agronomy

MDPI

Article

Protective and Curative Effects of *Trichoderma asperelloides* Ta41 on Tomato Root Rot Caused by *Rhizoctonia solani* Rs33

Ahmed A. Heflish [1], Ahmed Abdelkhalek [2], Abdulaziz A. Al-Askar [3,*] and Said I. Behiry [1]

[1] Agricultural Botany Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria 21531, Egypt; ahmed_heflish@yahoo.com (A.A.H.); said.behiry@alexu.edu.eg (S.I.B.)
[2] Plant Protection and Biomolecular Diagnosis Department, ALCRI, City of Scientific Research and Technological Applications, New Borg El Arab City, Alexandria 21934, Egypt; aabdelkhalek@srtacity.sci.eg
[3] Botany and Microbiology Department, Faculty of Science, King Saud University, Riyadh 11451, Saudi Arabia
* Correspondence: aalaskara@ksu.edu.sa; Tel.: +96-65-0346-1390

Abstract: Two molecularly identified tomato isolates, *Trichoderma asperelloides* Ta41 and *Rhizoctonia solani* Rs33, were characterized and antagonistically evaluated. The dual culture technique showed that Ta41 had a high antagonistic activity of 83.33%, while a light microscope bioassay demonstrated that the Ta41 isolate over-parasitized the pathogen completely. Under greenhouse conditions, the application of Ta41 was able to promote tomato plant growth and had a significant increase in plant height, root length, and shoot fresh, shoot dry, root fresh, and root dry weight. It also improved chlorophyll content and total phenol content significantly, both in protective and in curative treatments. The protective treatment assay exhibited the lowest disease index (16.00%), while the curative treatment showed a disease index of 33.33%. At 20 days post-inoculation, significant increases in the relative expression levels of four defense-related genes (PR-1, PR-2, PR-3, and CHS) were observed in all Ta41-treated plants when compared with the non-treated plants. Interestingly, the plants treated with Ta41 alone showed the highest expression, with relative transcriptional levels of CHS, PR-3, PR-1, and PR-2 that were, compared with the control, 3.91-, 3.13-, 2.94-, and 2.69-fold higher, respectively, and the protective treatment showed relative transcriptional levels that were 3.50-, 3.63-, 2.39-, and 2.27-fold higher, respectively. Consequently, the ability of Ta41 to promote tomato growth, suppress Rs33 growth, and induce systemic resistance supports the incorporation of Ta41 as a potential bioagent for controlling root rot disease and increasing the productivity of crops, including tomatoes.

Keywords: *Trichoderma asperelloides*; *Rhizoctonia solani*; tomato; biological control; defense-related genes

Citation: Heflish, A.A.; Abdelkhalek, A.; Al-Askar, A.A.; Behiry, S.I. Protective and Curative Effects of *Trichoderma asperelloides* Ta41 on Tomato Root Rot Caused by *Rhizoctonia solani* Rs33. *Agronomy* **2021**, *11*, 1162. https://doi.org/10.3390/agronomy11061162

Academic Editor: Noam Alkan

Received: 26 April 2021
Accepted: 4 June 2021
Published: 6 June 2021

1. Introduction

The tomato (*Lycopersicon esculentum* L.) is the second-most commonly consumed vegetable crop after the potato worldwide [1]. Several pathogens can infect tomato plants and cause diseases. Many common diseases that attack tomatoes are caused by fungi, bacteria, nematodes, and viruses [2,3]. Among the fungal pathogens causing several diseases in tomatoes, *Rhizoctonia solani* is the worst fungus that could damage tomato plants and reduce the yield [4–6].

R. solani is a destructive soil-borne pathogen that causes severe losses in many crops worldwide. *R. solani* does not form asexual spores (conidia) but reproduces a survival form called sclerotia [7], which considers a major cause of *R. solani* infection. The excessive use of chemical fungicides, the most common strategy used by farmers to control *R. solani*, poses severe risks to human health and the environment and leads to pathogen-resistant strains. Therefore, biological control has been used more frequently as an alternative for controlling plant diseases. Biological control is environment-friendly and effective in managing most plant fungal pathogens. Most research on disease control was done using different *Trichoderma* fungus strains [8–10]. Internal transcribed spacer (ITS), RNA

polymerase II subunit 2 (Rpb2), and translation elongation factor 1 alpha (Tef-1) genes are the most used molecular markers in phylogenetic analysis for the high throughput sensitive identification and characterization of *Trichoderma* spp. for the early screening of potential antagonists against soil-borne pathogens [11].

Trichoderma species use different mechanisms for pathogen inhibition, such as mycoparasitism via hydrolytic enzyme secretion, antibiosis via secondary metabolite production, competition for space and nutrients, promoting plant growth, and inducing plant systemic resistance mechanisms [12]. *Trichoderma* spp. are effective biocontrol agents due to the rapid multiplication or the tolerance of harsh conditions [13]. *Trichoderma* spp. have potent antagonism and mycoparasitic actions on plant pathogens, allowing them to reduce the incidence of plant diseases, and the main mechanism for *Trichoderma* species is hyperparasitism [14,15]. Many genes in *Trichoderma* spp. encoding extracellular proteases and oligopeptide transporters are expressed when contacts occur between *Trichoderma* spp. and the host-pathogen [16,17]. During the hyperparasitic process, cell wall degrading enzymes (CWDEs), i.e., glucanases, chitinase, and proteinases, can be secreted by *Trichoderma* spp. [18]. The secreted CWDEs can degrade the plant pathogen's cell wall [19]. The *Trichoderma* colonization of roots causes root hair growth and triggers defense activities, such as significant changes in a variety of metabolic pathways and the activation of genes involved in plant host defense, primarily through signaling pathways involving jasmonic acid and ethylene [20,21]. In *Arabidopsis*, colonization by *Trichoderma* fungus before infection by biotrophic or necrotrophic plant pathogens triggered an oxidizing status that enhanced resistance systemically [22].

Microbial communities have attracted much attention as an eco-acceptable and cost-effective disease resistance enhancement via induced systemic resistance (ISR) by releasing proteins, secondary metabolites, and plant growth stimulation for long-term crop production [23–25]. Secondary metabolites produced by *Trichoderma* spp. offer selective advantages in mechanisms such as competitiveness, symbiotic relationships, mineral transportation, growth production, sensing, and mycoparasitic behavior [26,27]. Recognizing the importance of screening new *Trichoderma* species with more potent antifungal activity for agricultural use, the current study aimed to evaluate the protective and curative activities of *Trichoderma asperelloides* Ta41 on tomato root rot caused by *Rhizoctonia solani* Rs33 under controlled greenhouse conditions. Moreover, the effects of Ta41 on the plant growth parameters, chlorophyll content, total phenol content, and expression levels of defense-related genes with or without Rs33 were estimated.

2. Materials and Methods

2.1. Sample Collection, Isolation, and Identification

Ten tomato plants (cv. Peto 86) showing root rot symptoms were collected from El-Behira governorate, Egypt. The phytopathogen was isolated from the 10 symptomatic samples and identified by cultural, morphological characteristics, and sequencing of the ITS, as described previously [6,28].

The *Trichoderma* isolate was isolated from soil rhizosphere samples collected from tomato cultivated areas, El-Behira governorate, Egypt. The serial dilution plate technique was used to isolate the antagonistic *Trichoderma* spp. using the *Trichoderma* specific medium (TSM). One milliliter of 1×10^{-3} dilution was poured onto a selective medium and the receipt described by Elad et al. [29]. The obtained culture was purified by the hyphal tip isolation technique and maintained on PDA slants for further identification processes. The identification was performed based on their morphological characteristics and molecular typing using ITS, Rpb2, and Tef-1 genes [30–33]. Primer sequences are given in Table 1. PCR reactions containing 0.5 µL of each primer pair (forward and reverse), 10 µL of 2x Taq Ready Mix (Enzynomics Inc., Daejeon, Korea), and 1 µL of template DNA, and the Milli-Q water was added up to a volume of 25 µL. Cycling was done using a Techne Prime Thermal Cycler (Cole-Parmer, Staffordshire, UK) as follows: an initial denaturation of 95 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and a

final extension step at 72 °C for 5 min. The PCR amplifications were sequenced, and the nucleotide sequences were aligned using MEGA 6 software. GenBank BLAST tool was used to compare the obtained sequences with those in the GenBank database.

Table 1. Primer nucleotide sequences used in this study.

Primer Name	Gene	Primer Direction	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	Tef-1	EF1-728F TEF1LLErev	CATCGAGAAGTTCGAGAAGG AACTTGCAGGCAATGTGG
Pathogenesis related protein-1	PR-1	Forward Reverse	GTTCCTCCTTGCCACCTTC TATGCACCCCCAGCATAGTT
Endoglucanase	PR-2	Forward Reverse	TATAGCCGTTGGAAACGAAG CAACTTGCCATCACATTCTG
Chitinase	PR-3	Forward Reverse	ATGGAGCATTGTGCCCTAAC TCCTACCAACATCACCACCA
Chalcone Synthase	CHS	Forward Reverse	CACCGTGGAGGAGTATCGTAAGGC TGATCAACACAGTTGGAAGGCG
Beta-actin	β-actin	Forward Reverse	TGGCATACAAAGACAGGACAGCCT ACTCAATCCCAAGGCCAACAGAGA

2.2. Dual Culture Technique

The *Trichoderma asperelloides* and *Rhizoctonia solani* isolates were grown on PDA individually for a week. After that, 5 mm mycelial plugs of the two fungal isolates *T. asperelloides* and *R. solani* were placed opposite each other on a PDA plate [34]. The antagonistic tests were conducted in triplicate, and hyphal interactions between the two isolates were studied. The radial growth of *R. solani* was measured (mm), and inhibition percentage was calculated as follows: Inhibition % $= \left[\frac{C-T}{C}\right] \times 100$, where C is the *R. solani* radial growth in the control, and T is the radial growth of *R. solani* in the presence of *T. asperelloides*. The mycoparasitic interacted regions on PDA were observed after 4 days under the 10x light microscope.

