

Short communication

Are plant cell wall hydrolysing enzymes of saprobe fungi implicated in the biological control of the *Verticillium dahliae* pathogenesis?

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ABSTRACT

We studied the possibility that the antagonistic saprobe fungi *Corioloopsis rigida*, *Trametes versicolor*, *Fusarium lateritium*, *Penicillium chrysogenum* and *Verticillium dahliae*-2379 may control *V. dahliae* disease through the inhibition of the activity of hydrolytic enzymes produced by the pathogen. These saprobe fungi were able to decrease the growth *in vitro* of *V. dahliae*. The exudates produced by these fungi seemed to be the main factor responsible for their antagonistic effect. The exudates of all the fungi tested had endopolymethylgalacturonase, endoglucanase and endoxyloglucanase activities. Exudates of *P. chrysogenum* and *V. dahliae*-2379 had higher hydrolytic activities than those of *V. dahliae*. The saprobe *V. dahliae*-2379 did not decrease the growth of the pathogen *V. dahliae* but decreased its harmful effect on the growth of tomato. However, no inhibition of the activities of the hydrolytic enzyme of the pathogenic *V. dahliae* by the non-pathogenic *V. dahliae*-2379 or by the other saprobe fungi was observed. Nevertheless, the protection of tomato by the five antagonistic saprobe fungi against *V. dahliae* disease could be carried out by the induction of plant defense by the hydrolytic enzymes produced by these fungi.

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1. Introduction

The destructive vascular wilt that *Verticillium dahliae* Kleb. causes in plants (Rodríguez et al., 2009) is well known. As chemical treatments have been unsuccessful in reducing disease severity, biological control using microorganisms under both field and greenhouse conditions has been proposed (Tjamos, 2000; Larena et al., 2003). Plant pathogenic microorganisms enter plant cells mainly by production of cell wall hydrolytic enzymes such as pectinases, cellulases and xyloglucanases (Walton, 1994). *V. dahliae* is able to produce pectinases and cellulases (Pegg and Brady, 2002). The production of pectolytic enzymes has been considered a prerequisite for virulence of *V. dahliae* (Carder et al., 1987; Bidochka et al., 1999). Inhibition of the pectinase activity of *V. dahliae* by plants has been found and seems to be related to a decrease in *V. dahliae* disease (Bulantseva et al., 2005). Endo polygalacturonase inhibition of plant pathogenic fungi by non-pathogenic fungi has also been observed (Roco and Pérez, 2001). However, no studies on inhibition of cell wall degrading enzymes of *V. dahliae* by bio-controller microorganisms have been done. Therefore, we studied the possibility that antagonistic saprobe fungi may control *V. dahliae*

disease through the inhibition of the activity of cell wall degrading enzymes produced by the pathogen.

2. Material and methods

The saprobe fungi *Corioloopsis rigida* (Berk. & Mont.) Murrill (CECT 20449), *Trametes versicolor* Pilát (A 136) from the Centro de Investigaciones Biológicas (Madrid, Spain), *Fusarium lateritium* Nees (BAFC 2317), *V. dahliae* Kleb. (BAFC 2379) from the Universidad de Buenos Aires (Argentina) and *Penicillium chrysogenum* Thom. (EEZ 10) from the Estación Experimental del Zaidín (Granada, Spain), were used. These fungi, except *V. dahliae* (BAFC 2379), were selected, from 45 soil or white rot saprobe fungi because of their antagonism against *V. dahliae* (EEZ 2), by using the technique described by Ortiz and Orduz (2001). *V. dahliae* was grown in plates of potato dextrose agar (PDA) for 3 days, then each antagonist fungus was transferred to PDA plates 3 cm apart from the *V. dahliae* colony. These plates were incubated at 25 °C for 11 d and the cultures were evaluated macroscopically every 48 h. There were 10 replicates for each treatment and plates with *V. dahliae* plus *V. dahliae* plugs were used as controls.

