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Suppressive effect of olive residue and saprophytic fungi on the growth of *Verticillium dahliae* and its effect on the dry weight of tomato (*Solanum lycopersicum* L.)

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Abstract

The saprophytic fungi *Aspergillus niger*, *Coriolopsis rigida, Fusarium lateritium*, *F. oxysporum*, *Mucor racemosus*, *Paecilomyces farinosu*s, *Penicillium chrysogenum*, *P. restrictum, Trametes versicolor, Trichoderma harzianum*, *T. pseudokoningii* and *T. viride* were able to decrease the growth *in vitro* of *Verticillium dahlie* in the presence of aqueous extract of olive residue. The conidia number of *V. dahliae* decreased when grown on aqueous extract of olive residue, autoclaved or filtered through 0.45 micron filters after culture of the fungi. These results suggest not only the predominance of suppressive substances of a biological nature, but also the existence of non-biological inhibitory substances. The olive residue decreased the negative effect of *V. dahliae* on shoot and root dry weight of tomato (*Solanum lycopersicum* L.), by the antifungal compounds present in the olive residue and by the antifungal substances produced by the antagonistic saprophytic fungi grown in this residue.

Keywords: crop residue, fungal antagonism, fungal exudates, phytotoxicity, antifungal compounds.

1. Introduction

Verticillium dahliae Kleb. is a destructive vascular wilt-causing fungus with worldwide distribution. In recent years, the spread of *Verticillium* wilt in crops, especially in olive orchards, caused by the soil-borne pathogen *V. dahliae*, is often related to intensive modern farming of highly productive cultivars, planted at high densities, usually irrigated, and under a mechanised system (Rodríguez *et al*., 2011). The disease causes serious economic losses to a large number of crops. There are no chemical treatments to control it. Thus, management strategies are focused on preventive measures; among those is the use of biological control practices, crop residues or compost soil amendments (Tjamos, 2000; Termorshuizen *et al*., 2006; Goicoechea *et al*., 2010). Several antagonistic fungi applied either alone or together with compost significantly reduced the inoculum density of *V. dahliae* in the soil (Lima *et al*., 2008; Pantelides *et al*., 2009; Pascual *et al*., 2009).

The importance of the olive mill industry in Mediterranean countries is well known and it is rapidly extending over other countries such as Australia, Chile, US and so on (D'Annibale *et al*., 2006). The two-phase extraction system is the most widely used technology to obtain oil and generates a semi-solid organic waste (alpeorujo) which is then dried and further extraction is carried out using solvents to obtain an extra yield of oil and a dry olive mill residue (Vlyssides *et al*., 1998). Due to its content of organic matter and mineral nutrients, this olive residue might be employed for agronomic purposes (Paredes *et al*., 1999; Bonanomi *et al*., 2006). Studies carried out with olive residue have shown that it has antimicrobial and phytotoxic properties (Moreno *et al*., 1987; Perez *et al*., 1992; Martín *et al*., 2002). A decrease in the phytotoxic effect of the olive residue can be achieved by inoculation with saprophytic fungi (Aranda *et al*., 2007; Sampedro *et al*., 2008). Saprophytic fungi are able to mobilize nutrients, to degrade phytotoxic substances and to promote a more efficient use of the nutrients by the plant (Fracchia *et al*., 2000). Some of the saprophytic fungi used in detoxification of olive residues, such as *Trichoderma*, and *Fusarium* have been used as antagonists against *V. dahliae* (Lima *et al*., 2008; Pantelides *et al*., 2009).

The aim of this work is to study the potential of olive residue to control Verticillium wilt of tomato, applied either alone or together with saprophytic fungi selected as antagonists of *V. dahliae*.

2. Material and methods

2.1 Materials

Olive mill dry residue was collected from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain) and stored at -20 ºC until use. The main characteristics of olive residue were as follows: total organic carbon, 58.5%; total nitrogen, 1.87%; total phosphorus, 0.21%; lignin, 24.7%; cellulose, 18%; hemicellulose, 11.4%; total phenols, 2.65%; total lipids, 0.2%. The most abundant elements, the concentration in g kg^{-1} olive residue were: potassium, 30.5; calcium, 13.6; magnesium, 3.8; iron, 1.1; sodium, 0.17; copper, 0.07; zinc, 0.06 and manganese, 0.04. 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 pH of the aqueous extract of the olive residue was 5.13. The extract obtained was filtered through several layers of cheesecloth; and this filtrate was used as a growth medium.

