



# Article Effective Applications of *Trichoderma* spp. as Biofertilizers and Biocontrol Agents Mitigate Tomato *Fusarium* Wilt Disease

Eman F. A. Awad-Allah<sup>1,\*</sup>, Amany H. M. Shams<sup>2</sup>, Amira A. Helaly<sup>3</sup> and Entsar I. M. Ragheb<sup>3</sup>

- <sup>1</sup> Soil and Water Sciences Department, Faculty of Agriculture, Alexandria University, Alexandria 21545, Egypt
- Plant Pathology Department, Faculty of Agriculture, Alexandria University, Alexandria 21545, Egypt
   Vogetable Crops Department, Faculty of Agriculture, Alexandria University, Alexandria 21545, Egypt
- <sup>3</sup> Vegetable Crops Department, Faculty of Agriculture, Alexandria University, Alexandria 21545, Egypt

Correspondence: eman.awadallah@alexu.edu.eg

Abstract: Eco-friendly and sustainable plant disease management employing *Trichoderma* spp. as bioagents is an economically feasible and ecologically sustainable approach. Therefore, their use in agriculture should be encouraged. The two main goals of the present study were to evaluate the abilities of two Trichoderma isolates to prevent Fusarium wilt disease, which is caused by Fusarium solani, in vitro and under greenhouse conditions, as well as their potential as biofertilizers to enhance cherry tomato growth and development. The results of a dual culture test revealed that T. viride and T. harzianum are antagonistic against the F. solani pathogen. The antagonism mechanisms include competition for nutrients and space, mycoparasitism, and antibiosis, according to scanning electron microscopy (SEM) findings. Additionally, T. harzianum reduced the mycelial growth of F. solani by 78.0%, whereas T. viride inhibited the growth by 61.2%, 10 days post-inoculation. In a greenhouse experiment, cherry tomato plants treated with each of these antagonistic Trichoderma isolates separately or in combination significantly suppressed Fusarium wilt disease, improved plant growth parameters, increased macro- and micronutrients uptake, and increased the content of photosynthetic pigments and total phenols. In conclusion, effective applications of Trichoderma isolates have the potential to mitigate Fusarium wilt disease, which is caused by F. solani in cherry tomato plants, while simultaneously promoting the growth and development of cherry tomatoes.



### 1. Introduction

*Fusarium* wilt is a destructive disease caused by fungal soil-borne pathogens such as Fusarium oxysporum or Fusarium solani that causes significant losses for many important vegetable and crop plants, including tomatoes, all over the world [1]. In Egypt, F. solani isolates were the most common soil-borne pathogenic fungi isolated from tomato plants in different governorates, causing damping-off and root rot diseases [2]. Early infections are characterized by stunted seedlings and yellowing of older leaves, followed by wilting that progresses up the stem until the foliage is killed and the stem decays, as well as brown vascular discoloration in stem tissue cross sections near the soil line [1]. In the last stages of the disease, the fungal pathogen generates white mycelia and plenteous macroconidia, and under adverse environmental conditions, chlamydospores develop from pathogen structures and conjoin into the soil [3]. Fusarium wilt disease is primarily produced by the spread of chlamydospores, which is the pathogen's primary mode of life and is the least controllable aspect of *Fusarium* wilt infection and can survive for more than 10–15 years [4]. Fusarium wilt does not transfer from plant to plant within a season since there is no spore generation above ground in the field, but it can be propagated via contaminated infected seeds [3,4]. Unfortunately, soil-borne fungi are challenging to manage because the soil system is a complex system with several paths that occur in a short period of time [5,6].



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**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Additionally, traditional disease control strategies, such as antifungal chemicals and crop rotation, are ineffective because spores may survive for many years and pesticide residues are hazardous to human health; hence, new and efficient control strategies are required [7]. Biological control of plant diseases looks to be the most promising approach to managing agricultural phytopathogens, as well as the most ecologically safe, cost-effective, and viable alternative to chemical control [8,9].

In agro-ecosystems, *Trichoderma* species are beneficial fungi from the Hypocreaceae family that improve soil health and crop development [10]. The majority of *Trichoderma* spp. investigated colonize the root surface or live as endophytes within root tissues [11]. However, some species can be isolated from plant aerial parts [11–13]. They have the ability to create mutualistic endophytic relationships with a wide range of plant species, increasing host growth in the face of biotic and abiotic stresses, and enhancing micro- and macronutrient uptake and use efficiency [11,12,14]. As a result, they should be encouraged and employed in the field as effective biofungicides and biofertilizers, decreasing the need for dangerous synthetic fungicides and fertilizers while simultaneously supporting ecofriendly and sustainable farming practices [15,16]. However, before they can be used in the field to prevent the spread of phytopathogens, their characteristics and mechanisms must be well understood. Furthermore, additional research is required to increase the efficacy and safety of these fungi. Therefore, the objectives of the current study were to (1) assess Trichoderma isolates' ability to control Fusarium wilt disease, which is caused by Fusarium solani, in vitro and under greenhouse conditions, and (2) assess their potential biofertilizers' effects on cherry tomato (Solanum lycopersicum L.) growth and development.

### 2. Materials and Methods

Assessment of the potential biocontrol of *Trichoderma* isolates against *Fusarium* wilt disease, caused by *Fusarium solani*, was performed in vitro and greenhouse conditions at the Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt. Additionally, the evaluation of *Trichoderma* isolates on the growth promotion of cherry tomatoes was conducted in pot experiments.

#### 2.1. Isolation of Pathogen and Antagonists

Cherry tomato plant samples *cv.* "Golden Cherry Hybrid" showing typical symptoms of *Fusarium* wilt disease were collected from a private farm in Borg El-Arab, Alexandria governorate, Egypt during the 2020 season as shown in Figure 1.

Different infected plant organs (wilted leaves and rotten roots) were chopped into small pieces (5 mm), their surface sterilized for 5 min in sodium hypochlorite (2%), rinsed three times with sterile distilled water for 5 min, and dried with a sterile filter paper, then transferred onto PDA plates (Potato-Dextrose Agar, HiMedia Laboratories MH096-500 G, India), and incubated at  $25 \pm 1$  °C for five days. Preliminary identification of the fungal isolates was carried out by morphological and microscopic examinations according to Leslie and Summerell [17]. The isolates identified as *F. solani* based on appearance, shape, and size of macro- and micro-conidia, were used in all experiments. *F. solani* identity was confirmed by using the polymerase chain reaction (PCR) technique, and nucleotide sequences were determined according to the method described below.

