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Bioresource Technology xxx (2006) xxx–xxx

BIORESOURCE TECHNOLOGY

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Solid-state cultures of *Fusarium oxysporum* transform aromatic components of olive-mill dry residue and reduce its phytotoxicity

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Received 23 June 2006; received in revised form 13 November 2006; accepted 13 November 2006
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Abstract

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The present study mainly investigated the ability of solid-state cultures of the non-pathogenic *Fusarium oxysporum* strain BAFC 738 to transform aromatic components to reduce the phytotoxicity in olive-mill dry residue (DOR), the waste from the two-phase manufacturing process. Lignin, hemicellulose, fats and water-soluble extractives contents of DOR colonized by the fungus for 20 weeks were reduced by 16%, 25%, 71% and 13%, respectively, while the cellulose content increased by 25%. In addition, the ethyl acetate-extractable phenolic fraction of the waste was reduced by 65%. However, mass-balance ultra-filtration and size-exclusion chromatography experiments suggested that the apparent removal of that fraction, mainly including 2-(3,4-dihydroxyphenyl)ethyl alcohol and 2-(4-hydroxyphenyl)ethyl alcohol, was due to polymerization. Mn-peroxidase and Mn-independent peroxidase activities were found in *F. oxysporum* solid-state cultures, while laccase and aryl alcohol oxidase activities were not detected. Tests performed with seedlings of tomato (*Lycopersicum esculentum* L.), soybean (*Glycine maximum* Merr.), and alfalfa (*Medicago sativa* L.) grown on soils containing 6% (w/w) of bioconverted DOR (kg soil)$^{-1}$ showed that the waste's phytotoxicity was removed by 20 weeks-old fungal cultures. By contrast, the same material exhibited a high residual toxicity towards lettuce (*Lactuca sativa* L.).
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Keywords: *Fusarium oxysporum*; Dry olive-mill residue; Phenols; Phytotoxicity

23
1. Introduction

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The olive oil industry has a significant environmental impact due to the production of huge amounts of either a highly polluted wastewater or a solid residue, depending on the olive oil extraction process. In the last two decades Spain, the greatest producer of olive oil at a worldwide level has witnessed the increasing replacement of the three-phase extraction process by the so-called two-phase system. The latter technology for olive oil extraction consists of a continuous centrifugation two-phase process that generates a liquid phase (olive oil) and a solid organic waste (alpeorujo), which is then dried and extracted with solvents to obtain an extra yield of oil and a dry residue (DOR) (Vlyssides et al., 1998).
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The two-phase extraction system yields about 800 kg of alpeorujo per ton of processed olives so that it has been calculated that the annual production of DOR in Spain approach four million tons (MAPA, 2002). Thus, the environmental risks associated with the production of huge amounts of DOR require the development of new technological procedures allowing a profitable and environmentally sound use of the waste.

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One of the main drawbacks to the biological degradation and/or upgrading of olive-mill residues by either aerobic or anaerobic processes is due to the presence of phenols, which may occur both in monomeric (Capasso et al., 1992; Sampedro et al., 2004a) and polymeric form (Sayadi et al., 2000). In fact, polyphenols have been shown to be responsible for

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0960-8524/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.biortech.2006.11.015

Please cite this article in press as: Sampedro, I. et al., Solid-state cultures of *Fusarium oxysporum* transform aromatic components of olive-mill dry residue and reduce its phytotoxicity, Bioresour. Technol. (2006), doi:10.1016/j.biortech.2006.11.015

several biological effects, which besides antibiosis (Ramos-Cormenzana et al., 1996) also include phytotoxicity (Capasso et al., 1992).

Due to its high content in organic matter and macronutrients such as potassium, the potential agronomic use of DOR, either as a fertilizer or an amendant, has been suggested, provided that the waste be detoxified through the biological removal of toxic phenols (Vlyssides et al., 1998).

In a previous study, the use of solid-phase extraction followed by liquid chromatography-mass spectrometry analyses led to the identification and quantitation of several phenolic glucosides in DOR, such as luteolin-7-*O*-glucoside, hydroxytyrosol-4-β-D-glucoside, oleuropein and salidroside (Sampedro et al., 2004b). Although these compounds were present in significant concentrations, which ranged from about 15 mmol kg^{-1} DOR (as for hydroxytyrosol-4-β-D-glucoside) to 0.34 mmol kg^{-1} DOR (as for luteolin-7-*O*-glucoside), they were totally o markedly depleted after two weeks incubation with *Fusarium oxysporum* BAFC 738 solid-state cultures (Sampedro et al., 2004b). A successive study showed that despite the extensive metabolization of these compounds, DOR, which had been incubated for two weeks with the same strain still exhibited a high phytoxicity (Sampedro et al., 2004a). In addition, it was shown that 20 weeks of fungal colonization were required to significantly remove toxicity from the waste (Sampedro et al., 2004a).

