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Use of nursery potting mixes amended with local *Trichoderma* strains with multiple complementary mechanisms to control soil-borne diseases

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ABSTRACT

This study was performed to isolate and characterize *Trichoderma* species from the rhizosphere of holm oak, olive and lavender in a nursery to select effective antagonistic and growth-promoting agents and use them to amend nursery plant growth substrates. Among the isolated *Trichoderma* species, three from olive (*T. asperellum* T2, *T. hamatum* T3, and *T. harzianum* T6), three from holm oak (*T. hamatum* T19, *T. asperellum* T20, and *T. virens* T21) and two from lavender (*T. asperellum* T12 *and T. harzianum* T14) were selected for the *in vitro* antagonism test against the plant pathogens *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Phytophthora nicotianae* and *P. cinnamomi*. The mycelial growth of each target species was differently and directly affected by each *Trichoderma* isolate and its volatile and non-volatile metabolites, suggesting multiple mechanisms in the antagonistic activity. The ability of *Trichoderma* isolates to trigger defense pathways in *Arabidopsis* was evaluated in an *in vitro* system by assessing the activation of the R2R3-MYB-like transcription factor gene *myb72*. The activation of *myb72* induced by the isolates was significantly different from the control, with *T. asperellum* T20 as the most effective. The application of a mixture of *Trichoderma* isolates decreased the root rot that was caused by the artificial inoculation of *R. solani* and *S. sclerotiorum* but to a lesser extent. The highest efficacy against the oomycete *P. nicotianae* was shown by *T. harzianum* T6 alone, which produced antibiotic compounds that were effective *in vitro* against oomycetes. The application of a combination of local antagonists through the amendment of potting soil may have potential in the sustainable production of nursery plants.

1. Introduction

Soil-borne diseases significantly decrease the quality and quantity of ornamental, horticultural and officinal plants. Most of the causal agents are polyphagous ubiquitous fungi, such as *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae* and many species of *Phytophthora* and *Pythium* (Pegg, 1989; Salerno et al., 1999; Mercado-Blanco et al., 2004; Motta and Annesi, 2006; Álvarez et al., 2007). Environmental conditions, such as high moisture and nutrient supplies, often promote the occurrence and spread of soil-borne pathogens. Disease management in nurseries is often based on the routine application of fungicides as fumigants for soil treatments and for the prevention of foliar and stem diseases (Dreistadt, 2001; Conte et al., 2006; Motta and Annesi, 2006), representing a potential for toxic residues to accumulate in the soil. Based on the recent European Regulation 1107/2009 concerning the placing of plant protection products on the market and the European Directive 2009/128/EC, which establishes a framework for community action to achieve the sustainable use of pesticides (Colla et al., 2012), an alternative control approach implementing the general principles of integrated pest management (IPM) is strongly required. In this context, the biological control of soil-borne diseases is an alternative to sustain high production with low impact (Antonelli et al., 2013).

Different soil microorganisms, including *Trichoderma* spp., colonize plant roots, forming rhizospheric microbiota, which have beneficial effects on the plant, influencing disease protection, plant growth and yield (Berg, 2009; Raaijmakers et al., 2009; Berendsen et al., 2012). The importance of the root microbiota in plant health is

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underlined by a body of experimental evidence, and it is becoming increasingly clear that plants control the composition of their microbiota (Hartmann et al., 2009; Berg and Smalla, 2009). The potential control of soil-borne diseases with antagonists, such as *Trichoderma* spp., has raised considerable research interest in recent decades (Hermosa et al., 2012). Some *Trichoderma* rhizosphere-competent strains increase plant growth potential and nutrient uptake, fertilizer-use efficiency, and the percentage and rate of seed germination and stimulate plant defenses (Shoresh et al., 2010; Hermosa et al., 2012). Moreover, the beneficial effects that have been obtained with *Trichoderma* spp. have sensible economic implications, such as shortening the plant production cycle in the nursery, thereby increasing the production capacity (Lo and Lin, 2002).

One method for obtaining effective biocontrol agents is to select the candidate *Trichoderma* species from areas of the plant and soil where these agents are expected to function in disease control and where they grow under conditions of temperature, moisture, soil microbial composition and nutrient availability that approximate those that are found in nature (Howell, 2003). Therefore, the success of a biological control program relies on the successful adaptation of a given biocontrol agent to the local environmental conditions in which it is supposed to work. Thus, the selection of bioagents should consider the efficacy toward the target pathogen along with the conditions in which the bioagents must develop. Another aspect to be considered is the impact of the application of an antagonistic strain on the non-target native microbial soil communities (Brimner and Boland, 2003; Cordier and Alabouvette, 2009).

This study was performed to characterize the antagonistic activity of *Trichoderma* isolates that naturally reside in the rhizosphere of holm oak, olive and lavender to select an array of effective biocontrol and growth-promoting isolates to amend nursery potting mix. To investigate the antagonistic mechanisms of selected *Trichoderma* species, the *in vitro* production of both volatile and non-volatile antifungal metabolites against a set of soil-borne pathogens with diverse lifestyles was detected. To test the ability of *Trichoderma* isolates to induce resistance in plants, the transcription of the *myb72* gene was evaluated in a *Trichoderma*–*Arabidopsis* system *in vitro*. Finally, a set of *Trichoderma* isolates were tested for their biocontrol efficacy *in vivo* versus the soil-borne pathogens *P. nicotianae*, *S. sclerotiorum* and *R. solani* in the soil mix of potted lavender plants.

2. Materials and methods

2.1. Soil sample collection and isolation of Trichoderma spp.

Samples were randomly collected from the rhizosphere of healthy holm oak (20 year old), olive (50 year old) and lavender (3 year old) that were grown in pots containing peat and pumice 1:1 with 2 kg m^{-3} lime and 3 kg m^{-3} Osmocote 10-11-18 NPK (Scotts Italia srl, Treviso, Italy) per year in a nursery located in the Province of Viterbo (42°27′00.9″N 12°05′52.9″E).

Samples were uprooted, and 2 kg of potting-mix was carefully transferred into sterile plastic bags that were covered with ice, transported to the laboratory and processed within 18–24 h. The samples were then homogenized and spread on paper to remove the plant material; then, the samples were air-dried, sifted with 2-mm mesh sieves, and stored at 4 °C until processing. Serial dilutions of soil samples were made to isolate *Trichoderma* spp. (Hirte, 1969). Determinations of the adequate soil suspension, dilution and aliquots to be added to the culture medium were performed as described by Vargas Gil et al. (2009). The soil suspension aliquots from each dilution were then transferred to Potato Dextrose Agar (Oxoid, Unipath Ltd, Basingstoke, England) and modified PDA (PDAm) through the addition of rose bengal (0.01 g L^{-1}), chloramphenicol (0.3 g L^{-1}), and streptomycin sulfate (0.01 g L^{-1}) after autoclaving the medium, adjusted to pH 6.0 (Vargas Gil et al., 2009). Based on colony morphology and microscopic observations, the frequently occurring species of the genus *Trichoderma* were selected and subjected to purification following subculturing.

