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Growth-Promoting and Protective Effect of *Trichoderma atrobrunneum* and *T. simmonsii* on Tomato against Soil-Borne Fungal Pathogens

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Abstract: *Trichoderma* fungi are promising candidates for biocontrol agents and plant growth promoters. *Trichoderma atrobrunneum* and *T. simmonsii* were evaluated for the control of soil-borne phytopathogenic fungi, in the present study. Dual culture tests with *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici* were used to conduct in vitro evaluation. In the presence of *Trichoderma*, phytopathogen's growth rate was inhibited up to 59.70% for *R. solani* and 42.57% for *F. oxysporum*. Greenhouse trials with potted tomato plants demonstrated that *Trichoderma* caused a significant increase of stem height and fresh stem weight in pathogen-inoculated plants, compared with the negative control (plants artificially inoculated with the phytopathogen only). Except for *T. simmonsii*, plant growth was not significantly enhanced by a *Trichoderma* presence in the positive control (healthy plants). The overall performance of the two *Trichoderma* species studied was equivalent to that of the *T. harzianum* T22 commercial strain. All the tested species were found to be effective in suppressing colony growth and disease development of the soil borne pathogens in dual cultures and potted plants, indicating that they could be used as biocontrol agents. Our findings are discussed in the context of enhancing endophytic microorganisms' application in crop production systems.

Keywords: fungi; endophytes; biostimulant; plant growth; *Rhizoctonia*; *Fusarium*

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1. Introduction

Control of crop fungal diseases has been achieved mainly by chemical fungicides, for centuries. These chemicals help farmers to preserve and increase crop quality and quantity all over the world [1,2]. However, their extensive and often irresponsible use over a very long period has caused very serious problems, such as pathogen resistance development, recurrence of secondary plant pathogens, environmental pollution, human health risks, etc. These side-effects have generated the drive to develop alternative crop protection tools and strategies, that are not only cost-effective and reliable but also more ecologically and human-friendly [1].

Biological control, the application of beneficial microorganisms to minimize the activities and population of plant pathogens is regarded as a reliable, cost-effective, environmental and human-friendly non-chemical approach to control plant pathogens [3–5]. Among biocontrol agents (BCAs), fungal antagonists play the most important role in controlling plant diseases, comprising a total of 300 species belonging to 13 classes and 113 genera [6].

Today, the genus *Trichoderma* is considered the most important fungal BCA [6,7]. Isolates of more than 25 *Trichoderma* species have been thoroughly studied and have shown great potential for significantly inhibiting phytopathogen growth [8]. *Trichoderma harzianum* is regarded the most widely used BCA for a variety of phytopathogens [9]. Moreover, many

strains of *Trichoderma* have often demonstrated excellent ability not only to produce several metabolites that play a major role in biocontrol efficiency [10] but also to adopt various mechanisms mainly competition, antibiotism and mycoparasitism [7,11].

Fungicidal is not the only action that have been recorded on *Trichoderma*, given that it may also act as plant growth promoter [12–18]. This dual beneficial effect of *Thrichoderma* (disease control and plant growth promotion) has been reported in many important crops, such as cucumber [19], melon [17], maize [20,21], soybean [20,22], wheat [20], lentil [20], tea [23], chickpea [24,25], common bean [26,27], muskmelon [28], potato [29] and tomato [30,31].

In the present study we tested newly discovered strains of *T. atrobrunneum* and *T. simmonsii*, two *Trichoderma* species that have not yet been very thoroughly studied. The first one has been evaluated as control agent against *Fusarium* wilt in cucumber and demonstrated both efficient protective effect and growth stimulation [32]. The same effect has been verified against *Rhizoctonia* root rot in cowpea seedlings [33]. The second species, *T. simmonsii*, has been studied for its plant growth promotion/and biocontrol activity against *Phytopthtora capsici* on bell pepper with promising results [34]. It also promoted soybean seed germination and seedling growth [35]. Moreover, several strains of both tested species showed signs of good antagonism against Armillaria pathogens in in vitro assays [36,37].

Our study aimed to evaluate biological control and plant growth promotion abilities of two *Trichoderma* isolates on tomato infected by soil borne fungal pathogens. The isolates were first assessed in vitro for their antagonism against *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* in dual culture tests. Further evaluation tests in plant growth took place in greenhouse trials on potted plants. Our findings are discussed in the context of enhancing endophytic microorganisms' application in crop production systems.

2. Materials and Methods

2.1. Isolation and Identification of Fungal Pathogens

Fusarium oxysporum f. sp. *lycopersici* was isolated from infected tomato plants in the glasshouse of the Department of Agrotechnology, Larissa, Central Greece. Stems of the infected tomato plant were chopped into small pieces, their surface sterilized in a 1% sodium hypochloride for 3 min, then rinsed in sterile distilled water. After the plant tissues were dried in a sterile filter, they were incubated in a PDA growth medium for 8 days in temperature of 25 °C under dark conditions (in incubators). After six days of incubation, small colonies of fungus appeared and were transferred to fresh PDA plates to obtain petri dishes with pure cultures of the pathogen.