As antagonistic fungi, *Trichoderma* isolates were isolated from soil rhizosphere samples collected from a healthy cherry tomato at a production farm in Borg El-Arab, Alexandria governorate, Egypt. For the isolation of antagonistic *Trichoderma* spp., the serial dilution plate technique was used, and soil samples were diluted into concentrations and vortexed. The supernatant was placed onto PDA plates with a 10 mg/mL stock solution of chloramphenicol (used to suppress bacterial growth in the medium) and incubated at 28 °C for 4 days before being transferred to a new plate containing PDA for another 5–7 days at 28 °C [18]. *Trichoderma* isolates were identified based on morphological characteristics and molecular typing employing the rDNA internal transcribed spacer (ITS), RNA polymerase II subunit (Rpb2), and translation elongation factor 1- $\alpha$  (Tef1- $\alpha$ ) genes [19,20].



**Figure 1.** Natural infection of tomato plants with *Fusarium* wilt disease under open field conditions at a private farm in Borg El-Arab, Alexandria governorate, Egypt.

# 2.2. Molecular Identification of Pathogen and Antagonists2.2.1. DNA Isolation and ITS Amplification of the Pathogen

The genomic DNA was isolated from fungal mycelium by the CTAB extraction method using standard protocols and the DNA concentration was adjusted to 50 ng [21]. The internal transcribed spacer (ITS) regions of rDNA were amplified using primers ITS1 and ITS4, PCR reactions containing a total volume of 25  $\mu$ L containing 3  $\mu$ L of template DNA, 12.5  $\mu$ L PCR Green Master Mix (Thermo Scientific<sup>TM</sup>), 0.5  $\mu$ L of each forward and reverse primers (10 pmol) and 8.5  $\mu$ L molecular grade water. Cycling was completed using a Techne Prime Thermal Cycler (Cole-Parmer, Staffordshire, UK) as follows: an initial denaturation at 95 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 2 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplified PCR products were electrophoresed on 1.5% agarose gel having 5  $\mu$ L ethidium bromide, in 0.5X Tris Acetate buffer at 140 Volt for 15 min, then UV light was used for visualization of results, and bands were photographed [22].

The amplified fragment of ITS1-5.8 s and ITS2 region (600–700 bp) of selected isolates were sent for sequencing (lab Technology, Scientific Services Company). Identification of isolates was confirmed by applying Basic Local Alignment Search Tool (BLAST search) on National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih. gov (accessed on 29 July 2022)) using the obtained sequences of the amplified regions. Alignments were completed by using Molecular Evolutionary Genetics Analysis (MEGA X) software. Phylogenetic tree was constructed using neighbor-joining (NJ) method by MEGA X software [23]. The obtained sequences were compared with different international fungal strains obtained from GenBank.

### 2.2.2. PCR-Amplification of (Tef1- $\alpha$ ) and (Rpb2) Genes of *Trichoderma* spp.

The PCR-amplification reactions were used to amplify the translation elongation factor 1- $\alpha$  (Tef1- $\alpha$ ) and RNA polymerase II subunit (Rpb2) fragments. Details of the primer names and sequences are shown in Table 1. PCR was performed on a PCR machine, and consisted of 0.5  $\mu$ L (10  $\mu$ M) for each primer (forward and reverse primers), 12.5  $\mu$ L *Taq* 2x ReadyMix (Enzynomics Inc., Daejeon, Korea), and 1  $\mu$ L template DNA, with Milli-Q water added up to a total volume of 25  $\mu$ L. Cycling was completed using Techne's extensive range of Prime Thermal Cyclers (Cole-Parmer, UK), the PCR settings for amplification of the Tef1- $\alpha$  gene

using specific primers were as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles each of 1 min at 94 °C, 1 min at 59 °C, 50 sec at 72 °C and, finally, 7 min at 72 °C [24]. The following protocol was used for the Rpb2 gene fragment: 3 min at 94 °C, 5 cycles each of 45 sec at 94 °C, 45 sec at 60 °C, and 2 min at 72 °C, followed by 5 cycles with the temperature decreasing by 1.0 °C per cycle from 58 °C to 54 °C, followed by 30 cycles at 54 °C, and finally, 10 min at 72 °C [25]. *Trichoderma* isolates were identified as *Trichoderma viride* ( $T_1$ ) and *Trichoderma harzianum* ( $T_2$ ).

<b>Table 1.</b> The primer names and	d sequences used in	PCR amplification	reactions.
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Primer Name	Gene	<b>Primer Direction</b>	Sequence (5'-3')	
Internal Transcribed Spacer	¶ ITS	ITS1/ITS4	TCCGTAGGTGAACCTGCGG/ TCCTCCGCTTATTGATATGC	
RNA polymerase II subunit 2	Rpb2	fRPB2-5f/fRPB2-7cr	GAYGAYMGWGATCAYTTYGG/ CCCATRGCTTGYTTRCCCAT	
Translation elongation factor $1-\alpha$	Tef1-α	EF1-728F/TEF1LLErev	CATCGAGAAGTTCGAGAAGG/ AACTTGCAGGCAATGTGG	

<sup>¶</sup> ITS region used in this study.

#### 2.3. Antagonistic Potential of Trichoderma Isolates against F. solani In Vitro

#### 2.3.1. Dual Culture Assay

The in vitro antagonistic activity was checked for *T. harzianum* and *T. viride* isolates against *F. solani* using the dual-culture test, according to Nofal et al. [26]. For a week, Petri dishes (9 cm) containing PDA medium were inoculated with 0.5 cm diameter mycelial discs of three isolates individually. Then, on a PDA plate, 0.5 cm mycelial plugs of the biocontrol *Trichoderma* isolates and *F. solani* pathogen were put opposite each other, while *F. solani* was inoculated alone as a control treatment. For each treatment, three replicates were kept and incubated for 5 days at  $27 \pm 2 \degree C$ , after that the antagonistic ability was assessed. Mycelia growth of pathogen was recorded, and the percentage inhibition was calculated using the formula: % RGI =  $(C - T)/C \times 100$ , where C = radial growth of the tested pathogen without the antagonist, and T = radial growth of the tested pathogen in dual culture with the antagonist [27].