2.2. Identification of Trichoderma isolates

The *Trichoderma* colonies that were isolated through the dilution plate count technique were identified based on colony morphological characters and micro-morphological traits and molecularly by analysis of the ITS region of the rDNA. The phenotypic characters were analyzed as reported by Samuels et al. (2002). Measurements and observations were taken from PDA and cornmeal agar +2% dextrose (CMD) (w/v) (Oxoid, Unipath Ltd). Micromorphological traits (shape and size of conidia, conidiophores, and non-fertile extensions) were recorded within 1 week from colonies that were grown on CMD at 25 °C and 12 h darkness/12 h cool white fluorescent light. Identification via analysis of micromorphological traits was performed as reported by Bissett (1991), Chaverri et al. (2003) and Samuels et al. (2010).

To confirm the morphological identification of *Trichoderma* spp., genomic DNA was extracted from the mycelium of each isolate using the DNeasy Plant Mini-kit (Qiagen, Milan, Italy) after growing in potato dextrose broth (PDB) for 1 week. Therefore, the ITS rDNA region was amplified with the universal primers ITS1 (5′-TCCGTAGGTGAACCTTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC- 3′) (White et al., 1990).

To differentiate the isolates of *T. harzianum*, the chitinase *ech42* gene was amplified with the primer pair Chit42-1a (5′-GCTYTCCATCGGTGGCTGGAC-3′) and Chit42-2a (5′-GGAGTTGG GGTAGCTCAGC-3′) (Kullnig-Gradinger et al., 2002) using the following amplification protocol: 1 min initial denaturation (94 °C), 30 cycles each of 1 min at 94 °C, 1 min at 62 °C, and 1 min at 74 °C, and a final extension period of 7 min at 74 °C. The amplicons were sequenced (Macrogen Inc., Seoul, Republic of Korea), and a basic local alignment search tool (BLAST, http://www.ncbi.nlm.nih.gov/) was used for sequence similarity searches.

2.3. Target soil-borne pathogens

In vitro characterization tests were performed using a panel of fungal and oomycete isolates representative of common soil-borne pathogens in nursery plants that included *S. sclerotiorum* Rs6 from lettuce, *R. solani* Ss10 from melon, *V. dahliae* Vd2 from *Arabidopsis thaliana* and Vd3 from chrysanthemum, *P. cinnamomi* Pc17 mating type A1 from beech and Pc24 mating type A2 from chestnut provided by the collection of fungal and oomycete pathogens of DIBAF, University of Tuscia, Italy, and *P. nicotianae* Pn28 and Pn698 from lavender kindly provided by the Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia. The pure cultures of fungal pathogens were maintained on PDA slants at 4 °C. *Phytophthora* isolates were maintained on carrot agar (CA) at 20 °C in the dark and subcultured at 4-week intervals.

2.4. In vitro antimicrobial assays and assessment of volatile and non-volatile antibiotics

Dual cultures were performed to determine the direct antagonistic activity and production of antifungal non-volatile and volatile metabolites. The experimental design was a randomized block with ten replicated plates per *Trichoderma*-pathogen combination and the experiment was repeated twice.

The *Trichoderma* isolates were evaluated for their potential to antagonize *in vitro* the panel of plant soil-borne pathogenic isolates using the dual culture technique as described by Morton and Stroube (1955). In detail, a mycelial disc (6 mm in diameter) obtained from the peripheral region of growing colonies of both *Trichoderma* and pathogenic isolates were placed simultaneously on PDA approximately 1 cm from the edge of 9-cm diameter Petri dishes at opposite sides. The Petri dishes containing the PDA that were inoculated with the tested pathogen alone served as the control. The plates were incubated at 24 °C, and measurements were taken after 5 days. At the end of the incubation period, radial growth was measured. Inhibition of average radial growth was calculated in relation to growth of the controls (Edington et al., 1971).

For non-volatile (diffusible) antibiotic assessment, each *Trichoderma* isolate was inoculated into 100 mL of PDB at 25 °C for 10 days. After incubation, the cultures were filtered through 0.22-mm filters (Merck Millipore, Darmstadt, Germany). One-milliliter aliquots of filtrates were placed in sterile Petri dishes, and 25 mL of 0.25-strength PDA at 45 °C was added. After solidification, a mycelial disc of each pathogen (6 mm in diameter) obtained from actively growing colonies was placed in the center of the agar plate.

The effect on pathogen mycelial growth of the volatile metabolites that were released from *Trichoderma* isolates was evaluated according to Dennis and Webster (1971). PDA plates were inoculated centrally with agar disks of *Trichoderma* isolates, and the lid of each dish was replaced by a bottom dish containing PDA that was newly inoculated with each pathogenic isolate. The two dishes were taped together with adhesive tape. Growth of the pathogenic isolates was recorded after 72 h. In the control, pathogenic isolates were cultured in the same way but without the *Trichoderma* isolates.

For both volatile and diffusible inhibitor tests, the percentage of inhibition was measured by dividing the difference between the radial growth of the control and antagonized cultures by the radial growth of the control and multiplied by 100. The experiments were repeated once to confirm the results.

2.5. Analysis of the root-specific transcription factor gene myb72

Trichoderma isolates were evaluated for their capacity to activate defense pathways in *Arabidopsis* (*Arabidopsis thaliana* Col-0) *in vitro*. Transcript accumulation of the root-specific transcription factor gene *myb72* (Van der Ent et al., 2008; Segarra et al., 2009) in seedlings that were challenged with *Trichoderma* was used as an indication of defense pathways activation.

Arabidopsis was grown as described by Contreras-Cornejo et al. (2009) with some modifications. The seeds were surface sterilized with sodium hypochlorite 3% (v/v) for 5 min. After three washes in sterile, distilled water, the seeds were germinated and grown on 9-cm diameter agar plates containing Hoagland's solid medium. There were twenty plates for each *Trichoderma–Arabidopsis* interaction, and each plate containing approximately 5 plants was placed vertically at an angle of 65° in a plant growth chamber at 21 °C and a 16 h light/8 h dark photoperiod. The experiment was repeated twice.

Root inoculation was performed by placing 50 μL *Trichoderma* spore suspensions (1×10^6 mL^{-1}) that were collected from 10-day-old colonies in 0.5-strength PDB (Sigma Chemical Co.) along each root of 4-day-old seedlings. The control seedlings received 50 μL of 0.5-strength PDB. The inoculated and control seedlings were cultured as described above. Total RNA was extracted from 100 mg (fresh weight) of frozen leaves and roots that were collected 72 and 96 h after inoculation with the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. Contaminating DNA was removed using DNA-free TM (Ambion, Austin, TX, USA). A PCR without reverse transcription was performed for each RNA sample to verify the absence of contaminating DNA. Forward and reverse primers that were specific for the *A. thaliana myb72* gene were designed based on the annotated sequence corresponding to AGI number AT1G56160 using the Primer 3 software (Rozen and Skaletsky, 2000). The resulting primers MYB72F and MYB72R had the following sequences: 5′-GATCAAAAACGTGTGGAACA-3′ and 5′-CCTCATGAAGCTCTGGTTTT-3′, respectively. Primers ACT8-F and ACT8-R (Salas-Marina et al., 2011) were used to amplify the *actin 8* gene that was used as a housekeeping gene. Reverse transcription (RT) was performed using ImProm-II™ Reverse Transcription System (Promega, Milan, Italy) following the manufacturer's instructions and using 50 ng of total RNA for each sample.