Thus, the aim of this work was to study the fate of free phenolics and the impact of colonization of *F. oxysporum* BAFC 738 on the overall chemical composition of DOR in order to gain further information on the chemical changes responsible for fungal detoxification. The possible implication of either extracellular phenol-oxidases or intracellular ring-cleaving enzymes was also investigated. In addition, further phytotoxicity tests were performed in order to assess whether the detoxification previously observed might be further extended to other plants of agronomic interest.

2. Methods

2.1. Materials

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 (10–15%, w/w) was increased by adding deionised water up to 25% prior to fungal incubation. Phenolic standards were purchased from Aldrich with the exception of 2-(3,4-dihydroxyphenyl)ethyl alcohol, which was a kind gift of Dr. Neri (University of Tuscia, Viterbo Italy). *Cis,cis*-muconic acid was from Acros Organics (Belgium), while 3-oxo-adipic acid was obtained from Sigma (Milan, Italy). All solvents were HPLC-grade and were obtained from BDH (Milan, Italy).

2.2. Microorganism and culture conditions

The saprobe fungus Fusarium oxysporum (Booth) BAFC Cult. No. 738, was isolated from rhizosphere soil and roots of maize cultivated in the Province of Buenos Aires (Argentina). The isolated fungus was maintained and periodically transferred in potato dextrose agar (PDA) slants at 4 °C. An aqueous suspension of *F. oxysporum* in sterile distilled water, containing approximately 7.5×10^3 spores mL^{-1} was prepared from cultures grown in PDA for one week at 28 °C. Glass jars containing 500 g of steam-sterilized DOR were inoculated with 3 mL of fungal spore suspension and incubated at 28 °C under stationary conditions for 0, 2 and 20 weeks. Appropriate controls were prepared by incubating non-inoculated sterilized DOR under the same conditions used for fungal cultures. From heretofore, these controls will be referred to as incubation controls. Fungal cultures and related inoculation controls were performed in triplicate.

2.3. Analytical determinations

Hemicellulose, cellulose and lignin were determined as previously reported (Giovannozzi Sermanni et al., 1994). Residual fats were determined gravimetrically after Soxhlet extraction with *n*-hexane for 8 h according to Commission Regulation (EEC) N. 2568 (1991). Determination of water-soluble extractives (WSE) was performed according to TAPPI T 207 om-88. Water-soluble phenols (WSP) were extracted from 1 g DOR with 10 mL acidified (pH 4.5) water for 4 h at 15 °C and analysed according to the method of Swain and Hillis (1959) using tyrosol as a standard. Acid-precipitable polymeric lignin (APPL) was obtained from aqueous extracts of DOR by using the procedure described by Crawford and Pometto (1988).

2.4. Biochemical determinations

Extra-cellular enzymes were extracted from DOR at 5 °C for 1 h using a modified procedure described by Criquet et al. (1999). Three grams of DOR were extracted with 30 mL 0.1 M acetate buffer pH 5.0 (buffer A) containing 5 mM $CaCl_2$, 0.05% Tween 80 and 3% insoluble polyvinylpolypyrrolidone. The aqueous suspension was centrifuged (11,000 × *g*, 30 min) and the resulting supernatant was dialy-filtrated with buffer A and 20-fold concentrated on a stirred cell equipped with PM 30 Diaflo membrane (Amicon, USA). The activity of β-glucosidase was determined according to the method of Hayano and Tubaki (1985). Pectinase, *endo*-β-1,4-xylanase, *endo*-β-1,4-glucanase, cellobiohydrolase activities were determined as described elsewhere (Giovannozzi Sermanni et al., 1997). Esterase and lipase activities were determined spectrophotometrically at 35 °C using either β-naphtylacetate or β-naphtylmyristate, respectively, as substrates as described elsewhere (Versaw et al., 1989). Tannin acyl hydrolase was assayed as described elsewhere (Sharma et al., 2000). Mn-peroxidase, Mn-independent peroxidase, laccase and aryl alcohol oxidase activities were assayed according to the method of Saparrat et al. (2000). The method described by Santos and Linardi (2004) was used to extract and subsequently assay catechol-1,2-

