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Detoxification of semisolid olive-mill wastes and pine-chip mixtures using *Phanerochaete flavido-alba*

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Abstract

Semisolid olive-mill residues, pine chips, and mixtures of both residues contain phytotoxic components capable of inhibiting germination and vegetative growth in plants. Solid-state cultures of *Phanerochate flavido-alba* on pine chips or mixtures of both residues reduce these phytotoxic effects in fermented substrates. The phenol and lipid contents in cultures detoxified by this fungus also decreases.

Keywords: *Phanerochaete flavido-alba*; *Phanerochaete*; Solid-state fermentation; Olive-oil residue; Pine chips; Pinus

1. Introduction

Olive-oil production is of great economic importance to the Mediterranean area. At present two processes are used for the extraction of olive oil: the ''three-phase'' and the ''two-phase''. Both systems generate large quantities of residue. Installations that recover olive oil via the ''three-phase system'' produce two main residues: a solid cake, and large amounts of an aqueous liquid known as olive-mill wastewater (OMW). In the ''two-phase'' system the volume of OMW produced is reduced because less water is used and much of that water and toxic substances are held within the solid olive cake, thus producing a semisolid residue (SOR). Both OMW and SOR contain phenols and olive lipids, and unless correctly disposed of constitute serious environmental hazards. The phenols contained within OMW are known to inhibit plant growth and furthermore are not easily colonized by bacteria (cf. Martínez et al., 1998). Recently there has been a significant increase in the number of olive mills using the ''two-phase system'' but still no reliable information is available concerning the properties of SOR, although extrapolations have been made from data relating to OMW from mills using the ''three-phase system''.

Efforts to decrease the environmental impact of olive-mill wastes include composting both the semisolid and liquid wastes, but the phytotoxic components in these wastes have led to only limited success. To detoxify SOR and make it suitable for agricultural use further investigations must be made into its toxic components, their effects, and efficient detoxification procedures. Information about the co-composting of OMW with agricultural lignocellulose residues has been published (Paredes et al., 2000) but no information is so far available about SOR composting.

Pine wood contains lipids (triglicerides, diglicerides, resin acids, long-chain free fatty acids and lesser quantities of sterols in *Pinus sylvestris*, Bosh-González et al., 1999; Dorado et al., 2000b), which all contribute to the toxicity of pine wood (Martinez-Iñigo et al., 1999).

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A number of authors have reported on white-rot fungal (WRF) growth on woody tissues in solid-state fermentations on pine-chip (PC) fermentations, with a decrease in their lipid content and overall toxicity (Gutierrez et al., 1999; Dorado et al., 2000a).

A few authors have dealt with the properties of OMW and the effect of WRF on these wastes (reviewed in Martínez et al., 1998). Since white-rot fungi detoxify OMW and lignocellulose materials, it would seem feasible than a WRF solid-state fermentation of SOR/PC mixtures might also detoxify these substrates. The inclusion of PC in the mixtures provides nutrients and enhances gas exchange and water retention, thus facilitating the degradation of the toxic compounds. Nevertheless, no studies have been published to date on the growth of WRF on SOR/PC mixtures with a view to detoxifying SOR.

It is known that *Phanerochaete flavido-alba* decolours, dephenolizes and detoxifies OMW (Ben Hamman et al., 1999) and that manganese peroxidase and laccase probably participate in this process (Perez et al., 1998; Ruiz et al., 2002). We describe here how *P. flavido-alba* cultures reduce the phenol and lipid contents of *Pinus halepensis* chips and SOR/PC mixtures and at the same time decrease their phytotoxic effects.

2. Materials and methods

2.1. Starting material

P. halepensis sapwood, hardwood and bark from pruning residues from the Sierra de Baza Nature Reserve (Granada, Spain) were milled and sieved to obtain 5 mm PC. SOR was provided by the "Casa Titos" olive mill, Granada, Spain. Table 1 shows the components in the starting material. Both PC and SOR were frozen at −20 °C until use. As a basis for the inocula some dry PCs were sterilized at 121 °C for 1 h. Before use in the cultures the PC were washed and sterilized at 121 °C for 1 h (thus increasing their water content to 70–80%). Each culture contained 264 g of milled PC (844 g after washing).

