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

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Abstract: The present study was undertaken to isolate and characterise *Trichoderma* species from the rhizosphere of holm oak, olive and lavender in nursery with the aim to select effective antagonistic and growth promoting agents and use them to amend nursery plant growth substrates. Among *Trichoderma* species isolated, three from olive (*T. asperellum* T2, *T. hamatum* T3, *T. harzianum* T6) and holm oak (*T. hamatum* T19, *T. asperellum* T20, *T. virens* T21), and two from lavender (*T. asperellum* T12 and *T. harzianum* T14) were selected for the in vitro tests of antagonism against the plant pathogens *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Phytophthora nicotianae* and *P. cinnamomi*. Mycelial growth of each target species was differently affected directly by each *Trichoderma* isolate and its volatile and non-volatile metabolites, suggesting multiple mechanisms involved in the antagonistic activity. The ability of *Trichoderma* isolates to trigger defence pathways in *Arabidopsis* was evaluated in an in vitro system by assessing the activation of the R2R3-MYB-like transcription factor gene *myb72*. Activation of *myb72* induced by the isolates was higher than control being *T. asperellum* T20 the most effective. The application of a mix of *Trichoderma* isolates was effective in decreasing root rot caused by artificial inoculation of *R. solani* and *S. sclerotiorum* though to a lesser extent. The highest efficacy against the oomycete *P. nicotianae* was shown by *T. harzianum* T6 alone, which was shown to produce antibiotic compounds in vitro effective against oomycetes. Results indicate that application of combination of local antagonists through the amendment of potting soil has a potential in the sustainable production of nursery plants.

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Dear Editors,

Please find enclosed a manuscript entitled: " **Use of nursery potting mixes amended with local *Trichoderma* strains with multiple complementary mechanisms to control soil-borne diseases** " which I am submitting for consideration of publication as an article in "Crop Protection".

Nursery soil-borne diseases represent a serious problem in Italy as well in other countries, therefore alternatives to conventional methods based on the use of fungicides are strongly needed. The use of potting mixes added with *Trichoderma* species locally isolated and with antagonistic and beneficial characters may be a sustainable alternative for nursery productions.

The paper findings provide an additional novel tool to be integrated in a sustainable management of nursery diseases. As such this paper should be of interest to a broad readership including those interested in biological control, sustainable soil management, plant protection, nursery production, horticulture.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

All authors have approved the manuscript and agree with its submission to "Crop Protection".

Sincerely,

Gabriele Chilosi

1 Use of nursery potting mixes amended with local *Trichoderma* strains with 2 multiple complementary mechanisms to control soil-borne diseases 3

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12 **Keywords:** induced resistance, *in vitro* evaluation, antagonism, nursery, biocontrol.

13

14 **Highlights:**

15 *Trichoderma* species were isolated from holm oak, olive, lavender rhizosphere in nursery.

16 The *Trichoderma* isolates showed differences and complementarity in modes of action.

17 Substrates added with selected *Trichoderma* were effective in decreasing root rot symptoms.

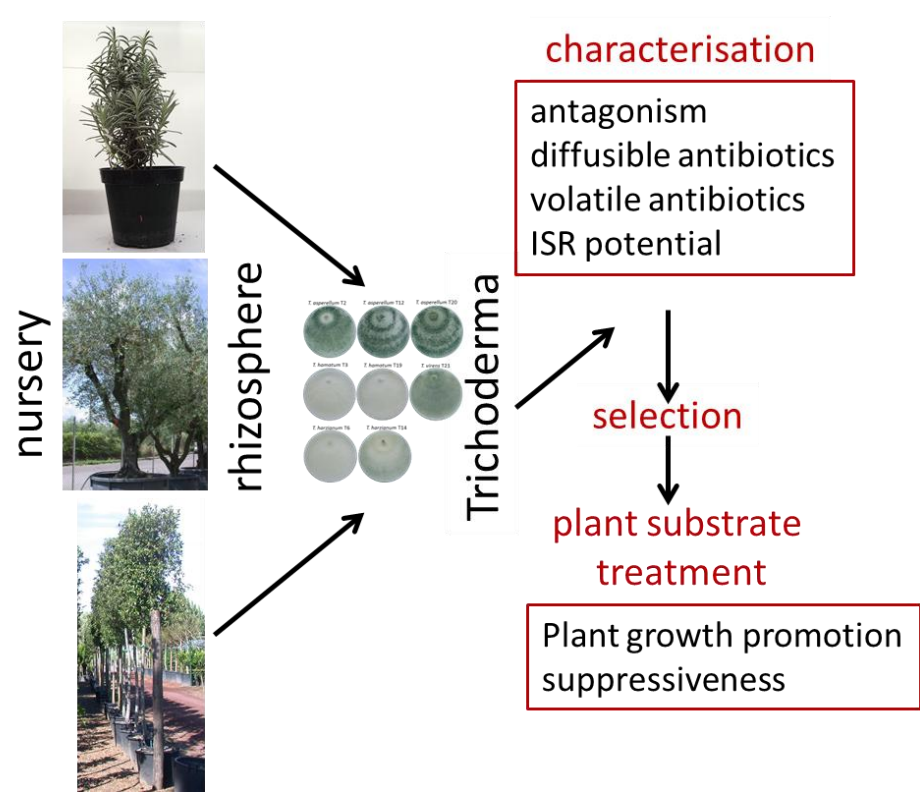
18 Local *Trichoderma* has a potential in the sustainable production of nursery plants.