#### 2.3.2. Interaction Zones in Dual Cultures by Scanning Electron Microscope

A scanning electron microscope (SEM) was used to study the interaction zone between the *F. solani* hyphae and the biocontrol agents *T. harzianum* and *T. viride* using dual culture, according to Nofal et al. [28]. At the Electron Microscope Unit, Faculty of Science, Alexandria University, Alexandria, Egypt, samples were examined for mycoparasitism and photographed at different magnifications using the JSM-IT200 SEM series (JEOL Ltd., Tokyo, Japan).

# 2.4. Effect of Trichoderma Isolates to Manage Tomato Fusarium wilt Disease under Greenhouse Conditions

#### 2.4.1. Inoculum of the Pathogen and Antagonists

All the fungal strains were individually cultured on PDA plates for 10 days at 25 °C. After incubation, sterile distilled water (SDW) was placed into the plates, and the conidia were scraped with a sterile glass rod, according to El-Komy et al. [29]. The spore suspensions were filtered to remove fungal hyphae, and the conidial suspension concentration was adjusted to  $1 \times 10^7$  conidia mL<sup>-1</sup> SDW using a hemocytometer. The inoculum concentrations were adjusted to  $10^3$  conidia g<sup>-1</sup> soil for *F. solani* and  $10^6$  conidia g<sup>-1</sup> of soil for *Trichoderma* isolates.

#### 2.4.2. Plant Materials, Experimental Conditions, and Design

A controlled greenhouse experiment was carried out to investigate the ability of *T. viride* ( $T_1$ ) and *T. harzianum* ( $T_2$ ), either alone or in mixture ( $TMix_{1+2}$ ), to suppress *F. solani* and boost cherry tomato growth. The compatibility of the two *Trichoderma* isolates was determined in vitro using the dual culture technique, with no inhibitory halos or overgrowth observed (data not shown).

Tomato "Golden Cherry" hybrid seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) for 1 min, rinsed three times for 5 min in sterile distilled water, and sown in polystyrene germination trays with 200 cavities on September 15, 2021. Seedlings were cared for with regular irrigation and practices for tomato production in a greenhouse. During the experiment, the growth conditions were average temperature of  $25 \pm 2$  °C; relative humidity of  $77 \pm 5\%$ ; and photoperiod of 14/10 h of light/dark. Plastic pots (20 cm inner diameter) were filled with a mixture of sterilized sandy loam–peat moss (2:1 v/v). The soil was artificially infested with *F. solani* by adding (10<sup>3</sup> conidia g<sup>-1</sup> soil) conidial suspension and kept for 7 days with regular irrigation for pathogen establishment.

At the time of transplant, 35-day-old cherry tomato seedlings with three true leaves were treated with the *Trichoderma* by using the root-dip method. Control cherry tomato plants ( $T_0$ ) were root-dipped in sterile water. A factorial combination of eight experimental treatments was arranged in a randomized complete block split-plot design (with and/or without *F. solani* infections as the main factor and four *Trichoderma* inoculations as subfactor) with five replicates for a total of 40 experimental plots. Cherry tomato roots were dipped in  $1 \times 10^7$  conidia mL<sup>-1</sup> for 15 min with different *Trichoderma* treatments ( $T_0, T_1, T_2$ , and  $TMix_{1+2}$ ) to allow the isolates to interact with the cherry tomato roots before being transplanted into soil pots (i.e., either with and/or without *F. solani* infested soil pots). The different *Trichoderma* treatments were repeated 15 and 30 days after transplanting (DAT), by watering each plant at the base with the spore suspension ( $10^6$  conidia g<sup>-1</sup> of soil). During the experiment, a surface drip irrigation system was employed in the greenhouse with a half-strength modified nutrient solution of Hoagland and Arnon as a base solution at a pH of 5.5~6.0, according to Hewitt [30]. Cherry tomato plants per treatment were grown for 45 DAT.

#### 2.5. Measurements

#### 2.5.1. Plant Disease Incidence (%)

Cherry tomato plants transplanted in soil previously infected with *F. solani* had clear disease symptoms, such as leaf yellowing and wilting, and then, plant disease incidence and severity were evaluated after 45 DAT.

The plant disease severity was recorded on a scale of 0–4 based on the degree of wilt as stated by Song et al. [31], where zero means no wilt symptoms, 1, 2, and 3 refer to varying degrees of wilt that reflect the scale of disease severity, and 4 means completely wilted plants as an indication of a complete infection. According to the following formula, plant disease incidence (%) is a parameter that incorporates both disease percentage and disease severity.

Disease incidence (%) = 
$$\left[\frac{(\Sigma \text{ scale } \times \text{ number of plants infected})}{(\text{highest scale } \times \text{ total number of plants})}\right] \times 100.$$

#### 2.5.2. Growth Parameters

Five cherry tomato plants were chosen randomly from each treatment at 45 DAT, for the fresh weights measurement of both roots and shoots. The plant materials were oven-dried at 65  $^{\circ}$ C for 48 h, and the dry weights were measured, and then ground in a stainless-steel mill and stored for analysis according to Chapman and Pratt [32]. The root-to-shoot ratio (R/S) was based on the ratio of root dry weight to shoot dry weight.

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#### 2.5.3. Leaves Chemical Composition

For mineral nutrient analysis, plant leaves were oven dried at 65 °C for 48 h, and N, P, K, Ca, Mg, Fe, Zn, and Cu contents were estimated. Total nitrogen was determined according to the method described by Jones Jr [33]. Total phosphorus was measured using the vanadate–molybdate method according to Page et al. [34]. Additionally, total potassium was measured according to the method described by Jones Jr [33]. In addition, calcium and magnesium contents were measured according to Chapman and Pratt [32]. Iron, zinc, and copper analysis were determined according to Jones Jr [33].

