finally, the phenolic extract was filtered through a 0.45 μm nylon filter, and 20 μL was injected into the chromatograph.

The HPLC system (Waters model 2690 Alliance, Waters Inc., Milford, MA) consisted of a pump, column heater, and autosampler modules, the detection being carried out with a 996 photodiode array detector. The system was controlled with Millenium^{32} software (Waters Inc.). A 25 cm × 4.6 mm i.d., 5 μm, Lichrosphere 100 (Merck, Darmstadt, Germany) column was used. Separation was achieved by gradient elution using an initial composition of 90% water (pH 2.5 adjusted with 0.15% phosphoric acid) and 10% methanol. The gradient has been described elsewhere (*28*).

Chromatograms were recorded at 280 nm, and compounds were identified by their retention time and absorption spectra from 200 to 400 nm. HPLC-MS was also used for peak identification. Sample extracts were analyzed using a ZMD4 mass spectrometer (Waters Inc.) equipped with an electrospray ionization ion source (ESI). The ion spray mass spectra in the negative-ion mode were obtained under the following conditions: capillary voltage, 3 kV; cone voltage, 20 V; extractor voltage, 12 V; desolvation temperature, 250 °C; and source temperature, 80 °C. A constant flow of 1 mL/min was used for each analysis with an approximately 4:1 split ratio (UV detector-MS).

pH Determination. This parameter was measured in the aqueous extract obtained during the phenolic analysis of DOR samples.

Color of the Dry Olive Residue. The external color was measured using a BYK-Gardner model 9000 color-view spectrometer (Silver Spring, MD). Interference by stray light was minimized by covering samples with a box, which had a matt black interior. Color was expressed in terms of the CIE L^*, a^*, b^* parameters calculated from the absorption spectra. The parameter L^* is a measure of lightness, from completely opaque (0) to completely transparent (100), a^* is a measure of redness (or $-a^*$ of greenness), and b^* is a measure of yellowness (or $-b^*$ of blueness).

RESULTS AND DISCUSSION

Figure 1 illustrates three representative HPLC chromatograms of phenolic extracts from untreated and treated DOR. They revealed that the main monomeric phenols in the DOR were hydroxytyrosol (3,4-dihydroxyphenylethanol), hydroxytyrosol glucoside, tyrosol (4-hydroxyphenylethanol), and salidroside (tyrosol glucoside), the chemical structures of which are shown in **Figure 2**. The presence of these four polyphenols, as well as oleuropein, rutin, and luteolin 7-glucoside, was confirmed by HPLC-MS. Indeed, the concentration of hydroxytyrosol glucoside was the highest among the monomeric phenols in the untreated solid (4792 mg/kg of dry olive pomace), followed by tyrosol glucoside, hydroxytyrosol, and tyrosol at 2014, 1824, and 682 mg/kg of dry olive residue, respectively. Hydroxytyrosol glucoside has also been reported as the main polyphenol in mature olives and OMW (*28*).

We did not detect other previously reported polyphenols in the olive residue by HPLC-MS, such as vanillic, *p*-coumaric, caffeic, and ferulic acids, and vanillin (*21*, *27*). Of course, this could be attributed to differences in raw material (olive variety) and processing, although we think that it could also be due to the hexane extraction and drying steps of DOR processing that could give rise to some polyphenol reduction in the solid. In contrast, most researchers have not found hydroxytyrosol and tyrosol glucosides in olive pomace or olive-mill wastewater because these compounds are not extracted with ethyl acetate, which has been the solvent most commonly used (*28*).

Interestingly, biotreatment of DOR for 20 weeks provoked the elimination of all monomeric phenols in all of the experi-