2.3. Greenhouse Assessments of R. solani, Samples Collection, and Plant Growth-Promoting Abilities of T. asperelloides

The effect of *T. asperelloides* isolates on the activity of *R. solani* and plant growth was evaluated based on a pot experiment under controlled greenhouse conditions (temperature: 28 ± 2 °C; humidity: 75 ± 5%; photoperiod: 14 light/10 dark h) The 4-week-old tomato seedlings (cv. Peto 86) were transplanted to plastic pots (20 cm in diameter) filled with sterile soil. After 5 days of seedlings transplanting, 10 mL of *T. asperelloides* inoculum with a concentration of 1×10^8 spores/mL was added to each pot as described by Fahmi et al. [35]. An *R. solani* inoculum was added to the pots at 1% (W:W). Treatments were distributed in the greenhouse with 5 replicates as follows: T1: tomato plant control inoculated with media-free microorganisms; T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application). Twenty days after inoculation with *R. solani*, tomato leaf samples from all treatments were collected to test the total phenolic compounds and defense-related genes as a response to different treatments under greenhouse conditions.

One month after transplanting, the plants screened for disease severity according to a 0–5 scale according to root browning appears on the root system [36], where 0 = No symptom, 1 = 0–25% root browning, 2 = 26–50% root browning, 3 = 51–75% root browning, 4 = 76–100% root browning, and 5 = plant death. Tomato plants were observed, and the disease index (DI) was calculated using the following formula:

$$\text{DI \%} = \frac{\sum \text{ of observed numerical rating}}{\text{Max. disease rating} \times \text{Total number of observed plants}} \times 100$$

The plants were also used to record the effect of *T. asperelloides* on the following growth parameters, plant height (cm), root length (cm), shoot and root fresh weight (g), shoot and root dry weight (g), and total chlorophyll content (SPAD-502 Plus, Konica Minolta, Inc., Tokyo, Japan).

2.4. Total Phenolic Compounds Content in Tomato Plants

To measure the Total Phenolic Compounds (TPCs) of tomato leaf samples collected from all treatments, the Folin–Ciocalteu (FC) method was chosen [37]. Ten milligrams of leaves extract were liquefied in 10 mL of ethanol to obtain 1 mg/mL as a final concentration. One hundred microliters of the liquefied extract were blended with 750 µL of the FC reagent. The liquid composite was allowed to remain at 25 °C for 5 min. After that, 750 µL of Na_2CO_3 was applied to the mixture, and the tube was gently shaken to combine it. The mix was measured after incubation for 1 h at 725 nm using an OPTIMA SP-300 spectrophotometer (Optima Inc., Tokyo, Japan). Gallic acid was used to establish a calibration curve. TPC was calculated as gallic acid equivalents (GAE, mg g-1 of extract).

2.5. Analysis of the Defense-Related Genes Using Quantitative Real-Time PCR (qRT-PCR)

2.5.1. Plant Total RNA Extraction and cDNA Synthesis

One hundred milligrams of tomato leaves collected at 20 days post-inoculation (dpi) were used as starting materials for total RNA extraction, using the guanidium isothiocyanate method [38]. The concentration and purity of the extracted RNA were estimated by using a Nano SPECTROstar (BMG Labtech, Ortenberg, Germany), while the RNA integrity was checked by agarose gel electrophoresis. For cDNA synthesis, two micrograms of DNase-treated total RNA were used as a template in a reverse transcription reaction, as described previously [39]. The cDNA samples were stored at −80 °C until use as a template in qRT-PCR.

2.5.2. qRT-PCR and Data Analysis

The transcriptional levels of 4 tomato defense-related genes, PR-1, PR-2, PR-3, and CHS (Table 1) were evaluated at 20 dpi using the qRT-PCR technique. The expression detected from the β-actin gene was used as an internal reference (Table 1). The experiments were run in triplicate reactions for each sample. The qRT-PCR was performed on a Rotor-Gene 6000 using SYBR Green PCR Master Mix [40,41]. The relative changes of the transcript level of each tested gene were measured according to the $2^{-\Delta\Delta CT}$ method [42].

2.6. Statistical Analysis

The obtained data were statistically analyzed by one-way ANOVA using the CoStat software. At the same time, the statistical differences in the mean were determined by Tukey's honest significant differences method (H.S.D.) at a $p \leq 0.05$ level of probability, and standard deviation ($\pm$ SD) was shown as a column bar. Columns with the same letter do not differ significantly. Compared to the control, relative transcript levels greater than 1 demonstrate an increase in gene transcription (upregulation), while values lower than 1 indicate a decrease in transcription levels (downregulation).

3. Results

3.1. Isolation and Identification of R. solani and T. asperelloides

Out of 10 *R. solani* isolates isolated from root rot diseased tomato plants, the most aggressive isolate of *R. solani* was chosen based on a pre-pathogenicity test (data not shown). The vegetative characteristics of *R. solani* showed that hyphae septate multinucleate and clamp connection, conidia, and rhizomorphs were never observed. Several *Trichoderma* isolates were obtained, and the best effective antifungal *Trichoderma* isolate (data not shown) was chosen to complete the study.

Molecular results confirmed the initial identification of the two confronting isolates used in this study. The sequence of amplified ITS regions (approximately 650 bp) *R. solani* Rs33 isolates were deposited in GenBank under accession no. MW664424. Comparing *R. solani* Rs33 nucleotide ITS sequence with those isolates of *R. solani* in the GenBank database (Figure 1) clarified that the highest homogeneity was 100% with the isolated *R. solani* from tomatoes in Egypt (MH687913). Minimum nucleotide sequence similarity (99.69%) with *R. solani* isolates from China (MH172598, MH172624, MH483966, and MH483967), Turkey (MT380171 and MT408040), Azerbaijan (MG654491), and Canada (MN313269) was found.

Figure 1. A phylogenetic diagram, degenerated by the neighbor-joining method showing the relationship between the *R. solani* Rs33 isolate and other *R. solani* isolates based on ITS sequences available in GenBank (NCBI).

Regarding the *Trichoderma* isolate, the three specific primers ITS1/ITS4, EF1-728F/TEF-1LLErevR, and RPB2-5F/RPB2-7cR successfully amplified approximately 650 bp, 500 bp, and 1050 bp, respectively. The NCBI-Blast alignment revealed that the *Trichoderma* isolate was highly similar to *T. asperelloides*. To describe species limits, phylogenetic analysis was carried out using the three-locus combined ITS, Tef-1, and Rpb2 datasets. The significance of each branch in alignment was indicated by bootstrap 2000 subsets (only values higher than 40% are indicated). A multiple sequence alignment in the neighbor-joining method using Mega 6 revealed that the relationship of almost all *Trichoderma* reference isolates could be clearly distinguished on the level of species and separated into different clusters and clades (Figure 2). The isolates *T. asperelloides* strain CEN1427 and *T. asperelloides* strain CEN1431 clustered with our isolate Ta41 (>98% similarity in three genes), so they should be classified within this species. Sequences of *T. asperelloides* Ta41 were analyzed and deposited in the GenBank database (accession no. MW797033, MZ269255, and MZ269254) for the three genes, respectively.