Seventy-one isolates from the genera: *Aspergillus* (11 species); *Coriolopsis* (2 species); *Fomes* (1 spe-

cies); *Fusarium* (13 species); *Mortierella* (3 species); *Mucor* (3 species); *Paecilomyces* (2 species); *Penicillium* (10 species); *Phanerochaete* (1 species)*; Phlebia* (2 species); *Pleurotus* (1 species)*; Poria* (3 species); *Pycnoporus* (1 species)*; Trametes* (4 species); *Trichoderma* (10 species) and *Wardomyces* (4 species) genera were selected to test their antagonism against *Verticillium dahliae* Kleb. EEZ2 (in vitro experiments). These fungi were grown on potato dextrose agar (PDA) slants at 25ºC for 7 days. Stock cultures of the fungi were stored in PDA slants at 4º C. Strains were provided by the Centro de Investigaciones Biológicas, CIB (Madrid, Spain), the University of Buenos Aires (Argentina) and by the fungal culture collection of the Estación Experimental del Zaidín (Granada, Spain).

2.2 In vitro experiments. Evaluation of saprophytic fungal antagonism to V. dahliae

The inhibitory ability of the saprophytic fungal strains against *V. dahliae* was evaluated by using the technique described by Ortiz and Orduz (2001). A plug of *V. dahliae* culture was transferred to a Petri dish containing PDA. After incubation in the darkness at 25 ºC a 4mm plug cut out from the margin of a 5-day-old colony was transferred to plates with 15 mL PDA amended with 1.5 mL of aqueous extract of olive residue. The plates were incubated for 3 days and after that each antagonist strain was transferred 3 cm apart from of the *V. dahliae*. The dual cultures were incubated for 11 days under the same conditions and tested for in-vitro antagonistic activity against *V. dahliae*. Ten replicates were set for each treatment and cultures were evaluated macroscopically every 48 h. Center-wise growth of antagonist and *V. dahliae*, determined as influenced mycelial growth radius (internal halo) and edge-wise growth, as free mycelial growth radius (external halo) was measured. Inhibition of fungal growth was calculated using the formula: (Mb − Ma / Mb)×100, where Ma=influenced mycelial growth, Mb=free mycelial growth. Plates with *V. dahliae* plus *V. dahliae* slants were used as control.

The percent mycelial growth inhibition of *V. dahliae* caused by antagonistic fungal strains was also evaluated on 15 mL PDA plus 1.5 mL of aqueous extract of olive residue as described before.

The antagonism of saprophytic fungi to *V. dahliae* grown in aqueous extract of olive residue was also evaluated. The saprophytic fungi and *V. dahliae* were grown individually in Erlenmeyer flasks (100 mL) containing 10 mL of aqueous extracts of the olive residue for 15 days at 25 ºC with orbital-shaking at 125 rpm. Each flask was inoculated by transferring a 4-mm plug cut out from the margin of a 5-day-old colony. The culture liquid was separated from the mycelium by centrifugation (8000 rpm 15 min) and the supernatant was retained. Tubes with 3 mL of aqueous extracts supernatant were autoclaved (120 ºC, 20 min) or either filtered by 0.45 µm pore size (Millipore). To these tubes was added to 9 mL of fresh aqueous extracts plus 1 mL of a suspension of $1x10^6$ conidia mL^{-1} of *V. dahliae*. The aqueous extract from olive residue without saprophytic fungi was used as control. Ten replicates tubes were prepared for each treatment. The tubes were incubated for 3 days at 25 ºC and the number of verticillate spore-bearing structures (conidia), and near-synchronous development of microsclerotia of *V. dahliae* was estimated using the Neubauer chamber (Neumann and Dobinson, 2003).