Species identification was carried out by the senior author through microscopic examination based on certain morphological characters like colony texture and pigmentation, conidial appearance and shape, the number of septa in macro-conidia, etc. [38,39].

Rhizoctonia inoculum was isolated from potted tomato seedlings, which were sown in soil infected with *Rhizoctonia*. The fungus was isolated from diseased tomato plants with visible symptoms 10 days post sowing. The fungus was isolated as above. Species identification was carried out by the senior author through the microscopic examination of the sclerotia, the shape of the cells, the hyphal branching and septa formation near it, the colony color, etc., from the fungal culture three weeks after incubation [40].

2.2. Isolation of Trichoderma Species

Trichoderma atrobrunneum was isolated in Serres (Northern Greece) from corn cobs, where green mold had developed between the kernels after a period of extreme rainfall with low temperatures and after severe insect damage. The fungus was transferred to a Petri dish, incubated for 7 days at 25 °C and kept as a pure culture.

Trichoderma simmonsii was isolated from soil sample collected from Devon Great Consols, a closed copper–arsenic mine near Tavistock in Devon, UK. The soil was heavily contaminated with copper and arsenic. Soil was air dried at 30 °C for three days, ground

and sieved. Fungi were isolated following the soil–plate method [41]. About 0.05 g of the dry soil was spread in Petri dishes with PDA and then incubated at 25 °C. Fungal colonies developed after 3 days and a small sample of the mycelium from each colony was transferred in a new dish to keep a pure culture for each fungus and incubated. From all the transferred fungal colonies, in one Petri dish the fungus was identified as *Trichoderma* spp. through microscopic examination based on colony growth and color, the pigmentation of hyphae and the arrangement of conidiophores and conidia [42].

A commercial strain of *Trichoderma harzianum* T22 (Trianum-P, Koppert B.V., Berkel en Rodenrijs, The Netherlands) was used as a positive control in our experiments. It was developed by spreading the granules in a Petri dish with PDA and incubated at 25 °C.

2.3. Molecular Characterisation of Trichoderma Species

Trichoderma isolates were subcultured several times on plates with Sabouraud dextrose agar (SDA) to ensure purity and monosporic cultures. Following the method outlined by Rogers and Bendich [43], the genomic DNA (gDNA) was extracted. Applying universal primer sets ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCG TAAC AAGG-3′), a fragment of the ITS I/5.8s/ITS II region of the ribosomal DNA (rDNA) was expanded. PCR reactions (30 µL) included 50 ng of template gDNA, 1.25 µL of each 10 pM oligonucleotide, 1 µL of 10 mM dNTPs, 1 µL of 2 U/µL Taq DNA polymerase (Minotech, Guangzhou, China), 1.5 µL of $MgCl_2$, and 2.5 µL of 10× PCR buffer. The PCR protocol for the amplification of ITS regions includes 31 cycles at 94 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s, followed by a final elongation at 72 °C for 5 min. PCR products were kept at 4 °C. The quantity and quality of PCR products were determined by gel electrophoresis using 2% agarose gel, which was stained with SYBR Safe DNA Gel Stain (Invitrogen, Waltham, MA, USA) and visualized under UV light (BIO-RAD, Molecular Imager Gel Doc XR System, Hercules, CA, USA).

The purification and the sequencing of the amplified products took place at Eurofins Genomics, Ebersberg, Germany. The similarity of the fungal DNA sequences in the present work with homologous sequences was matched using the Basic Local Alignment Search Tool (NCBI BLAST) [44]. The guidelines of Cai and Druzhinina [45] were adopted for the molecular identification of *Trichoderma* species. According to this protocol, a species of *Trichoderma* can be identified if its ITS sequence reaches at least one similarity value ≥76% to the sequences in the dataset and the two other DNA barcoding markers are highly similar to the corresponding sequences of the reference strain of one species as rpb2 ≥ 99% and tef1 ≥ 97%.

2.4. Dual Culture Tests

The dual culture method [46] was performed to determine the antagonistic effect of *Trichoderma* isolates against the pathogens *F. oxysporum* and *R. solani*. A small piece of mycelial disc (5 mm diameter) from the growing edge of the 7-day old fungal cultures (*Trichoderma* and phytopathogens) was placed on the opposite of the PDA Petri dish (size 90 × 15 mm), 10 mm from the edge of the plate and equal distances apart. All dishes were incubated at 25 °C. Control dishes were monocultures of *F. oxysporum* and *R. solani*, identical to treatment dishes but without the presence of *Trichoderma*.

The inhibition of the phytopathogen growth rate was recorded daily by measuring the radial growth of the pathogens for 6 days. To measure the radial growth over the PDA surface, the open-source Java freeware ImageJ 1.8v (http://rsbweb.nih.gov/ij/, accessed on 15 April 2021) was used after photography [47]. Photographs of examined dishes were taken daily with a stereo microscope (MOTIC SFC-11C N2GG, Motic Europe S.L.U., Barcelona, Spain), equipped with digital camera and connected with PC. Each treatment was replicated 6 times.