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Table 1
Changes in DOR's composition in water-soluble extractives, ash, fats and water insoluble polymers (*i.e.* hemicellulose, cellulose and lignin) along incubation with *Fusarium oxysporum*

Sample	Hemicellulose ($mg\,g^{-1}$ DOR)	Cellulose ($mg\,g^{-1}$ DOR)	Lignin ($mg\,g^{-1}$ DOR)	Fats ($mg\,g^{-1}$ DOR)	Water-soluble extractives ($mg\,g^{-1}$ DOR)	Ash ($mg\,g^{-1}$ DOR)
Incubation control[a]	133 ± 9a	139 ± 8a	255 ± 10a	21.5 ± 3a	365 ± 12a	93 ± 4a
F. oxysporum (two weeks)	136 ± 12a	161 ± 2b	248 ± 13a	20.5 ± 1a	310 ± 15b	116 ± 0b
F. oxysporum (20 weeks)	100 ± 7b	174 ± 11c	213 ± 13b	6.2 ± 0.5b	318 ± 18b	152 ± 8c

Data are the means ± standard deviation of three replicates. Column means followed by the same letter did not significantly differ each one another as assessed by the Tukey test ($P \leqslant 0.05$).
[a] Data reported refer only to two-weeks incubation, since no significant differences were obtained with 20-weeks incubation control.

155 dioxygenase, protocatechuate-3,4-dioxygenase and phenol 156 hydroxylase activities. Appropriate controls were performed 157 with heat-denatured extracts. Enzyme activities were 158 expressed as IU (g DOR)$^{-1}$. One IU was defined as the 159 amount of enzyme producing 1 µmoles product min^{-1} under 160 the assay conditions.

161 *2.5. Chromatographic analyses*

162 High-performance size-exclusion chromatography 163 (HPSEC) of APPL was performed as previously described 164 (D'Annibale et al., 2004). To determine the monomeric 165 composition of the phenolic fraction, the aqueous extract 166 containing WSP (15 mL) was acidified to pH 2.0 with few 167 drops of 6 N HCl and extracted with ethyl acetate 168 (15 mL × 3). Resorcinol (2 mg) was added as an internal 169 standard prior to extraction. The pooled organic phases 170 were dehydrated with Na_2SO_4 and dried under vacuum. To 171 quantify the degradation extent of the main DOR aromatic 172 components, the ethyl acetate residues were dissolved in 173 2 mL of methanol and analyzed by reversed-phase high- 174 performance liquid chromatography (RP-HPLC), as 175 described by D'Annibale et al. (2004).

176 *2.6. Ultra-filtration experiments*

177 Aqueous extracts containing WSP from DOR colonized 178 by the fungus or its related incubation control were sub- 179 jected to ultra-filtration on a stirred cell equipped with a 180 100-kDa PTHMK flat membrane (Waters Millipore, USA) 181 operated in a dialyfiltration mode and the concentration of 182 WSP was determined both in the permeate and in the reten- 183 tate fractions.

184 *2.7. Phytotoxicity experiments*

185 The soil used was a grey loam obtained from the field of 186 the Estación Experimental del Zaidín (Granada, Spain). 187 The soil had a pH of 8.1 in a 1:1 soil:water ratio. The P, N, 188 K, Fe, Mn, Cu and Zn were determined by methods of 189 Lachica et al. (1965). $NaHCO_3$-extractable P was 190 6.2 mg kg^{-1}, N was 2.5 mg kg^{-1}, K was 132 mg kg^{-1}, Fe was 191 9.6 g kg^{-1}, Mn was 110 mg kg^{-1}, Cu was 5.8 mg kg^{-1} and Zn 192 was 5.7 mg kg^{-1}. The soil texture was 358 g kg^{-1} sand, 193 436 g kg^{-1} silt, 205 g kg^{-1} clay and 18 g kg^{-1} organic matter.

194 DOR incubated with *F. oxysporum* for two and 20 weeks 195 and related incubation controls were sterilized and added 196 to soil pots at concentrations of 0 and 60 g kg^{-1} soil.