The growth of *V. dahliae* in the presence of exudates of the saprobe fungi was measured by the microplate reader technique (Ludwig and Boller, 1990). Fifty µl of potato dextrose broth (PDB) with approximately 2000 spores or mycelial fragments of *V. dahliae* and 50 µl of PDB with the exudates of 15-day-old saprobe fungal

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Table 1

Endopolymethylgalacturonase (EPMG), endoglucanase (EGN), and endoxyloglucanase (EXG) activities (units/mg protein $\times 10^{-3}$) of saprobe fungi and *V. dahliae* in presence or not of exudates of the saprobe fungi.

Treatment	EPMG	EGN	EXG
<i>C. rigida</i>	44.5 a	46.9 c	4.3 a
<i>T. versicolor</i>	31.2 a	49.8 c	6.5 a
<i>F. lateritium</i>	38.8 a	43.1 c	1.3 a
<i>P. chrysogenum</i>	197.9 b	42.1 c	1.5 a
<i>V. dahliae</i> -2379	255.5 c	43.1 c	4.6 a
<i>V. dahliae</i>	41.1 a	56.1 c	3.7 a
<i>V. dahliae</i> + <i>C. rigida</i> exudates	42.6 a	64.9 c	3.7 a
<i>V. dahliae</i> + <i>T. versicolor</i> exudates	40.3 a	56.4 c	1.2 a
<i>V. dahliae</i> + <i>F. lateritium</i> exudates	38.7 a	45.5 c	7.6 a
<i>V. dahliae</i> + <i>P. chrysogenum</i> exudates	491.4 c	47.2 c	28.3 b
<i>V. dahliae</i> + <i>V. dahliae</i> -2379 exudates	485.1 c	45.7 c	11.8 b

Each value is the mean of four replicates. Values sharing the same letter do not differ significantly according to Tukey's multiple range test ($P < 0.05$).

culture were added to the microplate wells and incubated at 25 °C. The mixture was equilibrated in the wells for 2 h before the first absorbance reading was taken. The absorbance of the well content was measured at 405 nm after 2 and 48 h with a microplate reader. The absorbance values were calculated by subtracting the values of the 2 h from those of the 48 h measurements. There were 4 replicates for each treatment.

To determine the hydrolytic enzyme activities, the saprobe fungi and the pathogen *V. dahliae* were grown in 100 ml of PDB during a fortnight at 25 °C. The culture liquid was separated from the mycelium by centrifugation (5000 \times g) and filtration (Whatman No.1 filter paper) and the supernatant was considered as the extracellular enzyme extract. Twenty five ml of supernatant from each saprobe fungal culture were mixed with 25 ml of supernatant from *V. dahliae* culture. Fifty ml supernatant of each fungal culture was also used. The endoglucanase (EC 3.2.1.4), endopolymethylgalacturonase (EC 3.2.1.15) and endoxyloglucanase (3.2.1.151) activities were measured by the viscosity method (Rejon-Palomares et al., 1996) using CMC, citrus pectin and xyloglucan from nasturtium seed as substrates. The reduction in viscosity was determined after 0 and 48 h incubation of the reaction mixture at 25 °C. The reaction mixture contained 1 ml of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5.0) and 0.2 ml of extracellular enzyme extracts. Autoclaved enzyme supernatants and autoclaved buffers were used as controls. Total proteins were measured (Bradford, 1976). There were 4 replicates for each treatment and enzyme.

The effect of the exudates of the saprobe fungi *C. rigida*, *T. versicolor*, *F. lateritium*, *P. chrysogenum* and *V. dahliae*-2379 on the growth of tomato (*Solanum lycopersicum* L. cv. Muchamiel) cultured in presence of *V. dahliae* was tested. The experiments were carried out in 0.3 l pots containing steam-sterilized grey loam soil (pH 8.1) and quartz sand mixed in a 1:1 ratio (v:v). Thirty ml of exudates from 15-day-old saprobe fungi cultured in PDB or 30 ml of an aqueous suspension of *V. dahliae* in sterile distilled water (7×10^6 spore ml⁻¹) were added to soil pots. Pots with PDB medium without fungi were used as control. Plants were grown in a greenhouse with natural light supplemented by Sylvania incandescent and cool-white lamps giving 400 nmol m² 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 with 10 ml Hewitt's nutrient solution (Hewitt, 1952). A 2 \times 6 full factorial randomized experimental design was used. The treatments were, on the one hand, uninoculated controls and soil pots inoculated with *V. dahliae* (named Pathogen); and, on the other hand, uninoculated controls and soil pots inoculated with exudates of: *P. chrysogenum*, *F. lateritium*, *T. versicolor*, *C. rigida*, and *V. dahliae*-2379 (named Biocontroller). Plants were inoculated at the time of transplanting. Plants were

harvested after 4 weeks and shoot and root dry weights were determined. Four replicate pots per treatment and one plant per pot were used.

