



Trichoderma asperellum is effective for biocontrol of Verticillium wilt in olive caused by the defoliating pathotype of *Verticillium dahliae*



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ARTICLE INFO

Article history:

Received 18 February 2016

Received in revised form

11 May 2016

Accepted 23 May 2016

Keywords:

Antibiosis

Biological control

Antagonism

Rhizosphere colonization

Growth promotion

'Picual' olive

Olea europaea

ABSTRACT

Verticillium wilt caused by a highly virulent, defoliating (D) pathotype of *Verticillium dahliae* is threatening olive production in Spain and other Mediterranean countries. This disease must be managed by an integrated strategy, in which biocontrol agents can play an important role. We have investigated the potential of *Trichoderma asperellum* strains for antagonism against *V. dahliae* and suppression of Verticillium wilt of olive caused by the D pathotype. First, we tested the antagonistic potential of *T. asperellum* strains Bt2, Bt3 and T25 against six *V. dahliae* isolates, four of the D and two of the nondefoliating (ND) pathotypes, in different *in vitro* assays. All *T. asperellum* strains overgrew the colonies of all *V. dahliae* isolates to a similar extent. However, extracellular compounds from strains Bt3 and T25 showed higher anti-*V. dahliae* activities than those of Bt2 in membrane assays. Also, growth of Bt2 was reduced by ND *V. dahliae* whereas that of Bt3 and T25 was not affected by *V. dahliae*-secreted compounds. *In planta* assays using strains Bt3 and T25, and 'Picual' olive plants, showed that the two *T. asperellum* strains significantly reduced the severity of symptoms and the standardized area under the disease progress curve caused by highly virulent D *V. dahliae*, but not the final disease incidence. Strain T25 significantly increased growth of 'Picual' plants and displayed higher ability for colonizing the olive rhizosphere and establishing endophytic infection in olive roots than Bt3.

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1. Introduction

Verticillium wilt caused by the asexual, vascular-colonizing, soilborne fungus *Verticillium dahliae* Kleb. (Inderbitzin et al., 2011; www.mycobank.org) is an important disease affecting olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) worldwide (Jiménez-Díaz et al., 2012). The fungus can survive in the soil by means of melanized microsclerotia without a host for at least 14 years (Wilhelm, 1955). Microsclerotia in soil germinate multiple times in response to root exudates. The resulting hyphae can penetrate the olive roots, grow across the root cortex, invade the xylem vessels

and form conidia that spread upward in the olive stem and give rise to extensive xylem colonization and functional impairment (Jiménez-Díaz et al., 2012). As a result, attacks by the disease can cause severe losses of fruit yield as well as tree death (Levin et al., 2003; Jiménez-Díaz et al., 2012). Fruit yield loss in affected Picual olive trees were estimated to amount 75–89% the third to fifth years after planting in an *V. dahliae*-infested soil (Levin et al., 2003). This confers Verticillium wilt in olive with a high socio-economic significance because of the extent of olive cultivation, which span over 10⁷ ha in more than 20 countries in temperate areas worldwide (FAO, 2012).

Verticillium wilt in olive was first observed in Spain in 1979 in orchards at the south near Córdoba, Andalusia. Subsequently, severe attacks by the disease occurred throughout that region. Recent assessment of disease prevalence in Andalusia indicated that more than 50% olive orchards were affected by Verticillium wilt (Ruiz

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Torres, 2010), and diseases surveys over 90 arbitrarily-chosen orchards in this region indicated 71% disease prevalence with a mean incidence of 20% in the affected orchards (López-Escudero et al., 2010). This makes *Verticillium* wilt one of main concerns for the Andalusian olive industry (Jiménez-Díaz et al., 2012; Areal and Riesgo, 2014).

The increase in distribution and importance of *Verticillium* wilt in olive in Andalusia has occurred together with the spread of a highly virulent, defoliating (D) *V. dahliae* pathotype that have displaced a previously existing nondefoliating (ND) one (Jiménez-Díaz et al., 2011, 2012). The D pathotype is characterized by its ability to cause a distinct defoliating syndrome, which involves early drop of symptomless, green leaves from individual olive twigs and branches that eventually gives rise to complete defoliation and necrosis. Conversely, the ND pathotype can cause extensive dieback of olive twigs and branches with leaf chlorosis and necrosis, but no leaf shedding, as well as flower mummification and necrosis of inflorescences (Navas-Cortés et al., 2008; Jiménez-Díaz et al., 2012).

Control of *Verticillium* wilt in olive is made difficult by the long-term survival of microsclerotia in soil, broad host range of the pathogen and lack of success of fungicide treatments in infected trees (Pegg and Brady, 2002; Jiménez-Díaz et al., 2012). Actually, application of an integrated strategy is required for the management of the disease because no control measure applied singly is fully effective for that purpose (Jiménez-Díaz et al., 2012). Thus, use of preplanting (i.e., eradication or reduction of pathogen inoculum in soil, use of *V. dahliae*-free planting material, protection of healthy planting material by root treatment with biocontrol agents, and use of resistant cultivars and rootstocks) and postplanting disease control measures are recommended for the effective management of *Verticillium* wilt in olive. Postplanting disease control measures include mainly cultural practices, soil solarization and treatment with biological control agents (Tjamos et al., 2004).