Table 1
Components in the starting material: *P. halepensis* chips and semisolid olive residue (SOR)

Component	Pine chips	SOR
Water (%)	13.5 ± 1.5	60.1 ± 0.5
C (%)	49.1 ± 0.6	50.5 ± 0.1
N (%)	0.26 ± 0.02	1.6 ± 0.1
H (%)	0.9 ± 0.11	7.4 ± 0.2
Phenolics[a]	0.39 ± 0.01	6.98 ± 0.42
Lipids[a]	13.5 ± 1.5	108.8 ± 0.13
Acid insoluble substances[a]	384 ± 27	507 ± 62

[a] As mg per gram dry weight. The acid-insoluble substances were quantified according to Klason lignin determination.

2.2. Inocula

Inocula were obtained from colonized PCs. *P. flavido-alba* FPL106507 was cultured in 100 ml YMPG medium (Bonnarme and Jeffries, 1990) in 1 l Erlenmeyer flasks, without shaking, at 30 °C for eight days. The cultures were then homogenized under sterile conditions in a blender and the homogenates (7 ml) used to inoculate a suspension of 95 g of washed and sterilized PC in 64 ml YMPG. Colonized PC was obtained after 30 days at 30 °C.

2.3. Cultures

Cultures were made by inoculating 10% colonized PC onto either washed PC substrate plus 43 ml extra water or SOR (132 g) plus PC (SOR/PC). The cultures were shaken manually and aerated every 24 h with sterile O_2 (Ben Hamman et al., 1999). After 60 days' culture at 30 °C the solid material was recovered and kept at −20 °C for chemical, antibacterial and phytotoxic studies. Abiotic replicas to be used as controls were made under the same conditions. The data represent mean values with standard deviations for three cultures and controls.

2.4. Determinations

The dry weight of 10 g aliquots was determined after drying at 105 °C for 24 h.

The content in phenols was determined in aqueous extracts (2 g culture material per 20 ml water). Suspensions were homogenized in a Gallenkamp orbital shaker (200 rpm for 30 min). The insoluble material was recovered by filtration through miracloth and then through 0.45 μm membrane filters. The total phenol content of the filtered solutions was estimated spectrophotometrically (Ribéreau-Gayon, 1968) using syringic acid as standard.

One gram of dried material was extracted with hexane for 6 h (soxhlet). The hexane was evaporated (105 °C) and the lipid content of the extracted material was measured gravimetrically.

The acid-insoluble content was determined according to Tappi's method (1993) (cf. Camarero Fernández, 1995). In brief, solid, dry, milled material was extracted with ethanol/toluene and measured gravimetrically (extractives). The water-soluble components were then extracted and measured gravimetrically. Finally, the dry material was digested with H_2SO_4 for Klason lignin determination. The Klason lignin content is referred to as acid-insoluble material.

Toxicity towards bacteria was determined in the aqueous extracts used to estimate the phenol content via the Microtox assay. The results are expressed as EC_{50} in toxic mg per gram of dry material (Microtox Manual, Microbics Corporation).

To determine phytotoxic effects solid samples were mixed in water (1/1 w/v), homogenized and filtered as for the determination of the phenol content. The aqueous extract and solid residue were separated by filtration. Phytotoxic effects were estimated in separate assays based upon seed germination with the aqueous extract in four plant species: alfalfa (*Medicago sativa* cv. Aragón), rape (*Brassica napus* cv. Kabel), maize (*Zea mays* cv. Bassano) and tomato (*Lycopersicon esculentum* cv. Tres Cantos), and also the decrease in vegetative growth in tomato plants with either the aqueous extract or the solid residue.

In the seed-germination assays the seeds were disinfected for 5 min in 5% sodium hypochlorite, washed in distilled water and germinated at 25 °C in Petri plates containing filter paper soaked either in aqueous extract or in the same volume of water (controls). Six plates (100 seeds each) were used per extract. The number of germinated seeds was determined after 2, 3 or 5 days, depending upon the species in question.

For the vegetative growth assays tomato plantules (1 cm radicule) were transferred aseptically to Magenta GA-7 (Sigma) recipients (five plants per recipient) containing 70 g of sterile vermiculite. Ten milliliter aqueous extract, 10 g solid residue or 10 ml water (control) were added per recipient. The incubation conditions and chamber have been described elsewhere (Caba et al., 2000). The plants were grown for 3 weeks and then dried for 24 h at 75 °C. Vegetative growth was estimated in terms of dry weight.