The inhibition growth rate (IGR) was calculated using the following Formula (1):

$$\text{IGR} = [(\text{CRG} - \text{TRG})/\text{CRG}] \times 100\%, \quad (1)$$

where CRG is the mean control radial growth, meaning the mean radial of the pathogenic fungal colony in the monoculture (in absence of *Trichoderma*), and TRG is the mean treatment radial growth, meaning the mean radial of the pathogenic fungal colony in the dual culture (in presence of *Trichoderma*).

2.5. Inoculum Preparation

PDA dishes with 7-day old pure monocultures of *F. oxysporum* and *Trichoderma* were used. After incubation, sterile water was poured into the plates and the mycelium was scraped with a sterile glass rod. The spore suspension was filtered and put into tubes, and the spore concentration was determined using a Neubauer hemocytometer (TIEFE 0.100 mm 1/400 9 mm). The concentration of the *F. oxysporum*, *T. harzianum*, *T. atrobrunneum* and *T. simmonsii* conidial suspensions were 1.08×10^8, 0.53×10^8, 4.04×10^8 and 2.33×10^8 conidia/mL, respectively.

For *R. solani* treatments, mycelium discs ~2 cm from *Rhizoctonia* 7-day old monocultures were used to inoculate plants.

2.6. Greenhouse Tests on Potted Tomato Plants

2.6.1. *Fusarium*

Tomato seeds (variety Campbell 33) were placed in small pots. The seedlings were individually transferred to 5 lt pots [23 cm diameter (top) × 17.4 cm deep × 17.5 cm diameter (base)] after 15 days. Two days after transplantation, each pot was inoculated with 4ml of the desired water conidial suspension (*F. oxysporum* and/or *Trichoderma*) according to experimental design. Tomato plants inoculated only with *Trichoderma* were used as positive control (*Trichoderma*-only plants), whereas plants inoculated only with *F. oxysporum* (*Fusarium*-only plants) and healthy plants without any fungus, were used as negative controls. There were 6 replications for each treatment.

Experimental plants were kept for 50 days under greenhouse conditions. After that time interval, stem height, stem and root fresh weight for each plant took place stem height was measured with a ruler and weights were recorded with a lab analytical balance (BT 2000 PCE Instruments UK Ltd., Southampton, UK) to assess the protective effect of *Trichoderma* species against *F. oxysporum* in potted tomato plants.

All pots were watered properly (1–2 times per week according to weather conditions) without water run-off.

2.6.2. *Rhizoctonia*

Two mycelium discs ~2 cm from 7-day old monocultures were used as the inoculum of *R. solani*. They were placed in the pots the same time the tomato seeds were sown. After 24 h, each pot was inoculated with 4 mL of the desired water conidial suspension of the *Trichoderma* strain according to experimental design. For each *Trichoderma* species, the concentration of the conidial suspensions was identical with above (in the case of *F. oxysporum*) and was measured 8.65×10^8, 6.09×10^8 and 3.9×10^8 conidia/mL for *T. harzianum* T22, *T. atrobrunneum* and *T. simmonsii*, respectively.

Tomato plants inoculated only with *Trichoderma* were used as positive control (*Trichoderma*-only plants), whereas plants only inoculated with *R. solani* (*Rhizoctonia*-only plants) and healthy plants without any fungus were used as negative controls. There were 6 replications for each treatment.

Experimental plants were kept for 30 days under greenhouse conditions. After that time interval, stem height and stem fresh weight for each plant took place (similarly with above) to assess the effect of the *Trichoderma* species against *R. solani* in potted tomato plants. Root fresh weight could not be measured for *R. solani* treated plants given that root development was minor due to the presence of *R. solani*. All pots were watered properly (1–2 times per week according to weather conditions) without water run-off.

2.7. Endophytic Re Isolation from Plant Tissues

Eight samples of tomato leaves, stems and roots were cut into 1-cm^2 diameter and 0.5-cm^2 thick discs in a laminar flow chamber. Measurements for endophytic colonization were made in the end of the experiment. The samples were surface sterilized by immersion in 96% ethanol solution for one minute, in 6% sodium hypochlorite solution for five minutes and, finally, in 96% ethanol solution for thirty seconds [48]. Sterile leaves, stems and roots samples were then inoculated SDA substrate using a sterile metal hook and incubated in the dark at 25 °C ± 2 and 80% humidity. The conidial growth sequence lasted 14 days at sealed Petri with parafilm.

The germination of *Trichoderma* conidia on the tomato leaves, stems and roots was evaluated using an optical microscope (40×) (type of microscope) and the number of samples which displayed fungal growth was measured. The above-mentioned process was completed inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD, Athens, Greece).

2.8. Statistical Analysis

Our data (radial growth, stem height, fresh stem and root weight) were subjected to analysis of variance in order to evaluate the significance of the differences among various treatments. Specifically, simple one-way ANOVAs were performed on data pooled from all treatments to test the significance of the effect of *Trichoderma* presence (a) in phytopathogen growth in Petri dishes and (b) in the development of diseased and healthy tomato plants. All statistical tests were performed by using SPSS v.27.0. [49].

3. Results

3.1. Molecular Characterization of Trichoderma Species

All *Trichoderma* isolates were molecularly characterized to species. The sequence of our strain, collected from the soil of an abandoned mine, had a 99.16% matched identity with *T. simmonsii*, while the other one recorded a 99.49% matched identity with *T. atrobrunneum* (Table 1).