197 The experiments were carried out in 0.3 L pots contain- 198 ing steam-sterilized soil and quartz sand mixed in a 1:1 199 ratio (v/v). Tomato (*Lycopersicum esculentum* L.), lettuce 200 (*Lactuca sativa*), soybean (*Glycine max*) and alfalfa (*Medi-* 201 *cago sativa*) pre-germinated seeds were selected for unifor- 202 mity prior to planting. Plants were grown in a greenhouse 203 with natural light supplemented by Sylvania incandescent 204 and cool-white lamps giving 400 nmol m^{-2} s^{-1} at 400– 205 700 nm; there was a 16–8 h light–dark cycle at 25–19 °C and 206 50% relative humidity. Plants were watered from below, 207 and fed with a nutrient solution at 10 mL per week (Hewitt, 208 1952). Plants were harvested after 4 weeks and dry matter 209 weight was determined

210 *2.8. Statistical treatment of data*

211 Data obtained were subjected to ANOVA and multi- 212 ple pair-wise comparisons were performed by the Tukey 213 test.

214 **3. Results**

215 Table 1 illustrates the impact of *F. oxysporum* growth on 216 lignin, cellulose, hemicellulose, residual fats and WSE con- 217 tents in DOR. After two weeks fungal incubation, DOR 218 composition did not change markedly with the exception of 219 WSE, which were reduced by about 15%. By contrast, in 220 DOR, where the fungus was grown for 20 weeks, both lig- 221 nin, hemicellulose and fats were degraded by about 16% 222 and 25% and 71%, respectively. In contrast, cellulose con- 223 tent apparently increased by about 25%, presumably due to 224 to the lower susceptibility of cellulose to biodegradation 225 than the hemicelluloses and thus resulting in a shift in the 226 relative proportion of biopolymers.

227 Table 2 shows that the WSP content in DOR incubation 228 controls was 9.9 mg (g DOR)$^{-1}$ dry weight and that ethyl 229 acetate-extractable phenols amounted to about 74% of 230 WSP. In addition, a mass-balance performed by ultra-filtra- 231 tion showed that the percent abundance of WSP contents 232 in MW (molecular weight) fractions higher than 100 kDa 233 (F1) and lower than 100 kDa (F2) were 18.9% and 81.1%, 234 respectively (Table 2).

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Table 2
Water-soluble phenols (WSP) contents in aqueous and ethyl acetate extracts and percent relative abundances of WSP in F1 (MW > 100 kDa) and F2 (MW < 100 kDa) aqueous fractions in DOR incubation controls (two weeks) and DOR colonized by *Fusarium oxysporum* for two and 20 weeks

Sample	Aqueous extract (mg g^{-1} DOR)	Percentage of relative abundance of:		Ethyl acetate extract (mg g^{-1} DOR)
		F1 fraction	F2 fraction	
Incubation control	9.87[a]	18.9[a]	81.1[a]	7.32[a]
F. oxysporum (two weeks)	10.60[a]	22.4[a]	77.6[a]	6.56[a]
F. oxysporum (20 weeks)	9.16[a]	53.1[b]	46.9[b]	2.57[b]

Column means followed by the same superscript letter did not significantly differ, as assessed by the Tukey test ($P = 0.05$).

F. oxysporum did not significantly affect the total WSP content in DOR, regardless of the incubation time. However, ethyl acetate-extractable phenols in DOR, where a 20-week-long fungal growth had occurred were reduced by 65%. In addition, the relative abundances of F1 and F2 fractions of WSP were shifted with respect to the incubation controls. In particular, the percent abundance of the F1 fraction increased from 18.9 (incubation control) to 53.1%. By contrast, the F2 fraction decreased from 81.1% (incubation controls) to about 47% (Table 2).

APPL content in incubation controls was as low as 20 mg (g DOR)$^{-1}$ and accumulated along the fungal incubation (44 and 112 mg (g DOR)$^{-1}$ after two and 20-weeks, respectively).

SEC–HPLC analysis of APPL from DOR incubation control showed the presence of a main MW fraction with K_{av} 0.279 (MW_{app} 36 kDa) and two minor ones with K_{av} 0 ($MW_{app} \geqslant 250$ kDa) and 0.53 ($MW_{app} = 9.3$ kDa), respectively (Fig. 1a). The MW distribution in APPL from DOR, where *F. oxysporum* had grown for 2 weeks was modified. In fact, the main fraction was shifted towards a lower K_{av} value (0.256) and higher MW_{app} (68 kDa) (Fig. 1b). In addition, an increase in absorbance of the MW fraction eluted at the void volume was observed. The SEC–HPLC profile of APPL recovered from 20-weeks-old fungal cultures showed the presence of a main peak with K_{av} 0.262 and MW_{app} 64 kDa (Fig. 1c).