A preliminary study indicated that some strains of *Trichoderma* spp. bear potential for biocontrol of *Verticillium* wilt in olive and thus for effectively contributing to the integrated management of the disease, but the mode of actions of those strains were not determined (Jiménez-Díaz et al., 2009). *Trichoderma* species (teleomorph *Hypocrea*) are filamentous fungi of worldwide distribution, including agricultural habitats, which are well-known biological control agents against plant pathogenic fungi, oomycetes and even nematodes (Lorito et al., 2010; Monte, 2001). The mechanisms of *Trichoderma*-based biocontrol rely mainly on the production of antibiotics and/or hydrolytic enzymes, as well as competition for nutrients and the systemic activation of plant defense responses (Harman et al., 2004; Hermosa et al., 2012; Shores et al., 2010). Most of *Trichoderma* strains used as active ingredient in commercial biocontrol products belong to selected rhizosphere-competent species such as *Trichoderma harzianum*, *Trichoderma atroviride*, *Trichoderma asperellum* and *Trichoderma virens* (Druzhinina et al., 2011). The use of *T. asperellum*-based products has grown exponentially in the last few years since this species was included in the list of biocontrol microorganisms for registration proposes in the European Union, as well as in Australia, Canada, China, India, New Zealand, South Africa, and the USA (Woo et al., 2014). *Trichoderma asperellum* was split from *Trichoderma viride* species complex using DNA sequencing (Lieckfeldt et al., 1999). More recently, multilocus genealogies of four genomic regions showed that *T. asperellum* sensu lato consists of two cryptic species, *T. asperellum* and *Trichoderma asperelloides* (Samuels et al., 2010). Strains identified as *T. asperellum* have been shown a wide spectrum for plant disease control (Mbarga et al., 2012; Narasimha-Murthy et al., 2013; Trillas et al., 2006).

In the present study, we have investigated the mode of action of different *T. asperellum* strains in the biocontrol of D *V. dahliae*, their

ability to colonize the rhizosphere of olive plants, and their capacity to suppress development of *Verticillium* wilt caused by the D pathotype.

2. Materials and methods

2.1. Antagonistic and pathogenic fungi

Trichoderma asperellum IMI 296237 (referred to as T25), *T. asperellum* ICC012 (referred to as Bt2) and *T. asperellum* Bt3 were used in this study. *Trichoderma* strains were routinely grown on potato dextrose agar (PDA, Difco, Scientific Laboratory Supplies) on Petri dishes at 25 °C in the dark and stored in test tubes at –80 °C in 30% glycerol.

Monoconidial cotton and olive isolates of *V. dahliae* previously typed to vegetative compatibility group (VCG), clonal lineage, and D and ND pathotypes, namely V-4I, V-T9 (listed as T9), V-138I, V-1477I, V-1558I and V-1900I, were used in this study (Collado-Romero et al., 2006; Korolev et al., 2001; Jiménez-Díaz et al., 2011; Jiménez-Gasco et al., 2014; Milgroom et al., 2014). Isolates V-T9, V-138I, V-1477I, and V-1900I belong to VCG1A and are of the D pathotype. Isolates V-4I and V-1558I are heterokaryon self-incompatible or belong to VCG2A, respectively, and both are of the ND pathotype. Plum extract agar (900 mL of distilled water, 20 g of agar, 100 mL of concentrated plum extract, 1 g of yeast, 5 g of lactose, pH 5.6–6.9; Talboys, 1960) cultures of these isolates covered with liquid paraffin were stored at 4 °C in the dark (Bejarano-Alcázar et al., 1996) and are deposited in the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain. Active cultures of isolates were obtained by placing small agar plugs from stock cultures on chlortetracycline-amended water agar (CWA; 1 L distilled water, 20 g agar, 30 mg chlortetracycline) and further subculturing on PDA. Cultures on PDA were grown for 4–10 days at 25 °C in the dark and used for assays and inoculum increase.

2.2. Antagonism assay

Confrontation assays (dual cultures) between *T. asperellum* strains Bt2, Bt3 and T25, and five *V. dahliae* isolates of the D (V-T9, V-1477I, V-1900I, V-138I) and ND (V-1558I) pathotypes, were carried out as previously described (Rubio et al., 2009) with some modifications. Briefly, *V. dahliae* was allowed to grow on a Petri dish at 25 °C in the dark for 4 days before placing a 5-mm diameter agar plug colonized by a *T. asperellum* strain at 2 cm from the border on the opposite side of the same plate on which the pathogen was grown. Cultures of the pathogen and the biocontrol tested strain growing alone were used as controls. Dual cultures were performed in triplicate and colony area of the pathogen was measured after 12 days of incubation at 25 °C in the dark. Results were expressed as percentage of colony growth inhibition.

2.3. Membrane antifungal assays

Two sets of membrane assays were performed. The first one included the same *T. asperellum* strains and *V. dahliae* isolates used for the above described antagonism assay. Growth assays on cellophane sheets and 14 kDa-cut-off dialysis cellulose membranes were carried out in triplicate as previously described (Rubio et al., 2009). The diameters of the fungal colonies were measured after 10 days of incubation at 25 °C in the dark. Results were expressed as the percentage growth inhibition of each *V. dahliae* isolate by each *T. asperellum* strain with respect to the mean colony diameters of each of fungi grown alone.

A second antifungal assay included the three *T. asperellum* strains and *V. dahliae* isolates D V-138I and ND V-4I, and it was carried out using 14 kDa-cut-off dialysis cellulose membranes. An agar plug cut off from the growing edge of a 10-day-old colony of a *V. dahliae* isolate was placed in the center of a Petri dish containing Czapek-Dox agar (CDA, Difco) and incubated at 25 °C for approximately 1 week until the colony reached a diameter of approximately 3 cm. Similarly, another agar plug cut from the growing edge of a 1-week-old colony of a *T. asperellum* strain was placed in the center of a Petri dish containing PDA covered with a sterile cellulose membrane, and it was allowed to grow at 25 °C for 1 day until the colony reached a diameter of ca. 3 cm too. The cellulose membrane bearing the *T. asperellum* colony was deposited on top of the *V. dahliae* CDA culture. These co-cultures were incubated at 25 °C for 1 week or until the *T. asperellum* strain reached the edge of the Petri dish. In parallel, cultures of *T. asperellum* and *V. dahliae* isolates grown alone, and developed on cellulose membrane or not, were used as controls. Every condition was tested in triplicate and results were expressed as the mean colony diameter increase (cm) recorded for the two fungi.