The percentage values were arcsine transformed for statistical analyses. Statistical procedures were carried out with the SPSS software, version 11.0 (SPSS Inc., 1989–2001). The data were analysed by one-way analysis of variance (ANOVA) and treatment means were compared using the standard errors of mean and Tukey's multiple range test. Statistical significance was determined at $P < 0.05$. Each experiment was repeated at least twice.

3. Results

The percent mycelial growth inhibition of the pathogen when *V. dahliae* was grown in the presence of *V. dahliae* was 2.8 (SE = 0.8). The percent mycelial growth inhibition of *V. dahliae* (4.4, SE = 2.1) did not differ significantly when it was grown in presence of *V. dahliae*-2379. However, the percent mycelial growth inhibition of the pathogen *V. dahliae* decreased significantly to 32.7 (SE = 4.7), 37.1 (SE = 4.9), 26.6 (SE = 6.1) and 32.1 (SE = 3.5) when it was grown in the presence of the saprobe fungi *C. rigida*, *T. versicolor*, *F. lateritium* and *P. chrysogenum*, respectively.

The growths of *V. dahliae* cultured in the absence (0.4 absorbance units, SE = 0.03) and presence of exudates of *V. dahliae*-2379 (0.35 absorbance units, SE = 0.08) did not differ significantly. The exudates of *C. rigida*, *T. versicolor*, *F. lateritium* and *P. chrysogenum* decreased the growth of *V. dahliae* to 0.052 (SE = 0.018), 0.081 (SE = 0.011), 0.11 (SE = 0.03) and 0.051 (SE = 0.02) absorbance units, respectively.

Table 1 show that all the fungi tested had endopolymethylgalacturonase, endoglucanase and endoxyloglucanase activities. The endopolymethylgalacturonase, endoglucanase and endoxyloglucanase activities of the saprobe fungi *C. rigida*, *T. versicolor*, *F. lateritium* did not differ significantly from those of the pathogen *V. dahliae*. However, *P. chrysogenum* and *V. dahliae*-2379 have higher

