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Contribution of hydrolytic enzymes produced by saprophytic fungi to the decrease in plant toxicity caused by water-soluble substances in olive mill dry residue

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Abstract We studied the influence of saprophytic fungi on the toxic effect that the water-soluble substances in dry residues from olive (ADOR) have on the growth of plants. All saprophytic fungi were able to decrease the phytotoxicity of ADOR, although the toxicity of this residue did not decrease in the same way. *Penicillium chrysogenum* was able to reduce the toxicity of ADOR when this residue was applied at the highest dose of 15%. *Fusarium lateritium*, *F. graminearum* and *Mucor racemosus* were able to reduce the toxicity of ADOR when this residue was applied at the intermediate doses. However, *F. oxysporum* decreased the phytotoxicity of ADOR only when the residue was applied at the lowest dose of 2.5%. All saprophytic fungi tested produce endoglucanase, endopolymethylgalacturonase and endoxiloglucanase when grown in the presence of ADOR. A close relationship was found between the decrease in the phytotoxicity of ADOR and the amount of hydrolytic enzymes produced by the saprophytic fungi. These results shows that hydrolytic enzymes can be important in the degradation of phytotoxic substances present in olive mill dry residue.

Introduction

The agroindustrial residues derived from the extraction of oil from the olive constitutes an environmental problem, due to its polluting capacity (Ramos-Cormenzana et al. 1995). In recent years, a wet residue (80% of the weight of the olive) has been obtained along with the oil, which is dried and extracted with solvents to obtain a dry residue. The olive mill dry residue is a lignocellulosic by-product with C, N and phenolic compounds (Nogales et al. 1999). This residue might be used as fertilizer due to

the high organic content (Nogales et al. 1999) but, like the majority of plant by-products, olive mill dry residue can be phytotoxic (Martín et al. 2002). The water-soluble substances in olive mill dry residue may be responsible for this phytotoxicity. It is known that water-soluble materials liberated from plant residues are highly phytotoxic (Ramos-Cormenzana et al. 1995).

Although contamination of soils with olive mill dry residue can be a serious problem, remediation may be possible using biological methods, such as bioremediation with saprophytic fungi. Saprophytic fungi are important and common components of the soil rhizosphere from which they obtain nutritional benefits in the form of inorganic compounds and exudates from the root (Dix and Webster 1995). These soil fungi are important, since they generally take part in the degradation of phytotoxic substances and also contribute to the best use of nutrients by the plant (Fracchia et al. 2000).

Phenolic compounds seem to be the main cause of the phytotoxic effect of plant residues (Capasso et al. 1992). However, other substances such as cell wall fragments (oligosaccharides) and glycoproteins have toxic effects on plant health (Bucheli et al. 1990). Some hydrolytic enzymes that degrade the plant cell wall have been shown to be involved in the detoxification of olive mill dry residue (Benitez et al. 2002). However, no studies have been carried out on the role of cellulase, pectinase and hemicellulase. Some saprophytic fungi are able to produce cellulases, pectinases and xyloglucanases, which are hemicellulases with a high hydrolytic activity on the plant cell wall (Tribak et al. 2002).

Thus, olive mill dry residue might contain water-soluble substances that may have a negative effect on plant growth. The aim of this work was to study the influence of saprophytic fungi on the effect that the water-soluble substances in dry residues from olive have on the growth of plants.

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Materials and methods

Materials

The saprophytic fungi *Fusarium oxysporum*, *F. lateritum*, *F. graminearum* (Booth 1977) and *Penicillium chrysogenum*, present in the rhizosphere soil and roots of maize cultivated in the Province of Buenos Aires (Argentina), and *Mucor racemosus*, present in ADOR, were isolated by the particle-washing method (Widden and Bisset 1972). The fungal isolates were transferred to tubes of 2% malt extract at 4 °C as stock cultures and stored in the collection of the Estación Experimental del Zaidín, Granada, Spain.

Olive mill dry residue was collected from an olive oil manufacturer (COLGRA S.L., Granada, Spain). Aqueous extracts from olive mill dry residue were obtained by orbital-shaking of olive mill dry residue with distilled water in a proportion of 1:2 (w/v) for 8 h. The suspension obtained was filtered through several layers of cheesecloth; and the ADOR filtrate was used as a growth medium. The fungi were grown in Erlenmeyer flasks (125 ml) containing 50 ml of ADOR for 15 days at 25 °C with orbital-shaking at 125 rpm. Each flask was inoculated by transferring a 3-mm plug cut out from the margin of a 5-day-old colony. The culture liquid was separated from the mycelium by centrifugation (8,000 g) and the supernatant was used to analyze the effect of ADOR on plant growth.