19

20 **Abstract**

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22 rhizosphere of holm oak, olive and lavender in nursery with the aim to select effective antagonistic
23 and growth promoting agents and use them to amend nursery plant growth substrates. Among
24 *Trichoderma* species isolated, three from olive (*T. asperellum* T2, *T. hamatum* T3, *T. harzianum*
25 T6) and holm oak (*T. hamatum* T19, *T. asperellum* T20, *T. virens* T21), and two from lavender (*T.*
26 *asperellum* T12 and *T. harzianum* T14) were selected for the *in vitro* tests of antagonism against the

1 plant pathogens *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Phytophthora*
2 *nicotianae* and *P. cinnamomi*. Mycelial growth of each target species was differently affected
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4 multiple mechanisms involved in the antagonistic activity. The ability of *Trichoderma* isolates to
5 trigger defence pathways in *Arabidopsis* was evaluated in an *in vitro* system by assessing the
6 activation of the R2R3-MYB-like transcription factor gene *myb72*. Activation of *myb72* induced by
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11 compounds *in vitro* effective against oomycetes. Results indicate that application of combination of
12 local antagonists through the amendment of potting soil has a potential in the sustainable production
13 of nursery plants.



Graphical abstract

1. Introduction

Soil-borne diseases are important cause of decreasing quality and quantity of nursery production of ornamental, horticultural and officinal plants. Most of the causal agents are polyphagous ubiquitous fungi such as *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, *Verticillium dahliae* and many species of *Phytophthora* and *Phytium* (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 nutrients supplies, often promote occurrence and spread of soil-borne pathogens. Disease management in nurseries is often based on the routinely applications 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. On the basis of the recent European Regulation 1107/2009, concerning the placing of plant protection products on the market, and the European Directive 2009/128/EC, which establish 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 needed. In this context, biological control of soil-borne diseases is an alternative to sustain high production with low impact (Antonelli et al., 2013). The potential of control of soil-borne diseases with antagonists such as *Trichoderma* spp. has raised considerable research interest in the last decades (Hermosa et al., 2012). Some *Trichoderma* rhizosphere-competent strains have been shown to increase, plant growth potential and nutrient uptake, fertilizer use efficiency, percentage and rate of seed germination and stimulation of plant defenses (Shoresh et al., 2010; Hermosa et al., 2012). Moreover, the beneficial effects obtained with *Trichoderma* spp. have sensible economic implications, such as the shortening the plant production cycle in the nursery, thereby the increase production capacity (Lo and Lin, 2002). The success of a biological control programme relies in the good adaptation of a given biocontrol agent to the local environmental conditions in which it is supposed to work. Thus, the selection of bioagents should take into account the efficacy toward the target pathogen along with the conditions where 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).

The present study was undertaken to characterise the antagonistic activity of *Trichoderma* isolates naturally resident in the rhizosphere of holm oak, olive and lavender with the aim to select an array of effective biocontrol and growth promoting isolates to be used to amend nursery potting mix. In order to investigate the antagonistic mechanisms of selected *Trichoderma* species, *in vitro* production of both volatile and non-volatile antifungal metabolites against a set of soil-borne pathogens with diverse lifestyles. 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 *Phytophthora nicotianae*, *S. sclerotiorum* and *R. solani* in 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 ys old), olive (50 ys old) and lavender (3 ys old) grown in pots containing peat and pumice 1:1 added with 2 kg m⁻³ lime and 3Kg m⁻³ Osmocote® 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 covered with ice and transported to the laboratory and processed within 18–24 h. Samples were then homogenized and spread on paper to remove plant material; then, they were air-dried, sifted with 2 mm mesh sieves, and stored at 4°C until processing. Serial dilutions of soil samples were made for isolation of *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 at pH 6.0 (Vargas Gil et al., 2009). Based on colony morphology and microscopic observations the frequently occurring species of genus *Trichoderma* were selected and subjected to purification following subculturing.

2.2 Identification of *Trichoderma* isolates

The *Trichoderma* colonies isolated from dilution plate count technique were identified on the basis of colonies morphological characters, micro-morphological traits and molecularly by the analysis of the ITS region of the rDNA. The phenotypic characters have been analysed as reported by Samuels et al. (2002). All measurements and observation have been taken from PDA and cornmeal agar +2 % dextrose (CMD) (w/v) (Oxoid, Unipath Ltd). Micro-morphological traits (shape and size of conidia, conidiophores, nonfertile extensions) were recorded within 1 week from colonies grown on CMD at 25°C and 12 h darkness/12 h cool white fluorescent light. Identification by analysis of micro-morphological traits was performed as reported by Bisset (1991), Chaverri et al. (2003) and Samuels et al. (2010).

To confirm morphological identification of *Trichoderma* spp. genomic DNA was extracted from mycelium of each isolate, after growing in PDB for 1 week, using the DNeasy Plant Mini-kit (Qiagen, Milan, Italy). 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 isolates of *T. harzianum* chitinase *ech42* gene was amplified with the primer pair Chit42-1a (5'-GCTYTCCATCGGTGGCTGGAC-3') and Chit42-2a (5'-GGAGTTGGGGTAGCTCAGC-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. Amplicons were sequenced (Macrogen Inc., Seoul, Republic of Korea) and the 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 characterisation tests were performed using a panel of fungal and oomycete isolates representative of common soil-borne pathogens in nursery plants (Table 1) that included *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Phytophthora cinnamomi*, provided by the collection of fungal and oomycete pathogens of DIBAF, University of Tuscia Italy, and *P. nicotianae* kindly provided by 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 darkness and subcultured at 4 week intervals.

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

Dual cultures were performed for determination of direct antagonistic activity and production of antifungal non-volatile and volatile metabolites. The experimental design was a randomized block with ten replicated plates per each *Trichoderma*-pathogen combination repeated twice. *Trichoderma* isolates were evaluated for their potential to antagonize *in vitro* the panel of plant soil-borne pathogenic isolates by the dual culture technique as described by Morton and Strouble (1955). In detail, a mycelial disc (6 mm diameter), obtained from the peripheral region of growing colonies of both *Trichoderma* and pathogenic isolates were placed simultaneously on PDA, about 1 cm from the edges of 9 cm diameter Petri-dishes at opposite sides. The Petri dishes containing the PDA inoculated with the tested pathogen alone served as control. All the plates were incubated at 24°C and measurements were taken after 5 days. At the end of incubation period, radial growth was

measured. The inhibition of average radial growth was calculated in relation to growth of controls (Edington et al., 1971).