2.4. In vivo assays in olive plants

The effect of *T. asperellum* strains Bt3 and T25 on development of Verticillium wilt and growth of olive plants was tested under controlled conditions using certified, 4-month-old plants of highly susceptible cv. Picual and highly virulent D *V. dahliae* isolate V-138I (Jiménez-Díaz et al., 2009, 2012). Own-rooted plants of “Picual” were propagated by Plantas Continental, S.A. (Posadas, Córdoba, Spain) by rooting leafy stem cuttings in a pasteurized potting mixture (peat: sand, 2:1, v/v) under mist conditions in plastic tunnels (Caballero and Del Río, 2010).

Inoculum of D *V. dahliae* consisted of infested cornmeal-sand mixture (CMS; sand: cornmeal: deionized water, 9:1:2, w/w) (Nene and Haware, 1980). Infested CMS was produced in Erlenmeyer flasks containing 400 g autoclaved (twice at 121 °C for 1.5 h) mixture and ten 5-mm diameter PDA discs from the growing edge of 7-day-old cultures of *V. dahliae* V-138I, and incubating at 25 °C in the dark for 4 weeks. The infested CMS was homogenized, allowed to desiccate in an incubator adjusted to 33 °C for 3 days and thoroughly mixed with a pasteurized soil mixture (clay loam: peat, 2:1, v/v; pH 8.4, 24% water holding capacity) at a rate of 3% (w/w) to reach an inoculum density of 1.3×10^6 colony forming units (cfu) g soil⁻¹ of *V. dahliae* V-138I as determined by dilution-plating on semi-selective NP-10 medium (Kabir et al., 2004).

Inocula of *T. asperellum* strains consisted of infested wheatbran cornmeal-sand mixture [WCMS; sand: wheatbran-cornmeal (1:1, w/w); deionized water, 9:1:2, w/w] or a conidia suspension. Inocula in WCMS were produced as described for *V. dahliae* V-138I, except that six agar plugs were used to infest the WCMS mixture and the infested mixture was incubated for 2 weeks. Thereafter, the *T. asperellum*-infested WCMS was mixed with the pasteurized soil mixture as above to reach an inoculum density of 1×10^7 cfu g soil⁻¹ as determined on a *Trichoderma*-selective medium (Elad et al., 1981). Conidial inoculum was obtained from 7-day-old cultures on PDA incubated as above. Conidia in sterile water scrapped from cultures were filtered through eight layers of sterile cheesecloth and inoculum concentration was adjusted to 1×10^7 conidia mL⁻¹ with sterile water using a haemocytometer.

Plants of “Picual” olive were inoculated with the Bt3 and T25 strains by transplanting them to 0.9 L (9 × 9 × 11 cm) disinfested plastic pots (one plant per pot) with either (i) the potting soil mixed with the infested WCMS or (ii) the potting soil mixed with sterile WCMS then drenching the soil around a plant with 60 mL of the 1×10^7 conidia mL⁻¹ suspension, followed by additional 20 mL of

sterile water to allow for conidia being washed down the soil profile. Control plants were treated similarly except for the absence of *T. asperellum* inoculum. Inoculated and control plants were incubated in a growth chamber adjusted to 22 ± 2 °C, 60–80% relative humidity and a 14-h photoperiod of fluorescent light of 360 μmol m⁻² s⁻¹ for 2 weeks. Thereafter, plants were carefully uprooted to retain most of rhizosphere soil, transplanted to *V. dahliae* V 138I-infested or –noninfested potting mixture in 1.5 L (11 × 11 × 13 cm) pots (one plant per pot), and incubated for 10 weeks in the growth chamber at same conditions of above, which are optimal for infection by D *V. dahliae* and development of Verticillium wilt in olive (Calderón et al., 2014). Plants were watered every 1–2 days as needed and fertilized weekly with 50 mL Hoagland’s nutrient solution (Hoagland and Arnon, 1950). Experiments consisted of a three-way factorial treatment design with *T. asperellum* treatment (control, Bt3, T25), antagonist application method (infested WCMS, soil drench) and D *V. dahliae* inoculation (inoculated, uninoculated). There were 10 replicated plants per treatment in a completely randomized design. The experiment lasted 12 weeks and was conducted twice with similar results.

The disease reaction in the plants was assessed by the incidence (percentage of plant showing disease symptoms) and severity of foliar symptoms. Symptoms were assessed on individual plants on a 0 to 4 rating scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant) at weekly intervals throughout the duration of experiments (Mercado-Blanco et al., 2004). Data on symptom severity were plotted over time and to obtain disease severity progress curves and the area under the curves standardized by duration of disease development in days (SAUDPC) was calculated using the trapezoidal integration method (Madden et al., 2007). Upon termination of experiments, 12 weeks after inoculation with *T. asperellum*, plants were excised at the soil level and the height (cm) of the main stem was measured for each plant. Also, infection by *V. dahliae* was determined in each plant by isolating the fungus on CWA medium. Four 1-cm-long stem pieces representative of the stem length were thoroughly washed under running tap water, the bark aseptically removed, and the pieces were surface-disinfested in 0.5% NaClO for 1.5 min, washed twice with sterile water and plated onto the medium. Cultures were incubated at 25 °C in the dark for 7 days. Colonies of *V. dahliae* were identified by microscopic observation of verticillate conidiophores and formation of microsclerotia (Inderbitzin et al., 2011).