3.2. Dual Culture Tests

The in vitro results showed a significant inhibition of mycelial growth of F. oxysporum after 4 days, caused by the presence of *Trichoderma*. As presented in Table 2, all *Trichoderma* isolates inhibited significantly the radial mycelial growth of *F. oxysporum* after 96 h (df = 3, 23; F = 66.78; $p < 0.0001$) and 120 h (df = 3, 23; F = 315.00; $p < 0.0001$) (Table 2). It should be noted that *T. atrobrunneum* caused significantly higher growth reduction on the phytopathogen even compared with the commercial *T. harzianum* strain (Table 2).

Trichoderma isolates showed an even stronger inhibitory effect on *R. solani*, causing a significant reduction in the phytopathogen's mean radial growth from the first day (df = 3, 23; F = 15.40; $p < 0.0001$) (Table 3). No significant differences in the growth inhibition of *R. solani* existed among the three tested *Trichoderma* species in almost all time intervals (Table 3).

Table 1. Molecular characterization of *Trichoderma* strains tested during present study.

Species	Collection Site	Blast ID Number	DNA Sequence	Identity (%)	GenBank Closest Hit
Trichoderma simmonsii	Devon, United Kingdom from soil of abandoned mine	GMMBSMTJ01R	GGAGGGCATTACCGAGTTTACA ACTCCCAAACCCAATGTGAA CGTTACCAAACTGTTGCCTCG GCGGGATCTCTGCCCCGGGT GCGTCGCAGCCCCGGACCAA GGCGCCCGCCGGAGGACCAA CCTAAAACTCTTATTGTATACC CCCTCGCGGGTTTTTTTATAAT CTGAGCCTTTCTCGGCGCCTCT CGTAGGCGTTTCGAAAATGAATC AAAACTTTCAACAACGGATCTCTT GGTTCTGGCATCGATGAAGAACG CAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCG AATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGT CCGAGCGTCATTTCAACCCTCGAAC CCCTCCGGGGGGTCGGCGTTGGGGAT CGGCCCTCCCTTAGCGGGTGGCCGTC TCCGAAATACAGTGGCGGTCTCGCCG CAGCCTCTCCTGCGCAGTAGTTTGCAC ACTCGCATCGGGAGCGCGGCGCGTCCA CAGCCGTTAAACACCCAACTTCTGAAAT GTTGACCTCGGATCAGGTAGGAATACC CGCTGAACTTAAGCATATCA	99.16	NR_137297.1
Trichoderma atrobrunneum	Serres, Greece from corn combs	GMMU60DF013	GGAGGGCATTACCGAGTTTACAACTC CCAAACCCAATGTGAACGTTACCAAA CTGTTGCCTCGGCGGGATCTCTGCCC CGGGTGCGTCGCAGCCCCGGACCAA GGCGCCCGCCGGAGGACCAACCAAA ACTCTTATTGTATACCCCCTCGCGGGT TTTTTTTATAATCTGAGCCTTCTCGGC GCCTCTCGTAGGCGTTTCGAAAATGA ATCAAAACTTTCAACAACGGATCTCTT GGTTCTGGCATCGATGAAGAACGCAG CGAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTG AACGCACATTGCGCCCGCCAGTATTCT GGCGGGCATGCCTGTCCGAGCGTCATT TCAACCCTCGAACCCCTCCGGGGGGTC GGCGTTGGGGATCGGCCCTGCCTTGGCG GTGGCCGTCTCCGAAATACAGTGGCGGT CTCGCCGCAGCCTCTCCTGCGCAGTAGTT TGCACACTCGCATCGGGAGCGCGGCGCGT CCACAGCCGTTAAACACCCAACTTCTGAGT TTGCACACTCGCATCGGGAGCGCGGCGCGT CCACAGCCGTTAAACACCCAACTTCTGA	99.49	NR_137298.1

Table 2. Mean radial growth (in cm) of *Fusarium oxysporum* f. sp. *lycopersici* in the presence or absence of *Trichoderma* sp. in dual culture tests.

Fungus	Time				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
F. oxysporum only [a]	0.57 ± 0.10 aE *	1.08 ± 0.12 aD	1.77 ± 0.15 aC	2.38 ± 0.15 aB	2.92 ± 0.10 aA
F. oxysporum and *T. harzianum* [b]	0.55 ± 0.10 aD	1.07 ± 0.12 aC	1.70 ± 0.17 aB	1.87 ± 0.08 bAB	1.90 ± 0.09 bA
F. oxysporum and *T. atrobrunneum*	0.55 ± 0.05 aC	1.05 ± 0.05 aB	1.60 ± 0.09 aA	1.65 ± 0.05 cA	1.68 ± 0.06 cA
F. oxysporum and *T. simmonsii*	0.55 ± 0.05 aD	1.05 ± 0.08 aC	1.75 ± 0.10 aB	1.93 ± 0.05 bA	1.95 ± 0.05 bA

[a]: negative control (phytopathogen monoculture), [b]: positive control, * means of the same column followed by the same small letter are not significantly different, means of the same row followed by the same capital letter are not significantly different (Tukey–Kramer HSD Test, $p < 0.05$), all tests were performed in Petri dishes with PDA at 25 °C, all treatments included six replications.

