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Phenolic removal of olive-mill dry residues by laccase activity of white-rot fungi and its impact on tomato plant growth

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Abstract

We studied the influence of the laccase activity of two white-rot fungi on the toxic effect of water-soluble substances from dry residues of olives (ADOR) on tomato plants. *Pycnoporus cinnabarinus* and *Coriolopsis rigida* decreased the phenol content of ADOR to 73% after 15 days. *P. cinnabarinus* and *C. rigida* produced laccase activity after 5 and 15 days, respectively, and the highest activity in both fungi was detected at 20 days. The treatment of ADOR with these white-rot fungi decreased the phytotoxicity of this residue on tomato plants. A close relationship was found between the amount of laccase produced, the decrease in phenol content of ADOR by the saprobic fungi, decrease of phytotoxicity of ADOR, and the increase in dry weight of tomato plants. These results show that phenol removal by the laccase activity of white-rot fungi can be important in the elimination of phytotoxic substances present in olive-mill dry residues.

Keywords: Laccase; Olive-mill dry residue; White-rot fungi; Phytotoxicity

1. Introduction

The two-phase centrifugation system of olive-mill extraction annually generates millions of tons of a solid byproduct called "alpeorujo." This wet byproduct is dried and extracted with solvents to obtain a dry residue that contains large quantities of polyphenols, carbohydrates, organic matter, and lignin. This residue could be used as a fertilizer because of its high organic content, either directly or after a composting process (Paredes et al., 2001). However, olive-mill dry residue contains a phytotoxic component capable of inhibiting microbial (Capasso et al., 1992; Ramos-Cormenzana et al., 1996) and plant growth (Martín et al., 2002). The phytotoxic effects of olive residues can be attributed mainly to their phenolic compounds (D'Annibale et al., 2004).

Although contamination of soils with olive-mill dry residue can be a serious problem, a solution may be possible using biological methods such as bioremediation with lignin-degrading fungi (Sampedro et al., 2004b). The lignin-degrading ability of white-rot fungi seems to be associated with the release of extracellular enzymes, which include mainly lignin-peroxidases, Mn-peroxidases, and laccase, and these enzymes could participate both in the removal of monomeric phenols and in the decolorization of olive residues (Perez et al., 1987; Kissi et al., 2001). In fact, laccase production by the efficient lignin degraders *Pycnoporus cinnabarinus* and *Coriolopsis rigida* (Eggert et al., 1996; Saparrat et al., 2002) has already been mentioned in certain studies.

The aim of this work was to study the removal of monomeric phenols from water-soluble substances of olive-mill dry residue using laccase enzymes produced by two white-rot fungi, and to determine their effect on plant growth.

2. Materials and methods

Olive-mill dry residue was collected from an "orujo" manufacturer (Sierra Sur S.L., Granada, Spain). Aqueous extracts from olive-mill dry residue were obtained by Soxhlet extraction with water in a 1:8 (w/v) proportion for 16 h. The suspension obtained (ADOR) was reduced to 300 mL and used as a growth medium for the fungi.

The fungi *P. cinnabarinus* and *C. rigida* were maintained in tubes of 2% malt extract at 4 °C as stock cultures. The fungi were grown in an

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Erlenmeyer flask (250 mL) containing 60 mL of ADOR for 30 days at 25 °C with orbital shaking at 125 rpm. Each flask was inoculated with homogenized mycelium from a 12-day-old culture grown in Czapek medium with 50% of ADOR. Every 5 days, an aliquot from the culture liquid was separated from the mycelium by centrifugation (8000*g*) and the supernatant was analyzed.

Total phenolic contents of ADOR were estimated according to Ribereau-Gayon (1968), using tannic acid as a standard, and expressed as grams per kilogram of olive-mill dry residue. Lacasse activity (EC 1.10.3.2) was assayed with 50 mM DMF (2,6-dimethoxyphenol) in 200 mM of acetate buffer pH 5.0 ($\varepsilon_{469} = 27\,500\,M^{-1}\,cm^{-1}$). One unit of enzyme activity was expressed as the amount of enzyme releasing 1 μmol of oxidized product per minute.

Thin-layer chromatography (TLC) analysis was performed using silica gel plates (20 × 20 cm, Silica Gel $60F_{254}$), a mixture of acetone-*n*-hexane 40:60 (v/v) as eluent and $FeCl_3$ (1%), as detector, followed by heating for 5 min at 105 °C.

The effect of ADOR on plant-shoot dry weight was carried out in 20 × 200 mm glass tubes with 15 mL of Hewitt nutrient solution (Hewitt, 1952). ADOR pre-treated with the fungi were added to the rooting medium at a final concentration of 0%, 0.5%, 5%, 10%, and 15%. The tubes were closed with cotton wool and autoclaved (121 °C for 20 min). Tomato seeds (*Lycopersicum esculentum Mill.*) were surface-sterilized and, after germination, selected for uniformity before planting. Plants were grown in a chamber with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 μE/m^2/s, 400–700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Plants were harvested after 15 days and shoot dry matter was determined.