For non-volatile (diffusible) antibiotics assessment, each *Trichoderma* isolate was inoculated into 100 ml potato dextrose broth at 25°C for 10 days. After incubation, the cultures were filtered through 0.22 mm filters (Merck Millipore, Darmstadt, Germany). One ml 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 were placed in the centre of the agar plate.

The effect on pathogen mycelial growth of released volatile metabolites 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 newly inoculated with each pathogenic isolate. The two dishes were taped together with adhesive tape. The growth of 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 inhibitors tests, the percentage of inhibition was measured by dividing the difference between the radial growth of control and antagonised cultures by the radial growth of the control and multiplied by 100. All experiments were repeated one time to confirm the results.

2.5 Analysis of root-specific transcription factor gene myb72

Trichoderma isolates were evaluated for their capacity to activate defence 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 challenged with *Trichoderma* was used as indication of defence pathways activation.

Arabidopsis was grown as described by Contreras-Cornejo et al. (2009) with some modifications. Seeds were surface sterilized with sodium hypochlorite 3% (v/v) for 5 min. After three washes in

sterilised distilled water, seeds were germinated and grown on 9 cm diameter agar plates containing Hoagland's solid medium. Plates were placed vertically at an angle of 65° in a plant growth chamber at 21 °C and 16 h of light/8 h photoperiod.

Root inoculation was performed by placing a 50 µl *Trichoderma* spore suspensions (1×10^6 ml⁻¹) collected from 10 day old colonies in 0.5 strength potato dextrose broth (PDB) (Sigma Chemical Co.) along each root of 4 day old seedlings. Control seedlings received 50 µl of 0.5 strength PDB. Inoculated and control seedlings were cultured at the environmental condition as above described.

Total RNA was extracted from 100 mg (fresh weight) of frozen leaves and roots collected 72 and 96 hours after inoculation with the RNeasy Plant Mini kit (Qiagen), following the manufacturer's instruction. Contaminating DNA was removed using DNA-free TM (Ambion, Austin, TX, USA). A PCR without reverse transcription was performed for each RNA sample in order to verify the absence of contaminating DNA. Forward and reverse primers 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 have 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 *actin 8* gene used as housekeeping gene. Reverse transcription (RT) was performed using ImProm-II™ Reverse Transcription System (Promega, Milan, Italy) following the manufacturer's instruction 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 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 µM of each forward and reverse primers and RNase-free water to a final volume of 15 µl. Each reaction was made in triplicate. Amplification conditions were the following: 15 min at 95 °C, then, 40 cycles at 94 °C for 15 s, 57 °C for 30 s and 72°C for 30 s for cDNA amplification. Relative expression analysis was determined

by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The qRT-PCR's were carried out on RNA extracted from two separate experiments. Relative expression value (REV) is reported as the number of fold increase of the transcript level compared to mock inoculated sample for each time point. The experiments were repeated one time to confirm the results.

2.6 Amendment of standard potting mix with *Trichoderma* local isolates

Candidate biocontrol *Trichoderma* isolates were selected for evaluation in a greenhouse trial of their potential for biocontrol of soil-borne pathogens of lavender plants upon their addition in potting mix. *T. hamatum* T3, *T. asperellum* T20, *T. harzianum* T6, *T. virens* T21 were selected for *in vivo* experiments on the basis of their performances in the *in vitro* experiments and inducing resistance potential described above. The selected *Trichoderma* isolates were used individually and as a combination of the four isolates.

The *Trichoderma* inoculum (Ti) was prepared utilizing 50 g of millet kernels imbibed with sterilised H₂O 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) cut from the edge of actively growing cultures and incubated for 10 days at 23±2 °C.

The potting mix (peat and pumice 1:1 added with 2 kg m⁻³ lime) was inoculated with 3g kg⁻¹ inoculum of Ti corresponding to approximately 2.5x10⁹ spores per kg substrate. The potting mix added with *Trichoderma* (PoMT⁺) and un-inoculated 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 grown in PoMT⁺ and PoMT⁻ and then inoculated with *R. solani* Rs6, *S. sclerotiorum* Ss10 and *P. nicotianae* Pn698 (Table 1). Fungal and oomycetes inoculum (Pi) was prepared on millet seeds as above described for *Trichoderma*.

1 The experimental plan was based on the combination of five *Trichoderma* treatments (Tricho⁺), five
2 *Trichoderma* uninoculated controls (Tricho⁻), three pathogen treatments (Path⁺), three pathogen
3 uninoculated controls (Path⁻). Treatments were arranged in a randomized complete block design
4 with four replicates. Each experimental unit consisted of ten plants. The trial was repeated one time
5 to confirm the results. Lavender plants 60 day old were transplanted and grown 1.5 L pot for 30
6 days in greenhouse at 21 °C±3. The inoculation with the target fungal and oomycete pathogens was
7 performed by mixing 30 g inoculum (5 cm depth) per kg of PoMT⁺ and PoMT⁻. Plants were grown
8 in ambient greenhouse conditions. Plants were harvested 90 days after transplanting, graded for
9 symptoms and measured for root development and biomass. After cutting the vines, the root
10 systems were carefully extracted from the pots. The soil was flushed with tap water, then the roots
11 were washed in distilled water. Thereafter, roots were measured and weighed. Symptoms on crown,
12 roots and aerial part of lavender inoculated plants were rated on the following scale: 0, healthy; 1,
13 30% lesions on roots with punctiform shape on roots, 30 % reduction of roots and aerial part
14 development; 2, 60 % root surface with extended lesions, 50 % reduction of roots and aerial part
15 development; 3, 80 % root surface with extended lesions, stunted root and aerial parts; 4, dead
16 plants. The severity of the disease symptoms was calculated as the mean of classes.