Real-time PCR (qPCR) experiments were performed using the *iCycleriQ* (Bio-Rad Laboratories, Hercules, CA, USA) and the QuantiTect® SYBR® Green PCR Kit (Qiagen) containing the fluorogenic dye SYBR® Green I. The reaction mixture contained 1 μL of cDNA, 7.5 μL of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 μM of each forward and reverse primer and RNase-free water to a final volume of 15 μL. Each reaction was made in triplicate. The amplification conditions were as follows: 15 min at 95 °C, 40 cycles at 94 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s for cDNA amplification. A relative expression analysis was performed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). The qRT-PCRs were carried out on RNA that was extracted from two separate experiments. The relative expression value (REV) was reported as the fold increase of the transcript level compared to the mock-inoculated sample for each time point. The experiments were repeated once to confirm the results.

2.6. Amendment of standard potting mix with Trichoderma local isolates

Candidate *Trichoderma* isolates were selected and evaluated in a greenhouse trial for their biocontrol potential against soil-borne pathogens of lavender plants. *T. hamatum* T3, *T. asperellum* T20, *T. harzianum* T6, and *T. virens* T21 were selected for *in vivo* experiments based on their performances in the *in vitro* experiments and their ability to induce resistance as described above. The selected *Trichoderma* isolates were used individually and as a combination of the four isolates.

The *Trichoderma* inoculum (Ti) was prepared using 50 g of millet kernels that were imbibed with sterilized H_2O in 250-mL Erlenmeyer flasks and autoclaved twice for 30 min at 121 °C. Each flask containing the substrate was inoculated with 10 agar discs (6-mm diameter) that were cut from the edge of actively growing cultures and incubated for 10 days at 23 ± 2 °C.

The potting mix that was represented by peat (grain size 10–30 mm, organic carbon 10% dry weight (dw), organic nitrogen 0.7 dw, organic matter 96% dw, pH 3.5) (Vigorplant, Lodi, Italy) and pumice 1:1 with 2 kg m^{-3} lime and Osmocote 10-11-18 NPK (Scotts Italia srl) was sterilized twice at 121 °C for 15 min and inoculated with 3 g kg^{-1} inoculum of Ti, corresponding to approximately 2.5×10^9 spores per kilogram of substrate. Inoculation of the *Trichoderma* mix was performed by mixing equal amounts of the inoculum of each *Trichoderma* isolate to produce 3 g kg^{-1} inoculum. The potting mixes that were added to *Trichoderma* (PoMT$^+$) and uninoculated controls (PoMT$^-$) were then incubated in sterile plastic bags for 15 days at 25 °C.

2.7. Biocontrol activity and effect on plant growth

Biocontrol activity and plant development were assessed on lavender (var. Hidcote) plants that were grown in PoMT$^+$ and

PoMT$^-$ and then inoculated with *R. solani* Rs6, *S. sclerotiorum* Ss10 and *P. nicotianae* Pn698. Fungal and oomycete inocula (Pi) were prepared on millet seeds as described above for *Trichoderma*.

The experimental plan was based on the combination of five *Trichoderma* treatments (Tricho$^+$), three *Trichoderma* uninoculated controls (Tricho$^-$), three pathogen treatments (Path$^+$), and three pathogen uninoculated controls (Path$^-$). The treatments were arranged in a randomized complete block design with four replicates. Each experimental unit consisted of ten plants. The trial was repeated once to confirm the results. Lavender plants that were 60 days old were transplanted and grown in a 1.5-L pot for 30 days in a greenhouse at 21 ± 3 °C. Inoculation with the target fungal and oomycete pathogens was performed by mixing 30 g of inoculum (5-cm depth) per kilogram of PoMT$^+$ and PoMT$^-$. The plants were grown under greenhouse conditions, harvested 90 days after transplanting, graded for symptoms and measured for root development and biomass. After cutting the vines, the root systems were carefully extracted from the pots. The soil was flushed with tap water; then, the roots were washed in distilled water. Thereafter, the roots were measured and weighed. The symptoms on the crown, roots and aerial parts of inoculated lavender plants were rated on the following scale: 0, healthy; 1, 30% lesions on the roots with punctiform shape, 30% reduction of the roots and aerial part development; 2, 60% of the root surface with extended lesions, 50% reduction of the roots and aerial part development; 3, 80% of the root surface with extended lesions, stunted root and aerial parts; and 4, dead plants. The disease index was calculated by the formula $DI = \Sigma (x_i\, n_i)/N$ (Groth et al., 1999).

The plant biomass (root and aerial dry weight per plant) was measured 90 days after transplanting on treated soil with both the *Trichoderma* isolates and target pathogens against controls that were represented by the substrate that was treated with not-inoculated millet and alternatively inoculated with *Trichoderma* isolates or target pathogens.

2.8. Statistical analysis

The data were subjected to a one-way analysis of variance using GraphPad Prism software (San Diego, CA). The Tukey test ($P = 0.05$) was used to compare means. Since no statistically significant