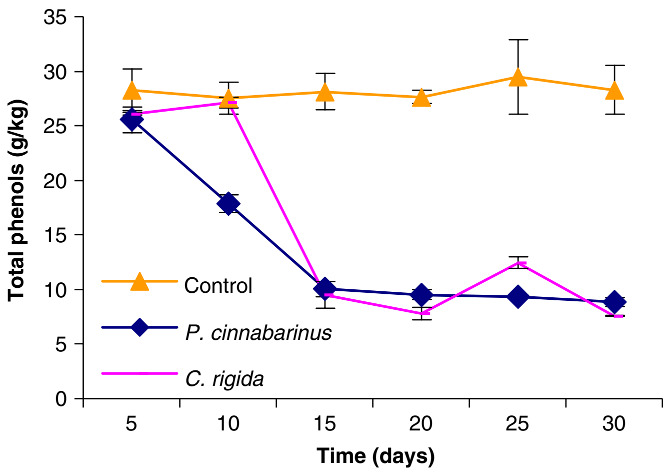

Fig. 1. Phenol content (g/kg DOR) of ADOR treated with *P. cinnabarinus* and *C. rigida* during different treatment times. Values are means of five replicates ±SE.

The mean values of five replicates per treatment were compared using the standard errors of means.

3. Results and discussion

Phenols are considered one of the main agents responsible for the toxic effect of wastes on plant health (Wang et al., 2002a). It is known that some microorganisms decrease the soil contamination provoked by toxic residues from plants (Moreno et al., 1990). One of the main causes of the detoxification effects of microorganisms is attributed to their capacity to metabolize phenolic compounds (Wang et al., 2002b). The initial phenolic content of ADOR was 26 g phenol/kg of olive-mill dry residue (Fig. 1). Treatment of ADOR for 30 days did not affect its phenolic content. *P. cinnabarinus* and *C. rigida* decreased the phenol content of ADOR after 5 and 10 days of treatment, respectively, reaching a maximum of 73% after 20 days. No further significant decrease was observed in the phenol content of ADOR by the fungi tested after 15 days culture in the presence of ADOR.

The TLC chromatograms of ADOR, incubated or not with the saprophytic fungi, revealed the presence of tyrosol and hydroxityrosol as the main monomeric phenols (Fig. 2). Interestingly, biotreatment of ADOR eliminated both monomeric phenols. *P. cinnabarinus* degraded tyrosol after 5 days of treatment whereas *C. rigida* required 15 days to remove the hydroxytyrosol. Marked differences in polyphenol removal and decolorization of olive byproducts by different saprobic fungi have been reported previously (Kissi et al., 2001; Sampedro et al., 2004b).

Laccase activity of ADOR increased slightly after 5–20 days of treatment with both fungi, but at 20 days the enzymatic activity declined (Fig. 3). It is widely known that the lignin-degrading ability of white-rot fungi is associated with the release of ligninolytic enzymes, including laccases,

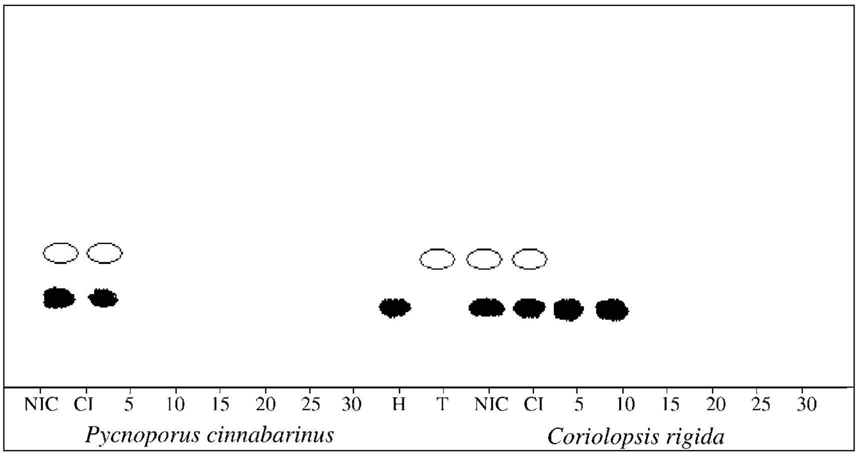

Fig. 2. TLC of ADOR, left to right: non-incubated control (NIC), control incubated (CI) with *P. cinnabarinus* and *C. rigida* for 5, 10, 15, 20, 25, and 30 days. Standards: Hydroxityrosol (H) and Tyrosol (T).