#### 2.5.4. Total Phenolic Contents

Total phenolic contents in cherry tomato leaves were determined using the Folin–Ciocalteu reagent as reported by Malik and Singh [35]. One gram of leaf sample was ground in 80% ethanol, and the homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant was kept, and the residue was re-extracted 5 times with the required volume of 80% ethanol, centrifuged, and the supernatants were collected together. The supernatant was evaporated nearly to dryness, and the residue was dissolved in 5 mL of distilled water. A 0.2 mL aliquot was pipetted into a clean test tube. The volume was made up to 3 mL with distilled water, and then 0.5 mL of Folin–Ciocalteu reagent was added. After 3 min, a 20% Na<sub>2</sub>CO<sub>3</sub> solution was added to each tube, which was then thoroughly and immediately mixed. The tubes were then placed in boiling water for one min, cooled, and the absorbance at 650 nm was measured against a blank. The total phenolic content was determined, and the results were expressed as mg of gallic acid per gram of fresh weight (mg g<sup>-1</sup> F.W.) using a spectrophotometric method.

#### 2.5.5. Analysis of Photosynthetic Pigments

Photosynthetic pigments (chlorophyll a, b, and total carotenoids) were determined in fresh leaves by using a Shimadzu spectrophotometer (Model UV-1208) according to the methods outlined by Horowitz [36]. Concentrations of chlorophyll a, b, and total carotenoids were expressed as mg/g, of fresh weight (FW).

#### 2.6. Statistical Analysis

Using CoStat computer software [37] (CoHort Software version 6.303, Monterey, CA, USA), experimental data were statistically analyzed by two-way analysis of variance according to Gomez and Gomez [38], and LSD at 0.05 level of significance was used for mean comparison.

#### 3. Results

#### 3.1. Identification of Pathogenic fungi F. solani and Biocontrol Agents Trichoderma Isolates

Three fungal species were isolated, one from infected plants identified as *F. solani*, and two *Trichoderma* isolates were isolated from soil samples collected from the rhizospheres' area. The sequences of amplified ITS regions were submitted to GenBank and given accession numbers: OP106576, OP106577, and OP106578 of *F. solani*, *T. harzianum*, and *T. viride*, respectively. The sequence identification was made considering results obtained from the NCBI Blast search engine (BLAST-N), depending on  $\geq$ 97% sequence identify to assign species identification.

The phylogenetic analysis of the ITS region for *F. solani* and other comparable GenBank isolates (data not shown) reveals a distinct clade with a bootstrap value of 91% between *F. solani* (OP106576) and the isolate of *F. solani* from tomatoes in Egypt (MT032385), while there was a low bootstrap value (78%) with *F. oxysporum* and *Fusarium* spp. isolates from India, Pakistan, and China.

Phylogenetic analysis of the *T. harzianum*, *T. viride*, and other *Trichoderma* reference isolates from GenBank (data not shown) indicates that clustering in one group of *T. harzianum* (OP106577) and (MW789612) from Egypt they share a high level of similarity in their genomes. Species *T. viride* (OP106578) was located within one clade with the isolates *T.*  *viride* (MW646450 and MH333256) from Austria and India grouped in a distinct clade with a bootstrap value of 89%. In accordance with identification with the ITS region, we observed the PCR amplification of (Tef1- $\alpha$ ) and (Rpb2) genes of *Trichoderma* spp. successfully amplified approximately 689 bp, and 1050 bp, respectively.

# 3.2. Antagonistic potential of Trichoderma Isolates against F. solani In Vitro 3.2.1. Dual Culture Assay

Through a dual culture test, the in vitro mycoparasitic activity of *T. viride* and *T. harzianum* against *F. solani* was demonstrated. The mycoparasitic nature of the *Trichoderma* isolates shows the growth over the *F. solani* pathogen, 10 days after inoculation at  $27 \pm 2 \degree C$ . Additionally, the results revealed that *Trichoderma* isolates were antagonistic to the *F. solani* pathogen that causes the *fusarium* wilt disease of cherry tomatoes, as shown in Figure 2.



**Figure 2.** The dual culture assay showing in vitro mycoparasitic and antagonistic activity of *Trichoderma* spp. (*T. viride and T. harzianum*) against the pathogen *F. solani* (inoculated simultaneously). (**A**,**B**) *F. solani* inoculated alone as control, (**C**,**D**) mycoparasitic activity of *T. viride* against *F. solani*, (**E**,**F**) mycoparasitic behavior of *T. harzianum* against *F. solani*, (**A**,**C**,**E**) pictures taken 5 days postinoculation, and (**B**,**D**,**F**) pictures taken 10 days post-inoculation.

Figure 3 shows the efficacy of *Trichoderma* isolates as biocontrol agents in significantly suppressing the mycelial growth of the *F. solani* pathogen. Through a dual culture assay, *T. harzianum* showed the maximum inhibitory action against the *F. solani* pathogen 5 and 10 days post-inoculation. *T. harzianum* reduced the mycelial growth of *F. solani* by 78.0%,

whereas *T. viride* inhibited the growth by 61.2%, 10 days post-inoculation. Moreover, the *T. harzianum* isolate showed greater inhibition against the *F. solani* pathogen than the *T. viride* isolate.



**Figure 3.** Bar graph represents the percentage of growth inhibition of the pathogenic mycelia of *F. solani* by *Trichoderma* spp. (*T. viride and T. harzianum*) 5 and 10 days post-inoculation, through dual culture assay. Error bars represent standard deviation of the mean.

3.2.2. Interaction Zone between F. Solani and Trichoderma by Scanning Electron Microscope

Scanning electron microscopy (SEM) images at different magnifications ( $500 \times$  and  $1000 \times$ ) show *T. viride* and *T. harzianum* mycoparasitism towards the pathogen *F. solani* (inoculated simultaneously). Figure 4A,B show higher growth of *F. solani* mycelium inoculated alone as control at different magnifications, and their mycelium growth is unchallenged by the biocontrol agents. Figure 4C–F shows overgrowth and heavy sporulation by both *Trichoderma* isolates on the colony of *F. solani* 10 days after inoculation at  $27 \pm 2$  °C using dual culture assays on PDA. The mycoparasitic behavior of *T. viride* and *T. harzianum* was observed in the form of adhesion, coiling, penetration, degradation, and deformation of the *F. solani* mycelium, as shown in Figure 4. In addition to the parallel growth of these antagonist isolates that were closely linked to the pathogen, these two *Trichoderma* isolates were able to overgrow and sporulate on the colony of *Trichoderma* isolates towards the *F. solani* pathogen. Based on SEM observations, the antagonism mechanisms of *Trichoderma* isolates towards the *F. solani* pathogen are competition for nutrients and space, mycoparasitism, and antibiosis.