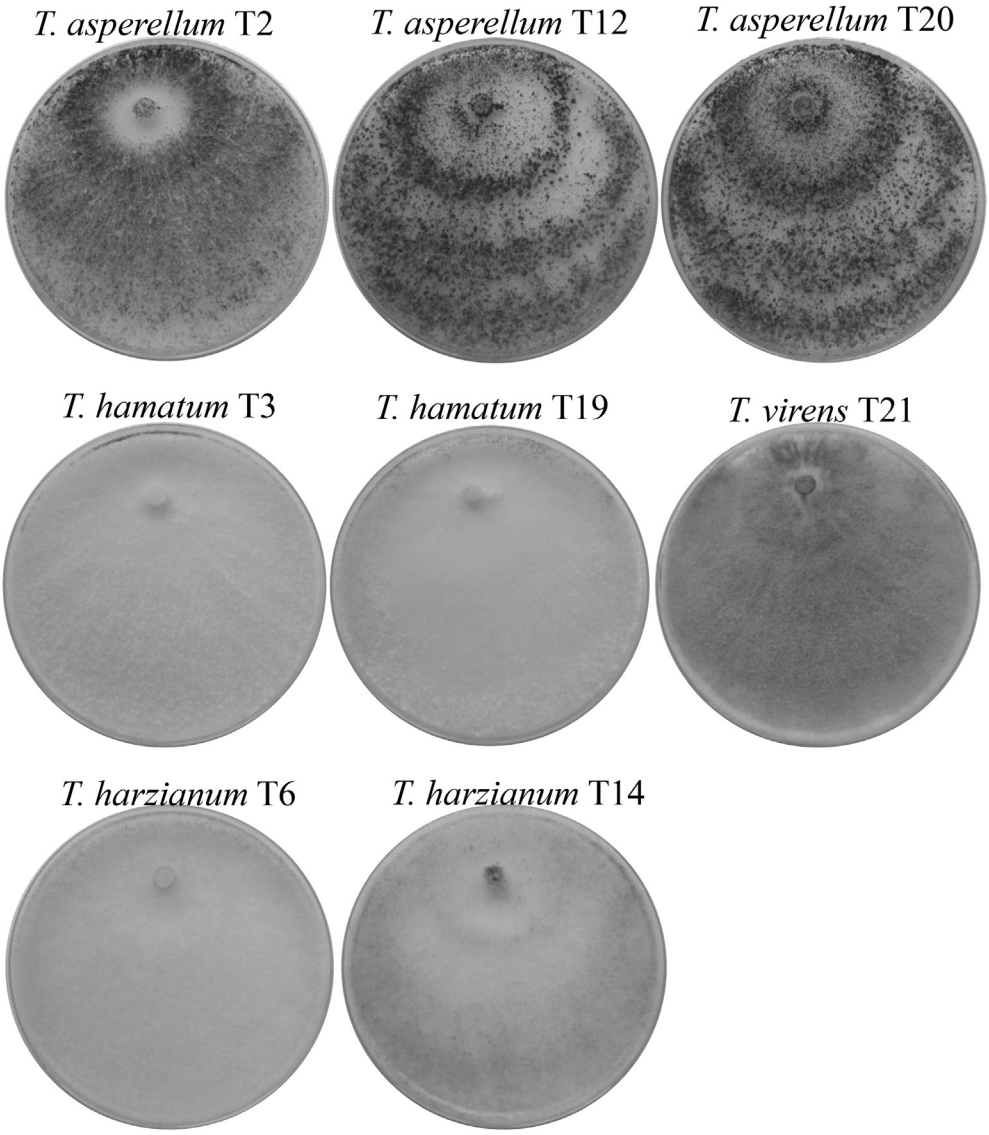

Fig. 1. Colony characters of *Trichoderma asperellum* T2, T12, and T20; *T. hamatum* T3 and T19; *T. harzianum* T6 and T14; and *T. virens* T21. The cultures were grown for 1 week on cornmeal agar +2% dextrose (CMD) at 25 °C under 12 h of cool white fluorescent light/12 h of darkness.

differences were found between data, a combined analysis was carried out of replicate experiments.

3. Results

3.1. Identification

Based on the phenotype characteristics of the colonies and their micromorphology, the *Trichoderma* isolates were identified as *T. asperellum*, *T. hamatum* and *T. harzianum* from olive tree; *T. asperellum*, *T. hamatum* and *T. virens* from holm oak; and *T. asperellum* and *T. harzianum* from lavender. Among the identified species, 8 isolates (*T. asperellum* T2, *T. hamatum* T3, and *T. harzianum* T6 from olive; *T. hamatum* T19, *T. asperellum* T20, and *T. virens* T21 from holm oak; and *T. asperellum* T12 *and T. harzianum* T14 from lavender) illustrating representative species that are associated with the rhizosphere of the tested plants were subjected to further characterization. As shown in Fig. 1, no particular morphological variation was noted among 1-week-old colonies belonging to the same species that were cultured on CMD. The representative microscopic features are illustrated in Fig. 2.

A molecular analysis confirmed the morphological identification. The multiple gene approach and the analysis of phenotypic characteristics successfully differentiated *T. harzianum* from *T. aureoviride*. The sequences for the representative isolates were deposited in the NCBI database (Table 1).

Table 1
Morphological and molecular identification of *Trichoderma* isolates illustrating the representative species associated with the rhizosphere of tested plants.

Isolate	Morphological identification	Genbank accession no.		Molecular identification
		ITS sequence	Chitinase sequence	
T2	*T. asperellum*	JF798504		*T. asperellum*
T3	*T. hamatum*	JF501656		*T. hamatum*
T6	*T. harzianum*	JF501658	JF501663	*T. harzianum*
T12	*T. asperellum*	JF501660		*T. asperellum*
T14	*T. harzianum*	JF501659	JF501662	*T. harzianum*
T19	*T. hamatum*	JF501657		*T. hamatum*
T20	*T. asperellum*	JF501661		*T. asperellum*
T21	*T. virens*	JF501655		*T. virens*

3.2. Antagonistic in vitro activity and inhibition by non-volatile and volatile compounds toward target pathogens

The results from the *in vitro* dual culture assay showed that the selected *Trichoderma* isolates inhibited with varying efficiencies the mycelial growth of the target fungal (*R. solani* Rs6, *S. sclerotiorum* Ss10, *V. dahliae* Vd2, Vd3) and oomycete (*P. cinnamomi* Pc17, Pc24, and *P. nicotianae* Pn28 and Pn698) pathogens. Generally, *Trichoderma* isolates displayed the highest inhibition of *S. sclerotiorum* Ss10 growth and, to a lesser extent, that of *R. solani* Rs6 (Table 2), but the percentage of inhibition of both of the *Verticillium* isolates was consistently lower. Among *Trichoderma* isolates, *T. asperellum* T12 and T20 significantly exerted inhibitory activity toward *R. solani* Rs6 along with *T hamatum* T3 against *S. sclerotiorum* Ss10. The *Trichoderma* isolates generally presented a moderate inhibition of all of the target oomycete pathogens (Table 3). In particular, *T. hamatum* T19 and *T. asperellum* T2 had statistically significant inhibition activity toward *P. cinnamomi* Pc17, and *T. hamatum* T3 and *T. asperellum* T2, T12 and T20 were the most effective against *P. cinnamomi* Pc24. *T. harzianum* T6 displayed significant inhibition activity toward *P. nicotianae* Pn28; in contrast, it had the lowest activity against *P. cinnamomi* Pc17, Pc24 and *P. nicotianae* Pn698.

The majority of the selected *Trichoderma* isolates produced non-volatile compounds with a low or absent inhibition activity of the hyphal growth of the target fungal pathogens, in contrast to *T. harzianum* T6, which exerted a statistically significant activity

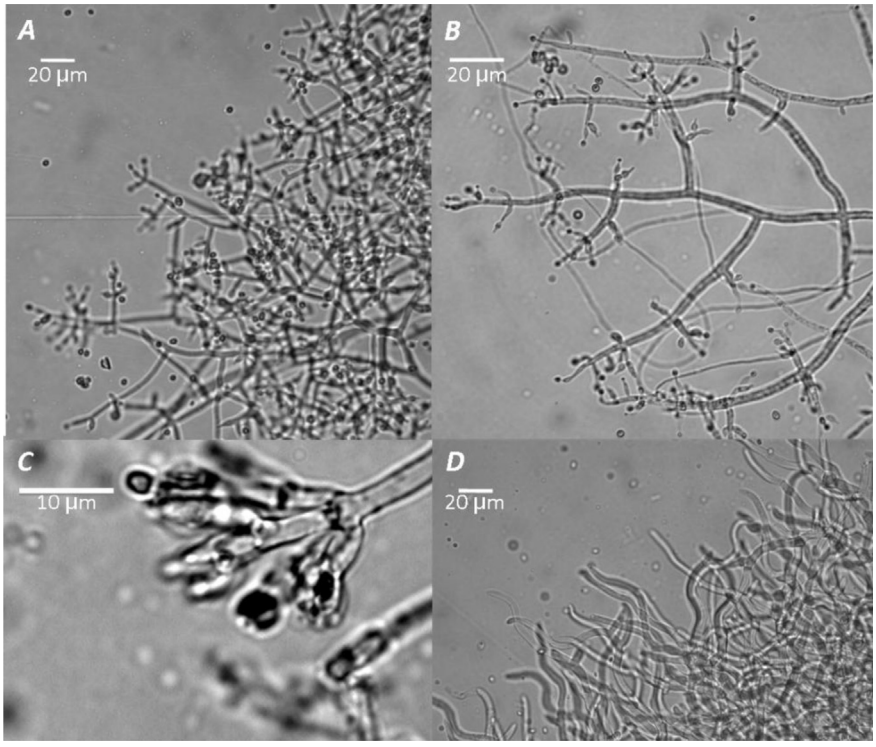

Fig. 2. Characters of the conidiophores of *Trichoderma harzianum* T6 (A) (20×), *T. asperellum* T12 (B) (20×), *T. virens* T21 (C) (40×); non-fertile conidiophore extensions of *T. hamatum* T3 (D) (20×).