Determination of phenolic content

The total phenolic content of aqueous extracts from ADOR was estimated according to Riberau-Gayon (1968), using tannic acid as a standard and was expressed as g kg^{-1} of ADOR.

Effect of aqueous extracts from ADOR on plant development

This experiment was carried out in glass tubes (20×200 mm) with 15 ml of Hewitt nutrient solution (Hewitt 1952). Aqueous extracts from olive mill dry residue after incubation with the saprophytic fungi were added to the rooting medium at final concentrations of 0, 2.5, 5, 10 and 15%. The tubes were closed with cotton wool and autoclaved at 120 °C for 20 min. Seeds of tomato (*Lycopersicon esculentum* L.) were surface-sterilized and, after germination, were selected for uniformity before planting. Plants were grown in a chamber under controlled conditions. Plants were harvested after 15 days and shoot dry matter was determined. Five replicates per treatment were carried out, making a total of 150 tubes.

Determination of enzyme activity of aqueous extracts from olive mill dry residue

The aqueous extracts of olive mill dry residue after incubation with the saprophytic fungi, extracted as described above, were assayed to determine endoglucanase (endo-GN; EC 3.2.1.4), endopoly-methylgalacturonase (endo-PMG; EC 3.2.1.15) and endoxyloglucanase (endo-XG) activities.

All hydrolytic activities were assayed by the viscosity method (Rejón-Palomares et al. 1996a), using carboxymethylcellulose, citrus pectin and xyloglucan from nasturtium seeds [*Tropaeolum majus* L.; extracted as described by McDougall and Fry (1989)], as substrates. The reduction in viscosity was determined at intervals of 0–30 min. The reaction mixture contained 1 ml of 0.5% substrate in 50 mM citrate–phosphate buffer (pH 5) with 0.2 ml of enzyme. Viscosity reduction was determined at 37 °C. Enzyme activity was expressed as the specific activity (units of activity per milligram of protein), calculated as: $\text{units of activity}/[\text{time (h)} \text{ for } 50\% \text{ viscosity loss}] \times 10^3$ (Rejón-Palomares et al. 1996b).

Total protein contents was determined by the method of Bradford (1976), using a Biorad kit with bovine serum albumin as the standard.

The data obtained for shoot dry matter and enzyme activities were subjected to ANOVA. The mean values of five replicates were compared using standard errors of means or Duncan's multiple range test ($P=0.05$).

Results and discussion

The application of ADOR, even at the lowest dose (2.5%), decreased the shoot dry weight of tomato (Fig. 1). None of the saprophytic fungi tested eliminated the phytotoxicity caused by ADOR. Nevertheless, all saprophytic fungi were able to decrease the phytotoxicity of ADOR when the inoculated residue was applied at a dose of 2.5%. The incubation of *F. oxysporum* in ADOR did not affect its inhibitory effect on the shoot dry weight of tomato when applied to the plant growth medium at a dose of 5%. However, incubation of the other saprophytic fungi decreased the phytotoxic effect of ADOR at this dose. Incubation of ADOR with *F. graminearum*, *M. racemosus* and *P. chrysogenum* decreased the phytotoxicity caused by a 10% dose, whereas only *P. chrysogenum* was able to decrease the phytotoxicity of ADOR when its residue was applied at a dose of 15%.

Phenols are considered one of the main agents responsible for the toxic effect of wastes on plant health (Wang et al. 2002). It is known that some microorganisms decrease the soil contamination produced 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. 2002). The initial phenol content of ADOR was 22.2 ± 2.2 mg phenol/g ADOR dry weight and was 19.8 ± 1.8 mg/g at 14 days of fungal incubation. No further significant decrease was observed in the phenol content of ADOR by any of the saprophytic fungi tested after 14 days of fungal culture in the presence of ADOR.

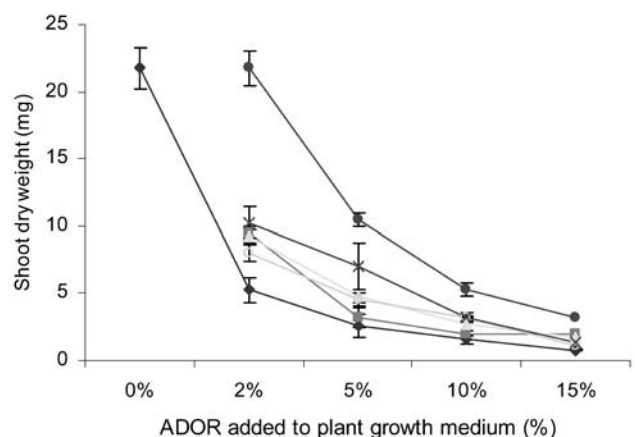


Fig. 1 Shoot dry weight (mg) of tomato (*Lycopersicon esculentum* L.) cultivated in the presence of aqueous extracts from olive mill dry residue (ADOR) incubated with different saprophytic fungi. ◆ Control, ■ *Fusarium oxysporum*, ▲ *F. lateritum*, ○ *F. graminearum*, × *Mucor racemosus*, ● *Penicillium chrysogenum*