The extent of rhizosphere and endophytic root colonization by the *T. asperellum* strains were assessed on four arbitrarily chosen olive plants per experimental antagonist treatment combination 2 and 12 weeks after inoculation. Plants were uprooted delicately from the pots and shaken gently to remove all but the most tightly adhering rhizosphere soil. The roots of a plant were cut into 1-cm segments, and 1 g of these segments were stirred in 20 mL of sterile water for 10 min and further sonicated for 10 min to remove bacteria and/or fungi from the roots. Serial dilutions of the washings were plated onto *Trichoderma*-selective medium (Elad et al., 1981) and incubated as above. Populations of *T. asperellum* were expressed as cfu g⁻¹ of fresh root tissue.

To determine the incidence of endophytic root colonization in *T. asperellum* inoculated plants, 12 arbitrarily selected roots from each of the four olive plants of above sampled 2 weeks after inoculation were surface disinfested in 75% ethanol and 1.5% NaClO for 3 min and 5 min, respectively, then washed twice with sterile water. The sampled roots were then allowed to dry on sterile filter paper for 10 min under a flow of sterile air and cut into 15-mm segments. These segments were placed horizontally onto the *Trichoderma*-selective medium in Petri dishes and incubated at 25 °C in the dark for 7 days. Results were expressed as percentage of root segments

that yielded colonies of *T. asperellum*.

2.5. Statistical analysis

Data from *in vitro* assays were analyzed by analysis of variance (ANOVA) using a General Linear Model implemented in SPSS v. 10 (SPSS Inc., Chicago, IL). Pairwise comparisons were made using Tukey's test at $P = 0.05$.

Data from *in vivo* assays were subjected to ANOVA using STATISTIC v10.0 (NH Analytical Software, Roseville, MN). Similarity among repetitions of the experiment tested by preliminary ANOVA using experimental runs as blocks allowed combining data for analyses. Means of *T. asperellum*-inoculated plants were compared with the non-inoculated control using the Dunnett's test at $P = 0.05$.

3. Results

3.1. Antifungal activity of *Trichoderma asperellum* strains against *Verticillium dahliae* isolates

The antagonistic behavior of *T. asperellum* strains Bt3, T11 and T25 against five isolates of *V. dahliae* was tested in dual cultures to select those with the highest biocontrol potential against Verticillium wilt in olive. All three strains of *T. asperellum* were able to overgrow and reduce the colony size ($P < 0.05$) of the tested *V. dahliae* isolates regardless of their belonging to the D or ND pathotype (Table 1). Overall, strains Bt2, Bt3, and T25 did not differ significantly ($P \geq 0.05$) in their ability to inhibit colony growth of the D and ND *V. dahliae* isolates.

The putative role of extracellular compounds as a mode of action in the antagonism of Bt2, Bt3 and T25 against the D and ND *V. dahliae* isolates was also evaluated. This was achieved by assaying the effect of the total compounds secreted by antagonistic isolates (cellophane sheet), or only molecules with a molecular weight lower than 14-kDa (dialysis membrane) on growth of *V. dahliae*. Results of the growth inhibition (expressed as percentage of control) of the five *V. dahliae* isolates by the three *T. asperellum* strains are summarized in Table 2. In general, the three *T. asperellum* strains inhibited growth of the five *V. dahliae* isolates, though higher inhibition was observed for strains Bt3 and T25 compared with Bt2 regardless of the tested *V. dahliae* isolates and type of membrane assay. The total compounds secreted by Bt3 and T25 displayed the highest ($P < 0.05$) inhibition against isolates V-138I and V-9T, respectively. Compounds with a molecular weight lower than 14-kDa from strain Bt3 determined a significantly ($P < 0.05$) higher inhibition against isolates V-138I, whereas those from strain T25 were more inhibitory against D isolate V-T9 and ND isolate V-1558I. These results indicate that *V. dahliae* is very sensitive to small compounds produced by strains Bt3 and T25 and that, at least in *in vitro* assays, these strains have the highest antagonistic potential against *V. dahliae*.

Table 1
Inhibition of *Verticillium dahliae* isolates colony growth on PDA after 12 days in dual culture with *Trichoderma asperellum* strains Bt2, Bt3 and T25.^a

	V-1558I	V-1477I	V-1900I	V-138I	V-T9
Bt2	67 ± 2.9	53 ± 3.5	40 ± 1.0	62 ± 6.7	66 ± 4.5
Bt3	68 ± 3.7	50 ± 3.1	46 ± 6.9	56 ± 3.0	68 ± 1.0
T25	71 ± 0.2	55 ± 5.3	44 ± 7.7	59 ± 7.1	74 ± 2.8

^a The pathogen was grown for 4 days before plating an agar plug colonized by *T. asperellum* opposite to *V. dahliae*. Results are expressed as the inhibition percentage with respect to the mean colony diameter of each fungus grown alone. Values are means of three replicates with the corresponding standard deviation.

To determine whether or not compounds secreted by either *V. dahliae* or *T. asperellum* may reciprocally affect each other growth, we confronted a colony from both fungi separated by a cellulose membrane. The three *T. asperellum* strains and the D V-138I and ND V-4I *V. dahliae* isolates were included in this assay. The development of colonies of V-138I and V-4I isolates when they were cultured on CDA medium under a cellulose membrane bearing a *T. asperellum* culture is shown in Fig. 1. The three strains of *T. asperellum* completely suppressed growth of V-138I (Fig. 1A). Conversely, growth of V-4I was reduced by 74, 65 and 87% by strains Bt2, Bt3 and T25, respectively, after 5 days and by 79% by strain T25 after 9 days (Fig. 1B). The growth of colonies of *T. asperellum* strains on a cellulose membrane over colonies of *V. dahliae* V-138I and V-4I is shown in Fig. 2. Initially, the rate of growth of *T. asperellum* strains was slowed down by the presence of V-138I under a cellulose membrane; however, that effect did not influence the final size of colonies, which was similar to the one reached in the control plates after 6 days of colony confrontation. In the case of interactions between strains of *T. asperellum* and *V. dahliae* V-4I, both the growth rate and final colony size of strain Bt2 were clearly reduced in the presence of this ND isolate under a cellulose membrane, whereas those of strains Bt3 and T25 were not affected. As a result of the several *in vitro* assays, strains Bt3 and T25 were selected to test their ability to suppress Verticillium wilt of olive by *in vivo* assays.