Nine aromatic compounds, two of which, including 3-(3,4-dimethoxyphenyl)-2-propenoic acid and 3-(4-methoxyphenyl)-2-propenoic acid, being non-phenolics, were identified in DOR. Their quantitation, performed by RP-HPLC analyses, showed that the most abundant components in DOR incubation control were 2-(3,4-dihydroxyphenyl) ethyl alcohol, 2-(4-hydroxyphenyl)ethyl alcohol and 1,2-benzenediol, the concentrations of which were 10.4, 6.4 and 1.5 μmoles (g DOR)$^{-1}$ (Table 3). DOR colonization by *F. oxysporum* for two weeks resulted in a partial reduction of some monocyclic components. In 20-weeks DOR samples colonized by the fungus, 2-(3,4-dihydroxyphenyl)ethyl alcohol, 1,2-benzenediol, 3-(3,4-dimethoxyphenyl)-2-propenoic acid and 3-(4-methoxyphenyl)-2-propenoic acid were totally depleted, the most recalcitrant compounds being 2-(4-hydroxyphenyl)ethyl alcohol and 3-(4-hydroxyphenyl)-2-propenoic acid (Table 3).

Extra-cellular enzyme production on DOR by F. oxysporum. Table 4 shows the extra-cellular enzyme composition in two and 20-week-old *F. oxysporum* solid-state cultures on DOR. Several hydrolytic activities involved in the depolymerization of plant cell wall polysaccharides were detected. With regard to these polysaccharidases, the highest activities were observed in two-week-old solid-state cultures *endo*-β-1,4-xylanase, *endo*-β-1,4-glucanase and cellobiohydrolase, which were found to be 1.48, 1.0 and 1.2 IU (g DOR)$^{-1}$. With regard to the other hydrolases, lipase and esterase activities increased with the incubation time reaching 0.05 and 0.18 IU (g DOR)$^{-1}$, respectively. By contrast, tannin acyl hydrolase and pectinase, the activities of which were detected in two-weeks-old fungal cultures were not found after 20 weeks incubation.

Besides hydrolases, two lignin-modifying oxidase activities were detected and included Mn-dependent peroxidase and Mn-inhibited peroxidase (Table 4). In contrast, nor laccase neither aryl alcohol oxidase activities were found, regardless of the incubation time. In addition, some intracellular activities involved in the metabolization of aromatics and including catechol-1,2-dioxygenase, protocatechuate-3,4-dioxygenase and phenol hydroxylase activities were not detected (data not shown).

Effect of F. oxysporumon DOR phytotoxicity. DOR incubation control, added at a rate of 60 g (kg soil)$^{-1}$, proved to be highly phytotoxic towards tomato, lettuce, soybean and alfalfa seedlings. With the exception of alfalfa, chlorotic symptoms were observed in all plants that had been grown on either untreated or DOR incubation controls. By contrast, under the same conditions, necrosis was observed only on tomato leaflets. With regard to growth, plant biomass production in the presence of that amount of waste was dramatically reduced and the extent of phytotoxicity did not significantly ($P = 0.05$) decrease throughout the incubation period (Table 5). For instance, shoot dry weights of tomato and lettuce plants grown in the presence of 20 weeks DOR incubation control were reduced by about 93% and 89% with respect to plants grown in soil without DOR. Regardless of the plants tested, the phytotoxicity of DOR, where the fungus had been grown for two week, was not reduced at all. By contrast, an evident detoxification was evident when growing tomato and soybean plants in soil in the presence of 6% DOR, where the fungus was grown for 20-weeks. In particular, shoots dry weight of soybean plants were significantly higher than those of plants grown in the absence