Table 2
Inhibition of growth of the fungal pathogens *Rhizoctonia solani* Rs6, *Sclerotinia sclerotiorum* Ss1, and *Verticillium dahliae* Vd2 and Vd3 by *Trichoderma* spp. in a dual culture experiment and by non-volatile and volatile compounds produced by the *Trichoderma* spp. isolated from the rhizosphere of nursery plants.

Pathogen / *Trichoderma*	Dual culture % inhibition[a]	Non volatile % inhibition	Volatile % inhibition
vs. Rs6			
T. asperellum T2	42.0 ± 10.1 cd	7.2 ± 2.1 c	67.8 ± 8.8 ab
T. hamatum T3	44.3 ± 8.6 cd	7.2 ± 2.9 c	61.8 ± 2.7 b
T. harzianum T6	33.5 ± 8.0 d	92.8 ± 0.0 a	54.3 ± 23.6 b
T. asperellum T12	58.0 ± 4.4 e	11.1 ± 2.1 c	79.2 ± 0.7 ab
T. harzianum T14	47.7 ± 10.8 abc	5.8 ± 1.6 cd	85.9 ± 2.4 a
T. hamatum T19	52.3 ± 5.9 abc	4.4 ± 1.9 cd	0.0
T. asperellum T20	56.8 ± 5.9 ab	5.8 ± 2.0 cd	77.1 ± 14.1 a
T. virens T21	44.3 ± 9.4 bcd	36.7 ± 6.3 b	61.8 ± 18.3 ab
vs. Ss10			
T. asperellum T2	62.0 ± 4.9 cd	47.2 ± 14.1 b	11.8 ± 20.4 d
T. hamatum T3	74.5 ± 1.8 a	0.0	85.7 ± 4.7 ab
T. harzianum T6	52.7 ± 1.7 e	100.0 ± 0.0 a	59.2 ± 14.2 c
T. asperellum T12	70.4 ± 1.5 ab	2.1 ± 4.1 c	90.0 ± 3.1 a
T. harzianum T14	65.7 ± 3.2 bcd	0.0	60.3 ± 9.7 c
T. hamatum T19	69.9 ± 2.8 abc	0.0	81.8 ± 14.3 abc
T. asperellum T20	74.1 ± 5.2 a	0.0	91.0 ± 0.7 a
T. virens T21	57.9 ± 4.1 ed	9.3 ± 9.1 c	82.4 ± 2.1 ab
vs. Vd2			
T. asperellum T2	34.8 ± 8.9 a	4.9 ± 0.0 c	65.2 ± 0.0 ab
T. hamatum T3	12.7 ± 11.2 b	13.4 ± 1.7 a	65.2 ± 15.1 abc
T. harzianum T6	34.8 ± 0.0 a	8.5 ± 3.1 abc	10.4 ± 4.6 d
T. asperellum T12	29.3 ± 10.9 a	6.1 ± 1.7 c	68.2 ± 0.0 a
T. harzianum T14	34.8 ± 0.0 a	10.6 ± 1.4 ab	43.9 ± 10.5 c
T. hamatum T19	38.4 ± 6.3 a	8.5 ± 1.7 abc	68.2 ± 0.0 ab
T. asperellum T20	37.5 ± 5.4 a	0.0	51.5 ± 22.9 bc
T. virens T21	23.9 ± 0.0 b	4.9 ± 4.2 bc	50.0 ± 21.7 b
vs. Vd3			
T. asperellum T2	30.2 ± 9.5 a	6.3 ± 0.9 b	62.2 ± 18.1 ab
T. hamatum T3	21.5 ± 11.1 ab	2.0 ± 2.4 d	42.4 ± 11.9 ab
T. harzianum T6	13.3 ± 7.7 b	3.6 ± 2.9 c	16.7 ± 8.2 c
T. asperellum T12	30.2 ± 9.5 a	9.8 ± 1.1 a	58.8 ± 5.2 a
T. harzianum T14	27.3 ± 5.8 a	8.6 ± 2.6 ab	63.9 ± 10.3 a
T. hamatum T19	33.1 ± 5.8 a	2.7 ± 2.0 c	nd
T. asperellum T20	18.6 ± 0.0 b	4.9 ± 0.0 c	33.0 ± 10.3 abc
T. virens T21	24.4 ± 6.7 ab	4.2 ± 2.2 c	43.3 ± 21.9 abc

[a] Means followed by the same letter within a column and pathogen are not significantly different at $P = 0.05$ according to Tukey's test.

Table 3
Inhibition of growth of the oomycete pathogens *Phytophthora cinnamomi* Pc17 and Pc24 and *P. nicotianae* Pn28 and Pn698 by *Trichoderma* spp. in a dual culture experiment and by non-volatile and volatile compounds produced by the *Trichoderma* spp. isolated from the rhizosphere of nursery plants.