3.2. Rhizosphere colonization by strains of *Trichoderma asperellum* in olive plants

Strains Bt3 and T25 successfully colonized the rhizosphere of "Picual" olive plants, but the extent of colonization was influenced by the inoculation method (Table 3). Two weeks after transplanting to the potting soil mixed with *T. asperellum*-infested WCMS, strain T25 infected 25% of the sampled root fragments and had reached a rhizosphere population of 4.4×10^6 cfu g⁻¹ fresh root tissue. Comparatively, strain Bt3 infected 6.3% of root fragments and had reached a rhizosphere population 2.75 times lower than that for strain T25. Inoculation by soil drench with a conidia suspension led to root infection only for strain Bt3 (8.3%) and resulted in rhizosphere populations for both strains of ca. one order of magnitude lower compared with those from infested soil, although the difference between the two strains increased to 3.5 times. Extending the incubation to 12 weeks led the rhizosphere population of the two strains to decrease by ca. one order of magnitude in plants inoculated by transplanting to infested soil. Conversely, in soil drench-inoculated plants the rhizosphere population of Bt3 did not change after 12 weeks incubation but that of the T25 strain decreased to reach a level similar to Bt3, which was ca 27% that of the rhizosphere population occurring 2 weeks after inoculation (Table 3).

3.3. Suppression of *Verticillium wilt* in olive by *Trichoderma asperellum*

The effect of treatment with *T. asperellum* strains Bt3 and T25 on development of Verticillium wilt caused by D *V. dahliae* V-138I in "Picual" olive is shown in Table 4. No symptoms developed in non-inoculated plants and plants treated with Bt3 or T25 strains. Symptoms of Verticillium wilt developed on plants inoculated only with *V. dahliae* V-138I or treated with Bt3 or T25 then inoculated with the pathogen. Symptoms in untreated control developed by 29.5 days after transplanting to the *V. dahliae*-infested soil mixture, and treatment with strains Bt3 or T25 significantly ($P < 0.05$) extended the incubation period by ca. 7 and 8 days, respectively. Symptoms consisted of early dropping of asymptomatic, green

Table 2

Colony growth inhibition of strains of *Verticillium dahliae* by metabolites from strains Bt2, Bt3 and T25 of *Trichoderma asperellum* grown on cellophane or a 14-kDa cut-off dialysis membrane (dialysis) for 10 days.^a

<i>V. dahliae</i> Isolate	Cellophane			Dialysis		
	Bt2	Bt3	T25	Bt2	Bt3	T25
V-1558I	48 ± 0.3 a	46 ± 4.2 a	52 ± 3.3 a	37 ± 0.5 c	72 ± 2.3 b	90 ± 6.0 a
V-1477I	43 ± 3.5 a	57 ± 2.6 a	43 ± 1.5 a	34 ± 3.0 b	58 ± 3.8 a	56 ± 1.9 a
V-1900I	28 ± 2.5 b	58 ± 1.4 a	52 ± 1.2 a	20 ± 1.6 b	48 ± 1.7 a	51 ± 2.1 a
V-138I	41 ± 1.3 b	95 ± 4.7 a	50 ± 2.4 b	27 ± 1.3 b	74 ± 2.4 a	34 ± 1.6 b
V-T9	21 ± 1.9 c	46 ± 1.7 b	63 ± 1.6 a	20 ± 1.0 c	50 ± 10.4 b	68 ± 2.2 a

^a Results are expressed as the inhibition percentage of each *V. dahliae* isolate by each *T. asperellum* strain with respect to the mean colony diameter of each fungus grown alone. Values are means of three replicates with the corresponding standard deviation. For each experimental data set (cellophane or dialysis membrane), values in the same row with different letters are significantly different according to Tukey's test ($P < 0.05$).

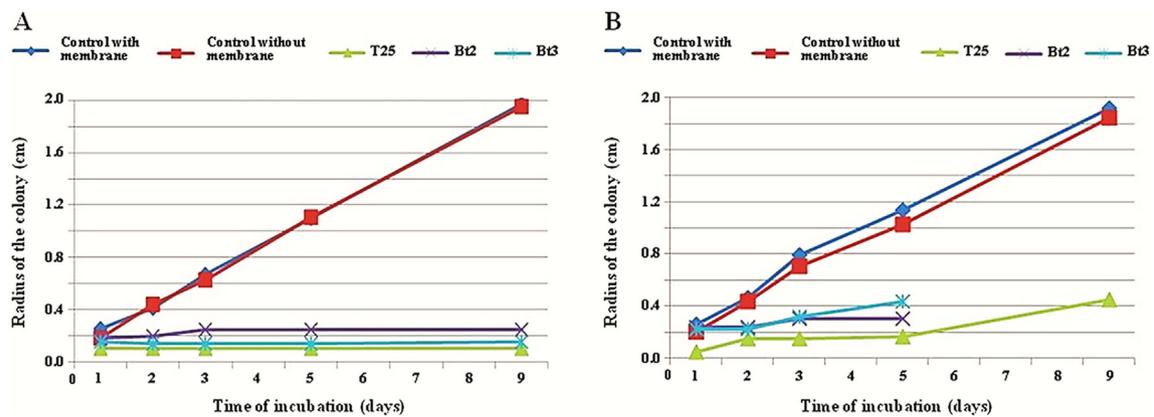


Fig. 1. Effect of compounds secreted from a colony of strains T25, Bt2 and Bt3 of *Trichoderma asperellum* placed on a cellulose membrane on growth of *Verticillium dahliae* defoliating isolate V-138I (A) and nondefoliating isolate V-4I (B) on Czapek-Dox agar under the cellulose membrane. The radius of colonies was recorded at the indicated time of incubation. Each data point is the mean of six replications.