Table 3. Mean radial growth (in cm) of *Rhizoctonia solani* in the presence or absence of *Trichoderma* sp. in dual culture tests.

Fungus	Time				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
R. solani only [a]	1.10 ± 0.09 aE *	3.03 ± 0.08 aD	5.17 ± 0.14 aC	6.57 ± 0.08 aB	7.82 ± 0.08 aA
R. solani and *T. harzianum* [b]	0.97 ± 0.05 bC	2.03 ± 0.16 bB	3.17 ± 0.08 bA	3.20 ± 0.09 bA	3.22 ± 0.10 bA
R. solani and *T.atrobrunneum*	0.88 ± 0.08 bcC	2.05 ± 0.05 bB	3.08 ± 0.08 bA	3.12 ± 0.12 bA	3.15 ± 0.10 bA
R. solani and *T. simmonsii*	0.85 ± 0.05 cC	1.95 ± 0.10 bB	3.15 ± 0.12 bA	3.28 ± 0.15 bA	3.30 ± 0.13 bA

[a]: negative control (phytopathogen monoculture), [b]: positive control, * means of the same column followed by the same small letter are not significantly different, means of the same row followed by the same capital letter are not significantly different (Tukey–Kramer HSD Test, $p < 0.05$), all tests were performed in Petri dishes with PDA at 25 °C, all treatments included six replications.

The inhibition growth rates (IGR) calculated during the present study are depicted in Figure 1. The highest IGR values for both *F. oxysporum* (42.57%) and *R. solani* (59.70%) were obtained by *T. atrobrunneum* after 120 h. Overall, for treatments with exposure time >96 h, *R. solani* proved to be much more vulnerable in the presence of *Trichoderma*, recording higher IGR values (50.00–59.70%) than *F. oxysporum* (18.88–42.57%) (Figure 1).

3.3. Greenhouse Tests

The effects of *Trichoderma* on tomato growth are presented in Figures 2 and 3. As expected, the growth of diseased tomato plants inoculated with phytopathogen only (brown bars) was dramatically reduced compared with the control and with *Trichoderma* treated plants in almost all cases (Figures 2 and 3).

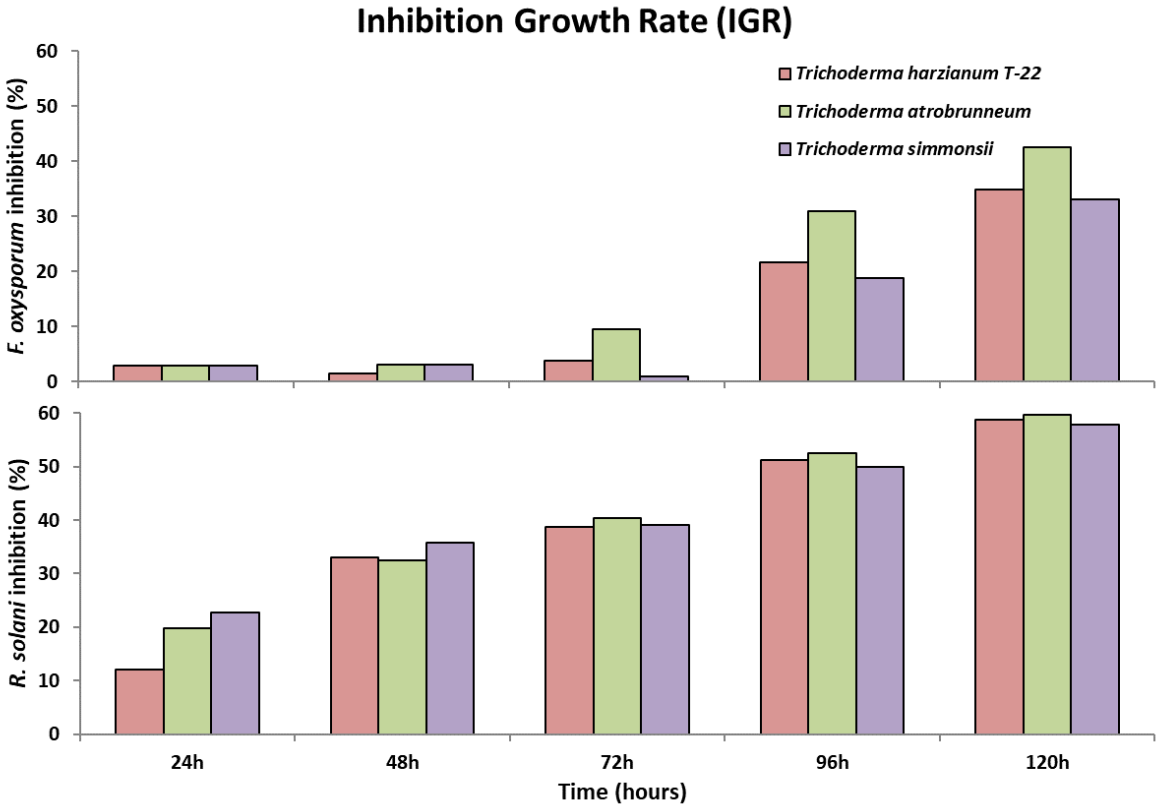

Figure 1. Inhibition growth rate (IGR) values of *F. oxysporum* (**up**) and *R. solani* (**down**) in dual culture tests with *Trichoderma* species (IGR = ((CRG − TRG)/CRG) × 100%, CRG: mean radial growth of the phytopathogen in monoculture, TRG: mean radial growth of the phytopathogen in treatment (dual culture with *Trichoderma*).