# 3.3. Effect of Trichoderma Isolates to Manage Tomato Fusarium wilt Disease under Greenhouse Conditions

#### 3.3.1. Plant Disease Incidence (%)

In this experiment, the biological control effects of the treatments on *Fusarium* wilt disease incidence (%) in cherry tomato plants grown in greenhouse conditions were demonstrated. According to Figure 5, cherry tomato plants with *F. solani* infection alone had the highest mean disease incidence value (95.2%). These infected plants displayed stunted seedlings, yellowing of older leaves, wilting, root rot, and a shorter main root, all of which might cause the plant serious harm or even death. The healthy control plants, on the

other hand, showed no symptoms. In comparison to untreated plants grown on infected soil, plants treated with various *Trichoderma* treatments had less severe symptoms and slower development of the disease. Disease incidence (%) decreased significantly when *T. viride* (57.1%) and *T. harzianum* (42.3%) were incorporated into the pathogen-infested soil, indicating that the application of these biocontrol agents could minimize the occurrence of disease (Figure 5). The *TMix*<sub>1+2</sub> treated plants, however, had the best control effectiveness of disease incidence (34%) overall. For both managing the *fusarium* wilt disease and promoting plant development in cherry tomato plants, it seems that a combination of the two *Trichoderma* isolates (*TMix*<sub>1+2</sub>) performs better than either isolate alone.



**Figure 4.** Mycoparasitic activity of *Trichoderma* isolates against *F. solani* in dual culture (inoculated simultaneously). Scanning electron microscope (SEM) images at different magnifications (500× and 1000×). (**A**,**B**) *F. solani* inoculated alone as control, (**C**,**D**) *T. viride* against *F. solani*, (**E**,**F**) mycoparasitic behavior of *T. harzianum* against *F. solani*. (→) *F. solani* hyphae, (→) *Trichoderma* spp. hyphae, (→) the *Trichoderma* hyphae coiled or attach to the *F. solani* hyphae, (→) degradation and deformation of *F. solani* hyphae are caused by *Trichoderma* spp. activity.



**Figure 5.** Disease incidence (%) at 45 DAT under greenhouse conditions after treating infected cherry tomato plants with different *Trichoderma* treatments, ( $T_0$ ), infected control with *F. solani*, ( $T_1$ ), *T. viride*, ( $T_2$ ), *T. harzianum*, and ( $TMix_{1+2}$ ), a mixture of the two *Trichoderma* isolates. Error bars represent the standard deviation of the mean. Different letter(s) above the error bars indicate statistically significant differences at ( $p \le 0.05$ ).

#### 3.3.2. Cherry Tomato Growth Parameters

The fresh and dry weights, g plant<sup>-1</sup>, of cherry tomato plants growing in greenhouse conditions with and/or without *F. solani-infested* soil as affected by different *Trichoderma* isolates alone or in a combination are shown in Table 2. Cherry tomato plants treated with either *T. harzianum* alone ( $T_2$ ) or in combination ( $TMix_{1+2}$ ), displayed significant effects on plant fresh and dry weights as well as a better root-to-shoot ratio compared to non-infected controls, regardless of the presence or absence of the *F. solani* pathogen. It is obvious that a larger root system (better root-to-shoot ratio) is nearly always a guarantee of more stable performance across all species, crops, and cultivars. Under infection stress, treatment of a mixture of the two *Trichoderma* isolates ( $TMix_{1+2}$ ) showed the highest increase in plant fresh and dry weights as well as a better root-to-shoot ratio. The combination of these two biocontrol agents, *T. viride* and *T. harzianum*, seems to have synergistic effects.

#### 3.3.3. Leaves Chemical Composition

Data in Table 3 shows the leaf chemical composition (%) of cherry tomato plants grown under greenhouse conditions with and/or without *F. solani-infested* soil and treated by different *Trichoderma* isolates alone or in a combination. It is obvious that the leaf chemical composition of cherry tomato plants, such as N, P, K, Mg, Ca, Fe, Zn, and Cu contents, was significantly affected by different *Trichoderma* isolates alone or in a mixture compared to non-infected controls. Regardless of the presence or absence of the *F. solani* infection, treatment of a mixture of the two *Trichoderma* isolates (*TMix*<sub>1+2</sub>) achieved the highest concentrations of N, P, K, Mg, Ca, Fe, Zn, and Cu in leaves of cherry tomato plants grown under greenhouse conditions.