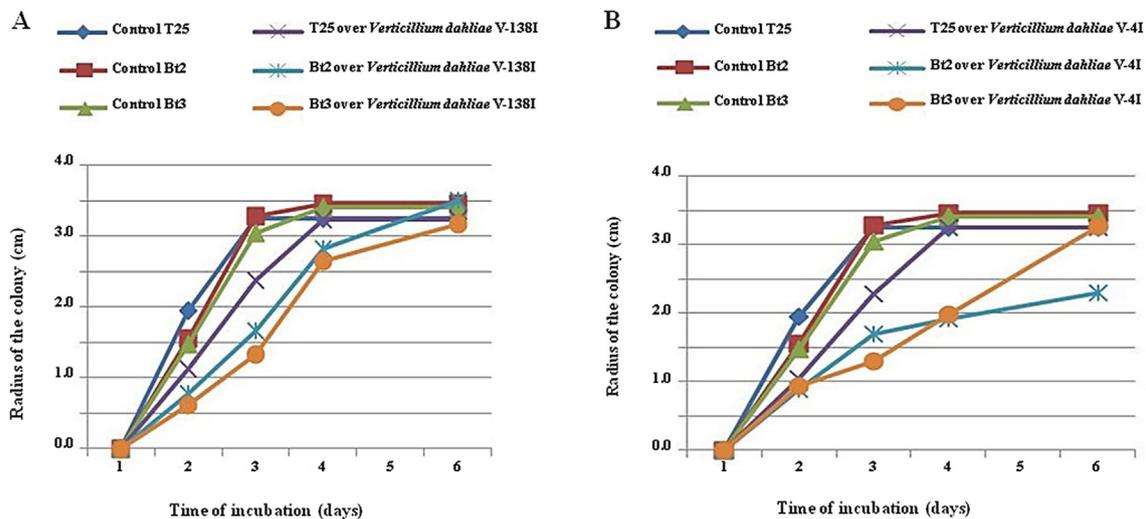


Fig. 2. Effect of compounds secreted from a colony of *Verticillium dahliae* defoliating isolate V-138I (A) and nondefoliating isolate V-4I (B) on Czapek-Dox agar, on growth of strains Bt2, Bt3 and T25 of *Trichoderma asperellum* on a cellulose membrane placed on top of *V. dahliae*. The radius of colonies was recorded at the indicated time of incubation. Each data point is the mean of six replications.

leaves from individual twigs that eventually resulted in complete defoliation, necrosis and death of the plant. These symptoms are typical for the defoliating syndrome caused by the *D. V. dahliae* pathotype (Jiménez-Díaz et al., 2012; Palomares-Rius et al., 2016). The method of treatment with *T. asperellum* had not an effect on the disease reaction and data from them were pooled together for further comparisons. By the end of the assay, 10 weeks after

inoculation with *V. dahliae* V-138I, 92.5% of *T. asperellum*-untreated “Picual” plants were affected with a mean severity of symptoms of 3.3 in the 0–4 rating scale. Treatment with strains Bt3 or T25 had not an effect on the disease incidence ($P > 0.05$) but significantly reduced ($P < 0.05$) the mean severity of symptoms and suppressed the SAUDPC by 43.2–48.4% (Table 4). There was no difference between strains Bt3 and T25 on the suppression of the disease.

Table 3
Rhizosphere colonization and endophytic infection by *Trichoderma asperellum* Bt3 and T25 in 4-month-old “Picual” olive plant 2 or 12 weeks after treatment.^a

Treatment	Rhizosphere population (cfu g ⁻¹ fresh root tissue) ^b (2 weeks)	Endophytic infection (% root fragments) ^c (2 weeks)	Rhizosphere population (cfu g ⁻¹ fresh root tissue) ^b (12 weeks)
Control (uninfested)	0.0	0.0	0.0
Bt3-infested WCMS	1.6 × 10 ⁶	6.3	1.9 × 10 ⁵
T25-infested WCMS	4.4 × 10 ⁶	25.0	4.5 × 10 ⁵
Control (water drench)	0.0	0.0	0.0
Bt3- conidial drench	2.0 × 10 ⁵	0.0	1.8 × 10 ⁵
T25- conidial drench	7.0 × 10 ⁵	8.3	1.9 × 10 ⁵

^a Plants were treated by transplanting to a potting soil mixed with *T. asperellum*-infested WCMS or by drenching the potting soil with a *T. asperellum* conidia suspension after transplanting. Plants were incubated at 22 ± 2 °C. Data are de mean of eight replicated plants per treatment combination.

^b Data are expressed as mean of colony-forming unit per g of soil. Determined by serial dilution of sonicated washings from 1 g of 1-cm-long root segments.

^c Data are expressed as percentage and referred to a total number of 96 root fragments tested per treatment (12 root fragments per plant, and eight plants).