2.3 Phytotoxicity experiments

The effect of the olive residue, alone and together with the saprophytic fungi *Penicillium chrysogenum*, *Fusarium lateritium* and *C. rigida,* on the growth of tomato (*Solanum lycopersicum* L. cv. Craigella susceptible to Verticillium wilt), cultured in presence of *V. dahliae* was tested. The soil was collected from the

field of the Estación Experimental del Zaidín (Granada, Spain). It was a loamy soil. The soil pH which was measured by glass electrode in a 1:1 soil:water suspension. The P ($NaHCO_3$-extractable), N, K, Fe, Mn, Cu and Zn were determined by methods of Lachica *et al*. (1965). The main characteristics of the soil are showed in Table 1. The experiments were carried out in 0.3 L pots containing steam-sterilized soil and quartz sand mixed in a 1:1 ratio (v/v). The saprophytic fungi incubated in potato dextrose broth (PDB) or in aqueous extract of olive residue were filter-sterilized as described above and 30 mL were added to soil-sand mixture. An aqueous suspension of *V. dahliae* in sterile distilled water, containing approximately $7x10^6$ spore mL^{-1} was prepared from cultures grown in PDA for one week at 25 °C and 30 mL of this suspension were added to pots with soil-sand mixture. The aqueous extract from olive residue or the PDB medium without fungi were used as control. Germinated tomato seeds were selected for uniformity prior to planting. Plants were grown in a greenhouse with natural light supplemented by Sylvania incandescent and cool-white lamps giving 400 nmol m^2 s^{-1} at 400–700 nm; there was a 16–8 h light–dark cycle at 25–19 °C and 50% relative humidity. Plants were watered from below, and fed with a nutrient solution at 10 mL per week (Hewitt, 1952). Pots without extract of olive residue or PDB application, as well as pots with soil-sand mixture inoculated only with an aqueous suspension of *V. dahliae* in sterile distilled water were also used. Plants were inoculated at the time of transplanting. Plants were harvested after 4 weeks and dry matter weight was determined.

Table 1. Chemical characteristics of soil sample.

pH	OM	N	P	K	Fe	Mn	Cu	Zn
	g/kg^{-1}	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
8.1	19.7	2.5	6.2	132	9.6	110	5.8	5.7

OM: Organic matter

2.4 Experimental design and statistical analysis

A design 2 x 2 x 4 full factorial completely randomized experimental design was used. We studied the three main factors: Amendment: extract of olive residue and PDB, pathogen fungus: control and *V. dahliae*; and biocontrol agent: control, *P. chrysogenum*, *F. lateritium* and C. *rigida*. Four replicate pots per treatment were used. The data were analysed by factorial analysis of variance with Amendment, pathogen fungus, biocontrol agent and their interaction as sources of variation. Statistical procedures were carried out with the SPSS software, version 11.0 (SPSS Inc., 1989–2001). Statistical significance was evaluated at alpha = 0.05. Data sets were tested for normality and equal variance (Kolmogorov Smirnov and Cochran's C test, respectively) and a log transformation was applied when significant departures from normality were found. Each experiment was repeated at least twice.

3. Results

The percent mycelial growth inhibition of *V. dahliae* grown in PDA medium in the presence of *V. dahliae* was 2.8±0.8. From the 71 saprophytic fungi tested, the percent mycelial growth inhibition of *V. dahliae*

increased significantly in presence of the saprophytic fungi *Aspergillus niger*-BAFC341 (7.9±1.2), *Coriolopsis rigida*-CECT20449 (35.2±4.9), *Fomes sclerodermus*-EEZ12 (5.5±1.4), *Fusarium lateritium*-BAFC2317 (30.2±3.2), *F. oxysporum*BAFC126 (8.5±2.5), *Mucor racemosus*-EEZ12 (12.6±3.7), *Paecilomyces farinosus*-BAFC8846 (25.7±4.3), *Penicillium chrysogenum*-EEZ10 (32.4±5.3), *P. restrictum*-BAFC512 (8.5±1.1), *Phanerochaete chrysosporium*-ATCC24725 (4.3±0.4), *Phlebia radiata*-CBS20448 (5.3±0.8), *Pleurotus ostreatus*-EEZ5 (6.1±0.3), *Trametes versicolor*-A136 (15.2±3.3), *Trichoderma harzianum*-BAFC8842 (9.2±2.3), *T. pseudokoningii*-BAFC8844 (12.4±1.3) and *T. viride*-BAFC8850 (11.3±1.2).