17 Plant biomass (root and aerial dry weight per plant) was measured 90 days after transplanting on
18 treated soil with both *Trichoderma* isolates and target pathogens, on controls represented by
19 substrate treated with not-inoculated millet and alternatively, inoculated with *Trichoderma* isolates
20 or target pathogens.

21

22 2.8. Statistical analysis

23 Data were subjected to one-way analysis of variance using GraphPad Prism software (San
24 Diego, CA). The Tukey test ($P \leq 0.05$) was used to compare means.

25

26

3. Results

3.1 Identification

Based on characteristics of phenotype of 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 species identified, 8 isolates, *T. asperellum* T2, *T. hamatum* T3, *T. harzianum* T6 from olive, *T. hamatum* T19, *T. asperellum* T20, *T. virens* T21 from holm oak and *T. asperellum* T12 and *T. harzianum* T14 from lavender, illustrating representative species associated to rhizosphere of the tested plants, were subjected to further characterisation. As shown in Fig. 1, no particular morphological variation was noted among 1 week colonies belonging to the same species cultured on CMD. Representative microscopic features are illustrated in Fig. 2.

Molecular analysis confirmed the morphological identification. The multiple gene approach and the analysis of phenotypic characteristics were successful to differentiate *T. harzianum* from *T. aureoviride*. Sequences for the representative isolates were deposited in the NCBI database (Table 2).

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

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 3), while the percentage of inhibition of both *Verticillium* isolates was shown to be consistently lower. Among *Trichoderma* isolates, *T. asperellum* T12 significantly exerted the strongest inhibitory activity toward *R. solani* Rs6, *S. sclerotiorum* Ss10. The *Trichoderma* isolates generally presented a

1 moderate inhibition of all the target oomycete pathogens (Table 4). In particular, *T. hamatum* T19
 2 and *T. asperellum* T2 had the strongest inhibition activity toward *P. cinnamomi* Pc17, while *T.*
 3 *hamatum* T3 and *T. asperellum* T2, T12 and T20 were the most effective against *P. cinnamomi*
 4 Pc24. *T. harzianum* T6 displayed the highest inhibition activity toward *P. nicotianae* Pn28,
 5 conversely, it had the lowest activity against *P. cinnamomi* Pc17, Pc24 and *P. nicotianae* Pn698.
 6 The majority of the selected *Trichoderma* isolates produced non-volatile compounds with a low or
 7 absent inhibition activity of the hyphal growth of the target fungal pathogens, apart from *T.*
 8 *harzianum* T6, which exerted a strong activity toward both *R. solani* Rs6 and *S. sclerotiorum* Ss10
 9 (Table 3). A moderate activity was also displayed by *T. asperellum* T2 toward *S. sclerotiorum* Ss10
 10 and *T. virens* T21 against *R. solani* Rs6. A different picture emerged when the oomycete pathogens
 11 were exposed to non-volatile compounds (Table 4). *T. harzianum* T6, *T. virens* T21 and, to a lesser
 12 extent, *T. hamatum* T3 produced highly effective non-volatile compounds.
 13 Volatile metabolites of the selected *Trichoderma* isolates showed high inhibition rate of *R. solani*
 14 Rs6 growth, with the significantly highest inhibition produced by *T. harzianum* T14, *T. asperellum*
 15 T20 and *T. virens* T21 (Table 3). The reduction of *S. sclerotiorum* Ss10 growth by volatile
 16 inhibitors produced by *T. hamatum* T3, T19, *T. asperellum* T12, T20 was shown to be remarkable,
 17 whereas *T. harzianum* T14 and *T. asperellum* T2 exhibited a moderate or poor production of
 18 effective volatiles, respectively. The volatile compounds produced by other *Trichoderma* species
 19 resulted moderately effective in inhibiting the growth of *Verticillium* Vd2 and Vd3. *Trichoderma*
 20 spp. produced volatile with no inhibitory effects on *P. nicotianae* Pn17, apart from *T. harzianum*
 21 T6, which was moderately effective (Table 4). Volatile metabolites of the selected *Trichoderma*
 22 isolates showed a moderate inhibition of *P. cinnamomi* Pc24 growth. Volatile substances produced
 23 by *T. asperellum* T2, T12, *T. hamatum* T3, T19 showed a consistent effect on *P. nicotianae* Pn28
 24 growth, whereas *P. nicotianae* Pn698 resulted less or not (T3) sensitive to these compounds.
 25

26 3.3 Expression analysis of *Arabidopsis thaliana* myb72 gene challenged with *Trichoderma* spp.

All *Trichoderma* spp. used to challenge *A. thaliana* plants were able to colonize Arabidopsis roots 72 hai (Fig. 3) and induced the expression of *myb72* gene at 72 and 96 hai in the roots (Fig. 4). *T. asperellum* T20 has shown the greater induction at 96 hai, with a REV of 18.2 significantly higher than the other isolates (Fig. 4). In the leaves of *A. thaliana* challenged with different *Trichoderma* spp. the expression of *myb72* gene was not induced (data not shown).

3.4 Bioassay and assessment of disease severity

The inoculation with the target pathogens (Path⁺) in Tricho⁻ plants resulted in development of severe symptoms (Fig. 5). The application of the *Trichoderma* mixed isolates was significantly effective in controlling *Rhizoctonia* root rot disease on lavender compared to each single *Trichoderma* isolate treatment and Tricho⁻ control. T21 and the mix of *Trichoderma* isolates have shown some effectiveness in containing *Sclerotinia* root rot. However, no significant differences were found among treatments.

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

3.5 Promotion of plant growth

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

Upon inoculation of the target pathogens on Tricho⁺ plants, root biomass was generally lower than that of Path⁻, although to a different extent. *T. harzianum* T6 and *Trichoderma* mix were found to

1 stimulate significantly lavender root weight inoculated with *R. solani* and *P. nicotianae*,
2 *Trichoderma* mix stimulated those infected with *S. sclerotiorum* (Fig. 6).