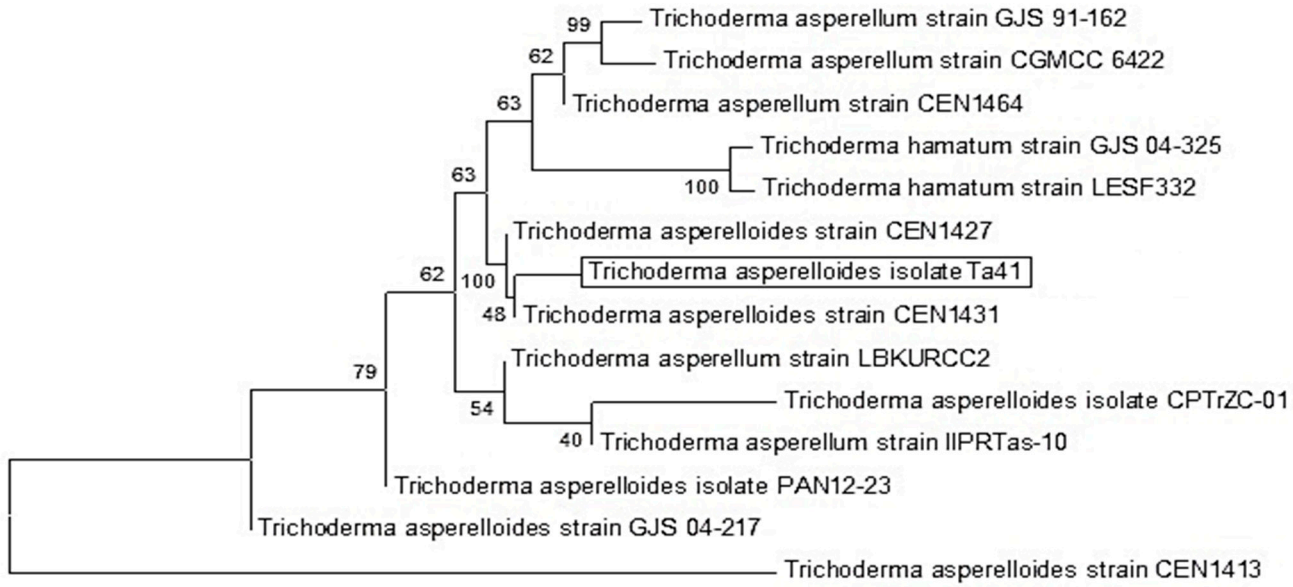

Figure 2. Neighbor-joining phylogenetic tree showing the relationship of *T. asperelloides* Ta41 isolate (displayed in a rectangle shape) among closely related *Trichoderma* isolates on partial concatenated sequences of ITS, Tef-1, and Rpb2 available in GenBank (NCBI).

3.2. Effect of T. asperelloides Ta41 on the Mycelial Growth of R. solani Rs33 In Vitro

The reduction of *R. solani* Rs33 growth in response to the *T. asperelloides* Ta41 isolate is presented in Figure 3. The growth of the Rs33 isolates inhibited significantly with Ta41 isolate in the dual culture technique with a value of 15 mm compared with the control. The percentage of growth inhibition in the confrontation assays raised values to 83.3%. 10× light microscope graphs of the Ta41 hyperparasite on the Rs33 cell wall were observed and describe the spore germination and penetration peg formation (Figure 3). The advanced stages of mycoparasitism of Ta41 started coiling around the Rs33 cell wall and tightly encircled the hyphae. The Ta41 isolate penetrated the hyphae of the Rs33 isolate and entered the mycelium. (Figure 3).

3.3. Disease Index of R. solani Rs33 on Tomato Plants In Vivo

Disease index was recorded 30 days after transplanting and was estimated according to the browning on the roots, regardless of its extent (using a 0–5 scale) depending on treatment (Figures 4 and 5). The Ta41 isolate (T3) application significantly reduced the disease index compared to the infested control plants (T2). The application of the Ta41 isolate as a pre-inoculation of the *R. solani* Rs33 isolate (T4) showed a low disease index (16.00%), followed by T5, the Ta41 isolate applied after inoculation with the Rs33 isolate (33.33%). The disease index was 81.00% with the *R. solani* Rs33 isolate alone (T2) compared to treatments that showed no symptoms—T1 and T3 (Figure 5).

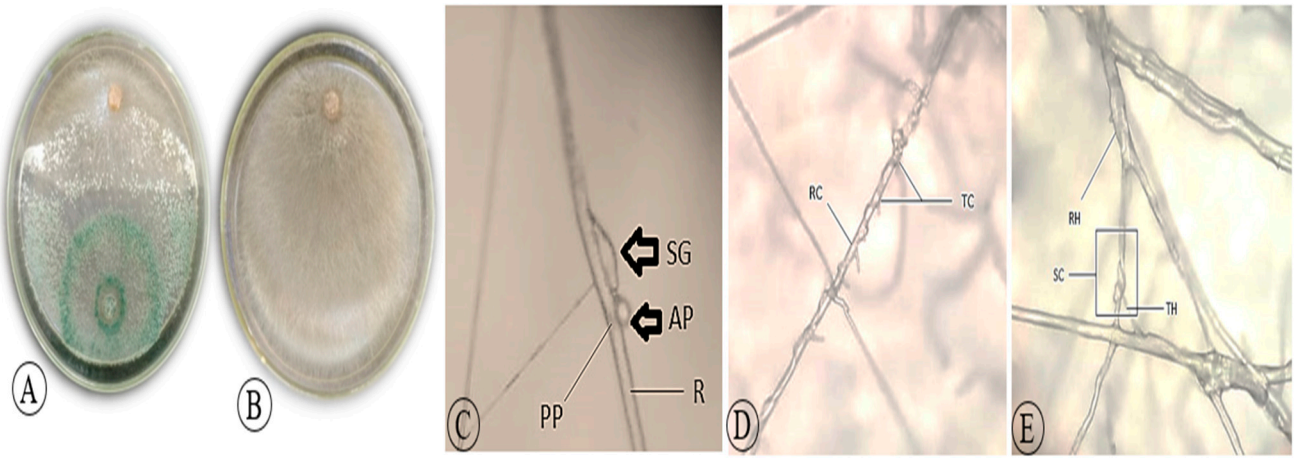

Figure 3. *Trichoderma asperelloides* Ta41 hyphae mycoparasitism on *R. solani* Rs33 (R) using a dual culture technique. (**A**) *T. asperelloides* Ta41 and *R. solani* Rs33 confronting each other; (**B**) *R. solani* Rs33 control; (**C**) 10X light microscope graphs of Ta41 penetrating the Rs33 (R) cell wall as a result of spore germination (SG), the formation of appressorium (AP), and the penetration peg (PP) (semi-visible); (**D**) Ta41 starting to coil (TC) around the Rs33 cell wall (RC); (**E**) Ta41 hyphae (TH) densely coiling and encircling the Rs33 hyphae as a supercoil (SC); the arrow (RH) points to the Ta41 thin hyphae in the Rs33 mycelium inside the cell walls.

Figure 4. Effect of *T. asperelloides* Ta41 on *R. solani* Rs33 on tomato plants under controlled greenhouse conditions. T1: tomato plant control inoculated with media-free microorganisms; T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application).

3.4. Effect of T. asperelloides Ta41 on Tomato Growth under Greenhouse Conditions

The efficacy of *T. asperelloides* Ta41 isolate against *R. solani* Rs33 on tomato plants was tested under controlled greenhouse conditions. Both the pre- and post-inoculation treatments of the Ta41 isolate significantly improved the growth of tomato plants. Application of the T3 treatment was found to be more effective in increasing chlorophyll content (39.00 SPAD unit), followed by the T4 application, which was found to be more effective than T5 (37.90 and 33.90 SPAD unit, respectively) without significant differences (Figure 6). On the other hand, the effect on plant height was significant due to the application of Ta41, which recorded 39.80 cm in T3, followed by T4 (34.20 cm). The root length increased with T3, T4, and T5 (18.4, 18.4, and 17.70 cm, respectively). These treatments displayed an increase in shoot fresh weight (16.4, 13.5, and 9.5 g, respectively) and root fresh weight (5.8, 5.6, and 4.5 g, respectively). Moreover, positive effects on the dry weight of the shoot were observed with T3 followed by T4 and T1 (3.50, 3.20, and 3.20 g, respectively) but without a

significant effect. The root dry weight of the tomato plants was affected by all treatments that contained Ta41 (T3, T4, and T5) significantly more than the other two treatments (T1 and T2) (Table 2).

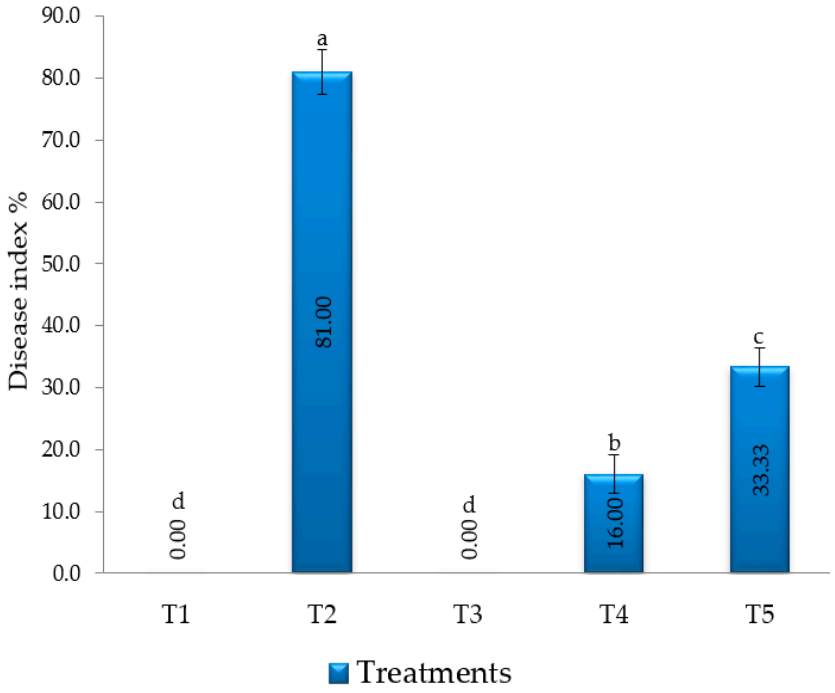

Figure 5. Disease index % in tomato caused by *R. solani* Rs33 under greenhouse conditions. T1: tomato plant control inoculated with media-free microorganisms; T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application). Data with the same letters are not significantly different at $p \leq 0.05$.