Figure 1 shows that *A. niger*, *C. rigida, F. lateritium*, *F. oxysporum*, *M. racemosus*, *P. farinosus*, *P. chrysogenum*, *P. restrictum, T. harzianum*, *T. pseudokoningii*, *T. versicolor* and *T. viride* were antagonistic against *V. dahliae* grown in PDA medium plus aqueous extract of dry olive mill residue. These fungi produced different degrees of inhibition of the *V. dahliae* mycelial growth. The saprophytic fungi *P. chrysogenum*, *F. lateritium*, *C. rigida* and *P. farinosus* showed the highest and statistically significant inhibitory effect on *V. dahliae* mycelial growth when compared to the other antagonist strains (Figure 1).

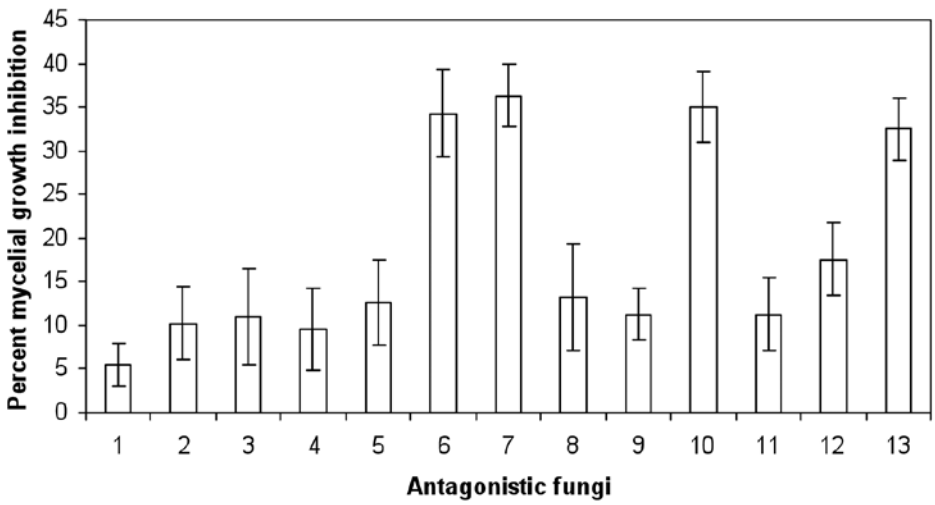

Figure 1. Percent mycelial growth inhibition of *Verticillium dahliae* in presence of *V. dahliae* and of antagonistic saprophytic fungi cultivated in PDA medium plus aqueous extract of olive residue. 1=*V. dahliae*, 2=*Trichoderma harzianum*, 3=*Aspergillus niger*, 4=*Trametes versicolor*, 5=*Trichoderma viride*, 6=*Penicillium chrysogenum*, 7=*Coriolopsis rigida*, 8=*Trichoderma pseudokoningii*, 9=*Mucor racemosus*, 10=*Fusarium lateritium*, 11=*Fusarium oxysporum*, 12=*Penicillium restrictum*, 13=*Paecilomyces farinosus*. The data are shown as mean ± standard error of the mean.

The aqueous extract of olive residue autoclaved or filtered with Millipore after its incubation with *V. dahliae* or without saprophytic fungi incubation (Control), had the same effect on the number of *V. dahliae* conidia and microsclerotia. Thus we included only the values of the control treatment in Figure 2. The filtered aqueous extract of olive residue autoclaved after its incubation with *T. viride*, *F. lateritium*, *F. oxysporum* or *C. rigida* reduced the number of conidia and microsclerotia of *V. dahliae* (Figure 2a). The filtered aqueous extract of olive residue after its incubation with *P. chrysogenum*, *F. lateritium* or *C. rigida* significantly reduced the number of *V. dahliae* conidia and microsclerotia (Figure 2b).