3

4 **4. Discussion**

5 Different soil microorganisms including *Trichoderma* spp. are able to colonize plant roots forming
6 the rhizospheric microbiota which has beneficial effects on the plant, thereby influencing disease
7 protection, plant growth and yield (Berg, 2009; Raaijmakers et al., 2009; Berensen et al., 2012).
8 The importance of the root microbiota in plant health is underlined by a body of experimental
9 evidences and it is becoming increasingly clear that the plant is able to control the composition of
10 its microbiota (Hartmann et al., 2009; Berg and Smalla, 2012). A method for obtaining effective
11 biocontrol agents is to select the candidate *Trichoderma* species from areas of the plant and soil
12 where it is expected to function in disease control, and where it is growing under conditions of
13 temperature, moisture, soil microbial composition and nutrient availability that approximate those
14 found in nature (Howell, 2003). Therefore, a screening of *Trichoderma* species was performed from
15 the rhizosphere of nursery plants with the aim to isolate and characterise potential biocontrol agents
16 for use in the same ecosystem where they were obtained. The expectation is that native antagonistic
17 species, alone or in combination, may have greater efficiency in terms of disease control, plant
18 growth stimulation and adaptability compared to those obtained from areas with diverse
19 environmental characteristics. *Trichoderma* isolates obtained in this manner have been identified by
20 means of morphological and molecular methods. A representative isolate of species isolated from
21 the rhizosphere of the three plant species have been then screened for their *in vitro* antagonistic
22 activity toward a set of target fungal and oomycete pathogens and compared in terms of
23 mechanisms of activity, such the production of non-volatile and volatile inhibitors and the capacity
24 to induce resistance. This functional approach brings some new information about the interactions
25 as well as the biocontrol potential that is harboured by the *Trichoderma* group (Anees et al., 2010).

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

4 Antagonistic activity has been performed against fungal and oomycete soil-borne pathogens,
5 important causative agents of nursery plant diseases and characterised by diverse lifestyles. *S.*
6 *sclerotiorum* is a necrotrophic ascomycete with an acidic behaviour by the production of oxalic acid
7 and polygalacturonases as virulence factors (Guimarães and Stotz, 2004; Cotton et al., 2003).

8 Conversely, *R. solani* is a necrotrophic basidiomycete with an alkaline behaviour and characterised
9 by the production of pectin lyase as predominant pectolytic enzyme (Bugbee, 1990). *Verticillium*
10 *dahliae* is a vascular pathogen is an important disease responsible for dramatic yield losses in many
11 crops (Tjamos, 2000). *P. nicotianae* and *P. cinnamomi* are heterotallic oomycete pathogens with a
12 worldwide distribution that causes disease in hundreds of plant species (Erwin and Ribeiro, 1996).

13 Differences found in mycelial growth inhibition caused by the antagonistic isolates of *Trichoderma*
14 spp. indicates that among these isolates there are physiological differences, and these variations
15 could be due to the mechanisms involved in the antagonistic activity toward pathogens with
16 different lifestyles. The mechanisms employed by *Trichoderma* species to affect biological control
17 are many and complex, and their use varies with the *Trichoderma* species, pathogen and host plant
18 involved (Howell, 2003). *S. sclerotiorum* Ss10 was the most sensitive pathogen fungi to all
19 *Trichoderma* isolates used. A high level of inhibition was also exerted toward *R. solani* Rs6,
20 confirming the sensitivity of this pathogen to *Trichoderma* species (Harman et al., 2004; Grosch et
21 al., 2006; Anees et al., 2010). *T. asperellum* T2, T12 and T20 provided significantly higher activity
22 than the other isolates, being T12 significantly the most effective. *T. asperellum* was confirmed to
23 be effective biocontrol agent against many soilborne plant pathogenic fungi, antagonising through
24 antibiosis, nutrient competition and cell wall-degrading enzymes and by inducing resistance in
25 plants (Liu et al., 2010).

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

8 Enhancement of basal resistance levels is a common reaction of plants to attempting attacks by
9 hypovirulent pathogens and root colonisation by non pathogenic rhizobacteria and rhizofungi
10 (Pieterse et al., 2002; Segarra et al., 2007), and is commonly referred to as systemic acquired
11 resistance (SAR) and induced systemic resistance (ISR), respectively (Van Loon, 2000). Using
12 *Arabidopsis thaliana* as a model plant species and rhizobacterial strain *Pseudomonas fluorescens*
13 WCS417r as ISR inducer, it was found by microarray analysis that the R2R3-MYB-like
14 transcription factor gene *myb72* is specifically activated in the roots upon colonisation by WCS417r
15 (Verhagen et al., 2004; Van der Ent et al., 2008). Analysis of *myb72* mutant plants revealed that
16 *myb72* is required also for the onset of ISR mediated by *T. asperellum* against a set of pathogens
17 (Segarra et al., 2009). In this research we have set up an *in vitro* system to assess the ability of
18 *Trichoderma* spp. to activate the expression of transcription factor gene *myb72* on *A. thaliana*
19 seedlings. By this model, we found that the isolates tested are all capable to activate early signaling
20 of ISR. However, the level of expression of the isolates tested was found to be significantly
21 different, being *T. asperellum* T20 the most effective. This result suggests that rhizospheric
22 *Trichoderma* population is capable of activating the defence pathway by similar basic mechanisms,
23 but different amplitude depending on the species tested. It is likely that this ability has an ecological
24 role to colonise and benefit plants across a range of species and environmental conditions.

25 A combination of biological control agents is more likely to have a greater variety of traits
26 responsible for suppression of pathogens and for promoting plant growth. It has been shown that

combination of antagonistic microorganisms including *Trichoderma* spp. can provide the most effective suppression of soil-borne diseases by fungal and oomycete pathogens tested in controlled conditions (Spadaro and Gullino, 2005). The present data indicate that the suppressive capacity of isolates combination seems to be related to the lifestyle of the target pathogen. A higher decrease of disease incidence resulted when selected *Trichoderma* and their antagonistic/ISR traits were combined against the fungal pathogens *R. solani* and *S. sclerotiorum* on lavender. By contrast, the highest suppressive efficacy was determined not by isolates combination, but by the single *T. harzianum* T6 against the oomycete *P. nicotianae*. This might be due to the high efficiency of diffusible antibiotics produced by T6 against oomycete pathogens as indicated by the *in vitro* characterization tests and in an environment presumably not competitive with other isolates. Taken together, present results indicate that application of combination of local antagonists through enrichment of potting mixes has a great potential in the sustainable production of nursery plants.