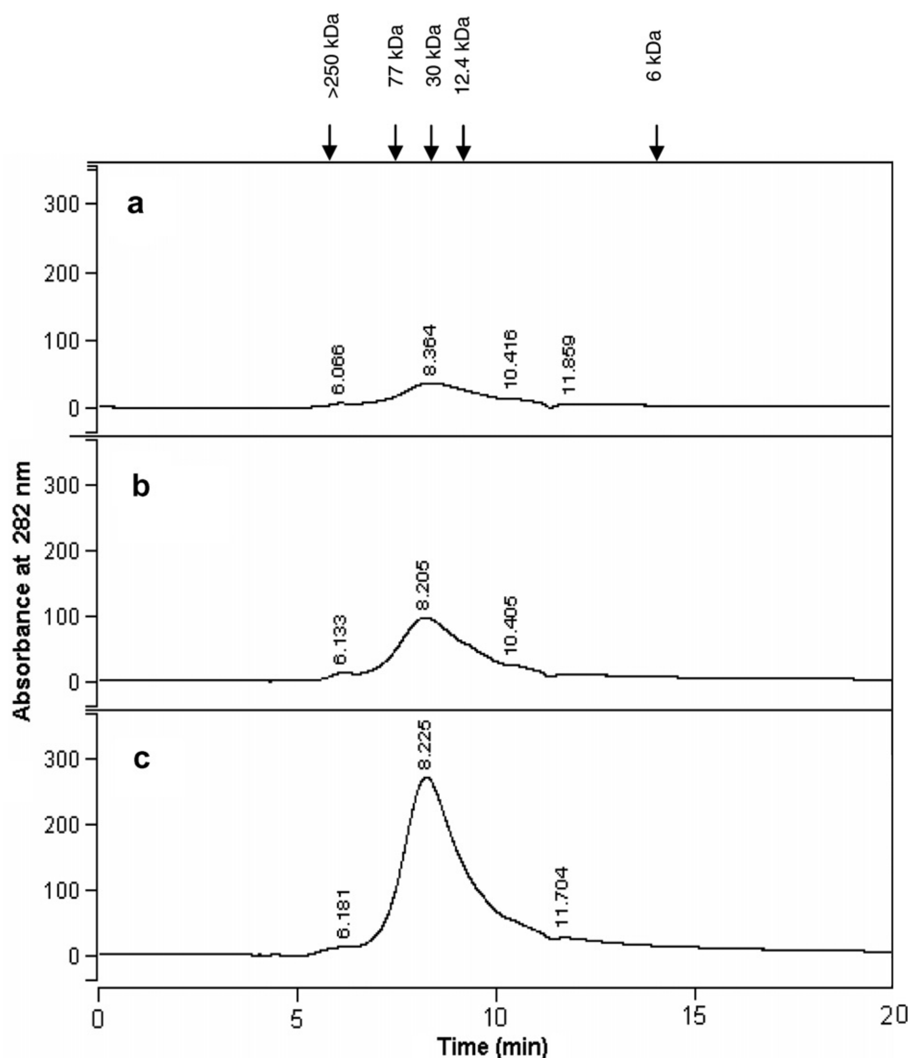

Fig. 1. SEC–HPLC profiles at 282 nm of APPL recovered from either DOR incubation control (a) or treated with *Fusarium oxysporum* for two weeks (b) or 20 weeks (c). The column was calibrated with the following standards: blue dextran (>250 kDa, $R_T = 6.06$ min); ovotransferrin (77 kDa, $R_T = 8.05$); carbonic anhydrase (30 kDa, $R_T = 8.39$ min); cytochrome c (12.4 kDa, $R_T = 9.22$ min) and aprotinin ($R_T = 14.22$ min, 6 kDa). Elution times of molecular weight standards are indicated by the arrows above plots.

Table 3
Concentration of phenolic compounds (expressed in μmoles g^{-1}) in DOR incubation control (two weeks) and after two and 20 weeks incubation with *Fusarium oxysporum*

Parameter	DOR incubation control	*F. oxysporum* on DOR	
		two weeks	20 weeks
2-(3,4-Dihydroxyphenyl)ethyl alcohol	10.4 ± 0.9	6.88 ± 0.3	n.d.
1,2-Benzenediol	1.5 ± 0.1	n.d.	n.d.
2-(4-Hydroxyphenyl)ethyl alcohol	6.4 ± 0.2	5.88 ± 0.3	0.3 ± 0.03
4-Hydroxy-3-methoxybenzoic acid	0.02	n.d.	0.01
4-Methyl-1,2-benzenediol	0.3 ± 0.02	0.08 ± 0.01	0.05
3-(4-Hydroxyphenyl)-2-propenoic acid	0.09 ± 0.004	0.06 ± 0.005	0.04 ± 0.02
3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid	0.16 ± 0.04	0.04	0.03
3-(3,4-Dimethoxyphenyl)-2-propenoic acid	0.3 ± 0.02	0.4 ± 0.05	n.d.
3-(4-Methoxyphenyl)-2-propenoic acid	0.02	0.02	n.d.

n.d. – Not detected.