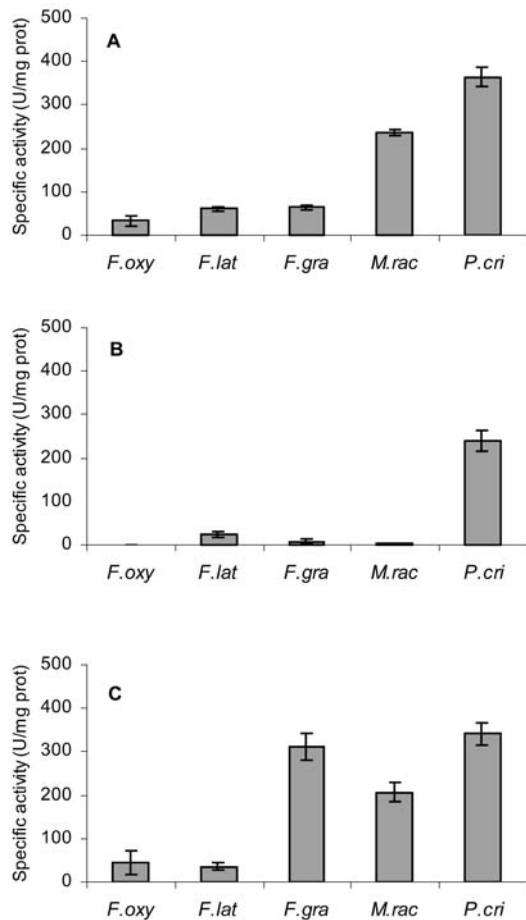


Fig. 2 Endoglucanase (A), endopolymethylgalacturonase (B) and endoxyloglucanase (C) activities produced by different saprophytic fungi grown on aqueous extracts from olive mill dry residue. *F. oxy* *F. oxysporum*, *F. lat* *F. lateritum*, *F. gra* *F. graminearum*, *M. rac* *M. racemosus*, *P. cri* *P. chrysogenum*. Bars show standard errors of means

In spite of the importance of hydrolytic enzymes on the detoxification of plant residues (Benitez et al. 2002), there is not information available about the role of endo-GN, endo-PMG and endo-XG on the phytotoxicity of wastes. All saprophytic fungi tested produced endo-GN, endo-PMG and endo-XG when grown in the presence of ADOR (Fig. 2). *F. oxysporum* produced the lowest quantity of hydrolytic enzymes, especially endo-PMG, which did not reach 1 unit /mg of protein. *F. lateritum* produced endo-XG activity similar to that of *F. oxysporum*, but had higher endo-GN and endo-PMG activities. Incubation of *F. graminearum* and *M. racemosus* in ADOR produced more endo-GN and endo-XG activities than those of *F. oxysporum* and *F. lateritum*. Finally, *P. chrysogenum* produced the highest activity of all three enzymes. These results indicate that there was a correlation ($r=0.92$; $P=0.008$) between the decrease in the phytotoxicity of ADOR (Fig. 1) and the production of hydrolytic enzymes by the saprophytic fungi (Fig. 2). In fact, *F. oxysporum*, which produced the lowest quantity of

hydrolytic enzymes, was not able to decrease the phytotoxicity of ADOR at a dose of 5%. Incubation with *F. lateritum*, which produced more hydrolytic enzymes than *F. oxysporum*, decreased the phytotoxicity of ADOR when applied to the plant growth medium at a dose of 5%. *F. graminearum* and *M. racemosus*, with an intermediate production of hydrolytic enzymes, decreased the phytotoxicity of ADOR at doses of 5% and 10%. *P. chrysogenum*, which produced the highest quantity of hydrolytic enzymes, was able to decrease the phytotoxicity of ADOR at 15%.

Our results show that hydrolytic enzymes can be important in the process of degrading the phytotoxic substances present in ADOR. However, we cannot exclude the possibility that fungi grown in ADOR may produce secondary metabolites that somehow favor plant growth independent of the ADOR effect. A detailed study under way in our laboratory will shed further light on the process of the degradation of phytotoxic substances in ADOR.

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