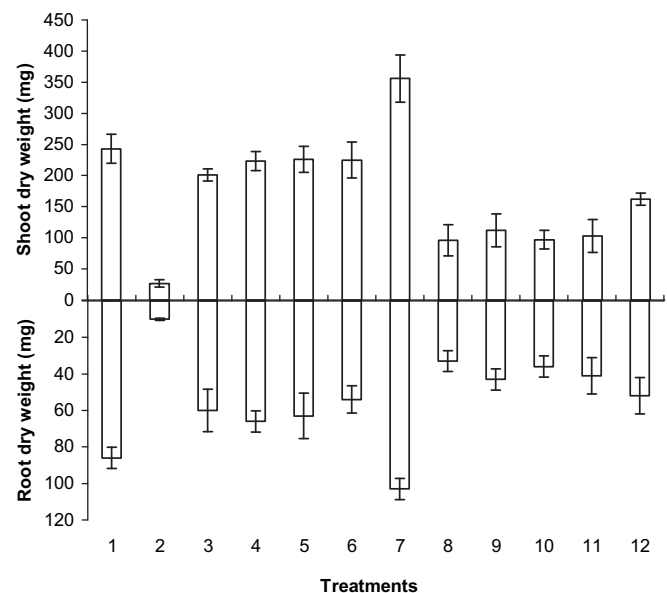


Fig. 1. Effect of *Verticillium dahliae* and exudates of saprobe fungi on shoot and root dry weight of tomato (*Solanum lycopersicum* L.). 1 = Uninoculated control; 2 = Plants inoculated with *V. dahliae*; 3, 4, 5, 6 and 7 = Plant inoculated with exudates of *Coriopsis rigida*, *Trametes versicolor*, *Fusarium lateritium*, *Penicillium chrysogenum* and *V. dahliae*-2379 respectively; 8, 9, 10, 11 and 12 = Plants inoculated with *V. dahliae* plus exudates of *C. rigida*, *T. versicolor*, *F. lateritium*, *P. chrysogenum* and *V. dahliae*-2379 respectively. The data are shown as mean \pm standard error of the mean.

endopolymethylgalacturonase activity but similar endoglucanase and endoxyloglucanase than the other fungi tested. The exudates of *P. chrysogenum* and *V. dahliae*-2379 increased the endopolymethylgalacturonase and the endoxyloglucanase activities of the pathogenic fungus *V. dahliae*. However, the exudates of *C. rigida*, *T. versicolor* and *F. lateritium* did not increase the endopolymethylgalacturonase, endoglucanase and endoxyloglucanase activities of the pathogenic fungus *V. dahliae*.

The results of factorial ANOVA showed significant differences in the population means of the response variables (shoot and root dry weight) to the all levels of Pathogen and Biocontroller main factors ($P < 0.001$). The contrasts between the entire factors Pathogen and Biocontroller were statistically significant ($P < 0.001$) for all the response variables.

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 (Fig. 1). The highest significant increase in the shoot and root dry mass of tomato was found with inoculation with the saprobe *V. dahliae*-2379. As Fig. 1 shows, the shoot and root dry weight of tomato inoculated with *V. dahliae* plus the exudates of all the saprobe fungi tested were significantly higher than plants inoculated with *V. dahliae* alone ($P < 0.001$).

4. Discussion

Five saprobe antagonistic fungi from 5 genera tested decrease the growth *in vitro* of *V. dahliae* disease. However, the fact that the saprobe *V. dahliae*-2379 did not decrease the growth of the pathogen *V. dahliae* but decreased its negative effect on the growth of tomato indicates other mechanisms than competition in the interaction between pathogenic and non-pathogenic species of *Verticillium* were in play. We observed that the exudates produced by these antagonistic saprobe fungi seem to be the main ones responsible for their antagonistic effect. The exudates of all antagonistic fungi tested had endopolymethylgalacturonase, endoglucanase and endoxyloglucanase activities. *V. dahliae*-2379 and *P. chrysogenum* had higher hydrolytic activities than *V. dahliae*. A close relationship between the production of the hydrolytic enzymes pectinases and the virulence of *V. dahliae* has been found (Leal and Villanueva, 1962; Carder et al., 1987; Bidochka et al., 1999). However, in spite of the production of the pectinase endopolymethylgalacturonase and the other plant cell wall degrading enzymes endoglucanase and endoxyloglucanase by the saprobe fungi tested, no pathogenic effect of these fungi on tomato growth was observed. On the other hand, inhibition by plants of the pectinase activity of *V. dahliae* has been proposed as a possible mechanism of inhibition of *V. dahliae* disease (Bulantseva et al., 2005). However, the lack of inhibition of the hydrolytic enzyme activities of the pathogenic *V. dahliae* by the non-pathogenic *V. dahliae*-2379, as well as by the other saprobe fungi, indicates us that the antagonistic effect of the saprobe fungi works through other mechanisms. Nevertheless, it is known that fungal enzymes hydrolyse plant cell wall components generating oligo-fragments

which could act as elicitors of plant defense against pathogens (Lagaert et al., 2009). Therefore, the hydrolytic enzymes produced by the five antagonistic saprobe fungi tested could hydrolyse tomato root cell wall components generating low-molecular-weight compounds which could act as elicitors of plant defense against *V. dahliae* disease. Induced plant systemic resistance is a possible other mechanism involved in the action for selected non-pathogenic antagonists as happens with *Fusarium* spp. (Larkin and Fravel, 1999) and will be investigated by other techniques such as the split plants root systems (Khaosaad et al., 2007).

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