Pathogen / *Trichoderma*	Dual culture % inhibition[a]	Non volatile % inhibition	Volatile % inhibition
vs. Pc17			
T. asperellum T2	31.8 ± 3.7 ab	16.9 ± 0.0 c	0.0
T. hamatum T3	26.9 ± 3.2 bc	19.0 ± 2.4 c	0.0
T. harzianum T6	10.0 ± 6.1 d	100.0 ± 0.0 a	32.3 ± 8.9
T. asperellum T12	26.9 ± 6.2 bc	0.0	0.0
T. harzianum T14	10.7 ± 8.2 d	6.2 ± 5.3 d	0.0
T. hamatum T19	35.1 ± 0.0 a	0.0	0.0
T. asperellum T20	17.2 ± 9.7 cd	10.2 ± 9.0 c	0.0
T. virens T21	22.1 ± 6.5 abc	72.8 ± 4.4 b	0.0
vs. Pc24			
T. asperellum T2	27.9 ± 7.4 ab	0.0	13.7 ± 1.8 c
T. hamatum T3	35.3 ± 17.3 ab	50.5 ± 4.7 b	3.9 ± 0.0 d
T. harzianum T6	12.6 ± 2.7 c	100.0 ± 0.0 a	32.3 ± 8.9 a
T. asperellum T12	33.8 ± 2.9 a	5.0 ± 1.5 d	21.9 ± 2.5 ab
T. harzianum T14	25.0 ± 5.6 b	0.0	0.0
T. hamatum T19	27.9 ± 11.1 ab	0.0	14.1 ± 3.3 c
T. asperellum T20	33.8 ± 2.9 a	24.5 ± 1.7 c	22.7 ± 12.2 abc
T. virens T21	23.5 ± 8.3 b	100.0 ± 0.0 a	17.8 ± 1.5 bc
vs. Pn28			
T. asperellum T2	20.5 ± 16.1 cd	6.7 ± 2.4 e	65.2 ± 0.0 ab
T. hamatum T3	20.5 ± 0.0 c	13.0 ± 1.8 d	65.2 ± 15.1 abc
T. harzianum T6	43.4 ± 5.3 a	100.0 ± 0.0 a	10.4 ± 4.6 d
T. asperellum T12	32.8 ± 1.0 bd	19.8 ± 3.1 c	68.2 ± 0.0 a
T. harzianum T14	26.1 ± 6.6 bc	23.0 ± 1.6 c	43.9 ± 10.5 c
T. hamatum T19	23.3 ± 5.7 c	9.3 ± 6.0d e	68.2 ± 0.0 ab
T. asperellum T20	26.1 ± 11.4 bc	11.7 ± 4.4 d e	51.5 ± 22.9 bc
T. virens T21	29.0 ± 5.7 bc	37.1 ± 1.6 b	50.0 ± 21.7 b
vs. Pn698			
T. asperellum T2	21.4 ± 10.1 a	7.5 ± 4.4 d	14.6 ± 0.0 b
T. hamatum T3	15.0 ± 1.0 a	17.0 ± 2.5 c	0.0
T. harzianum T6	9.8 ± 5.2 a	100.0 ± 0.0 a	7.1 ± 1.9 c
T. asperellum T12	21.4 ± 10.1 a	6.4 ± 6.1 d	11.0 ± 5.2 b
T. harzianum T14	14.3 ± 0.0 a	12.2 ± 9.7 d	18.7 ± 3.7 b
T. hamatum T19	15.3 ± 1.0 a	32.9 ± 9.3 c	31.7 ± 6.5 a
T. asperellum T20	19.0 ± 8.2 a	11.1 ± 7.4 d	12.2 ± 11.2 b
T. virens T21	15.0 ± 1.0 a	52.3 ± 6.4 b	30.9 ± 1.4 a

[a] Means followed by the same letter within a column and pathogen are not significantly different at $P = 0.05$ according to Tukey's test.

toward both *R. solani* Rs6 and *S. sclerotiorum* Ss10 (Table 2). A moderate activity was also displayed by *T. asperellum* T2 against *S. sclerotiorum* Ss10 and *T. virens* T21 against *R. solani* Rs6. A different picture emerged when the oomycete pathogens were exposed to non-volatile compounds (Table 3). *T. harzianum* T6, *T. virens* T21 and, to a lesser extent, *T. hamatum* T3 produced highly effective non-volatile compounds.

The volatile metabolites of the selected *Trichoderma* isolates showed a high inhibition rate of *R. solani* Rs6 growth with a significant inhibition produced by *T. harzianum* T14; *T. asperellum* T2, T12, and T20; and *T. virens* T21 (Table 2). The reduction of *S. sclerotiorum* Ss10 growth by the volatile inhibitors that were produced by *T. hamatum* T3 and T19, *T. asperellum* T12 and T20 and *T. virens* T21 was remarkable, whereas *T. harzianum* T6 and T14 exhibited a moderate and *T. asperellum* T2 a poor production of inhibitory volatile metabolites. The volatile compounds that were produced by other *Trichoderma* species moderately effectively inhibited the growth of *Verticillium* Vd2 and Vd3. *Trichoderma* spp. produced volatile compounds with no inhibitory effects on *P. nicotianae* Pn17 apart from *T. harzianum* T6, which was moderately effective (Table 3). The volatile metabolites of the selected *Trichoderma* isolates exhibited a moderate inhibition of *P. cinnamomi* Pc17 growth. The volatile substances that were produced by *T. asperellum* T2 and T12 and *T. hamatum* T3 and T19 produced a consistent effect on *P. nicotianae* Pn28 growth that was significantly different than other isolates, whereas *P. nicotianae* Pn698 was less or not (T3) sensitive to these compounds.

3.3. Expression analysis of the Arabidopsis thaliana myb72 gene challenged with Trichoderma spp.

All of the *Trichoderma* spp. that were used to challenge *A. thaliana* plants colonized Arabidopsis roots 72 h after inoculation (hai) (Fig. 3) and induced the expression of the *myb72* gene in the roots at 72 and 96 hai (Fig. 4). *T. asperellum* T20 had the greatest induction at 96 hai, with a REV of 18.2 that was significantly different from that of the other isolates (Fig. 4). In the leaves of *A. thaliana* that were challenged with different *Trichoderma* spp., the expression of the *myb72* gene was not induced (data not shown).

3.4. Bioassay and assessment of disease severity

Un-inoculated control plants ($Tricho^{-}$ $Path^{-}$) were healthy, with no apparent rot symptoms throughout the duration of the experiment (data not shown). Inoculation with the target pathogens ($Path^{+}$) in the $Tricho^{-}$ plants resulted in the development of severe symptoms (Fig. 5). Application of *Trichoderma* mixed isolates was significantly effective in controlling *Rhizoctonia* root rot disease on

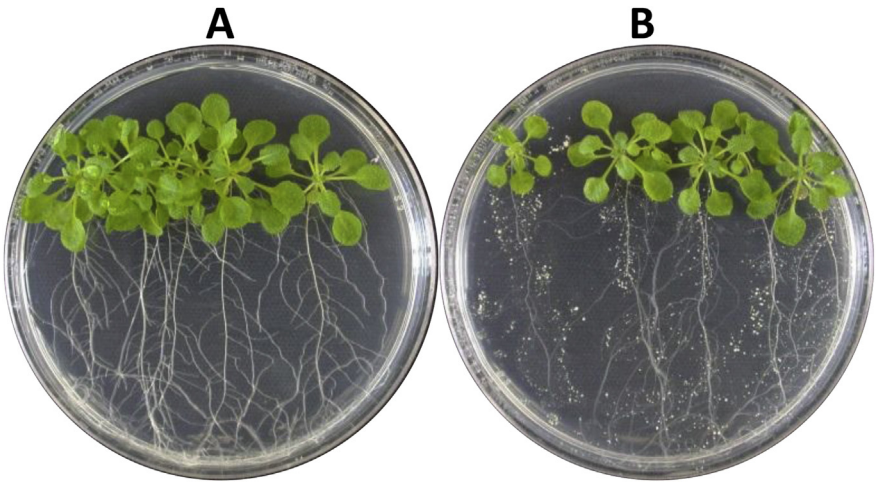

Fig. 3. *Arabidopsis* grown *in vitro* on Hoagland's medium (25 days) and inoculated with *Trichoderma hamatum* T3 (72 h after inoculation). A, non-inoculated control; B, inoculated with T3.

lavender compared to each single *Trichoderma* isolate treatment and Tricho$^-$ control. T21 and the mix of *Trichoderma* isolates had some effectiveness in containing *Sclerotinia* root rot. However, no significant differences were found among treatments.

Among the tested isolates, T6 was significantly effective in controlling *Phytophthora* root rot compared to other treatments.