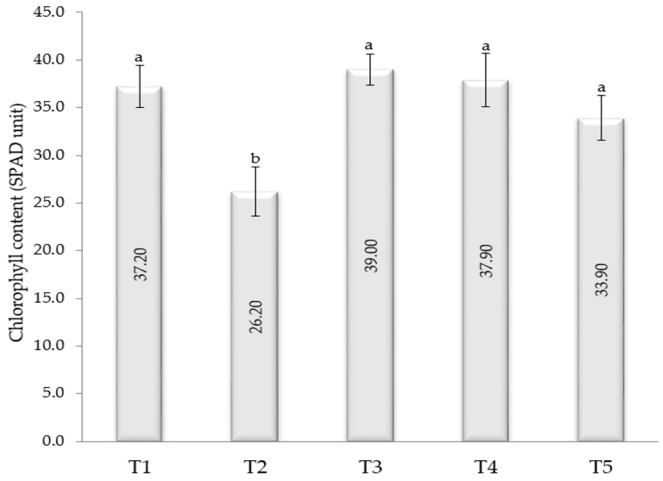

Figure 6. Chlorophyll content (SPAD unit) of tomato plants under greenhouse conditions as affected by *T. asperelloides* Ta41 with or without *R. solani* infection. T1: tomato plant control inoculated with media-free microorganisms; T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application). Data with the same letters are not significantly different at $p \leq 0.05$.

Table 2. Effect of different treatments on growth parameters of tomato plants under greenhouse conditions.

Treatment	Plant Height (cm)	Root Length (cm)	Shoot Fresh Weight (g)	Shoot Dry Weight (g)	Root Fresh Weight (g)	Root Dry Weight (g)
T1	33.4 ± 4.39 ab	9.9 ± 0.74 b	7.6 ± 1.97 c	3.2 ± 0.73 a	2.2 ± 0.89 c	1.6 ± 0.50 b
T2	26.6 ± 7.30 b	5.5 ± 0.79 c	6.7 ± 2.48 c	2.9 ± 0.57 a	1.9 ± 0.40 c	1.2 ± 0.36 b
T3	39.8 ± 3.34 a	18.4 ± 3.20 a	16.4 ± 3.97 a	3.5 ± 0.38 a	5.8 ± 1.29 a	2.6 ± 0.49 a
T4	34.2 ± 7.98 ab	18.4 ± 1.68 a	13.5 ± 3.97 ab	3.2 ± 0.25 a	5.6 ± 1.14 ab	2.5 ± 0.28 a
T5	33.0 ± 9.05 ab	17.7 ± 4.97 a	9.5 ± 3.10 bc	3.0 ± 0.32 a	4.5 ± 0.83 b	2.4 ± 0.13 a

T1: tomato plant control (media-free microorganisms); T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application). Data with the same letters are not significantly different at $p \leq 0.05$.

3.5. Total Phenolic Compound Content

Total phenol content (TPC) was accumulated in high concentrations in plants in the T4 (51.4 GAE mg/g dry extract) and T5 (33.6 GAE mg/g dry extract) treatments, as well as in T2 (36.8 GAE mg/g dry extract), compared to their corresponding control, T1 (13.2 GAE mg/g dry extract). All plants treated with T3 showed a low accumulation of total phenol (26.8 GAE mg/g dry extract) compared to the control and the other treatments.

3.6. Transcriptional Levels of Defense-Related Genes

At 20 dpi, significant increases in the relative expression levels of four defense-related genes (PR-1, PR-2, PR-3, and CHS) were observed in the treated plants when compared with the non-treated plants ($p \leq 0.05$). Compared to the control (T1), a significant upregulation of PR-1 was observed in all treatments, while the infested plants in T2 showed downregulation (Figure 7). The highest relative expression level (2.94-fold higher than the control) was reported in T3, followed by the protective (T4) and curative (T5) treatments with expression levels that were 2.39- and 1.46-fold higher, respectively (Figure 6). Like PR-1, the transcriptional level of PR-2 exhibited upregulation in all plants treated with *T. asperelloides* Ta41 (Figure 7). The greatest expression level (2.69-fold higher than the control) was reported in T3, followed by T5 (2.42-fold higher) and T4 (2.12-fold higher). On the other hand, the inoculated plants with T2 showed a downregulation, with a relative expression level that was 0.94-fold lower than the control (Figure 7). Regarding PR-3 expression, significant upregulation was found in all treatments compared to the control (Figure 7). The highest transcriptional level (3.13-fold higher than the control) was observed with T3, followed by T4, T5, and T2 with relative expression levels that were 2.63-, 2.32-, and 1.43-fold higher, respectively, higher than the control (Figure 7). Similar to PR-3, the expression level of CHS exhibited upregulation in all treatments compared to the control. The expression level of T3 was the most extraordinary (3.91-fold higher than the control), while the levels for the T4, T5, and T2 treatments were 3.50-, 2.81-, and 1.52-fold higher, respectively (Figure 7).

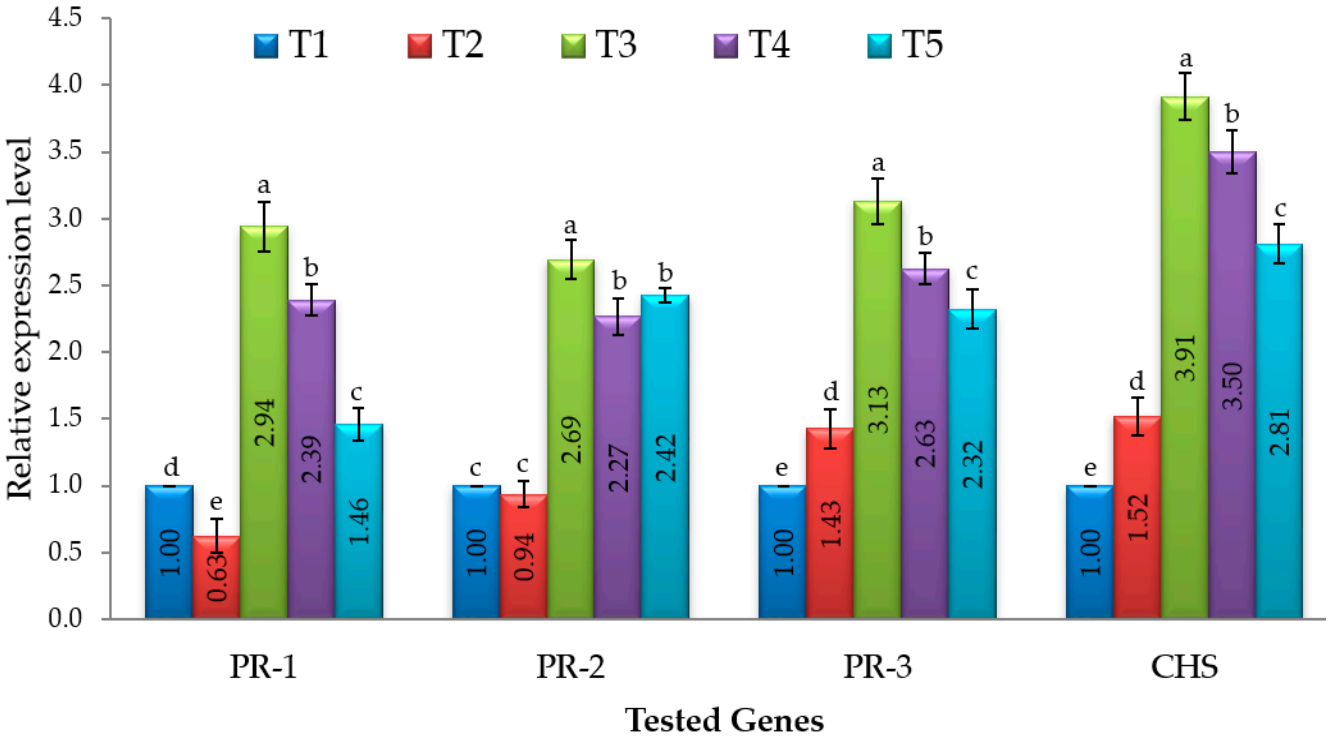

Figure 7. A histogram shows the relative expression levels of the PR-1, PR-2, PR-3, and CHS genes at 20 dpi in the assay of different treatments. T1: tomato plant control inoculated with media-free microorganisms; T2: tomato plants inoculated with *R. solani* only; T3: tomato plants inoculated with *T. asperelloides*; T4: tomato plants inoculated with *T. asperelloides* 48 h before inoculation with *R. solani* (protective application); T5: tomato plants inoculated with *T. asperelloides* 48 h after inoculation with *R. solani* (curative application). Data with the same letters are not significantly different at $p \leq 0.05$.