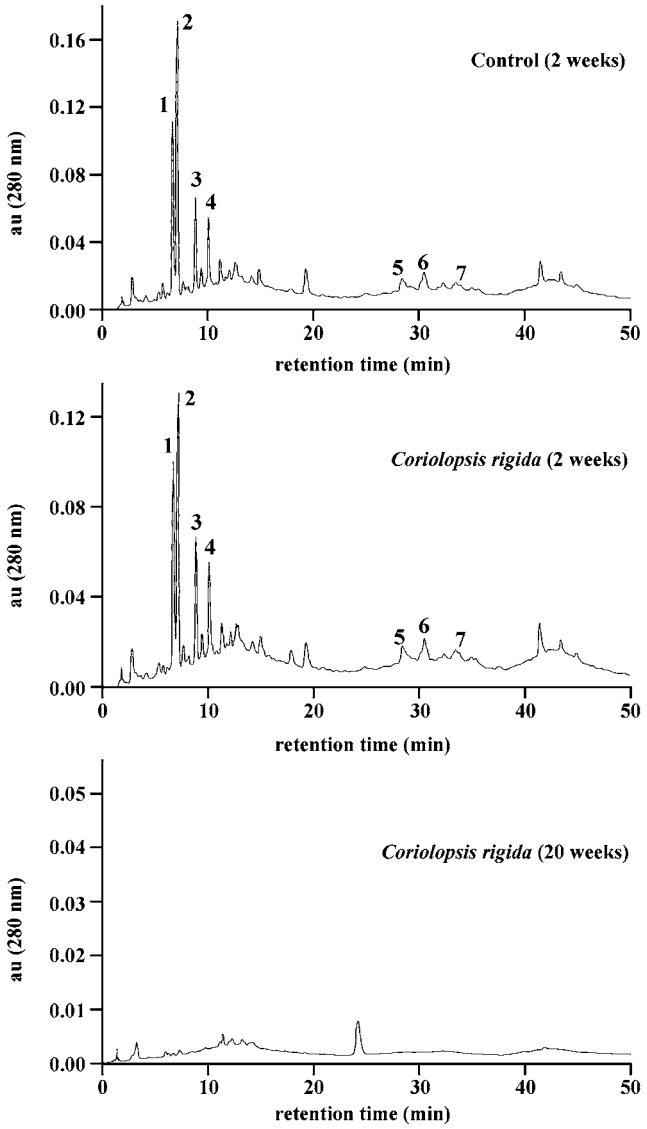

Figure 1. HPLC chromatograms of phenolic compounds in DOR biotreated for 2 and 20 weeks with *C. rigida* and untreated DOR (control). Peaks: (1) hydroxytyrosol 4-β-D-glucoside; (2) hydroxytyrosol; (3) salidroside; (4) tyrosol; (5) luteolin 7-*O*-glucoside; (6) rutin; (7) oleuropein.

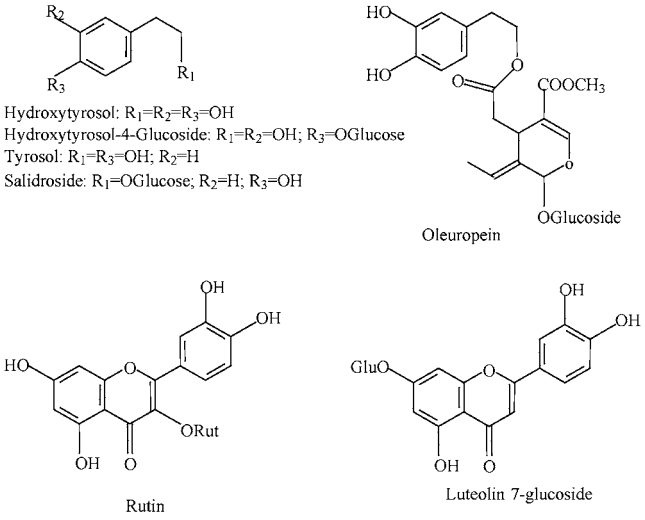

Figure 2. Chemical structure of the monomeric phenols identified in DOR.

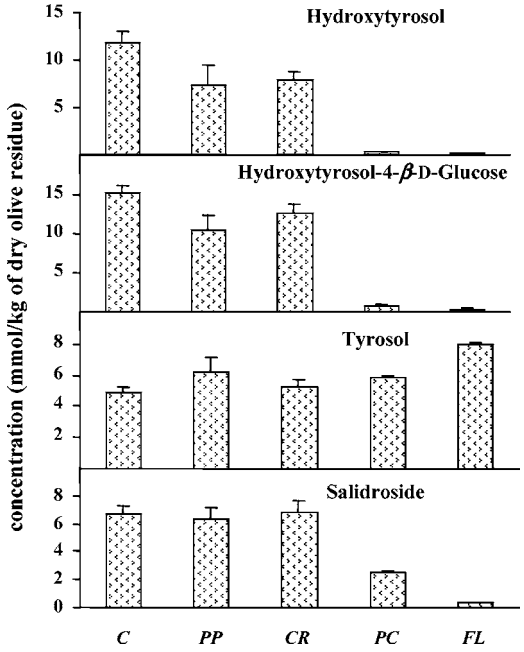

Figure 3. Main monomeric phenols in DOR treated for 2 weeks with *Pl. pulmonarius* (PP), *C. rigida* (CR), *Py. cinnabarinus* (PC), and *F. lateritum* (FL). Data in untreated DOR have also been included (C). Error bars are standard deviation ($n = 2$).