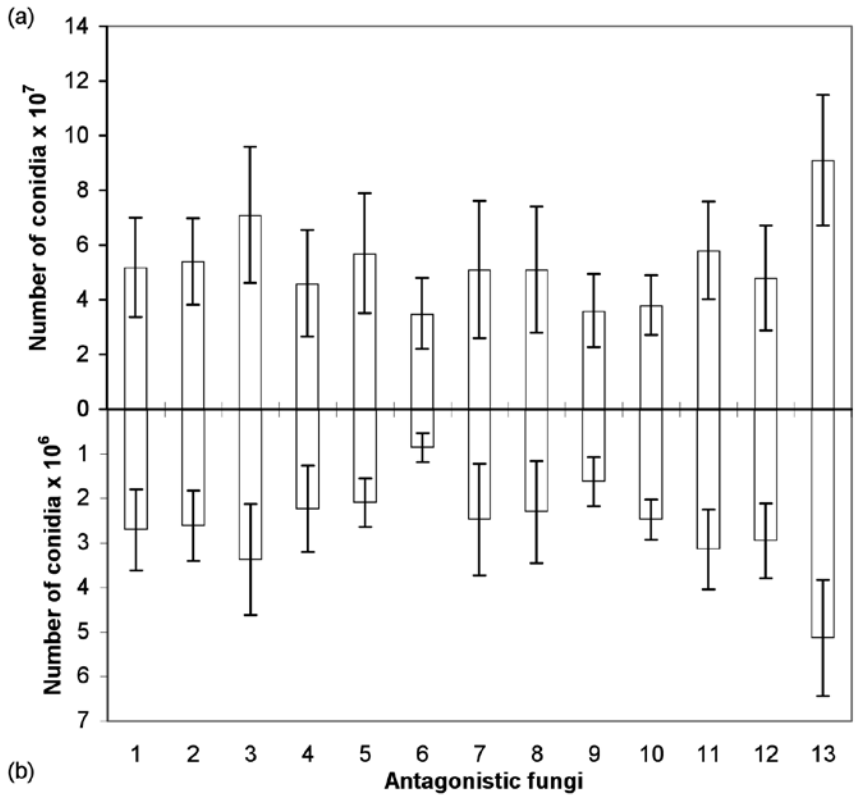

Figure 2. Number of *Verticillium dahliae* conidia grown in (a) autoclaved or (b) filtered aqueous extract of olive residue after its incubation with of the antagonistic fungi. 1=*Trichoderma harzianum*, 2=*Aspergillus niger*, 3=*Trametes versicolor*, 4=*Trichoderma viride*, 5=*Penicillium chrysogenum*, 6=*Coriolopsis rigida*, 7=*Trichoderma pseudokoningii*, 8=*Mucor racemosus*, 9=*Fusarium lateritium*, 10=*Fusarium oxysporum*, 11=*Penicillium restrictum*, 12=*Paecilomyces farinosu*s, 13=Uninoculated control. The data are shown as mean ± standard error of the mean.

Symptoms of *Verticillium* wilt were observed during the experiment and vascular browning was found in the plant tissues. However, the influence of the pathogen was measured as the reduction of shoot and root dry weight of the plant infected with the pathogen in comparison to the untreated control. As Table 2 shows, there were significant differences in the means of the response variables (shoot dry weight), to all the levels and main factors (Amendment, p=0.00063; pathogen fungus, p=0.00087 and BCA, p=0.00058) tested. The contrasts between the factors amendment, pathogen fungus and BCA (p=0.00063), between the factors amendment and pathogen fungus (p=0.00065), as well as between the factors amendments and BCA (p=0.025) were statistically significant for both the response variables. The application of aqueous extract of olive residue decreased the shoot and root dry weight of tomato (Figure 3). The decrease of the shoot and root dry weight of tomato caused by inoculation with *V. dahliae* suspended in water or in PDB were significantly greater than when the pathogen was inoculated in a suspension of an aqueous extract of olive residue. The saprophytic fungi *P. chrysogenum*, *F. lateritium* and *C. rigida* were able to decrease the toxicity of aqueous extract of olive residue on tomato, but none of them were able to eliminate the phytotoxicity caused by this residue. The saprophytic fungi cultivated in aqueous extract of olive residue or PDB decreased, but did not eliminate, the negative effect caused by *V. dahliae* on the shoot and root dry weight of tomato The shoot and root dry weight of tomato inoculated with *V. dahliae* grown in aqueous extract of olive residue plus exudates of the saprophytic fungi, were similar to plants grown in presence of aqueous extract of olive residue. However, the shoot and root dry weights of tomato inoculated with PDB, were higher than those of plants inoculated with *V. dahliae* grown in PDB plus exudates of the saprophytic fungi.