3. Results

P. flavido-alba did not grow on SOR when the fungus was inoculated from cultures grown in laboratory culture media. It did however thrive when PC colonized by the fungus were transferred to SOR/PC mixtures. Fungal growth did not significantly decrease the substrate weight, lignin content, extractives or hydrosoluble material as quantified by Klason lignin determination. Nevertheless, significant decreases in phenols and lipids were detected in the cultures on PC and SOR/PC mixtures (Fig. 1). The antibacterial effect of PC extracts also decreased after fungal growth.

Aqueous extracts from PC and from SOR/PC mixtures inhibited the germination rate in alfalfa, maize, and particularly in tomato and rape (Fig. 2). The shoots of seeds germinated in both aqueous extracts were shorter than those germinated in water. Aqueous extracts from cultures on PC and on SOR/PC mixtures, on the other hand, inhibited germination to a lesser extent (Fig. 2; no inhibition was detected in the germination of alfalfa or maize seeds). This decrease in the phytotoxicity of the aqueous extracts from both cultures was observed as increased shoot length.

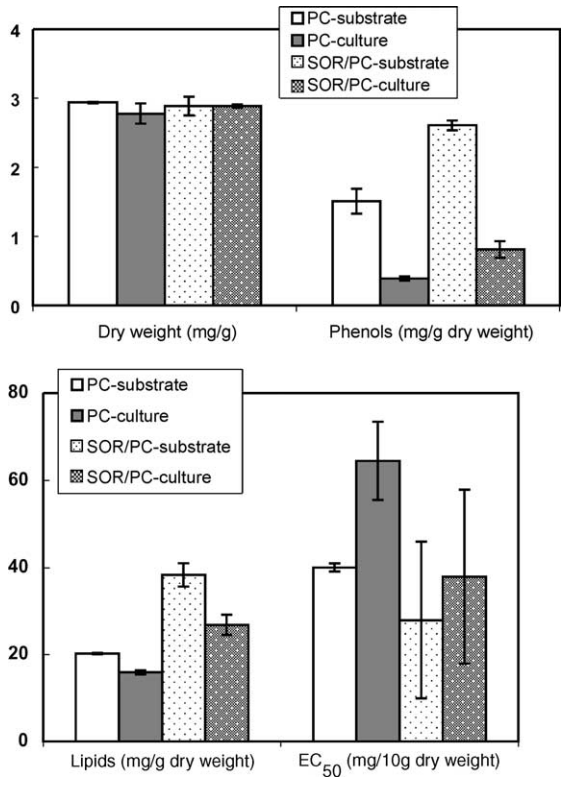

Fig. 1. Components and bacterial toxicity of *P. halepensis* chips and olive-oil-mill-residue mixtures from *P. flavido-alba* cultures and abiotic controls. Values are means of three replicates ± SD.

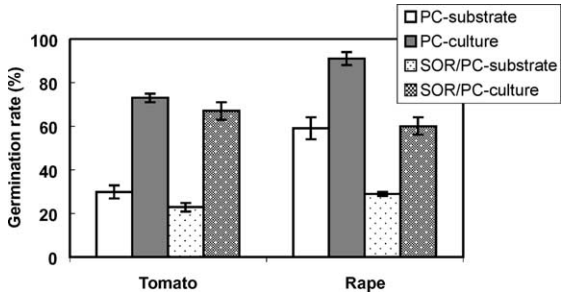

Fig. 2. Germination rate of tomato and rape seeds (as percentages compared to controls) in the aqueous extracts of *P. flavido-alba* cultures on *P. halepensis* chips (PC) and olive-oil-mill-residue mixtures with pine chips SOR/PC. Mean value ± error.