ments carried out (data not shown). This period of time seemed to be long enough to degrade polyphenols in the olive residue, but, in contrast, 2 weeks was too short. It must be stressed that the degradation ability of white-rot fungi is dependent on oxygen and, therefore, the porosity of the matrix is a very important parameter that influences in many cases the developments of the experiments. Hence, some authors found a ~70% decrease of total polyphenols in olive pomace treated for 10 weeks with *Phanerochaete flavido-alba* (*7*), but a similar trend was reported in OMW treated with *Pleurotus ostreatus* for only 2 weeks (*15*). In fact, *Pha. chrysosporium* was more effective than *Pl. ostreatus* in the latter experiment. Therefore, marked differences in polyphenol removal and decolorization of olive byproducts have been reported (*17*). Accordingly, in the present study different behaviors among saprobic fungi on monomeric phenols removal in DOR after 2 weeks of incubation were found.

Py. cinnabarinus and *F. lateritum* almost eliminated hydroxytryosol and its glucoside from the solid, whereas tyrosol was the most recalcitrant polyphenol (**Figure 3**). These results were expected from previous works because *o*-diphenols such as hydroxytyrosol were more rapidly degraded than monophenols in OMW (*20*, *31*), and this tendency was confirmed for most of the fungi tested in DOR.

On the other hand, *Lentinula edodes* took 10 days to eliminate half of the tyrosol content in OMW (*19*), and *Azotobacter vinelandii* needed only 7 days to completely remove this compound from the same substrate (*13*). Among the saprobic fungi tested, only *Pha. chrysosporium* completely removed tyrosol from DOR after 2 weeks of incubation. This fungus has also been found to be superior to others in removing monophenols from maize stover, such as syringic, vanillic, *p*-hydroxybenzoic, and *p*-coumaric acids (*31*). It is supposed that enzymes released by fungi during incubation in olive residues first oxidize and polymerize monomeric phenols but, to our knowledge, there is only one paper describing the transformation of tyrosol during fungus treatment (*32*). These researchers report that tyrosol is converted into a dimeric tetracyclic ketone, but they do not report other further reactions.

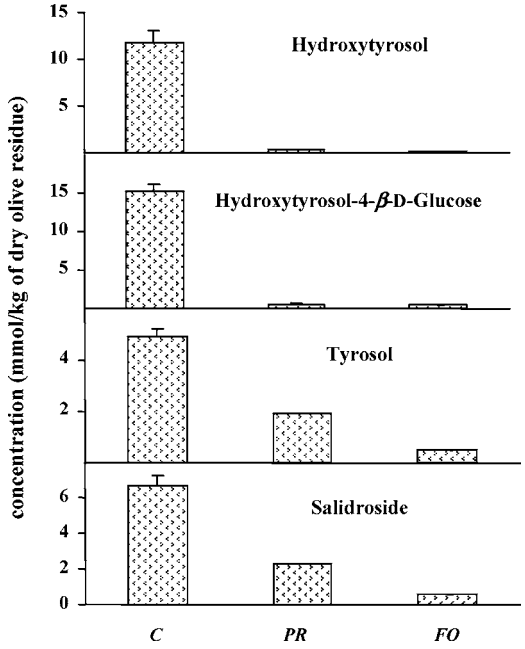

Figure 4. Main monomeric phenols in DOR treated for 2 weeks with *Phl. radiata* (PR) and *F. oxysporum* (FO). Data in untreated DOR have also been included (C). Error bars are standard deviation ($n = 2$).

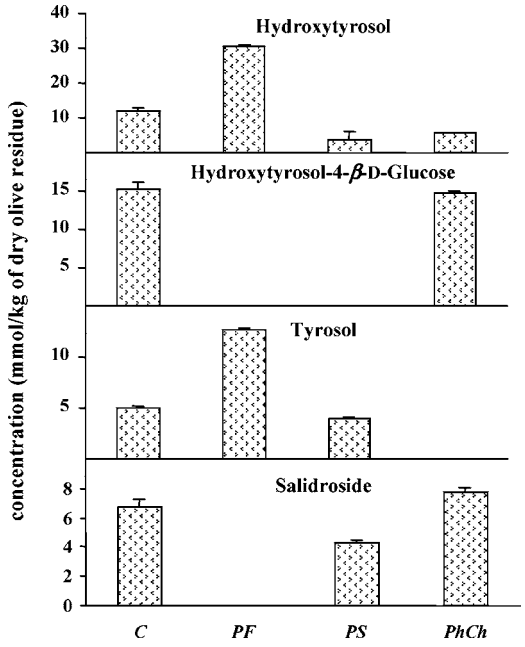

Figure 5. Main monomeric phenols in DOR treated for 2 weeks with *Pa. farinosus* (PF), *Po. subvermispora* (PS), and *Pha. chrysosporium* (PhCh). Data in untreated DOR have also been included. Error bars are standard deviation ($n = 2$).