Table 2. Significance of the main treatment effects and their interactions based on factorial analysis of variance.

	F-values						
	A	**P**	**BCA**				
	Amendment	**Pathogen fungus**	**Biocontrol Agent**	**A x P**	**A x BCA**	**P x BCA**	**A x P x BCA**
Shoot dry weight	30.82***	235.37***	8.45***	29.46 ***	3.51*	10.69***	10.85***

A: soil pot with Amendment; P: Pathogen fungus; BCA: Biocontrol agent.
*: $p < 0.05$
***: $p < 0.001$

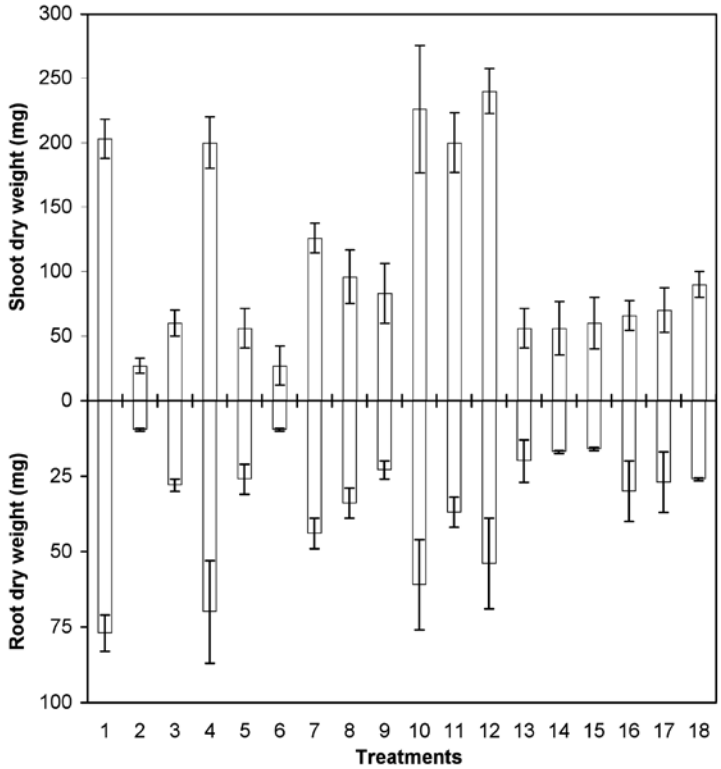

Figure 3. Shoot and root dry weight of tomato (*Solanum lycopersicum* L.) inoculated with *Verticillium dahliae* and cultivated in presence of exudates from the antagonistic saprophytic fungi grown in olive residue or in potato dextrose broth (PDB) medium.1=Uninoculated controls, 2=Plants inoculated *V. dahliae*, 3=Plants inoculated with aqueous extract of olive residue, 4=Plants inoculated with PDB, 5=Plants inoculated with aqueous extract of olive residue plus *V. dahliae*, 6=Plants inoculated with PDB plus *V. dahliae*, 7, 8 and 9=Plants inoculated with exudates of *Penicillium chrysogenum*, *Coriolopsis rigida* and *Fusarium lateritium* grown in aqueous extract of olive residue, respectively, 10, 11 and 12=Plants inoculated with exudates of *P. chrysogenum*, *C. rigida* and *F. lateritium* grown in PDB, respectively, 13, 14 and 15=Plants inoculated with *V. dahliae* plus exudates of *P. chrysogenum*, *C. rigida* and *F. lateritium* grown in aqueous extract of olive residue, respectively, 16, 17 and 18=Plants inoculated with *V. dahliae* plus exudates of *P. chrysogenum*, *C. rigida* and *F. lateritium* grown in PDB, respectively. The data are shown as mean ± standard error of the mean.