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Figure captions

Fig. 1. Colony characters of *Trichoderma asperellum* T2, T12, T20, *T. hamatum* T3, T19, *T. harzianum* T6, T14 and *T. virens* T21. Cultures were grown 96 h on cornmeal agar +2% dextrose (CMD) at 25°C under 12 h cool white fluorescent light/12 h darkness.

Fig. 2. Characters of conidiophores of *Trichoderma harzianum* T6 (A), *T. asperellum* T12 (B), *T. virens* T21 (C); nonfertile conidiofore extensions of *T. hamatum* T3.

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

Fig. 4. Transcript level of *myb72* gene in *Arabidopsis thaliana* Col-0 roots challenged with *Trichoderma asperellum* (T2, T12, T20), *T. hamatum* (T3, T19), *T. harzianum* (T6, T14), *T. virens* (T21) at 72 (■) and 96 (▣) hours after inoculation. REV = Relative expression value. Error bars represent standard error. Different letters denote significant differences at post-ANOVA Tukey test ($P<0.05$).

Fig. 5. Control of *Rhizoctonia solani* Rs6 (A), *Sclerotinia sclerotiorum* Ss10 (B), *Phytophthora nicotianae* Pn698 (C) by selected *Trichoderma* isolates and un-inoculated (Tricho⁻) on lavender plants 90 days after inoculation. Bars with different letters are significantly different (post-ANOVA Tukey test ($P<0.05$)).

Fig. 6. Root dry weight per plant of lavender plants 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; D, inoculated with *Phytophthora nicotianae* Pn698, 90 days after inoculation. Bars with different letters are significantly different (post-ANOVA Tukey test ($P<0.05$)).