Treat	ments	Free	Fresh Weight (g Plant <sup>-1</sup> )			Dry Weight (g Plant <sup>-1</sup> )			
F. solani Infection	Trichoderma Isolates	Shoots	Roots	Whole Plant	Shoots	Roots	Whole Plant	Root-Shoot Ratio	
Without	$\begin{array}{c} T_0\\T_1\\T_2\\TMix_{1+2}\end{array}$	$\begin{array}{c} 132 \pm 1.67 \text{ e} \\ 155 \pm 1.68 \text{ d} \\ 184 \pm 1.67 \text{ b} \\ 199 \pm 2.51 \text{ a} \end{array}$	$\begin{array}{c} 78\pm 0.71 \text{ e} \\ 103\pm 1.82 \text{ d} \\ 128\pm 1.67 \text{ b} \\ 136\pm 1.52 \text{ a} \end{array}$	$\begin{array}{c} 210 \pm 1.52 \text{ e} \\ 257 \pm 3.42 \text{ d} \\ 311 \pm 1.79 \text{ b} \\ 334 \pm 3.35 \text{ a} \end{array}$	$\begin{array}{c} 64 \pm 2.07 \text{ f} \\ 70 \pm 0.84 \text{ e} \\ 86 \pm 2.51 \text{ b} \\ 95 \pm 1.82 \text{ a} \end{array}$	$40 \pm 1.78 \text{ e} \\ 50 \pm 0.70 \text{ d} \\ 69 \pm 1.67 \text{ b} \\ 85 \pm 2.50 \text{ a} \end{cases}$	$\begin{array}{c} 103 \pm 1.95 \ \mathrm{f} \\ 120 \pm 1.48 \ \mathrm{e} \\ 154 \pm 1.30 \ \mathrm{b} \\ 179 \pm 2.39 \ \mathrm{a} \end{array}$	$\begin{array}{c} 0.63 \pm 0.01 \text{ d} \\ 0.71 \pm 0.02 \text{ c} \\ 0.80 \pm 0.01 \text{ b} \\ 0.89 \pm 0.03 \text{ a} \end{array}$	
With	$\begin{array}{c} T_0\\T_1\\T_2\\TMix_{1+2}\end{array}$	$\begin{array}{c} 105 \pm 1.82 \ {\rm f} \\ 130 \pm 1.87 \ {\rm e} \\ 156 \pm 1.81 \ {\rm d} \\ 175 \pm 1.82 \ {\rm c} \end{array}$	$\begin{array}{c} 55 \pm 1.81 \text{ f} \\ 80 \pm 1.82 \text{ e} \\ 103 \pm 0.71 \text{ d} \\ 120 \pm 1.87 \text{ c} \end{array}$	$\begin{array}{c} 159 \pm 3.11 \text{ f} \\ 210 \pm 1.82 \text{ e} \\ 260 \pm 0.89 \text{ d} \\ 295 \pm 2.19 \text{ c} \end{array}$	$\begin{array}{c} 50 \pm 0.71 \text{ h} \\ 59 \pm 0.70 \text{ g} \\ 73 \pm 0.71 \text{ d} \\ 84 \pm 2.61 \text{ c} \end{array}$	$\begin{array}{c} 25\pm1.79~{\rm g}\\ 36\pm1.67~{\rm f}\\ 51\pm0.71~{\rm d}\\ 66\pm2.51~{\rm c} \end{array}$	$\begin{array}{c} 75 \pm 2.17 \text{ h} \\ 95 \pm 2.07 \text{ g} \\ 124 \pm 0.71 \text{ d} \\ 150 \pm 3.67 \text{ c} \end{array}$	$\begin{array}{c} 0.50 \pm 0.02 \ \mathrm{f} \\ 0.61 \pm 0.01 \ \mathrm{e} \\ 0.70 \pm 0.01 \ \mathrm{c} \\ 0.79 \pm 0.02 \ \mathrm{b} \end{array}$	

**Table 2.** Plant growth promoting effects of different *Trichoderma* treatments on cherry tomato plants cultivated in greenhouse conditions either with and/or without *F. solani* infested soil.

( $T_0$ ): control; ( $T_1$ ): *T. viride*; ( $T_2$ ): *T. harzianum*; and (*TMix*<sub>1+2</sub>): a mixture of the two *Trichoderma* isolates. Means in each column, followed by the same alphabetical letter(s) in common, are not significantly different at  $p \le 0.05$ . Values are expressed as mean  $\pm$  SD (n = 5).

**Table 3.** Leaf chemical composition (%) of cherry tomato plants grown under greenhouse conditions with and/or without *F. solani* infested soil, as affected by different *Trichoderma* treatments.

Trea	tments	Leaf Chemical Composition of Cherry Tomato Plants							
F. solani Infection	Trichoderma Isolates	(%)				(µg/g), of D.W.			
		Ν	Р	К	Ca	Mg	Fe	Zn	Cu
Without	$\begin{array}{c} T_0\\T_1\\T_2\\TMix_{1+2}\end{array}$	$\begin{array}{c} 3.58 \pm 0.02 \text{ f} \\ 3.99 \pm 0.02 \text{ d} \\ 4.47 \pm 0.08 \text{ b} \\ 4.71 \pm 0.03 \text{ a} \end{array}$	$\begin{array}{c} 0.31 \pm 0.01 \ f \\ 0.37 \pm 0.01 \ d \\ 0.46 \pm 0.01 \ ab \\ 0.47 \pm 0.02 \ a \end{array}$	$\begin{array}{c} 3.00 \pm 0.01 \text{ f} \\ 3.38 \pm 0.01 \text{ d} \\ 3.78 \pm 0.04 \text{ a} \\ 3.80 \pm 0.03 \text{ a} \end{array}$	$\begin{array}{c} 0.97 \pm 0.02 \ \mathrm{f} \\ 1.38 \pm 0.02 \ \mathrm{d} \\ 1.76 \pm 0.04 \ \mathrm{a} \\ 1.78 \pm 0.04 \ \mathrm{a} \end{array}$	$\begin{array}{c} 0.43 \pm 0.01 \ e \\ 0.64 \pm 0.02 \ c \\ 0.71 \pm 0.01 \ b \\ 0.75 \pm 0.03 \ a \end{array}$	$\begin{array}{c} 63 \pm 0.71 \text{ e} \\ 75 \pm 1.79 \text{ c} \\ 86 \pm 2.51 \text{ b} \\ 98 \pm 3.65 \text{ a} \end{array}$	$\begin{array}{c} 35 \pm 0.71 \text{ e} \\ 47 \pm 1.82 \text{ c} \\ 52 \pm 1.79 \text{ b} \\ 58 \pm 2.86 \text{ a} \end{array}$	$\begin{array}{c} 6.24 \pm 0.09 \text{ f} \\ 7.12 \pm 0.08 \text{ d} \\ 8.33 \pm 0.15 \text{ b} \\ 9.15 \pm 0.26 \text{ a} \end{array}$
With	$\begin{array}{c} T_0\\T_1\\T_2\\TMix_{1+2}\end{array}$	$\begin{array}{c} 3.21 \pm 0.02 \ g \\ 3.79 \pm 0.01 \ e \\ 4.25 \pm 0.02 \ c \\ 4.42 \pm 0.07 \ b \end{array}$	$\begin{array}{c} 0.22 \pm 0.03 \ \text{g} \\ 0.33 \pm 0.01 \ \text{e} \\ 0.41 \pm 0.02 \ \text{c} \\ 0.45 \pm 0.01 \ \text{b} \end{array}$	$\begin{array}{c} 2.81 \pm 0.02 \ g \\ 3.22 \pm 0.01 \ e \\ 3.51 \pm 0.02 \ c \\ 3.73 \pm 0.04 \ b \end{array}$	$\begin{array}{c} 0.82\pm 0.02~g\\ 1.12\pm 0.02~e\\ 1.57\pm 0.01~c\\ 1.69\pm 0.03~b \end{array}$	$\begin{array}{c} 0.37 \pm 0.01 \ f \\ 0.46 \pm 0.02 \ e \\ 0.55 \pm 0.02 \ d \\ 0.69 \pm 0.04 \ b \end{array}$	$\begin{array}{c} 47\pm 0.70\ g\\ 56\pm 0.71\ f\\ 71\pm 3.19\ d\\ 84\pm 3.17\ b\end{array}$	$\begin{array}{c} 22 \pm 0.70 \text{ g} \\ 30 \pm 1.87 \text{ f} \\ 41 \pm 1.79 \text{ d} \\ 50 \pm 1.78 \text{ b} \end{array}$	$\begin{array}{c} 5.13 \pm 0.09 \text{ g} \\ 6.59 \pm 0.06 \text{ e} \\ 7.55 \pm 0.07 \text{ c} \\ 8.49 \pm 0.23 \text{ b} \end{array}$