3.5. Promotion of plant growth

The promotion of root and aerial growth by isolated *Trichoderma* was measured in each combination of Tricho$^+$/Tricho$^-$ – Path$^+$/Path$^-$ plants. As both the dry and fresh weights were similarly affected, only the data on dry weight are reported. Inoculation with the combination of *Trichoderma* isolates resulted in a significant increase in the root biomass compared to that of the Tricho$^-$ control and each *Trichoderma* isolate (Fig. 6). No significant differences with respect to the biomass of the aerial part were found (data not shown).

Upon inoculation of the target pathogens on Tricho$^+$ plants, the root biomass was generally lower than that of Path$^-$, although to a different extent. *T. harzianum* T6 and *Trichoderma* mix significantly stimulated lavender root weight when inoculated with *R. solani and P. nicotianae*, *and Trichoderma* mix stimulated those that were infected with *S. sclerotiorum* (Fig. 6).

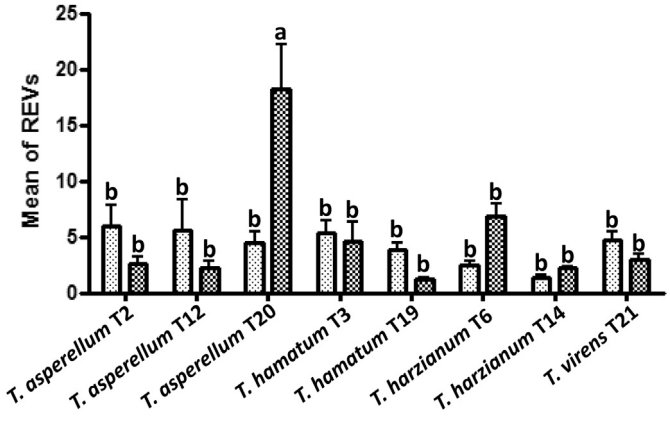

Fig. 4. Transcript level of the *myb72* gene in *Arabidopsis thaliana* Col-0 roots that were challenged with *Trichoderma asperellum* (T2, T12, and T20), *T. hamatum* (T3 and T19), *T. harzianum* (T6 and T14), *T. virens* (T21) at 72 (▨) and 96 (▩) hours after inoculation. REV = Relative expression value. The error bars represent the standard error. Different letters denote significant differences at $P = 0.05$ according to Tukey's test.

4. Discussion

In this study, *Trichoderma* species from the rhizosphere of nursery plants were screened to isolate and characterize potential biocontrol agents for use in the same ecosystem in which they were obtained. The *Trichoderma* isolates that were obtained in this manner were identified by means of morphological and molecular methods. Representative isolates of the species that were isolated from the rhizosphere of the three plant species were then screened for their *in vitro* antagonistic activity toward a set of target fungal and oomycete pathogens and compared in terms of mechanisms of activity, such as the production of non-volatile and volatile inhibitors and the capacity to induce resistance. This functional approach brings new information about the interactions as well as the biocontrol potential that is harbored by the *Trichoderma* group (Anees et al., 2010).

Using the dilution plate count technique, we isolated and identified three rhizospheric *Trichoderma* species from olive (*T. asperellum*, *T. hamatum*, and *T. harzianum*), holm oak (*T. asperellum*, *T. hamatum*, and *T. virens*) and two from lavender (*T. asperellum and T. harzianum*).

The antagonistic activity was measured against fungal and oomycete soil-borne pathogens, important causative agents of nursery plant diseases that are characterized by diverse lifestyles. *S. sclerotiorum* is a necrotrophic ascomycete with an acidic behavior in the production of oxalic acid and polygalacturonases as virulence factors (Guimarães and Stotz, 2004; Cotton et al., 2003). In contrast, *R. solani* is a necrotrophic basidiomycete with an alkaline behavior and is characterized by the production of pectin lyase as a predominant pectolytic enzyme (Bugbee, 1990). *V. dahliae* is a vascular pathogen that is responsible for dramatic yield losses in many crops (Tjamos et al., 2000). *P. nicotianae* and *P. cinnamomi* are heterothallic oomycete pathogens with a worldwide distribution that cause disease in hundreds of plant species (Erwin and Ribeiro, 1996).

The differences that were found in mycelial growth inhibition that were caused by the antagonistic isolates of *Trichoderma* spp. indicate that, among these isolates, there are physiological differences, and these variations may be due to the mechanisms that are involved in the antagonistic activity toward pathogens with

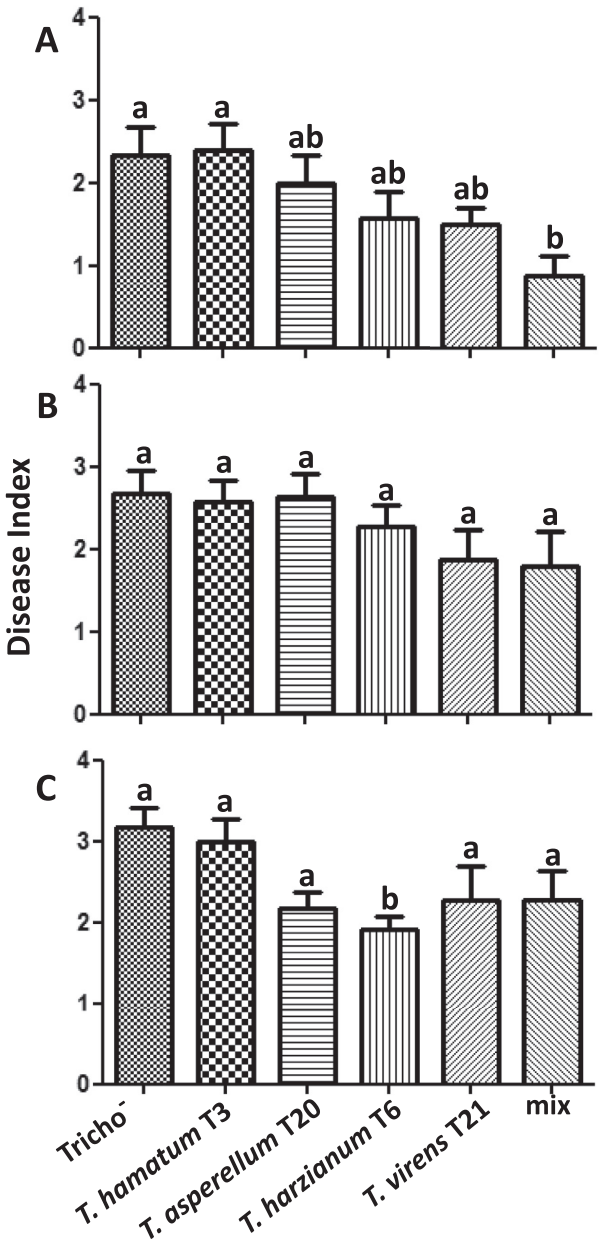

Fig. 5. Control of root rot caused by the artificial inoculation of *Rhizoctonia solani* Rs6 (A), *Sclerotinia sclerotiorum* Ss10 (B), and *Phytophthora nicotianae* Pn698 (C) by selected *Trichoderma* isolates and un-inoculated (Tricho$^-$) on lavender plants 90 days after inoculation. Different letters denote significant differences at $P = 0.05$ according to Tukey's test. According to the formula of Groth et al. (1999) the disease index of un-inoculated controls (Path$^-$) was equal to zero and therefore not displayed as a bar in the graph.

different lifestyles. The mechanisms that are used by *Trichoderma* species to affect biological control are many and complex, and their use varies with the *Trichoderma* species, pathogen and host plant that are involved (Howell, 2003). *S. sclerotiorum* Ss10 was the most sensitive pathogenic fungus to all of the used *Trichoderma* isolates. When *Trichoderma* isolates were tested in dual culture, a high level of inhibition was also exerted toward *R. solani* Rs6, confirming the sensitivity of this pathogen to *Trichoderma* species (Harman et al., 2004; Grosch et al., 2006; Anees et al., 2010). *T. asperellum* T2, T12 and T20 provided a significantly higher activity than that of the other isolates ($P = 0.05$). *T. asperellum* is an effective biocontrol agent against many soil-borne plant pathogenic fungi, antagonizing through antibiosis and nutrient competition and by inducing resistance in plants (Liu et al., 2010).