Pl. pulmonarius and *C. rigida* were slightly active in removing monomeric phenols from DOR, the concentration of tyrosol and its glucoside remaining constant after 2 weeks of incubation and that of hydroxytyrosol slightly decreasing. In contrast, *Phl. radiata* and specially *F. oxysporum* were the most active because they were able to eliminate hydroxytyrosol and its glucoside and most tyrosol and its glucoside (**Figure 4**). As special cases, we have plotted the behaviors of *Po. subvermispora*, *Pha. chrysosporium*, and *Pa. farinosus* in **Figure 5**. The first fungus completely degraded hydroxytyrosol glucoside, 90% of hydroxytyrosol, and, to a lesser extent, tyrosol and its glucoside. Similarly, *Pha. chrysosporium* preferably eliminated

Table 1. Content in Luteolin 7-Glucoside, Rutin, and Oleuropein of Untreated and Treated DOR with Nine Saprobic Fungi for 2 Weeks

sample	luteolin 7-glucoside (mmol/kg)	rutin (mmol/kg)	oleuropein (mmol/kg)
control	0.341 (0.018)[a]	0.378 (0.005)	0.763 (0.065)
Pl. pulmonarius	0.220 (0.058)	0.290 (0.051)	0.399 (0.074)
C. rigida	0.297 (0.052)	0.293 (0.025)	0.429 (0.103)
Py. cinnabarinus	0.069 (0.004)	0.202 (0.017)	0.220 (0.018)
F. lateritum	nd	0.060 (0.003)	0.124 (0.006)
Phl. radiata	0.043 (0.012)	0.130 (0.005)	0.200 (0.015)
F. oxysporum	nd	nd	nd
Pa. farinosus	0.111 (0.048)	0.445 (0.066)	0.524 (0.023)
Po. subvermispora	0.096 (0.001)	0.240 (0.022)	0.391 (0.088)
Pha. chrysosporium	0.418 (0.064)	0.426 (0.071)	0.647 (0.088)

[a] Standard deviation is given in parentheses ($n = 2$). nd, not detected.

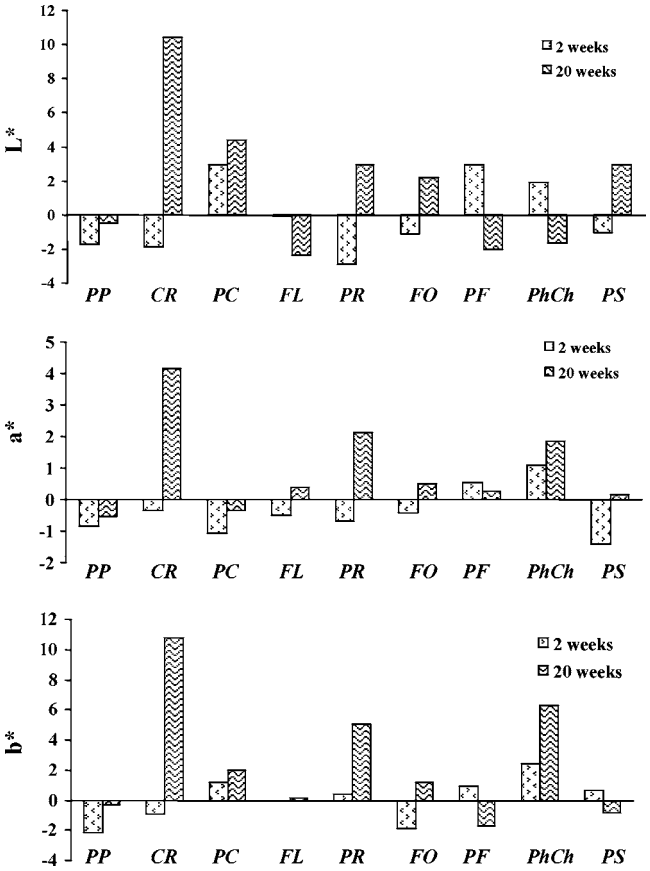

Figure 6. Barrs represent the differences in color parameters (L^*, a^*, b^*) among nontreated and treated DOR with nine saprobic fungi for 2 and 20 weeks: *Pl. pulmonarius* (PP), *C. rigida* (CR), *Py. cinnabarinus* (PC), *F. lateritum* (FL), *Phl. radiata* (PR), *F. oxysporum* (FO), *Pa. farinosus* (PF), *Po. subvermispora* (PS), and *Pha. chrysosporium* (PhCh).

the simple phenols tyrosol and hydroxytyrosol, whereas their glucoside concentrations remained constant. At this point, it must stressed that all monomeric phenols were removed from DOR by all of the saprobic fungi after 20 weeks of incubation. However, the different behavior of the fungi after 2 weeks of incubation is important in order to optimize the future biotreatment of DOR.