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327 of the waste (91.6 *vs.* 71.6, respectively). The lowest extent 328 of detoxification was observed for lettuce grown on 6% 20-329 week-old DOR, the biomass of which, albeit being higher than the related incubation control (60.9 *vs.* 22 mg, respec- 330 tively), was lower than that obtained in the absence of 331 DOR (Table 5). 332

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Table 4
Extracellular enzyme activities produced by *Fusarium oxysporum* after two and 20 weeks incubation on dry olive-mill residue

Enzyme	Enzyme activity IU (g DOR)$^{-1}$	
	Two weeks	20 weeks
Pectinase	0.27	n.d.
Endo-β-1,4-xylanase	1.48	0.75
Endo-β-1,4-glucanase	1.0	0.86
Cellobiohydrolase	1.2	1.17
β-Glucosidase	0.07	0.07
Lipase	0.03	0.05
Esterase	0.01	0.18
Tannin acyl-hydrolase	7.3×10^{-3}	n.d.
Mn-peroxidase	5×10^{-3}	3×10^{-3}
Mn-inhibited peroxidase	5×10^{-3}	1×10^{-3}
Laccase	n.d.	n.d.
Aryl-alcohol oxidase	n.d.	n.d.

Data are the means of three determinations. Standard deviation of data was less than 7%.
n.d. – Not detected.

333 ## 4. Discussion

334 Several species belonging to the genus *Fusarium* have 335 been isolated from both soils and industrial effluents char- 336 acterized by the presence of either phenols or aromatic 337 hydrocarbons (Atagana, 2004; Santos and Linardi, 2004). 338 These isolates have been shown to be highly effective in the 339 detoxification of solid and liquid matrices contaminated by 340 phenols (Atagana, 2004; Mendonça et al., 2004). For this 341 reason, these fungi appear to be good putative candidates 342 to perform the detoxification of a polyphenols-containing 343 solid waste, such as DOR.

344 In the present study, *F. oxysporum* BAFC 738 was found 345 to be able to grow on DOR under static conditions and in 346 the absence of both nutritional supplements and forced aer- 347 ation. Total WSP content in DOR was not significantly 348 affected by *F. oxysporum* after two weeks growth, with the 349 exception of a slight reduction of ethyl-extractable phenols.

350 The impact of fungal colonization on DOR's aromatic 351 compounds was evident after 20-weeks incubation. The 352 65% reduction of ethyl acetate-extractable phenols 353 appeared to be due to their polymerisation rather than to 354 ring cleavage reactions. This could be inferred by the nota- 355 ble increase in the relative abundance of the phenolic MW 356 fraction above 100-kDa (fraction F1) with respect to the 357 related incubation control. Both RP-HPLC and GC–MS 358 analyses did not show the presence of typical ring fission 359 products such as *cis,cis*-muconic acid, carboxymuconic 360 acids and their related lactone derivatives and 3-oxo-adi- 361 pate (data not shown). In addition, the activities of some 362 intra-cellular enzymes involved in the hydroxylation and 363 ring cleavage of substituted phenols, were not detected, 364 regardless of the incubation time. However, it is worth men- 365 tioning that some studies reported the capability of several 366 *Fusarium* species to grow on phenols as the sole carbon 367 source and detected catechol-1,2-dioxygenase or protoca- 368 techuate-3,4-dioxygenase activity suggesting the presence 369 of the ring fission pathway of the *ortho*-type (Santos and 370 Linardi, 2004).

371 The marked increase in acid-precipitable polymeric lig- 372 nin (APPL) content in DOR, where *F. oxysporum* had 373 grown for 20 weeks with respect to the incubation control 374 and the change in the MW distribution could be attributed 375 either to a partial fragmentation of lignin or to the forma- 376 tion of oligomers through oxidative coupling of monocyclic 377 phenolic components triggered by some extracellular oxi- 378 dases (Crawford and Pometto, 1988; Giovannozzi Ser- 379 manni et al., 1991). However, it has been previously shown 380 that APPL, being a water-soluble plant cell wall degrada- 381 tion intermediate, is a lignin-carbohydrate complex rather 382 than lignin alone (Crawford and Pometto, 1988; Gio- 383 vannozzi Sermanni et al., 1991). Therefore, the above men- 384 tioned increase in the APPL content might be due to a 385 concerted action of both polysaccharidases and lignin- 386 modifying enzymes.