Table 4
Effect of root treatment with strains Bt3 and T25 of *Trichoderma asperellum* on development of Verticillium wilt in “Picual” olive grown in soil infested with the defoliating *Verticillium dahliae* isolate V-1381.^a

<i>T. asperellum</i> treatment	Incubation period (days) ^b	Disease incidence (%)	Symptoms severity (0–4)	Standardized area under disease progress curve
Untreated	29.5	92.5	3.3	2.13
Bt3	36.9*	87.5	2.3*	1.10*
T25	37.1*	95.0	2.4*	1.21*

^a Plants were treated by transplanting to a potting soil mixed with *T. asperellum*-infested WCMS or by drenching with a *T. asperellum* conidia suspension after transplanting. Plants were incubated at 22 ± 2 °C for 2 weeks; then, plants were inoculated by transplanting to soil infested with *V. dahliae* V-1381. The method of treatment with *T. asperellum* did not influence the disease reaction and data from them were pooled. Data are de mean of 40 replicated plants per treatment combination. Significant differences between mean of a treatment and the untreated control ($P \leq 0.05$) and are indicated by *.

^b Time to first symptom.

3.4. Olive growth promotion by *Trichoderma asperellum*

The effect of treatment with *T. asperellum* strains Bt3 and T25 on growth of olive plant is shown in Table 5. Infection by D.V. *dahliae* V-1381 reduced by 51.1–69.1% the mean stem height of a plant. Compared with untreated plants, treatment with strains of *T. asperellum* increased the mean stem height of *V. dahliae*-uninfected and -infected plants regardless of the method of application of the antagonist. However, that effect was significant ($P < 0.05$) only for strain T25. Treatment with this latter strain by transplanting to infested soil or drenching with a conidia suspension increased the mean stem height of uninfected plants by 126.1% and 25.1%, respectively, and by 82.6 and 140.7%, respectively, that in *V. dahliae*-infected plants. The effect of T25 on growth of olive plants was significantly higher ($P < 0.05$) than that of strain Bt3 in all cases except for uninfected plants drenched with a conidia suspension (Table 5).

4. Discussion

The biocontrol assays carried out in the present work revealed that strains of *T. asperellum* have antifungal activity against *V.*

dahliae via different mechanisms. Mycoparasitism is an ancestral feature in *Trichoderma* (Druzhinina et al., 2011). Also, it has been reported that the mycoparasitic potential of *Trichoderma* spp. varies depending upon the strains being confronted (Atanasova et al., 2013). We found that *T. asperellum* is able to overgrow D and ND isolates of *V. dahliae*. Since some systemic fungal pathogens such as formae speciales of *Fusarium oxysporum* are not mycoparasitized by *T. asperellum* and other *Trichoderma* spp. (Pérez et al., 2015; Taghdi et al., 2015), it would be interesting to explore the specific mycoparasitic potential of the hydrolytic activities induced by *V. dahliae* in *T. asperellum*.

Antibiosis is a biocontrol mechanisms against *V. dahliae*, as indicated by results of membrane assays with the production of diffusible compounds in the absence of the pathogen host (Table 2). The higher growth inhibition of *V. dahliae* recorded for Bt3 and T25 strains compared with that for strain Bt2 in the two membrane assays could be associated with a stronger antifungal activity of those two strains against *V. dahliae*. This pathogen must be very sensitive to small compounds produced by strains Bt3 and T25, since large inhibition of *V. dahliae* V-1558I, V-1477I and V-T9 by Bt3 and T25 was observed in the dialysis membrane assay, and only small molecules secreted by *Trichoderma*, able to pass through the

Table 5
Effect of 12-week treatment with strains Bt3 and T25 of *Trichoderma asperellum* on the main stem height (cm) of olive cv. Picual in soil uninfested or infested with the defoliating *Verticillium dahliae* isolate V-1381 (Vd).^a

<i>T. asperellum</i> treatment	Uninfested WCMS–/Vd noninoculated	Infested CMS/Vd inoculated	Water drench/Vd-noninoculated	Conidial drench/Vd inoculated
Untreated	17.6	8.6	19.1	5.9
Bt3	28.0	11.4	23.0	8.2
T25	39.8*	15.7*	23.9*	14.2*
(<i>P</i> for contrast) ^b				
Bt vs. T25	0.0245	0.0149	ns	0.0008

^a Plants were treated by transplanting to a potting soil mixed with *T. asperellum*-infested WCMS or by drenching with a *T. asperellum* conidia suspension after transplanting. Plants were incubated at 22 ± 2 °C for 2 weeks; then plants were inoculated by transplanting to soil infested with *V. dahliae* V-1381. Data are de mean of 20 replicated plants per treatment combination.

^b Probability for the *t* statistic of linear single-degree-of freedom-contrast; ns: not significant ($P > 0.05$). Significant differences between mean of a treatment and the untreated control ($P \leq 0.05$) are indicated by *.

14 kDa-cut-off, were present in the culture medium. This agrees with results from a previous study, which showed that low molecular weight compounds are the major contributors to antifungal activity of *T. asperellum* strains against the fungal plant pathogens *Rhizoctonia solani*, *Botrytis cinerea* and *F. oxysporum* (Taghdi et al., 2015). The percentage inhibition of ND *V. dahliae* V-15581 calculated for dialysis membrane growing Bt3 and T25 strains were markedly higher than that for the cellophane assay. Since small molecules can also pass through the cellophane membrane, other high molecular weight compounds released by *T. asperellum* may be acting as facilitators of pathogen's fitness.

Secreted metabolites play a major role in mycoparasitism by *Trichoderma* spp. because they interact synergistically with cell wall hydrolytic enzymes, thus facilitating the disruption of the host's structures (Schirmböck et al., 1994; Lorito et al., 2010). However, we speculate that many of *T. asperellum* genes encoding hydrolytic enzymes may have not been induced during these membrane assays because there was no contact between the assayed *T. asperellum* strains and *V. dahliae*, and *Trichoderma*-based biocontrol is due mainly to the production of hydrolytic enzymes and/or metabolites (Harman et al., 2004; Lorito et al., 2010).