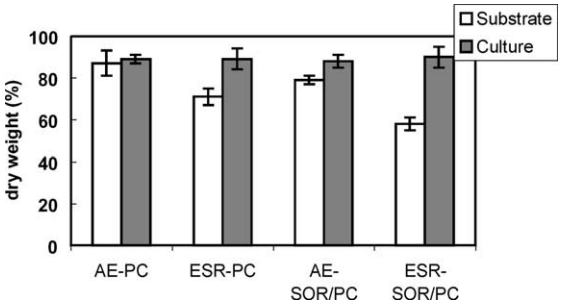

Fig. 3. Dry weight of tomato-plant vegetative growth (as percentages compared to controls) with components of *P. flavido-alba* cultures on *P. halepensis* chips (PC) and olive-oil-mill-residue mixtures with pine chips (SOR/PC). Mean ± error. AE: aqueous extract. ESR: extracted solid residue.

The effect of both the aqueous extracts and the water-extracted solids from PC and SOR/PC substrates on vegetative growth in tomatoes was determined (see Section 2). The phytotoxic effects of PC and SOR/PC mixtures were detected as a decrease in the dry weight of the plants. Water-extracted solids from PC (Fig. 3 ESR-PC) and SOR/PC mixtures (Fig. 3 ESR-PC) yielded plants with a lower dry weight than those grown in aqueous extracts from PC (Fig. 3 AE-PC) or SOR/PC mixtures (Fig. 3 AE-SOR/PC). No significant decrease in the dry weight of plants grown with water-extracted solids from *P. flavido-alba* cultures was observed.

4. Discussion

PC and PC/SOR mixtures contain phytotoxic components. These phytotoxins include water-soluble and relatively water-insoluble components. *P. flavido-alba* decreased the phenol and lipid content of both PC and SOR/PC mixtures whilst at the same time yielding a less phytotoxic substrate.

A significant quantity of phenols were recovered in the aqueous extracts of PC. Pine wood phenols are complex mixtures of compounds (Fengel and Wegener, 1984). Furthermore, the phenols in OMW are responsible for its phytotoxic and antibacterial properties (reviewed in Martínez et al., 1998). We have reported that the phenol content of OMW detoxified by *P. flavido-alba* is decreased (Ben Hamman et al., 1999). Our research demonstrates that *P. flavido-alba* also dephenolizes the semisolid olive residue in solid-state cultures. *P. flavido-alba* cultures in PC and SOR/PC mixtures resulted in aqueous extracts containing about 70% less phenolic compounds, which inhibited seed germination to a much lesser degree than PC or SOR/PC mixtures (an improvement of more than 40% in tomato plants). These results suggest that the phytotoxic effect of the aqueous extracts is due at least in part to their phenol content. This agrees with findings by Blum et al. (2000) and Caspersen et al. (2000) that phytotoxicity decreases concomitantly with a drop in the phenolic content caused by microbial activity.

Small quantities of olive lipids are discharged in SOR (Table 1). Although no relationship has so far been described between SOR lipids and phytotoxicity, the antimicrobial effects of olive-oil and pine-wood lipids is well established. Fungi, including white-rot fungi, are already being used to decrease the woody lipid content and offset the technical and environmental problems caused by these substances in the paper- and pulp-making industry (Gutierrez et al., 1999). Fungal degradation of wood lipids decreases the toxicity of effluents in the paper industry (Dorado et al., 2000a). Basidiomycete white-rot fungi are generally more efficient detoxifiers than ascomycetes (Sierra-Alvarez et al., 2000). With some exceptions the most easily degradable wood lipids are triglycerides (Dorado et al., 1999a) and free fatty acids (Dorado et al., 1999b). *P. flavido-alba* solid-state cultures on SOR/PC mixtures decrease the total lipid content by reducing the content in triglycerides, the most abundant lipids in these mixtures (Linares et al., 2002).

In summary, our results reveal that the phytotoxic components of PC and SOR/PC mixtures include substances easily dissolved in water, such as phenols, and a rather more water-insoluble lipid fraction. *P. flavido-alba* diminishes the phenol and lipid contents of both, PC and SOR/PC mixtures, thus producing a substrate with lower antibacterial and phytotoxic effects. To our knowledge this is the first report concerning the use of white-rot fungi to detoxify SOR. Since SOR detoxification has been obtained in mixtures with lignocellulose material containing toxic compounds (PCs) it seems conceivable than SOR mixtures with less toxic lignocellulose residues, such as wheat straw for example, might be more easily detoxified by *P. flavido-alba*, thus facilitating either the disposal or recycling of these olive-mill residues.

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