In the *Fusarium* experiment, the differences in growth parameters among control and treated plants were significant in all cases (stem height: df = 7, 47; F = 8.34; $p < 0.0001$; fresh stem weight: df = 7, 47; F = 9.14; $p < 0.0001$; root fresh weight: df = 7, 47; F = 4.61; $p = 0.0007$). Generally, the presence of *Trichoderma* in *F. oxysporum* diseased plants (dark yellow bars) managed to stop the growth reduction in most cases, given that there were not any significant differences with the healthy control plants. Another interesting conclusion is that our Trichogramma strains (*T. atrobrunneum* and *T. simmonsii*) performed better than the commercial *Trichoderma* strain (T-22), reaching higher growth parameters values. However, differences were not always significant (Figure 2).

As far as the *R. solani* experiment is concerned, differences among various control and treated plants were significant (stem height: df = 7, 47; F = 8.48; $p < 0.0001$; fresh stem weight: df = 7, 47; F = 11.61; $p < 0.0001$). Plants inoculated with *T. simmonsii* (healthy or diseased) demonstrated the highest growth values, exceeding even the healthy plants' growth (red bars) (Figure 3). Similarly to *F. oxysporum* plants, the inoculation with *Trichoderma* in *R. solani* diseased plants managed to stop the plant growth reduction in most cases.