4. Discussion

The application of plant-growth-promoting fungi (PGPFs) as biocontrol agents is a safe substitution for undesirable chemical fungicide use and is considered a sustainable and environmentally friendly alternative [43,44]. Among PGPFs, *Trichoderma* spp. are highly efficient antagonist fungi and possess biocontrol activity on many phytopathogenic fungi such as *R. solani*, *S. rolfsii*, and *V. dahliae*, among others [45,46]. The results of ITS molecular characterization of the isolated pathogen from root rot tomato plants revealed that the pathogen was *Rhizoctonia solani* Rs33. For *Trichoderma* isolate identification, we performed a full multigenic analysis with three markers: ITS, Tef-1, and Rpb2. Among the sequenced markers, the most informative gene was Tef-1, followed by Rpb2 and ITS. The phylogram based on the concatenation of three gene sequences confirmed the identification of *Trichoderma asperelloides* Ta41 among reference sequences, based on their clustering with reference taxa [47,48].

Our results indicate an ability of the bioagent Ta41 to stop the progress of the pathogen Rs33 by up to 83.33% as Ta41 grew three times faster than Rs33 in the early in vitro experiments conducted in this study. Ramírez-Cariño et al. [49] reported that *T. asperelloides* has a major advantage over the pathogens *A. alternata* and *F. oxysporum* in the competition for space and nutrients due to its faster growth rate. *T. asperelloides* Ta41 prevented pathogen development and triggered hyperparasitism, mostly on phytopathogens, using a dual culture technique. 10× microscopic photographs of the interaction assays were used to confirm this mechanism. The images indicate that, when Ta41 and Rs33 interact, the Ta41 hyphae wrap around the Rs33 hyphae. Mycoparasitism is a complicated process involving recognition, invasion, and eventual penetration, and pathogen destruction [50]. The findings of this study are aligned with those of Alamri et al. [51], who found that *T. harzianum* parasitized *R. solani* hyphae by winding around them and then secreting

secondary metabolites such as lytic enzymes to weaken and destroy the target cell wall and promote the feed intake of nutrients.

Similarly, several authors mentioned the role of secondary metabolites of several strains from *Trichoderma* spp. to control several plant pathogens, especially in the rhizosphere. *Trichoderma* spp. and *R. solani* interacted with many mechanisms to destroy the hyphae cell wall and the membrane permeability. *T. asperelloides* has been shown to suppress the incidence of *R. solani*, which causes damping-off disease in beans and induces defensin genes in cucumber seedlings against *Pseudomonas syringae* pv. *lachrimans* [52]. Furthermore, *Trichoderma* spp. has been confirmed to treat *Sclerotinia sclerotiorum* in soybean crops [45] effectively. Doley et al. [53] found that *T. asperelloides* decreased the prevalence of *Sclerotium rolfsii* pathogen in peanut plants. There are no other studies in the literature about using *T. asperelloides* to control *R. solani* in tomato plants besides this study.

Under controlled greenhouse conditions, the antifungal protective (T4) and curative (T5) treatments of Ta41 against Rs33 were evaluated in this study. Ta41 application significantly improved the tomato growth parameters, including shoot and root length, the fresh and dry weight of shoots and roots, and chlorophyll content, with a significant difference compared to the control (T1) and the infected tomato plants (T2). Our results showed that Rs33 could be inhibited by treating tomato plants with Ta41, either before or after the challenge with Rs33. These results agree with the enhancement of shoot and root growth, as reported previously [54,55]. The obtained data are also in agreement with those found by Yedidia and coworkers, who reported a stronger effect with treatment by *T. harzianum* on cucumber plants, which increased the root length by 75%, the shoot length by 95%, and the dry weight by 80% compared to the control plants [56]. The application of *T. harzianum* and *T. asperellum*, increased the chlorophyll content in melon-treated plants [57,58].

As an organic fertilizer, *Trichoderma* can degrade soil nutrients and enhance plant photosynthesis, resulting in improving plant growth; plants and several microorganisms can synthesize indole acetic acid (IAA) [59,60]. The indole acetic acid hormone is essential for root and shoot growth in plants and is considered a key regulator for root hair and lateral root development [46,61]. Studies have shown that *Trichoderma* spp. from various geographical areas can produce IAA and encourage plant growth such as cucumber, tomato, and bitter gourd [46,61]. Contreras-Cornejo et al. [62] revealed that the secretion of IAA by *Trichoderma* spp. can significantly improve plant and lateral root growth. *Trichoderma* spp. can produce volatile and non-volatile secondary metabolites including 6-*n*-pentyl-6H-pyran-2-one (6PP), viridin, gliotoxin, harziandione, harzianopyridone, and peptaibols [27,63], which, as plant-growth promoters, have a significant effect [8,55]. Secondary metabolites of the T22 and T39 commercial strains of *T. harzianum* and *T. atroviride* P1, as well as *T. harzianum* A6, also significantly affected plant-growth promoters [64].

Through invading the epidermis, *Trichoderma* spp. colonize plant roots that are usually associated with and triggering plant metabolism by changing gene expression [62,65]. In the current study, the impact of Ta41 on the relative expression levels of four defense-related genes (PR-1, PR-2, PR-3, and CHS) at 20 dpi were evaluated. It was reported that, when the plant contacts with a pathogen, a mechanism of SAR is activated, while when it interacts with a non-pathogen organism, a mechanism for ISR was activated [66,67]. During the *Trichoderma*–plant interaction, various secondary metabolites induce PR proteins' expression that triggers plant defense mechanisms against the pathogen [68,69].

PR-1, the salicylic acid (SA) marker gene, is a crucial regulator of SAR and could be an indicator for early defense response in plants [70,71]. Meanwhile, the increasing resistance of plants is often associated with PR-1 induction and SA content accumulation [72,73]. In the present study, the tomato plants challenged with Rs33 only (T2) showed downregulation of PR-1, with a relative expression level 0.63-fold lower than the control (T1). Interestingly, the tomato plants inoculated with Ta41 only (T3) showed the highest expression level (2.94-fold higher), followed by those of the protective (T4) and curative (T5) treatments with relative transcriptional levels 2.39- and 1.46-fold higher than the control (T1). Consequently, we suggest that Ta41 may produce an elicitor metabolite molecule that

induces the immune defense system resulting in SAR activation. The upregulation of PR1 could be related to SAR activation and the ISR status [74]. Thus, it could be deduced that the *T. asperelloides* Ta41 can modulate the response of the plant, increase resistance, and prevent the suppression of defense genes caused by *R. solani* Rs33.

PR-2 proteins have β-1,3-glucanase activity, are involved in pathogenic defense and different physiological plant functions, and are induced mainly by SAR and SAR inducers, such as SA [75–77]. In the current study, all Ta41-treated tomato plants (T3, T4, and T5) showed an upregulation of PR-2 compared with the control (T1). On the other hand, the decrease in the PR-2 expression of T2 plants (0.94-fold lower than the control) does not show a significant difference with respect to the control. The activity of PR-2 increased in plants treated with Ta41, which could be due to the elicitors (the oligosaccharides released) of the plant response and the fungal secondary metabolism [9]. This finding is consistent with other studies indicating that *Trichoderma* strain colonization causes an increase in PR-2 transcript in plants [22,78,79]. It has been documented that increasing 1,3-glucanase activity in the cell wall increased the amount of oligosaccharides released, acting as elicitors of plant defense responses and/or a secondary metabolism of the bioagents [9,78].

PR-3, which encodes a chitinase enzyme that catalyzes the hydrolysis of chitin, is a fungicide that protects plants from fungal infestations by inhibiting fungal growth [79,80]. The ability of each *Trichoderma* strain to inhibit *R. solani* growth may be due to differences in mycoparasitism activity through the secretion of enzymes, e.g., chitinase, that degrade the fungal cell wall [79]. In mycoparasitic action, chitinase is one of the most critical extracellular lytic enzymes [81]. In the present study, the tomato plants challenged with RS33 only or treated with Ta41 exhibited upregulation of the PR-3 gene. The highest relative expression (3.13-fold higher than the control) was shown in T3 plants, followed by that of the T4, T5, and T2 plants (2.63-, 2.32- and 1.43-fold higher, respectively) (T1). The obtained results show the role of PR-3 in increasing plant resistance against fungal infection. The colonization of roots with *Trichoderma* species promotes leaf tissue for the enhanced activation of many defensin genes, including PR-3, which results in higher resistance to pathogens [68,82].

The accumulation of phenolic compounds in exposure to *Trichoderma* species has been correlated with oxidative biochemical defense against pathogenic fungi. Yedidia et al. [83] reported that the roots invaded and conquered by *T. harzianum* showed a high defense against harmful organisms, and this was correlated with changes in the accumulation of phenolics. In the T4 treatment, the phenolic content was nearly four-fold higher than the content in the control, while in T3 or T5, it was 2.78 and 2.54-fold higher. Additionally, in the pathogen attack treatment (T2), the phenolic concentrations were lower than those found in all other treatments but still higher, by about two-fold, than those of the untreated plants (T1). Therefore, phenolics accumulating in *Trichoderma*-treated plants can serve as electron and hydrogen donors, preventing root tissue from oxidative damage throughout pathogen attacks [84]. Our TPC results are consistent with protection from *R. solani* phytopathogen in tomatoes. This was also found by Ortega-García et al. [85] in their studies on onions.