4. Discussion

Most of the studies on antagonistic microorganisms against *V. dahliae* have been focussed on soil fungi belonging to *Trichoderma*, *Paecylomices, Penicillium*, *Fusarium* and *Talaromyces* genera (Tjamos and Fravel, 1995; Berg *et al*., 2005; Lima *et al*., 2008; Naraghi *et al*., 2008; Pantelides *et al*., 2009). We found isolates belonging to the genera *Aspergillus*, *Coriolopsis*, *Fomes*, *Mucor*, *Phanerochaete*, *Phlebia, Pleu-*

rotus and *Trametes* that also have antagonistic effect against *V. dahliae in vitro* and can be useful in reducing *Verticillium* wilt in conditions where other fungi were not effective antagonists. Exudates produced by fungi antagonistic to *V. dahliae* seem to be the responsible of the antagonistic effect of crop residues on the density of *V. dahliae* in the soil and on the *Verticillium* wilt (Lima *et al*., 2008; Pantelides *et al*., 2009). The antagonistic fungi *A. niger*, *C. rigida F. lateritium*, *F. oxysporum*, *M. racemosus*, *P. chrysogenum*, *P. restrictum, P. farinosu*s, *T. versicolor, T. harzianum*, *T. pseudokoningii*, and *T. viride* were able to decrease the growth *in vitro* of *V. dahlie* the presence of aqueous extract of olive residue. Several studies have demonstrated the loss of disease suppression of composts following sterilisation or pasteurisation (Cotxarrera *et al*., 2002; Reuveni *et al*., 2002). Therefore, in these instances the suppressive effect was predominantly biological rather than chemical or physical in nature (Noble and Coventry, 2005; Malandraki *et al*., 2008). We found that the aqueous extract of olive residue filtered after its incubation with *C. rigida, F. lateritium*, *P. chrysogenum* and *P. farinosu*s decreased the number of *V. dahliae* conidia and microsclerotia indicating the presence of suppressive substances of biological origin. However, we also found that the aqueous extract of olive residue autoclaved after its incubation with *C. rigida*, *F. lateritium*, *F. oxysporum* and *T. viride* decreased the number of *V. dahliae* conidia and microsclerotia. On the other hand, our results shows that the aqueous extract of olive residue non-inoculated with saprophytic fungi also decreased the negative effect of *V. dahliae* on the growth of tomato. These results indicate the existence of other kind of thermoresistant inhibitory compounds in the aqueous extract of olive residue. In fact, studies carried out with olive residues have shown that it has notable antimicrobial properties mainly due to its phenolic content (Moreno *et al*., 1987; Perez *et al*., 1992). The existence of some saprophytic fungi that can change the chemical structure of the phenolic compounds of the olive residue have been also found (Aranda *et al*., 2007; Sampedro *et al*., 2007). However, the possibility of the implication of the phenolic compounds in the antagonistic effect of the aqueous extract of olive residue on *V. dahliae* remains to be investigated. The exudates produced by the saprophytic fungi grown in the aqueous extract of olive residue also decreased the pathogenesis of *V. dahliae* against tomato. The possible joint actions between the antifungal compounds that have the aqueous extract of olive residue and the antifungal substances produced by the saprophytic fungi grown in this olive residue, may explain why the effectiveness of this residue with the fungal exudates on the damage caused by *V. dahliae* on tomato was higher than of the PDB with the antagonist fungal exudates.

On the other hand, it is known that the olive residue is toxic to plants and that the content of monomeric phenols of the olive residue seems to be responsible for phytotoxic properties (Martín *et al*., 2002). Reductions of the phytotoxic effect of olive residue by the use of saprophytic fungi that decrease its phenolic content have been found (Aranda *et al*., 2007; Sampedro *et al*., 2008). The saprophytic fungi *P. chrysogenum*, *F. lateritium* and *C. rigida,* which were antagonistic in vitro to *V. dahliae*, also decrease the phytotoxicity of aqueous extract of olive residue on shoot and root dry weight of tomato.

4. Conclusions

We demonstrated in this work, that the olive residue decreased the negative effect of *V. dahliae* on the growth of tomato (*Solanum lycopersicum* L.), by the antifungal compounds present in the olive residue and by the antifungal substances produced by the antagonistic saprophytic fungi grown in this residue. The use of olive residues incubated with specific isolates of

several saprophytic fungi open possible ways towards its use in agriculture and the possibility of its future use in the biological control of *Verticillium* wilt of tomato; however, more investigation is necessary.

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