The most surprising behavior found was that of *Pa. farinosus*. This fungus hydrolyzed the phenolic glucosides into their simple phenols hydroxytyrosol and tyrosol, as can be observed in **Figure 5**. The amount of hydroxytyrosol in DOR after 2 weeks of incubation with this microorganism was the sum of its initial concentration (12 mM) and that formed from hydroxytyrosol

Table 2. pH of the Aqueous Extract of Untreated and Treated DOR with Nine Saprobic Fungi for 2 and 20 Weeks

time (weeks)	sample	pH
2	control	5.33 (0.04)[a]
2	*Pl. pulmonarius*	5.64 (0.01)
2	*C. rigida*	5.34 (0.04)
2	*Py. cinnabarinus*	6.54 (0.13)
2	*F. lateritum*	7.09 (0.01)
2	*Phl. radiata*	6.87 (0.08)
2	*F. oxysporum*	7.68 (0.06)
2	*Pa. farinosus*	4.85 (0.01)
2	*Po. subvermispora*	5.75 (0.04)
2	*Pha. chrysosporium*	2.60 (0.01)
20	control	5.15 (0.15)
20	*Pl. pulmonarius*	5.93 (0.08)
20	*C. rigida*	5.38 (0.06)
20	*Py. cinnabarinus*	5.45 (0.05)
20	*F. lateritun*	9.21 (0.11)
20	*Phl. radiata*	8.47 (0.11)
20	*F. oxysporum*	7.95 (0.11)
20	*Pa. farinosus*	8.88 (0.05)
20	*Po. subvermispora*	9.03 (0.03)
20	*Pha. chrysosporium*	4.58 (0.01)

[a] Standard deviation is given in parentheses ($n = 2$).

glucoside hydrolysis (15 mM), and the same can be applied for tyrosol. It seems, therefore, that *Pa. farinosus* released a great amount of β-glucosidase enzyme able to break the glycosidic bond of the phenolic glucosides. This result could appear as a scientific curiosity, but it will probably open a new field to use the byproducts AL and DOR as a good source of phenolic antioxidants, in particular, hydroxytyrosol. It has been proposed that the first step to obtain free hydroxytyrosol from OMW or DOR should be the hydrolysis of the glucoside by mineral acids or steam explosion (*2*), and the biotreatment with this fungus could be a very attractive alternative because it is a process with low energy and mineral acid consumption.

As to oleuropein and the flavonoids luteolin 7-glucoside and rutin (**Table 1**), most of the fungi reduced their concentration in DOR after 2 weeks of incubation; these reductions were almost complete after 20 weeks.

It is important to note that all assays were run in duplicate, and changes in DOR polyphenols could be considered as significant because standard error values were in general low.

Monomeric phenols are well-known as toxic substances against plant germination, and the polymeric fraction of OMW and DOR also participates in the toxic activity of these byproducts. Due to the difficulty in characterization of this polymeric fraction, researchers have correlated depolymerization with decolorization of the olive byproducts (*11*, *17*, *19*, *23*) and, in some way, with the detoxification degree. The differences in color (L^*, a^*, and b^* parameters) among untreated and treated DOR for 2 and 20 weeks with the nine saprobic fungi are plotted in **Figure 6**. Overall, most fungi decolorized the solid, the effect being more pronounced after 20 weeks of growth. The highest increase in the color parameters of the biotreated DOR was provoked by *C. rigida*, followed by *Phl. radiata*, *Py. cinnabarinus*, and *Pha. chrysosporium*. A broad screening of 46 fungi on their ability to decolorize OMW disclosed obvious differences among them but also reported the latter four fungi as good in thee decolorization of OMW (*18*). An explanation for the differences in decolorization is not easy, and an attempt to correlate decolorization of OMW with production of extracellular enzymes by fungi failed (*18*). It was found in this work that the pH of DOR biotreated for 20 weeks with *C. rigida*, *Py. cinnabarinus*, and *Pha. chrysosporium* was similar to the control and lower than DOR biotreated by other fungi, except *Pl. pulmonarius* (**Table 2**). It is known that the production of extracellular enzymes by fungi decreases as pH increases (*33*) and, therefore, this could be an explanation for the higher decolorization activity of fungi growing at a low pH.

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