CHS, the initial enzyme of the pathway of flavonoids, converts the *p*-coumaroyl CoA to naringenin chalcones and is considered a strict precursor required for plant flavonoids [41,86]. Among the four tested genes, CHS exhibited the highest expression, with relative transcriptional levels in T3, T4, T5, and T2 that were 3.91-, 3.50-, 2.81-, and 1.52-fold higher than those of the control. In earlier studies, the overexpression of CHS was found to result in a high accumulation of flavonoid and isoflavonoid compounds that showed broad antifungal activity against a variety of fungal phytopathogens [87–90]. Consequently, the treatment of tomato plants with Ta41, in protective or in curative treatments, may increase the number of many flavonoid compounds. Thus, Ta41 could be useful as a biocontrol agent against *R. solani* infections. However, for future field applications, more research is required.

5. Conclusions

The current study aims to assess the in vitro biological control activities and in vivo activation of defense response of tomato by *T. asperelloides* either with or without *R. solani*, given the significance of implementing new *Trichoderma* species with much more efficient antimicrobial activities for agriculture applications. The application of *T. asperelloides* Ta41 was able to promote tomato plant growth and caused a significant increase in plant height, root length, and shoot fresh, shoot dry, root fresh, and root dry weight. It also improved chlorophyll content and total phenol content significantly, both in protective or in curative treatments. The protective treatment assay exhibited the lowest disease index. At 20 dpi, significant increases in the relative expression levels of four defense-related genes (PR-1, PR-2, PR-3, and CHS) were observed in all Ta41-treated plants compared to untreated plants.

Author Contributions: Conceptualization, A.A.H., S.I.B., and A.A.; methodology, A.A.H., S.I.B., and A.A.; software, A.A. and S.I.B.; formal analysis, A.A.; investigation, A.A.H. and S.I.B.; writing—original draft preparation, A.A.H., S.I.B., and A.A.; writing—review and editing, A.A., S.I.B., and A.A.A.-A.; funding acquisition, A.A.A.-A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work throughout the research group project (RGP-1440-094).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data reported here is available from the authors upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Georgé, S.; Tourniaire, F.; Gautier, H.; Goupy, P.; Rock, E.; Caris-Veyrat, C. Changes in the contents of carotenoids, phenolic compounds and vitamin C during technical processing and lyophilisation of red and yellow tomatoes. *Food Chem.* **2011**, *124*, 1603–1611. [CrossRef]
2. Roux, F.; Voisin, D.; Badet, T.; Balagué, C.; Barlet, X.; Huard-Chauveau, C.; Roby, D.; Raffaele, S. Resistance to phytopathogens e tutti quanti: Placing plant quantitative disease resistance on the map. *Mol. Plant Pathol.* **2014**, *15*, 427–432. [CrossRef]
3. Mickelbart, M.V.; Hasegawa, P.M.; Bailey-Serres, J. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nat. Rev. Genet.* **2015**, *16*, 237–251.
4. Morsy, E.M.; Abdel-Kawi, K.A.; Khalil, M.N.A. Efficiency of Trichoderma viride and Bacillus subtilis as biocontrol agents gainst Fusarium solani on tomato plants. *Egypt. J. Phytopathol.* **2009**, *37*, 47–57.
5. Nikraftar, F.; Taheri, P.; Rastegar, M.F.; Tarighi, S. Tomato partial resistance to Rhizoctonia solani involves antioxidative defense mechanisms. *Physiol. Mol. Plant Pathol.* **2013**, *81*, 74–83. [CrossRef]
6. Heflish, A.I.A.I.; Singh, N.; Raina, S.; Buttar, D.S. Evaluation of Trichoderma isolates against Rhizoctonia solani and Rhizoctonia oryzae causing sheath blight of rice. *Plant Dis. Res.* **2017**, *32*, 36–46.
7. Li, S.; Peng, X.; Wang, Y.; Hua, K.; Xing, F.; Zheng, Y.; Liu, W.; Sun, W.; Wei, S. The effector AGLIP1 in Rhizoctonia solani AG1 IA triggers cell death in plants and promotes disease development through inhibiting PAMP-triggered immunity in Arabidopsis thaliana. *Front. Microbiol.* **2019**, *10*, 2228. [PubMed]
8. Vinale, F.; Sivasithamparam, K.; Ghisalberti, E.L.; Marra, R.; Woo, S.L.; Lorito, M. Trichoderma–plant–pathogen interactions. *Soil Biol. Biochem.* **2008**, *40*, 1–10. [CrossRef]
9. Druzhinina, I.S.; Seidl-Seiboth, V.; Herrera-Estrella, A.; Horwitz, B.A.; Kenerley, C.M.; Monte, E.; Mukherjee, P.K.; Zeilinger, S.; Grigoriev, I.V.; Kubicek, C.P. Trichoderma: The genomics of opportunistic success. *Nat. Rev. Microbiol.* **2011**, *9*, 749–759. [CrossRef] [PubMed]
10. El-Benawy, N.M.; Abdel-Fattah, G.M.; Ghoneem, K.M.; Shabana, Y.M. Antimicrobial activities of Trichoderma atroviride against common bean seed-borne Macrophomina phaseolina and Rhizoctonia solani. *Egypt. J. Basic Appl. Sci.* **2020**, *7*, 267–280. [CrossRef]
11. Jaklitsch, W.M. European species of Hypocrea part II: Species with hyaline ascospores. *Fungal Divers.* **2011**, *48*, 1–250. [CrossRef]
12. Chao, W.; Zhuang, W. Evaluating effective Trichoderma isolates for biocontrol of Rhizoctonia solani causing root rot of Vigna unguiculata. *J. Integr. Agric.* **2019**, *18*, 2072–2079.
13. Tapwal, A.; Pandey, H. In vitro evaluation of Trichoderma species for virulence efficacy on Botryodiplodia palmarum. *Curr. Life Sci.* **2016**, *2*, 86–91.