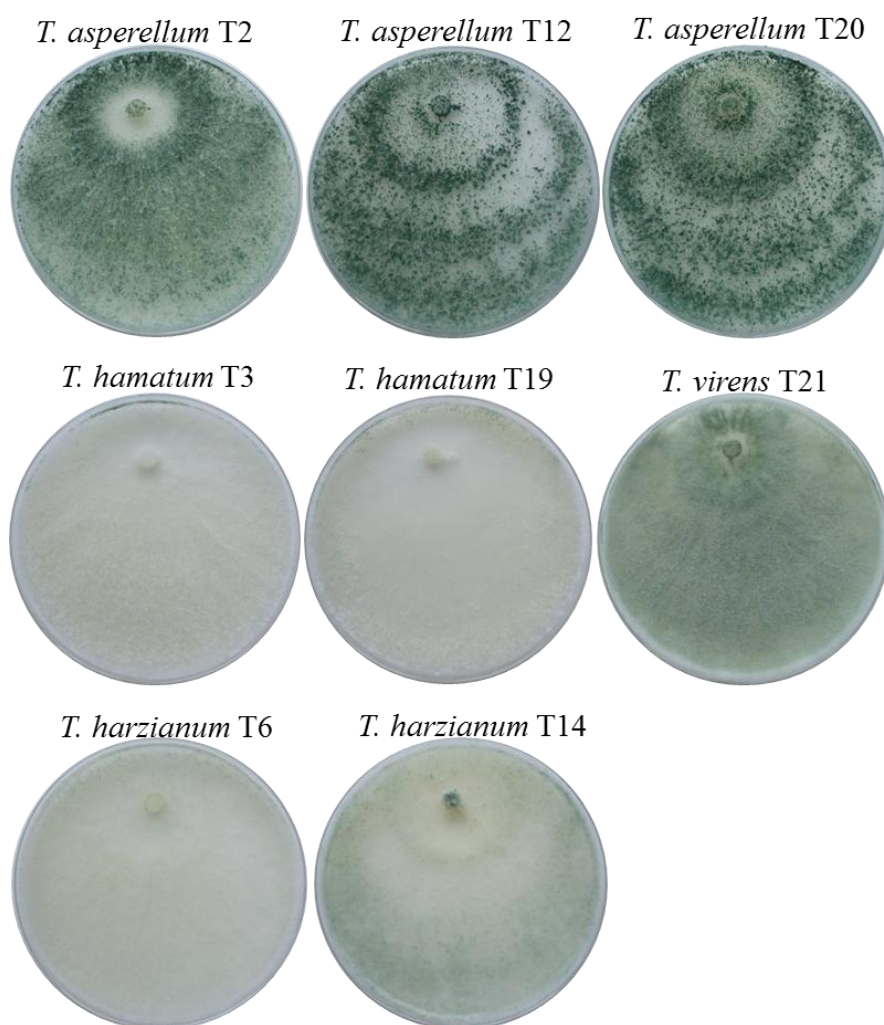


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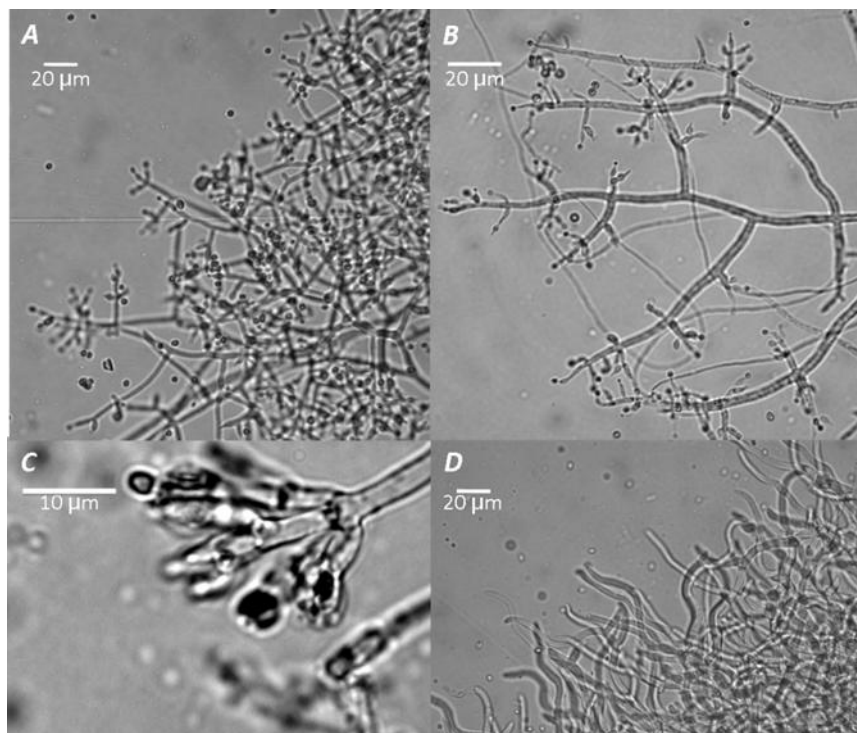


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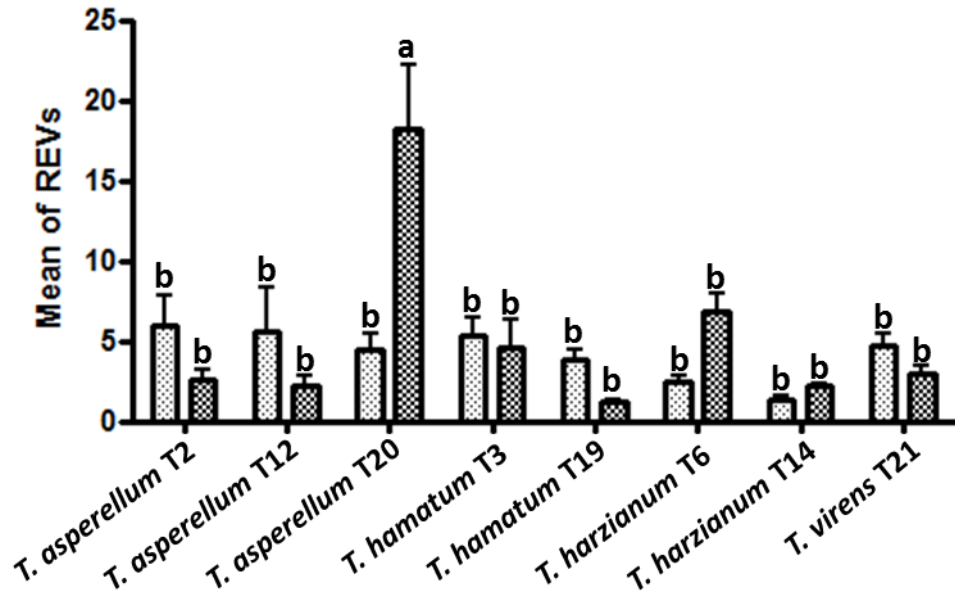


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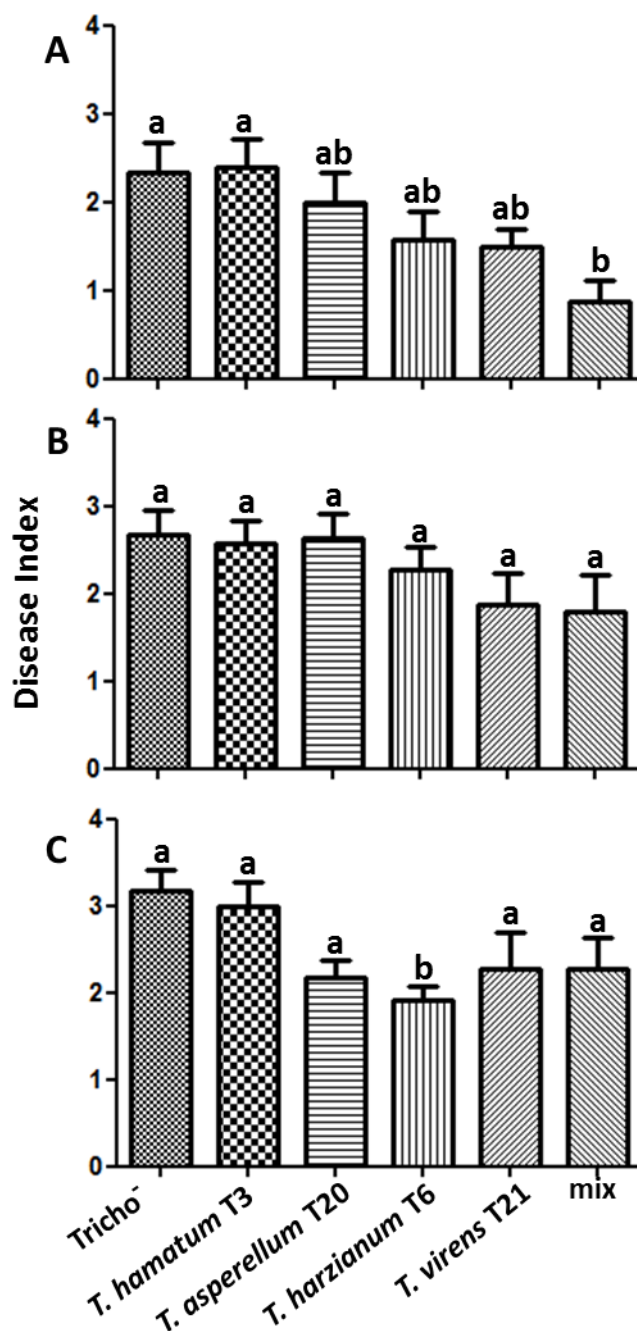