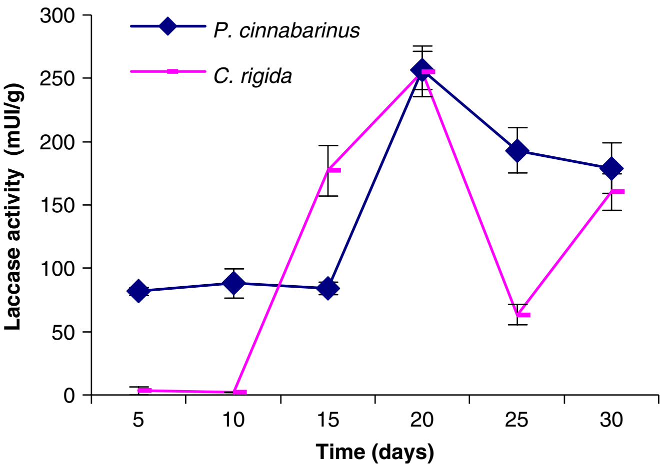

Fig. 3. Laccase activity (mUI/g) of ADOR treated with *P. cinnabarinus* and *C. rigida* during different treatment times. Values are means of five replicates ±SE.

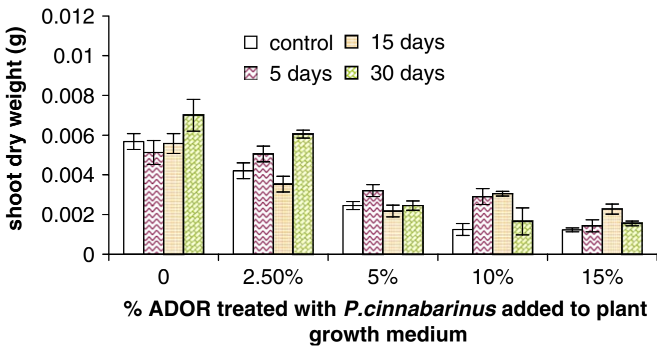

Fig. 4. Shoot dry weight (g) of tomato (*Lycopersicum esculentum* Mill.), cultivated in the presence of various concentrations of ADOR, treated for various times with *P. cinnabarinus*. Values are means of five replicates ±SE.

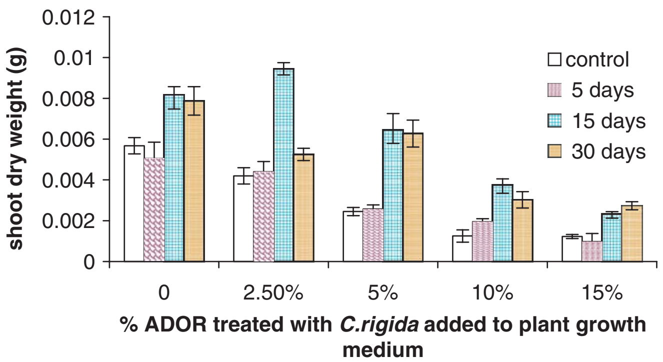

Fig. 5. Shoot dry weight (g) of tomato (*Lycopersicum esculetum* Mill.), cultivated in the presence of various concentrations of ADOR, treated for various times with *C. rigida*. Values are means of five replicates ±SE.

and these enzymes could participate both in the removal of monomeric phenol and the decolorization of olive residues (Ruiz et al., 2002; Martinez et al., 2005). Previous work has also shown that the white-rot fungi *C. rigida* and *P. cinnabarinus* possess laccases capable of degrading a wide variety of pollutants (Eggert et al., 1996; Saparrat et al., 2002). These results are in line with those reported for the role of white-rot fungi enzyme activities in the bioconversion of ''alpeorujo'' (Jaounai et al., 2005).

The application of ADOR, even at the lowest dose (2.5%), decreased the shoot dry weight of tomato plants (Figs. 4 and 5). The saprophytic fungi decreased the phytotoxicity of all the doses of ADOR used. *C. rigida* reduced phytotoxicity after 15 days, whereas *P. cinnabarinus* decreased it after 5 days.

It is worth pointing out that olive-mill dry residue incubated for 20 weeks with several saprophytic fungi significantly increased the biomass production of *Lycopersicum esculetum* (Sampedro et al., 2004a). A significant reduction of olive-mill dry residue phytotoxicity by saprobic fungi has also been obtained with other plants, such as soybean (*Glycine maximum* L.) (Martín et al., 2002).

A close relationship was found between laccase activity, decrease in phenol content of ADOR by the white-rot fungi, and increase in shoot dry weight of plants. These results show that phenol elimination by the lacasse activity of white-rot fungi can be important in the elimination of the phytotoxicity of olive-mill dry residues.

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