(*T*<sub>0</sub>): control; (*T*<sub>1</sub>): *T. viride*; (*T*<sub>2</sub>): *T. harzianum*; and (*TMix*<sub>1+2</sub>): a mixture of the two *Trichoderma* isolates. Means in each column, followed by the same alphabetical letter(s) in common, are not significantly different at  $p \le 0.05$ . Values are expressed as mean  $\pm$  SD (n = 5).

The beneficial roles of *Trichoderma* isolates in cherry tomato nutrition, resistance, and development are graphically represented in Figure 6. *Trichoderma* isolates can stimulate cherry tomatoes' development while also protecting them from pathogenic attacks. Furthermore, different *Trichoderma* isolates can boost root growth and development, confer abiotic and biotic stress tolerance, and improve micro- and macronutrient absorption and utilization efficiency, resulting in higher crop productivity.

#### 3.3.4. Total Phenolic Contents and Photosynthetic Pigments

Total phenolic content and photosynthetic pigments (chlorophyll a, b, and total carotenoids) of cherry tomato plants cultivated in greenhouse conditions with and/or without *F. solani* infected soil, as affected by different *Trichoderma* treatments, are shown in Table 4. We found that different *Trichoderma* treatments significantly improved chlorophyll content and total phenol content, regardless of the presence or absence of the *F. solani* infection. In general, total phenol levels were significantly higher in infected cherry tomato plants treated with various *Trichoderma* treatments than in healthy cherry tomato plants. Cherry tomato plants treated with a mixture of the two *Trichoderma* isolates (*TMix*<sub>1+2</sub>) showed the highest total phenolic content and photosynthetic pigments compared to non-infected controls.



**Figure 6.** Graphical diagram representing the beneficial roles of *Trichoderma* spp. in cherry tomato nutrition and growth.

**Table 4.** Total phenolic content and photosynthetic pigments (chlorophyll a, b, and total carotenoids), of cherry tomato plants grown under greenhouse conditions with and/or without *F. solani* infested soil, as affected by different *Trichoderma* treatments.

Treatments						
	Trichoderma Isolates	Chlo	orophyll (mg/g), of		Total Phenolic	
F. solani Infection		а	b	Total Chlorophyll	(mg/g), of F.W.	(mg/g), of F.W.
Without	$T_0$	$1.63 \pm 0.09 \text{ d}$	$0.70 \pm 0.03 \text{ d}$	$2.33 \pm 0.11 \text{ d}$	$0.30\pm0.01~\mathrm{e}$	$0.67\pm0.04$ h
	$T_1$	$1.85\pm0.10~{\rm c}$	$0.86\pm0.02~\mathrm{c}$	$2.71\pm0.13~{\rm c}$	$0.47\pm0.01~{\rm c}$	$1.51\pm0.10~{ m f}$
	$T_2$	$2.10\pm0.10~b$	$1.12\pm0.03~\mathrm{b}$	$3.22\pm0.12~\mathrm{b}$	$0.59\pm0.03~\mathrm{b}$	$2.10\pm0.05~\mathrm{e}$
	$TMix_{1+2}$	$2.35\pm0.09~a$	$1.30\pm0.04~\mathrm{a}$	$3.64\pm0.13~\text{a}$	$0.68\pm0.03~\mathrm{a}$	$2.41\pm0.10~c$
With	T <sub>0</sub>	$1.25\pm0.10~{ m f}$	$0.50\pm0.04~{ m f}$	$1.74\pm0.12~{ m f}$	$0.23\pm0.01~{ m f}$	$1.20\pm0.06~{ m g}$
	$T_1$	$1.54\pm0.06~\mathrm{e}$	$0.67\pm0.03~\mathrm{e}$	$2.20\pm0.06~\mathrm{e}$	$0.35\pm0.02~\mathrm{d}$	$2.27\pm0.08~{ m d}$
	$T_2$	$1.90\pm0.07~{ m c}$	$0.88\pm0.03~\mathrm{c}$	$2.78\pm0.06~\mathrm{c}$	$0.46\pm0.01~{ m c}$	$2.65\pm0.10~\mathrm{b}$
	$TMix_{1+2}$	$2.15\pm0.10~b$	$1.10\pm0.02~b$	$3.25\pm0.13b$	$0.57\pm0.03b$	$3.12\pm0.19~\text{a}$

( $T_0$ ): control; ( $T_1$ ): *T. viride*; ( $T_2$ ): *T. harzianum*; and (*TMix*<sub>1+2</sub>): a mixture of the two *Trichoderma* isolates. Means in each column, followed by the same alphabetical letter(s) in common, are not significantly different at  $p \le 0.05$ . Values are expressed as mean  $\pm$  SD (n = 5).

#### 4. Discussion

In the current study, we found that *T. viride* and *T. harzianum* were antagonistic to the *F. solani* pathogen that causes the *Fusarium* wilt disease in cherry tomatoes. A dual culture test revealed that *Trichoderma* isolates have in vitro mycoparasitic activity against *F. solani*. Within 10 days of inoculation, these two *Trichoderma* isolates were able to overgrow

and sporulate on the colony of the *F. solani* pathogen, as well as parallel growth of these antagonist isolates closely associated with the pathogen. Furthermore, scanning electron microscopy revealed the coiling of *Trichoderma* isolates around *F. solani* hyphae, degradation and deformation of pathogen hyphae, and parallel growth of *Trichoderma* isolates closely connected with the *fusarium* wilt pathogen. Based on these findings, the antagonistic *Trichoderma* isolates investigated in the current study might be a source of novel biological fungicides, especially against the *fusarium* wilt pathogen *F. solani*, while avoiding the negative effects of chemical fungicides.