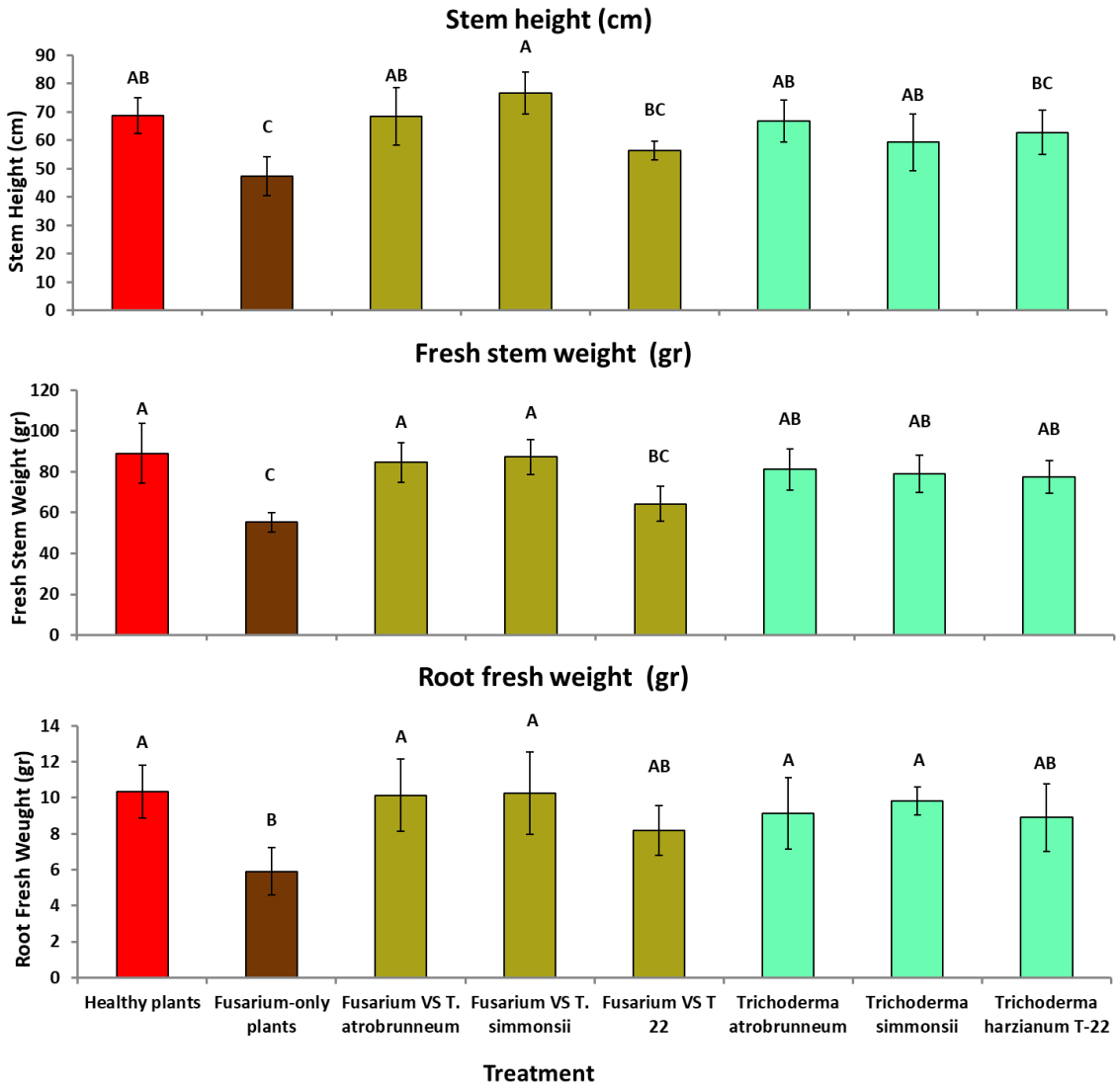

Figure 2. Effects of *Trichoderma* treatments on the growth of healthy and *F. oxysporum*-inoculated tomato plants. Columns of the same chart followed by the same letter are not significantly different (Tukey–Kramer HSD Test, $p < 0.05$), all tests were performed in potted greenhouse plants, all treatments included six replications, DAS 50 days.

Both tested *Trichoderma* strains stimulated plant growth mainly in the diseased plants (*Fusarium*- and *Rhizoctonia*-only plants) but not always in the healthy ones, considering that, in most treatments (except for *T. simmonsii* in the *R. solani* experiment, Figure 3), growth parameters of *Trichoderma*-only plants (green bars) were either lower or not significantly increased compared with those of healthy plants (red bars) (Figures 2 and 3).

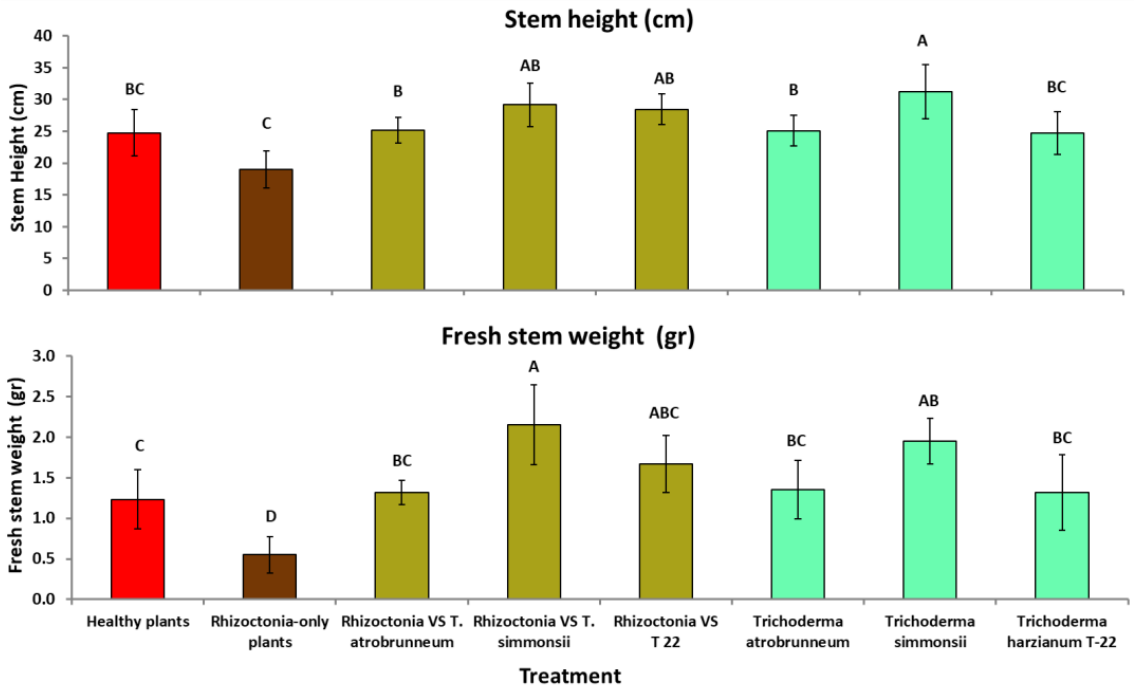

Figure 3. Effects of *Trichoderma* treatments on the growth of healthy and *R. solani*-inoculated tomato plants. Columns of the same chart followed by the same letter are not significantly different (Tukey–Kramer HSD Test, $p < 0.05$), all tests were performed in potted greenhouse plants, all treatments included six replications, DAS 30 days.

3.4. Re-Isolation of Trichoderma from Leaves, Stems and Roots on SDA Substrate

The establishment of *Trichoderma* endophytes in tomato plants was evaluated at the end of the greenhouse trials. Successful re-isolations of the fungus were obtained from all treated plants (at least one sample form the plant was colonized). On the other hand, no *Trichoderma* was detected in control plants. In total, about 90% of leaf, 70% of stem and 85% of roots samples from treated plants were successfully colonized by *Trichoderma*.