14. Kredics, L.; Chen, L.; Kedves, O.; Büchner, R.; Hatvani, L.; Allaga, H.; Nagy, V.D.; Khaled, J.M.; Alharbi, N.S.; Vágvölgyi, C. Molecular tools for monitoring Trichoderma in agricultural environments. *Front. Microbiol.* **2018**, *9*, 1599. [CrossRef] [PubMed]
15. Halifu, S.; Deng, X.; Song, X.; Song, R.; Liang, X. Inhibitory Mechanism of Trichoderma virens ZT05 on Rhizoctonia solani. *Plants* **2020**, *9*, 912. [CrossRef]
16. Sharma, V.; Salwan, R.; Sharma, P.N. Differential response of extracellular proteases of Trichoderma harzianum against fungal phytopathogens. *Curr. Microbiol.* **2016**, *73*, 419–425. [CrossRef]
17. Stappler, E.; Dattenböck, C.; Tisch, D.; Schmoll, M. Analysis of light-and carbon-specific transcriptomes implicates a class of G-protein-coupled receptors in cellulose sensing. *Msphere* **2017**, *2*, e00089-17. [CrossRef] [PubMed]
18. Berini, F.; Caccia, S.; Franzetti, E.; Congiu, T.; Marinelli, F.; Casartelli, M.; Tettamanti, G. Effects of Trichoderma viride chitinases on the peritrophic matrix of Lepidoptera. *Pest Manag. Sci.* **2016**, *72*, 980–989. [PubMed]
19. Xiong, H.; Xue, K.; Qin, W.; Chen, X.; Wang, H.; Shi, X.; Ma, T.; Sun, Z.; Chen, W.; Tian, X. Does soil treated with conidial formulations of Trichoderma spp. attract or repel subterranean termites? *J. Econ. Entomol.* **2018**, *111*, 808–816. [CrossRef] [PubMed]
20. Contreras-Cornejo, H.A.; López-Bucio, J.S.; Méndez-Bravo, A.; Macías-Rodríguez, L.; Ramos-Vega, M.; Guevara-García, Á.A.; López-Bucio, J. Mitogen-activated protein kinase 6 and ethylene and auxin signaling pathways are involved in Arabidopsis root-system architecture alterations by Trichoderma atroviride. *Mol. Plant Microbe Interact.* **2015**, *28*, 701–710. [CrossRef]
21. Martínez-Medina, A.; Van Wees, S.C.M.; Pieterse, C.M.J. Airborne signals from Trichoderma fungi stimulate iron uptake responses in roots resulting in priming of jasmonic acid-dependent defences in shoots of Arabidopsis thaliana and Solanum lycopersicum. *Plant. Cell Environ.* **2017**, *40*, 2691–2705. [CrossRef] [PubMed]
22. Salas-Marina, M.A.; Silva-Flores, M.A.; Uresti-Rivera, E.E.; Castro-Longoria, E.; Herrera-Estrella, A.; Casas-Flores, S. Colonization of Arabidopsis roots by Trichoderma atroviride promotes growth and enhances systemic disease resistance through jasmonic acid/ethylene and salicylic acid pathways. *Eur. J. Plant Pathol.* **2011**, *131*, 15–26. [CrossRef]
23. Elsharkawy, M.M.; Shimizu, M.; Takahashi, H.; Hyakumachi, M. Induction of systemic resistance against Cucumber mosaic virus by Penicillium simplicissimum GP17-2 in Arabidopsis and tobacco. *Plant Pathol.* **2012**, *61*, 964–976. [CrossRef]
24. Abdelrahman, M.; Abdel-Motaal, F.; El-Sayed, M.; Jogaiah, S.; Shigyo, M.; Ito, S.; Tran, L.-S.P. Dissection of *Trichoderma longibrachiatum*-induced defense in onion (*Allium cepa* L.) against *Fusarium oxysporum f.* sp. *cepa* by target metabolite profiling. *Plant Sci.* **2016**, *246*, 128–138. [CrossRef] [PubMed]
25. Keswani, C.; Bisen, K.; Singh, V.; Sarma, B.K.; Singh, H.B. Formulation technology of biocontrol agents: Present status and future prospects. In *Bioformulations: For Sustainable Agriculture*; Springer: Berlin, Germany, 2016; pp. 35–52.
26. Liu, M.; Sun, Z.; Zhu, J.; Xu, T.; Harman, G.E.; Lorito, M. Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from Trichoderma atroviride. *J. Zhejiang Univ. Sci.* **2004**, *5*, 133–136. [CrossRef]
27. Reino, J.L.; Guerrero, R.F.; Hernández-Galán, R.; Collado, I.G. Secondary metabolites from species of the biocontrol agent Trichoderma. *Phytochem. Rev.* **2008**, *7*, 89–123. [CrossRef]
28. Salem, M.Z.M.; Behiry, S.I.; EL-Hefny, M. Inhibition of Fusarium culmorum, Penicillium chrysogenum and Rhizoctonia solani by n-hexane extracts of three plant species as a wood-treated oil fungicide. *J. Appl. Microbiol.* **2019**, *126*, 1683–1699. [CrossRef]
29. Elad, Y.; Chet, I.; Henis, Y. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* **1981**, *9*, 59–67. [CrossRef]
30. Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]
31. Abdelkhalek, A.; Behiry, S.I.; Al-Askar, A.A. Bacillus velezensis PEA1 Inhibits Fusarium oxysporum Growth and Induces Systemic Resistance to Cucumber Mosaic Virus. *Agronomy* **2020**, *10*, 1312. [CrossRef]
32. Jiang, H.; Zhang, L.; Zhang, J.; Ojaghian, M.R.; Hyde, K.D. Antagonistic interaction between Trichoderma asperellum and Phytophthora capsici in vitro. *J. Zhejiang Univ. B* **2016**, *17*, 271–281. [CrossRef]
33. Samuels, G.J.; Dodd, S.L.; Gams, W.; Castlebury, L.A.; Petrini, O. Trichoderma species associated with the green mold epidemic of commercially grown Agaricus bisporus. *Mycologia* **2002**, *94*, 146–170. [CrossRef]
34. Fahmi, A.I.; Al-Talhi, A.D.; Hassan, M.M. Protoplast fusion enhances antagonistic activity in *Trichoderma* spp. *Nat. Sci.* **2012**, *10*, 100–106.
35. Verma, M.; Brar, S.K.; Tyagi, R.D.; Surampalli, R.Y.; Valero, J.R. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochem. Eng. J.* **2007**, *37*, 1–20. [CrossRef]
36. Abdeljalil, N.O.-B.; Vallance, J.; Gerbore, J.; Bruez, E.; Martins, G.; Rey, P.; Daami-Remadi, M. Biocontrol of Rhizoctonia root rot in tomato and enhancement of plant growth using rhizobacteria naturally associated to tomato. *J. Plant Pathol. Microbiol.* **2016**, *7*, 1–8. [CrossRef]
37. Velioglu, Y.; Mazza, G.; Gao, L.; Oomah, B.D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113–4117. [CrossRef]
38. Abdelkhalek, A.; Sanan-Mishra, N. Differential expression profiles of tomato miRNAs induced by tobacco mosaic virus. *J. Agric. Sci. Technol.* **2019**, *21*, 475–485.
39. Abdelkhalek, A.; Ismail, I.A.I.A.; Dessoky, E.S.E.S.; El-Hallous, E.I.E.I.; Hafez, E. A tomato kinesin-like protein is associated with Tobacco mosaic virus infection. *Biotechnol. Biotechnol. Equip.* **2019**, *33*, 1424–1433. [CrossRef]

40. Abdelkhalek, A.; Al-Askar, A.A. Green Synthesized ZnO Nanoparticles Mediated by Mentha Spicata Extract Induce Plant Systemic Resistance against Tobacco Mosaic Virus. *Appl. Sci.* **2020**, *10*, 5054. [CrossRef]
41. Abdelkhalek, A.; Dessoky, E.S.; Hafez, E. Polyphenolic genes expression pattern and their role in viral resistance in tomato plant infected with Tobacco mosaic virus. *Biosci. Res.* **2018**, *15*, 3349–3356.
42. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
43. Abdelkhalek, A.; Hafez, E. Plant Viral Diseases in Egypt and Their Control. In *Cottage Industry of Biocontrol Agents and Their Applications*; Springer: Berlin, Germany, 2020; pp. 403–421.
44. Sharma, A. Fungi as Biological Control Agents. In *Biofertilizers for Sustainable Agriculture and Environment*; Springer: Berlin, Germany, 2019; pp. 395–411.
45. Lamenew, F.; Mekonnen, H.; Gashaw, T. Biocontrol potential of trichoderma and yeast against post harvest fruit fungal diseases: A review. *World News Nat. Sci.* **2019**, *27*, 153–173.
46. Kotasthane, A.; Agrawal, T.; Kushwah, R.; Rahatkar, O. V In-vitro antagonism of Trichoderma spp. against Sclerotium rolfsii and Rhizoctonia solani and their response towards growth of cucumber, bottle gourd and bitter gourd. *Eur. J. Plant Pathol.* **2015**, *141*, 523–543. [CrossRef]
47. Chaverri, P.; Branco-Rocha, F.; Jaklitsch, W.; Gazis, R.; Degenkolb, T.; Samuels, G.J. Systematics of the Trichoderma harzianum species complex and the re-identification of commercial biocontrol strains. *Mycologia* **2015**, *107*, 558–590. [CrossRef]
48. Inglis, P.W.; Mello, S.C.M.; Martins, I.; Silva, J.B.T.; Macêdo, K.; Sifuentes, D.N.; Valadares-Inglis, M.C. Trichoderma from Brazilian garlic and onion crop soils and description of two new species: Trichoderma azevedoi and Trichoderma peberdyi. *PLoS ONE* **2020**, *15*, e0228485.
49. Ramírez-Cariño, H.F.; Guadarrama-Mendoza, P.C.; Sánchez-López, V.; Cuervo-Parra, J.A.; Ramírez-Reyes, T.; Dunlap, C.A.; Valadez-Blanco, R. Biocontrol of Alternaria alternata and Fusarium oxysporum by Trichoderma asperelloides and Bacillus paralicheniformis in tomato plants. *Antonie van Leeuwenhoek* **2020**, *113*, 1247–1261. [CrossRef]
50. Benítez, T.; Rincón, A.M.; Limón, M.C.; Codon, A.C. Biocontrol mechanisms of Trichoderma strains. *Int. Microbiol.* **2004**, *7*, 249–260. [PubMed]
51. Alamri, S.; Hashem, M.; Mostafa, Y.S. In vitro and in vivo biocontrol of soil-borne phytopathogenic fungi by certain bioagents and their possible mode of action. *Biocontrol Sci.* **2012**, *17*, 155–167. [CrossRef]
52. Gal-Hemed, I.; Atanasova, L.; Komon-Zelazowska, M.; Druzhinina, I.S.; Viterbo, A.; Yarden, O. Marine isolates of Trichoderma spp. as potential halotolerant agents of biological control for arid-zone agriculture. *Appl. Environ. Microbiol.* **2011**, *77*, 5100–5109. [CrossRef]
53. Doley, K.; Dudhane, M.; Borde, M.; Jite, P.K. Effects of glomus fasciculatum and trichoderma asperelloides in roots of groundnut (cv. western-51) against pathogen Sclerotium rolfsii. *Int. J. Phytopathol.* **2014**, *3*, 89–100. [CrossRef]
54. Harman, G.E. Myths and dogmas of biocontrol changes in perceptions derived from research on Trichoderma harzinum T-22. *Plant Dis.* **2000**, *84*, 377–393. [CrossRef] [PubMed]
55. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. Trichoderma species—Opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56. [CrossRef] [PubMed]
56. Yedidia, I.; Srivastva, A.K.; Kapulnik, Y.; Chet, I. Effect of Trichoderma harzianum on microelement concentrations and increased growth of cucumber plants. *Plant Soil* **2001**, *235*, 235–242. [CrossRef]
57. Martínez-Medina, A.; Roldán, A.; Pascual, J.A. Performance of a Trichoderma harzianum bentonite–vermiculite formulation against Fusarium wilt in seedling nursery melon plants. *HortScience* **2009**, *44*, 2025–2027. [CrossRef]
58. Tchameni, S.N.; Sameza, M.L.; O'donovan, A.; Fokom, R.; Mangaptche Ngonkeu, E.L.; Wakam Nana, L.; ETOA, F.-X.; NWAGA, D. Antagonism of Trichoderma asperellum against Phytophthora megakarya and its potential to promote cacao growth and induce biochemical defence. *Mycology* **2017**, *8*, 84–92. [CrossRef]
59. Casimiro, I.; Marchant, A.; Bhalerao, R.P.; Beeckman, T.; Dhooge, S.; Swarup, R.; Graham, N.; Inzé, D.; Sandberg, G.; Casero, P.J. Auxin transport promotes Arabidopsis lateral root initiation. *Plant Cell* **2001**, *13*, 843–852. [CrossRef] [PubMed]
60. Singh, N.; Raina, S.; Singh, D.; Ghosh, M.; Heflish, A. Exploitation of promising native strains of Bacillus subtilis with antagonistic properties against fungal pathogens and their PGPR characteristics. *J. Plant Pathol.* **2017**, 27–35.
61. Halifu, S.; Deng, X.; Song, X.; Song, R. Effects of two Trichoderma strains on plant growth, rhizosphere soil nutrients, and fungal community of Pinus sylvestris var. mongolica annual seedlings. *Forests* **2019**, *10*, 758. [CrossRef]
62. Contreras-Cornejo, H.A.; Macías-Rodríguez, L.; Cortés-Penagos, C.; López-Bucio, J. Trichoderma virens, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiol.* **2009**, *149*, 1579–1592. [CrossRef]
63. Vey, A.; Hoagland, R.E.; Butt, T.M. Toxic metabolites of fungal biocontrol agents. *Fungi Biocontrol Agents Prog. Probl. Potential* **2001**, *1*, 311–346.
64. Vinale, F.; Sivasithamparam, K.; Ghisalberti, E.L.; Marra, R.; Barbetti, M.J.; Li, H.; Woo, S.L.; Lorito, M. A novel role for Trichoderma secondary metabolites in the interactions with plants. *Physiol. Mol. Plant Pathol.* **2008**, *72*, 80–86. [CrossRef]
65. Shoresh, M.; Harman, G.E. Differential expression of maize chitinases in the presence or absence of Trichoderma harzianum strain T22 and indications of a novel exo-endo-heterodimeric chitinase activity. *BMC Plant Biol.* **2010**, *10*, 1–11. [CrossRef]