Several studies have revealed that the majority of root, shoot, and postharvest diseases are controlled by *Trichoderma* spp., which have antagonistic abilities based on the activation of a number of biocontrol mechanisms [16,39]. According to Benítez et al. [40], Trichoderma spp. either exert indirect biocontrol against fungal soil-borne pathogens by competing for nutrients and space, affecting environmental conditions, fostering plant development, enhancing plant defense mechanisms, and inducing antibiosis or direct biocontrol through mycoparasitism. Trichoderma spp. start the production of hydrolytic or lytic enzymes during mycoparasitic interactions, such as glucanase, chitinase, and protease, which break down the chitin polymers of the fungal pathogen cell wall [41,42]. In addition, Trichoderma may produce antibiotics or low-molecular-weight diffusible substances such tricholin, harzianic acid, peptaibols, viridin, 6-pentyl-pyrone, and heptelidic acid, all of which prevent the growth of other microorganisms [39]. These indirect and direct biocontrol mechanisms may work together, and their impact in the biocontrol process is controlled by Trichoderma spp., crop plant, and environmental factors such as nutrient availability, pH, temperature, and iron content [40]. For these reasons, *Trichoderma* spp. can be utilized as efficient biofungicides and alternative agents against fungal soil-borne pathogens [43].

Our present research in a greenhouse found that a combination of the two *Trichoderma* isolates ( $TMix_{1+2}$ ) showed greater antifungal activity as a result of their synergistic effect, resulting in slower growth of *F. solani* and better disease control compared to their separate application. Our findings also showed that *T. viride* and *T. harzianum* could stimulate cherry tomato development, but that their mixture was more efficient.

*Trichoderma* spp. are employed as successful biostimulants, biofertilizers, plant growth enhancers, and efficient biocontrol agents against a range of fungal diseases [44]. They are applied by foliar spray, soil application, seed treatment, bio-priming, and seedling dipping [45,46]. Additionally, for the control of agricultural plant pests and diseases as well as for the promotion of plant growth, mixtures of *Trichoderma* strains perform better than individual strains [45,47]. According to several studies, combining biocontrol agents with various modes of action (such as competition and the formation of antagonistic or parasitic/reparasitic chemicals) may be one explanation for the synergistic effects of these biocontrol agents [48,49].

The current study found that different *Trichoderma* isolates alone or in a combination significantly affected the leaf chemical composition of cherry tomato plants, such as N, P, K, Mg, Ca, Fe, Zn, and Cu contents, when compared to non-infected controls. Biofertilizers increase soil nutrients, making them more accessible to crops; consequently, utilizing microorganisms as bio-inoculants is seen as the best eco-friendly alternative to chemical fertilizers for plant development and soil fertility [50,51]. Scientists reported that mineral nutrients have a substantial impact on plant diseases and in many cases, they are the first line of defense against disease, and balanced nutrition is a factor that promotes plant disease resistance [52–54]. The effects of mineral nutrients on plant disease may be linked to effects on plant development, cell walls and tissues, biochemical composition, host growth rate, and pathogen growth rate [55]. As successful biofertilizers, *Trichoderma* spp. have the ability to provide important nutrients to crops in sufficient quantities to enhance crops without negatively impacting the environment [51,56]. *Trichoderma* spp. can also help to accelerate the composting process and improve compost humification [57]. Furthermore, root colonization by *Trichoderma* spp. increases root growth and development, which imme-

diately leads to improved nutrient absorption and translocation in the shoots, resulting in greater plant biomass through the effective use of N, P, K, and micronutrients [57–59].

Regardless of the presence or absence of *F. solani* infection, our research found that different *Trichoderma* treatments significantly enhanced chlorophyll content and total phenol content. In response to pathogenic microbes, plants create a wide range of secondary metabolites, including phenolics, which are generated via the shikimate and phenyl-propanoid pathways [60]. Phenolics can be deposited in the cell wall as an important first line of defense against fungal penetration and infection [61]. In addition, the accumulation of phenolic compounds at the site of infection has been proven to limit fungal activity by interacting with proteins and causing enzymatic dysfunction, resulting in pathogen restriction and disease spread inhibition to surrounding plant tissues [62,63]. According to Yedidia et al. [64], plant roots colonized by *T. harzianum* exhibited alterations in the synthesis of antimicrobial phenolics associated with plant defense against plant diseases. When pathogens attack plant tissues, accumulated phenolic compounds act as electron and hydrogen donors, protecting plant tissues from oxidative stress [65]. *Trichoderma* also creates peptides, proteins, and low molecular weight compounds that are involved in biochemical resistance to infections and cause resistance in plants [66].

In summary, our findings showed that two *Trichoderma* isolates significantly suppressed *F. solani* in vitro. The greenhouse experiment revealed that *Trichoderma* isolates, applied separately or as a mixture, were either directly protective against the *F. solani* pathogen or indirectly related to the plant's defense mechanism. As a result, this study suggests employing two *Trichoderma* isolates as biocontrol agents for *Fusarium* wilt disease and to promote the development of cherry tomato plants. Additionally, the efficacy of *Trichoderma* isolates in managing *Fusarium* wilt disease in field conditions should be further investigated and explored. In addition, further study is needed to identify undiscovered microbial communities for specific applications, since plant growth-promoting microbial treatments are helpful for promoting plant growth and development as well as providing disease resistance.

#### 5. Conclusions

Our current research revealed that two *Trichoderma* isolates, *T. viride* and *T. harzianum*, are antagonistic to the *F. solani* pathogen. The antagonism mechanisms include competition for nutrients and space, mycoparasitism, and antibiosis. In the greenhouse experiment, we found that treating cherry tomato plants with each of these antagonistic *Trichoderma* isolates separately was effective in preventing *Fusarium* wilt disease incidence. However, when the two *Trichoderma* isolates ( $TMix_{1+2}$ ) were mixed, they showed a synergistic impact and enhanced disease management as compared to when they were used separately. Additionally, two *Trichoderma* isolates applied separately or in combination significantly improved plant growth parameters, increased macro- and micronutrient uptake, and increased the content of photosynthetic pigments and total phenols. As a result, using *Trichoderma* isolates as effective biofungicides and biofertilizers can minimize the need for dangerous synthetic fungicides and chemical fertilizers while simultaneously supporting ecologically friendly and sustainable farming practices.

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