387 As a matter of fact, Mn-peroxidase and Mn-indepen- 388 dent peroxidase activities were detected in *F. oxysporum* 389 solid-state cultures on DOR (present study). By contrast, 390 neither laccase nor aryl alcohol oxidase activities were 391 detected. It is widely known that phenols oxidation, cata-

Table 5
Shoot dry weights (mg) of tomato (*Lycopersicum esculentum* L.), lettuce (*Lactuca sativa*), soybean (*Glycine max*) and alfalfa (*Medicago sativa*) plants grown either in control soil (Pot minus DOR) or in soil amended with 6% (w/w) DOR incubation control or colonized by *Fusarium oxysporum* for variable incubation times (0, 2 and 20 weeks)

Plant	Treatment	Pots minus DOR	Incubation time of DOR (weeks)		
			0	2	20
Tomato	Incubation control	146.5^{b}	9.4^{a}	9.8^{a}	9.3^{a}
	F. oxysporum	148.3^{b}	10.1^{a}	16.6^{a}	182.5^{b}
Lettuce	Incubation control	176.5^{c}	19.3^{a}	18.1^{a}	22.2^{ab}
	F. oxysporum	177.9^{c}	17.3^{a}	50.7^{ab}	60.9^{b}
Soybean	Incubation control	71.6^{c}	33.9^{a}	32.8^{a}	32.4^{a}
	F. oxysporum	74.6^{c}	41.0^{ab}	52.6^{b}	91.6^{d}
Alfalfa	Incubation control	56.1^{cd}	17.4^{a}	19.2^{a}	18.7^{a}
	F. oxysporum	58.3^{d}	20.1^{a}	29.5^{b}	48.9^{c}

For each plant, values within a row sharing the same superscript letter did not significantly differ as determined by the Tukey test ($P = 0.05$).

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lyzed or mediated by these enzymes, generates aryloxyradicals, which are susceptible to undergo non-enzymatic coupling reactions with the consequent formation of oligomers. Several studies have reported the occurrence of lignin-modifying oxidases in *Fusarium* sp. liquid cultures (Regalado et al., 1999; Saparrat et al., 2000) and a laccase-like phenoloxidase has been purified and partially characterized (Curir et al., 1997). The partial transformation of monocyclic phenols into polymeric products in 20-weeks-old cultures might be the basis for the reduction in phytotoxicity of the waste mainly observed towards tomato and soybean plants. In fact, it has been suggested that the enhanced degree of polymerisation of phenoloxidases reaction products with respect to their parent compounds might result in a dramatic decrease in their accessibility *via* the plant cell membrane (Hulzebos et al., 1991). As a matter of fact, quantitative structure/activity relationships (QSAR) studies have shown that the toxicity of phenols is mainly correlated with their lipophilicity expressed by the octanol/water partition coefficient $K_{o/w}$, a parameter, which is generally in strict relation with the ability of compounds to pass through the biological membrane (Wang et al., 2001). In general, phytotoxicity of monomeric phenols towards higher plants is mainly exerted *via* alterations of water uptake (Lyu and Blum, 1990), metabolism of phytohormones, such as auxins (Tomaszewski and Thimann, 1966) and abscissic acid (Li et al., 1993) and photosynthesis (Mersie and Singh, 1993).

It is worth pointing out that DOR and its incubation controls, albeit used as amendants at a 6% concentration, significantly depressed biomass production of tomato, lettuce, soybean and alfalfa plants, as it is illustrated in Table 5. This study confirmed that two weeks incubation with *F. oxysporum* did not remove phytotoxicity, regardless of the plants tested. A high residual toxicity toward lettuce plants was still evident, when 20-week-old fungal-treated DOR was used. This is not surprising due to the high susceptibility of lettuce to inhibition by phenols, which makes it an optimal test plant in allelopathy (Yamane et al., 1992) and QSAR studies (Hulzebos et al., 1991). By contrast, the phytotoxicity of DOR towards tomato and soybean was completely suppressed by incubation with *F. oxysporum* for 20 weeks.

These data suggest that the aerobic treatment with the non-pathogenic *F. oxysporum* strain BAFC 738 is able to reduce phytotoxicity of DOR and that, albeit at distinct extents, detoxification is observed with several plants of agronomic interest. More detailed studies will be required to explain the significant increase in biomass shoots observed for soybean plants grown on soil amended with 6% fungal-treated DOR.

Acknowledgements

This study was financed by the Comisión Interministerial de Ciencia y Tecnología (CICYT) through projects AGL2004-00036 and CO3-048.

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