The biocontrol potential of strains Bt2, Bt3 and T25 against *V. dahliae* was confirmed in the antagonist-pathogen interaction assay using *V. dahliae* isolates representative of the D and ND pathotypes. Results from this assay showed that mycelial growth of *V. dahliae* can be reduced by *T. asperellum* without a physical contact, and as discussed above the extent of that reduction varies depending upon the interacting strains of the two fungi. In particular, we noticed that growth of Bt2 can be reduced by ND *V. dahliae* isolate V-41. Thus, strains Bt3 and T25 were selected for further assays on antagonism against different D and ND *V. dahliae* isolates, as well as to assess their ability to suppress Verticillium wilt in olive caused by D *V. dahliae*. Although it is not possible to extrapolate the biocontrol activity of a given strain under laboratory conditions to natural environments, results from *in vitro* antagonism served to select potential biocontrol agents against particular pathogens (Hermosa et al., 2000; Taghdi et al., 2015).

For *in vivo* assays we purposely selected the highly susceptible and widely grown cv. Picual and the highly virulent D pathotype of *V. dahliae* (Jiménez-Díaz et al., 2009, 2012; Barranco, 2010). That, together with environment highly conducive for Verticillium wilt in olive (Calderón et al., 2014; Jiménez-Fernández et al., 2016; Palomares-Rius et al., 2016), led to severe disease in untreated plants (Table 4). In spite of that, results from the assays showed that prior root colonization by either strain Bt3 or T25 have a potential to significantly suppress development of Verticillium wilt in olive. This potential did not allow for a reduction in the incidence of the disease, but led to a delay of disease onset together with reduction of the severity of symptoms and total amount of disease (SAUDPC) (Table 4). These results extend our knowledge of *Trichoderma* behavior against *V. dahliae* under conditions conducive to severe Verticillium wilt in olive, as it was observed with a *Trichoderma gamsii* formulation both in growth chambers and artificially infested field microplots (Jiménez-Díaz et al., 2009).

Successful root colonization is considered a major prerequisite for the beneficial effects of *Trichoderma* spp. on plants not only regarding antagonistic activities and plant growth promotion by the biocontrol agents but also for inducing systemic resistance (Harman et al., 2004; Morán-Díez et al., 2009; Shores et al., 2010; Hermosa et al., 2012; Rubio et al., 2014). In our assays, the T25 strain showed a better ability for colonizing the rhizosphere of "Picual" olive compared with Bt3, and it was able to establish endophytic root infection in them as a difference from Bt3. The rhizosphere colonization of "Picual" plants by *T. asperellum* also appeared to be influenced by the method of application. Data indicate that a

thorough infestation of the potting soil with the WCMS substrate colonized by the antagonist allowed for an increased colonization of the olive rhizosphere compared with drenching the soil with a conidia suspension.

In the present work, it was found that only the *T. asperellum* T25 strain induced a significant growth promotion in olive plants (Table 5). This effect could be due to the higher ability for rhizosphere colonization of strain T25, which was 2.5–3.5 times higher than that of strain Bt3 by 2 weeks after treatment, depending upon the mode of application. Although the rhizosphere population of the two strains decreased by 10 weeks later, such a difference still held when inoculum of antagonist in WCMS was mixed with the potting soil. Conversely, by that time, the population of T25 in the rhizosphere of plants treated by drenching the soil with a conidia suspension decreased to less than 30% to reach a population density similar to that of Bt3, which had remained stable over the 10 weeks of incubation. This suggests that the carrying substrate plays an important role in the establishment of the antagonist in the rhizosphere and its effect on plant growth promotion. Previous reports have shown that growth responses are influenced by the *Trichoderma* strain (Rubio et al., 2012) and the plant's genetic background (Tucci et al., 2011), as well as that positive and negative growth responses occurred in tomato plants after treatment with *Trichoderma*. Interestingly enough, the T25-induced growth promotion effect in olive plants also developed in *V. dahliae*-infected plants under conditions for severe Verticillium wilt, thus facilitating that plants counteracted to a degree harmful effects from the pathogen. Under disease pressure, it was expected that growth is suppressed and defenses are activated to balance energy cost (Hermosa et al., 2012; Kazan and Manners, 2012). Results on disease suppression by strains Bt3 and T25 under high Verticillium wilt pressure suggest that *V. dahliae* V-1381 was antagonized by both strains and plants were able to continue investing in growth. Thus, the growth improvement of *V. dahliae*-infected plants by *T. asperellum* is another parameter of disease suppression because it counteracts the stunting caused by the pathogen.

In conclusion, results of the present study support the hypothesis that strains of *T. asperellum* can establish successfully in the olive rhizosphere and bear biocontrol potential against Verticillium wilt in olive under conditions in the pathosystem for severe disease. This makes them a suitable mean of control for the integrated management of the disease, which merits further assessment in olive orchards under natural conditions. This assessment should include a strategy of repeated applications in the orchard, as done in a preliminary study with other *Trichoderma* strains (Jiménez-Díaz et al., 2009), which can further improve the results.

Acknowledgments

This work was supported by projects from the "Consejería de Innovación, Ciencia y Empresa, Regional Government of Andalusia (P10-AGR 6082) and the Spanish Government MINECO (AGL2012-40041-CO2), and co-financed with FEDER funds from the European Union. ICC was supported by a predoctoral fellowship of CICE, Regional Government of Andalusia (project P10-AGR 6082). Authors have no conflict interest to declare.

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