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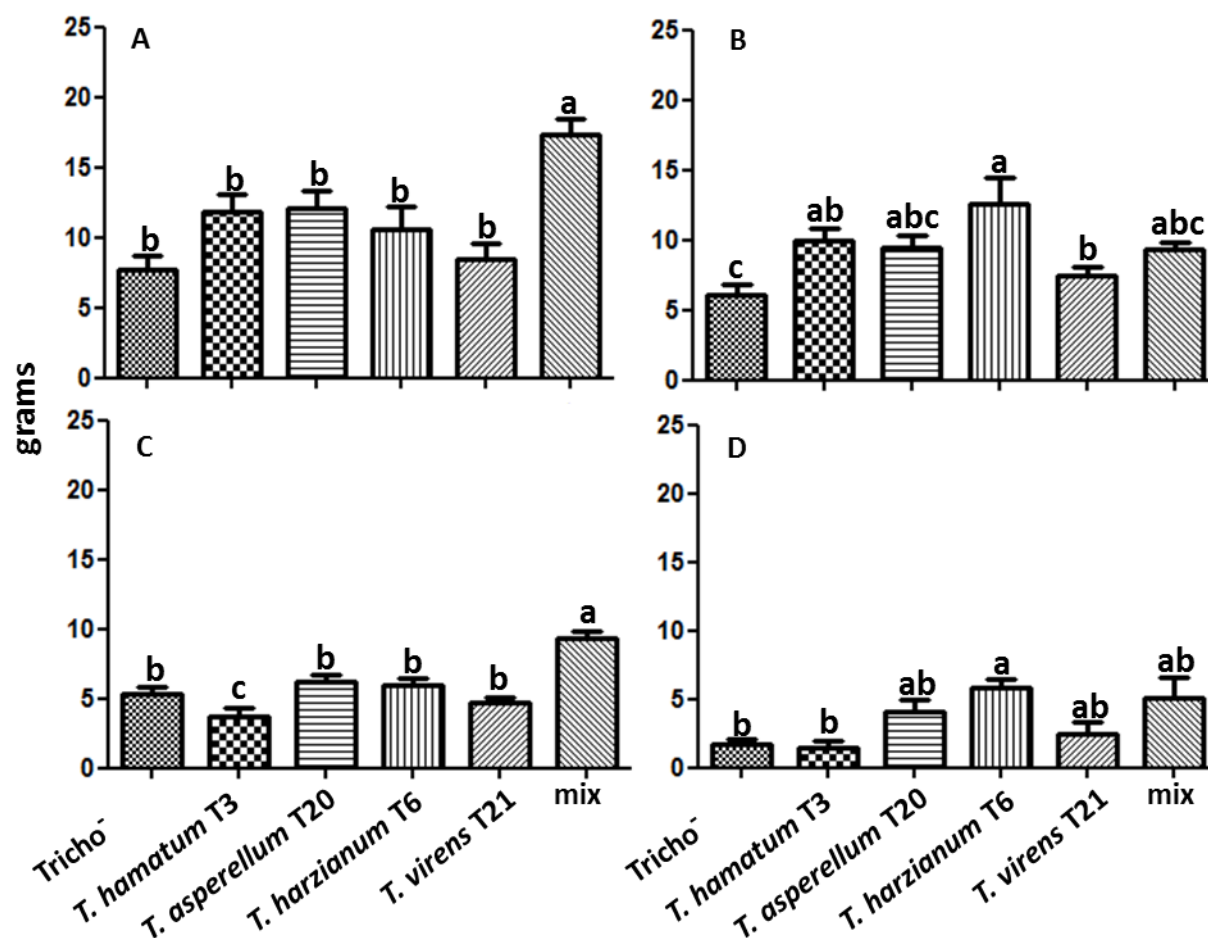


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Tab. 1. Isolates of fungal and oomycete pathogens tested in the antimicrobial assays and assessment of volatile and non-volatile antibiotics.

species	isolate	host	mating type	source
<i>Rhizoctonia solani</i>	Rs6	lettuce		DIBAF, University of Tuscia, Italy
<i>Sclerotinia sclerotiorum</i>	Ss10	melon		DIBAF, University of Tuscia, Italy
<i>Verticillium dahliae</i>	Vd2	<i>Arabidopsis thaliana</i>		DIBAF, University of Tuscia, Italy
<i>Verticillium dahliae</i>	Vd3	chrysanthemum		DIBAF, University of Tuscia, Italy
<i>Phytophthora cinnamomi</i>	Pc17	beech	A1	DIBAF, University of Tuscia, Italy
<i>Phytophthora cinnamomi</i>	Pc24	Chestnut	A2	DIBAF, University of Tuscia, Italy
<i>Phytophthora nicotianae</i>	Pn28	lavander		UPV, Valencia, Spain
<i>Phytophthora nicotianae</i>	Pn698	lavander		UPV, Valencia, Spain

Tab. 2. Morphological and molecular identification of the *Trichoderma* isolates illustrating the representative species associated to rhizosphere of the tested plants.

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

Table 3.

Inhibition of growth of the fungal pathogens *Rhizoctonia solani* Rs6, *Sclerotinia sclerotiorum* Ss1, *Verticillium dahliae* Vd2 and Vd3 by dual cultures analysis and non-volatile and volatile compounds produced by *Trichoderma* species isolated from rhizosphere of nursery plants. Means followed by the same letter are not significantly different at $P \leq 0.05$ by one way ANOVA (Tukey).

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

Table 4.

Inhibition of growth of the oomycete pathogens *Phytophthora cinnamomi* Pc17 and Pc24, and *P. nicotianae* Pn28 and Pn698 by dual cultures analysis and non-volatile and volatile compounds produced by *Trichoderma* species isolated from rhizosphere of nursery plants. Means followed by the same letter are not significantly different at $P \leq 0.05$ by one way ANOVA (Tukey).

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