The tested *Trichoderma* isolates exerted a different and often complementary mycelial growth inhibition by volatile and nonvolatile compounds, indicating that the mechanisms that are involved in the inhibition of target pathogens are different and complement each other among and within the different *Trichoderma* species when isolates are grouped according to the rhizosphere from which they were isolated. For example, the data indicate a marked intraspecific difference between the isolates of *T. harzianum* T14 and T6 in terms of the production of active non-volatile and volatile inhibitors of target pathogens.

The enhancement of basal resistance levels is a common reaction of plants to attempted attacks by hypovirulent pathogens and root colonization by non-pathogenic rhizobacteria and rhizofungi (Pieterse et al., 2002; Segarra et al., 2007) and is commonly referred to as systemic acquired resistance (SAR) and induced systemic resistance (ISR), respectively (Van Loon, 2000). Using *A. thaliana* as a model plant species and the rhizobacterial strain *Pseudomonas fluorescens* WCS417r as an ISR inducer, it was found by microarray analysis that the R2R3-MYB-like transcription factor gene *myb72* is specifically activated in the roots upon colonization by WCS417r (Verhagen et al., 2004; Van der Ent et al., 2008). An analysis of *myb72* mutant plants revealed that *myb72* is required for the onset of ISR mediated by *T. asperellum* against a set of pathogens (Segarra et al., 2009). In this study, we set up an *in vitro* system to assess the ability of *Trichoderma* spp. to activate the expression of the transcription factor gene *myb72* on *A. thaliana* seedlings. Using this model, we found that the tested isolates activate the early signaling of ISR. However, the level of expression of the tested isolates was significantly different, with *T. asperellum* T20 being the most effective. This result suggests that the rhizospheric *Trichoderma* population can activate the defense pathway through similar basic mechanisms but different amplitudes depending on the tested species. It is likely that this ability has an ecological role to colonize and benefit plants across a range of species and environmental conditions.

A combination of biological control agents is more likely to have a greater variety of traits that are responsible for the suppression of pathogens and for promoting plant growth. A combination of antagonistic microorganisms, including *Trichoderma* spp., can provide the most effective suppression of soil-borne diseases by fungal and oomycete pathogens as tested under controlled conditions (Spadaro and Gullino, 2005). A significant decrease in disease index resulted when selected *Trichoderma* and their antagonistic/ISR traits were combined against the fungal pathogen *R. solani* on lavender. However, the values of the *in vitro* antagonistic assay and inhibition by non-volatile and volatile compounds toward *R. solani* were mostly lower than were those related to *S. sclerotiorum*. It is likely that the suppressive capacity of the combination of isolates and their cumulative or synergistic mechanisms are related to the characteristics and lifestyle of the target pathogen. In contrast, the greatest suppressive efficacy was determined not by isolate combination but by the single *T. harzianum* T6 against the oomycete *P. nicotianae*. This result might be due to the high efficiency of diffusible antibiotics that are produced by T6 against oomycete pathogens as indicated by the *in vitro* characterization tests and under an environment that is presumably not competitive with other *Trichoderma* isolates. These data confirm that promising results in the *in vitro* assay are not always good indicators of positive antagonistic effects *in vivo* (Martínez-Medina et al., 2014).

Taken together, these results indicate that the application of a combination of local antagonists through the enrichment of potting

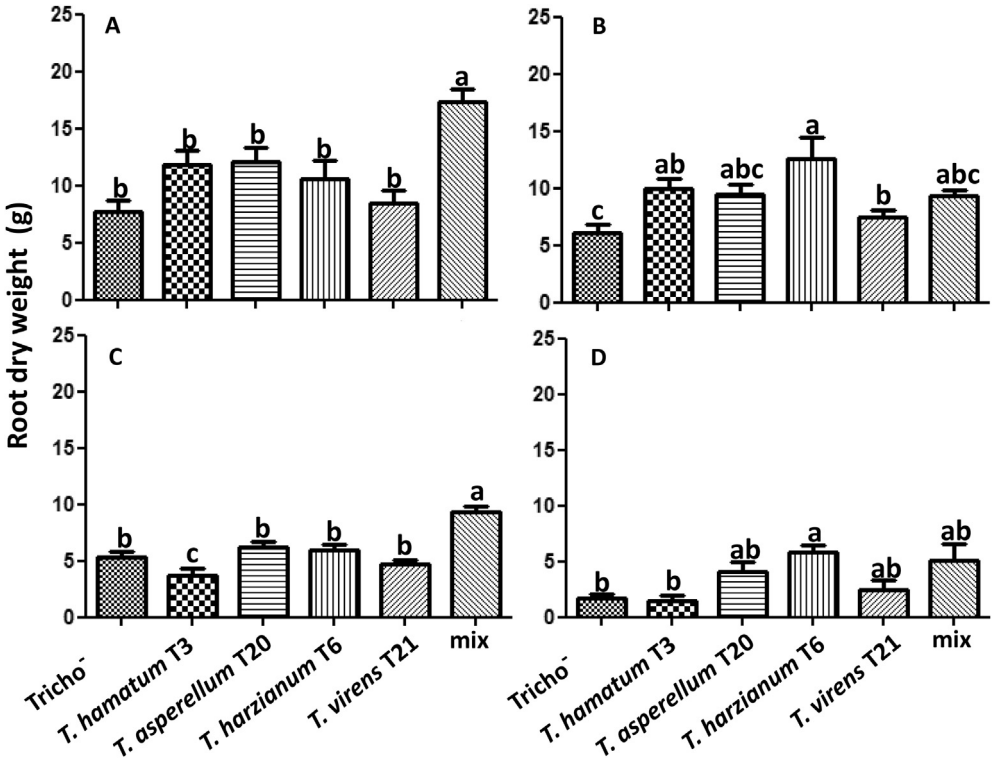

Fig. 6. Root dry weight per plant of the lavender plants that were treated with selected *Trichoderma* isolates and un-inoculated (Tricho$^-$). A, control un-inoculated with pathogens; B, inoculated with *Rhizoctonia solani* Rs6; C, inoculated with *Sclerotinia sclerotiorum* Ss10; and D, inoculated with *Phytophthora nicotianae* Pn698, 90 days after inoculation. The bars with different letters are significantly different (post-ANOVA Tukey test ($P < 0.05$)).

mixes has great potential in the sustainable production of nursery plants.

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