4. Discussion

Actions and methods for promoting sustainability in agricultural production systems, such as integrated pest management (IPM) and organic farming, have been greatly boosted by the urgent need to preserve the environment and human health from the harmful effects of agrochemicals [50]. The use of BCAs, which are based on living microbes or their metabolites and suppress plant diseases, is a key component of these techniques [5,50,51]. The efficiency of a wide variety of non-pathogenic fungi against soil-borne plant diseases has been the subject of significant research [52]. These control measures are in line with the European Green Deal (EGD) that aims to increase organic farming from 8% to 25% and reduce chemical pesticide application by 50% by 2030 [53].

Endophytic beneficial fungi that are naturally present in the plant ecosystem have been utilized for plant disease control for decades [54,55]. They manage to eliminate fungal phytopathogens in the plant mainly by competing for food sources, producing metabolites that inhibit the pathogen growth and by occupying specific sites in the plant first [55–57]. Among those antagonistic microorganisms, *Trichoderma* is by far the most promising and well-studied genus [58], having been investigated for more than 100 years for its beneficial effects. A plethora of scientific publications and reviews, including dozens of strains, have studied its mechanisms and interactions with plants ([11,59,60] and others), its antibiotics and metabolites ([59,61] and others), its action as biocontrol agent against various pathogens ([6,25,62] and others) and as biostimulant ([12–15,25] and others).

Trichoderma has demonstrated great effectiveness against soil-borne plant diseases. This has been demonstrated for *R. solani*, not only in dual culture tests [63–65] but also in field experiments, including crops like sugar beet [63], potato [66], cucumber [67], bean [68], tobacco [69], maize [70], cowpea [33] and tomato [33]. It has been well documented that *Trichoderma* suppresses *R. solani* through mycoparasitism and the production of antifungal compounds [66,71,72]. Moreover, various *Trichoderma* species have shown great potential in suppressing tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* both in vitro [73–79] and in vivo [75–78,80,81].

We examined new strains from two species that have not been thoroughly investigated, especially against soil-borne pathogens. In a very recent study, a strain of *T. atrobrunneum* showed an inhibition rate 39.1 and 47.5% in dual culture tests with two *F. oxysporum* isolates after 5 days [32]. Despite the different strain, this conclusion is very close with our results (42.57% IGR after 5 days). Another strain of the same species inhibited the growth of *R. solani* by 58.9% [33] and the growth of *Colletotrichum lagenarium* by 95% [82] after 9 days.

As far as *T. simmonsii* is concerned, three strains caused 65.7, 68.6 and 81.9% inhibition in the growth of *R. solani* in dual culture tests after 9 days [33]. Our relevant value (57.78% after 5 days) does not differ notably, given that the exposure time was much shorter. In the same study, one strain stimulated cowpea plant growth significantly, increasing root and aerial part weight compared with *R. solani*-only control plants. Similar growth promotion by *T. simmonsii* in tomatoes was observed in the present study.

Tested *Trichoderma* strains showed differentiated potential for plant growth promotion given that their presence in healthy plants did not result in a significant increase in the development of tomato aerial parts and root system in most of the treatments. Generally, the ability of *Trichoderma* to promote plant growth under various biotic and abiotic stress conditions has been well established in numerous recent and older studies [12–19,25,30]. Plant growth mechanisms are stimulated by the presence of *Trichoderma*, include phosphorous mobilization, by extracellular phosphatases and the production of indole-3-acetic acid derivatives [83]. Although we did not verify that *T. atrobrunneum* may act effectively as a plant growth promoter, other strains of this species caused very high K, P and Zn solubilization [82], like other *Trichoderma* species [84–87].

The only verified case of biostimulant action in the present study was that of *T. simmonsii* in the *R. solani* experiments. The fact that the same *Trichoderma* strain caused significant growth increase in tomato plants only in the second experiment may be attributed to the early inoculation (one day after sow) compared with late inoculation (15 days after sow) in the first experiment. Similar growth promoting effects for this *T. simmonsii* have been reported in the shoot and total biomass of rice seedlings [88], ball peppers [34] and soybeans [35].

Our *Trichoderma* isolates enhanced tomato growth in the presence of *F. oxysporum* or *R. solani*, but in the absence of the pathogen they did not promote plant growth, in most cases. This finding supports the theory that many antagonistic microbes help the plant to grow indirectly by preventing the pathogen's harmful effects and lowering disease intensity [89–91].

Even though a plethora of *Trichoderma*-based products are commercially available, most of them are concentrated on a very limited number of well-known species [9,92–94]. Many *Trichoderma* species remain undiscovered or uninvestigated. In the present study, two new *Trichoderma* strains of not so well-studied species were investigated for the first time as promising biocontrol agents against *Fusarium* wilt and foot rot in tomato. Although our results clearly showed their biocontrol potential, the exact mechanism should be elucidated and studied in depth to avoid the occurrence where strains that perform well in lab conditions fail to perform effectively in the field [95–99].

5. Conclusions

In conclusion, the evaluation of our endophytic *Trichoderma* strains revealed their notable biocontrol capability and their usage as biocontrol agents against fungal infections appears to be a promising approach. More research into the practical issues of large-scale production and formulation, on the other hand, is needed to develop a biocontrol product that is stable, effective, secure and increasingly successful. Endophytic fungal antagonists are a viable non-chemical alternative technique for long-term disease management on a wide range of plants and crops.

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