66. Hermosa, R.; Rubio, M.B.; Cardoza, R.E.; Nicolás, C.; Monte, E.; Gutiérrez, S. The contribution of Trichoderma to balancing the costs of plant growth and defense. *Int. Microbiol* **2013**, *16*, 69–80.
67. Mukherjee, P.K.; Horwitz, B.A.; Herrera-Estrella, A.; Schmoll, M.; Kenerley, C.M. Trichoderma research in the genome era. *Annu. Rev. Phytopathol.* **2013**, *51*, 105–129. [CrossRef]
68. Hermosa, R.; Viterbo, A.; Chet, I.; Monte, E. Plant-beneficial effects of Trichoderma and of its genes. *Microbiology* **2012**, *158*, 17–25. [CrossRef]
69. Malmierca, M.G.; Barua, J.; McCormick, S.P.; Izquierdo-Bueno, I.; Cardoza, R.E.; Alexander, N.J.; Hermosa, R.; Collado, I.G.; Monte, E.; Gutiérrez, S. Novel aspinolide production by T richoderma arundinaceum with a potential role in B otrytis cinerea antagonistic activity and plant defence priming. *Environ. Microbiol.* **2015**, *17*, 1103–1118. [CrossRef]
70. Hoegen, E.; Strömberg, A.; Pihlgren, U.; Kombrink, E. Primary structure and tissue-specific expression of the pathogenesis-related protein PR-1b in potato. *Mol. Plant Pathol.* **2002**, *3*, 329–345. [CrossRef] [PubMed]
71. Abdelkhalek, A.; Salem, M.Z.M.; Ali, H.M.; Kordy, A.M.; Salem, A.Z.M.; Behiry, S.I. Antiviral, antifungal, and insecticidal activities of Eucalyptus bark extract: HPLC analysis of polyphenolic compounds. *Microb. Pathog.* **2020**, *147*, 104383. [CrossRef] [PubMed]
72. Dempsey, D.A.; Shah, J.; Klessig, D.F. Salicylic acid and disease resistance in plants. *CRC. Crit. Rev. Plant Sci.* **1999**, *18*, 547–575. [CrossRef]
73. Abdelkhalek, A.; Al-Askar, A.A.; Hafez, E. Differential induction and suppression of the potato innate immune system in response to Alfalfa mosaic virus infection. *Physiol. Mol. Plant Pathol.* **2020**, 101485. [CrossRef]
74. Manganiello, G.; Sacco, A.; Ercolano, M.R.; Vinale, F.; Lanzuise, S.; Pascale, A.; Napolitano, M.; Lombardi, N.; Lorito, M.; Woo, S.L. Modulation of tomato response to Rhizoctonia solani by Trichoderma harzianum and its secondary metabolite harzianic acid. *Front. Microbiol.* **2018**, *9*, 1966. [CrossRef]
75. Van Wees, S.C.M.; Luijendijk, M.; Smoorenburg, I.; Van Loon, L.C.; Pieterse, C.M.J. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. *Plan. Mol. Biol.* **1999**, *41*, 537–549. [CrossRef]
76. Newman, M.; Von Roepenack-Lahaye, E.; Parr, A.; Daniels, M.J.; Dow, J.M. Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J.* **2002**, *29*, 487–495. [CrossRef]
77. Abdelkhalek, A. Expression of tomato pathogenesis related genes in response to Tobacco mosaic virus. *JAPS J. Anim. Plant Sci.* **2019**, *29*, 1596–1602.
78. Mayo, S.; Gutierrez, S.; Malmierca, M.G.; Lorenzana, A.; Campelo, M.P.; Hermosa, R.; Casquero, P.A. Influence of Rhizoctonia solani and Trichoderma spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* **2015**, *6*, 685. [CrossRef]
79. Eslahi, N.; Kowsari, M.; Zamani, M.r.; Motallebi, M. The profile change of defense pathways in phaseouls vulgaris L. by biochemical and molecular interactions of Trichoderma harzianum transformants overexpressing a chimeric chitinase. *Biol. Control* **2021**, *152*, 104304. [CrossRef]
80. Abo-Zaid, G.; Abdelkhalek, A.; Matar, S.; Darwish, M.; Abdel-Gayed, M. Application of Bio-Friendly Formulations of Chitinase-Producing Streptomyces cellulosae Actino 48 for Controlling Peanut Soil-Borne Diseases Caused by Sclerotium rolfsii. *J. Fungi* **2021**, *7*, 167. [CrossRef] [PubMed]
81. Markovich, N.A.; Kononova, G.L. Lytic enzymes of Trichoderma and their role in plant defense from fungal diseases: A review. *Appl. Biochem. Microbiol.* **2003**, *39*, 341–351. [CrossRef]
82. Yedidia, I.; Benhamou, N.; Chet, I. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agentTrichoderma harzianum. *Appl. Environ. Microbiol.* **1999**, *65*, 1061–1070. [CrossRef]
83. Yedidia, I.; Shoresh, M.; Kerem, Z.; Benhamou, N.; Kapulnik, Y.; Chet, I. Concomitant induction of systemic resistance to Pseudomonas syringae pv. lachrymans in cucumber by Trichoderma asperellum (T-203) and accumulation of phytoalexins. *Appl. Environ. Microbiol.* **2003**, *69*, 7343–7353. [CrossRef]
84. Singh, B.N.; Singh, A.; Singh, S.P.; Singh, H.B. Trichoderma harzianum-mediated reprogramming of oxidative stress response in root apoplast of sunflower enhances defence against Rhizoctonia solani. *Eur. J. Plant Pathol.* **2011**, *131*, 121–134. [CrossRef]
85. Ortega-García, J.G.; Montes-Belmont, R.; Rodríguez-Monroy, M.; Ramírez-Trujillo, J.A.; Suárez-Rodríguez, R.; Sepúlveda-Jiménez, G. Effect of Trichoderma asperellum applications and mineral fertilization on growth promotion and the content of phenolic compounds and flavonoids in onions. *Sci. Hortic.* **2015**, *195*, 8–16. [CrossRef]
86. André, C.M.; Schafleitner, R.; Legay, S.; Lefèvre, I.; Aliaga, C.A.A.; Nomberto, G.; Hoffmann, L.; Hausman, J.-F.; Larondelle, Y.; Evers, D. Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress. *Phytochemistry* **2009**, *70*, 1107–1116. [CrossRef] [PubMed]
87. Dao, T.T.H.; Linthorst, H.J.M.; Verpoorte, R. Chalcone synthase and its functions in plant resistance. *Phytochem. Rev.* **2011**, *10*, 397–412. [CrossRef] [PubMed]
88. Abdelkhalek, A.; Al-Askar, A.A.; Behiry, S.I. Bacillus licheniformis strain POT1 mediated polyphenol biosynthetic pathways genes activation and systemic resistance in potato plants against Alfalfa mosaic virus. *Sci. Rep.* **2020**, *10*, 1–16. [CrossRef]

89. Martínez, G.; Regente, M.; Jacobi, S.; Del Rio, M.; Pinedo, M.; De La Canal, L. Chlorogenic acid is a fungicide active against phytopathogenic fungi. *Pestic. Biochem. Physiol.* **2017**, *140*, 30–35. [CrossRef] [PubMed]
90. Kanwal, Q.; Hussain, I.; Latif Siddiqui, H.; Javaid, A. Antifungal activity of flavonoids isolated from mango (*Mangifera indica* L.) leaves. *Nat. Prod. Res.* **2010**, *24*, 1907–1914. [CrossRef] [PubMed]