Print ISSN: 0375-9237
Online ISSN: 2357-0350

EGYPTIAN JOURNAL OF BOTANY (EJBO)

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PUBLISHED BY

Protective and curative applications of some *Trichoderma* species to tomato plants infected by tomato mosaic virus

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Trichoderma has been used as an eco-friendly biocontrol agent for different crop species. Not only does it enhance plant resistance, but it also improves plant growth. Therefore, in this study, *Trichoderma viride* and *Trichoderma harzianum* were used as protective and curative treatments to trigger the systemic defense of tomato plants against tomato mosaic virus (ToMV). *T. viride* isolate Tvd44 and *T. harzianum* isolate ThDA66 were identified and molecularly characterized before being used as antiviral agents against ToMV. The HPLC analysis results of Tvd44 and ThDA66 culture filtrates revealed that the most detected active biomolecules had a range of biological activities, including antiviral capabilities. The foliar spraying application of Tvd44 or ThDA66 filtrates against ToMV-inoculated tomato plants significantly improved plant growth parameters. Different *Trichoderma* treatments improved tomato plants' chlorophyll content, phenolics, flavonoids, and DPPH. Additionally, the antioxidant enzymatic activities of tomato plants that were ToMV-inoculated and non-inoculated were enhanced compared to those of untreated plants. Moreover, different *Trichoderma* treatments exhibited a decrease in the oxidative stress markers in all treatments compared to plants that were inoculated with ToMV alone. The q-PCR analysis of genes from different pathways, including *PAL-1*, *CHS*, *POD*, *PR-2*, *JERF3*, and *WRKY19*, showed significant gene expression in all *Trichoderma* treatments, showing systemic resistance. In conclusion, the present investigation proved the effective role of *Trichoderma* as a biocontrol agent against ToMV in tomato plants.

Keywords: ToMV, *Trichoderma* spp., HPLC, antioxidant enzymes, defense genes pathways

ARTICLE HISTORY
Submitted: December 20, 2023
Accepted: March 29, 2024

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DOI: 10.21608/ejbo.2024.265650.2679

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INTRODUCTION

The tomato belongs to the *Solanaceae* family and is a major vegetable crop that has recently gained importance and is now farmed worldwide. It is considered an important food and the second-largest crop after potatoes (Hanssen and Lapidot, 2012; Alkowni et al., 2019). Tomato mosaic virus (ToMV) belongs to the *Virgaviridae* family and the *Tobamovirus* genus (Lefkowitz et al., 2018). Although it can infect a variety of hosts, in the field, it primarily affects vegetable plants in the *Solanaceae* family (Xu et al., 2021). ToMV can cause inhibition of growth, mosaic, and distortion in leaves (Broadbent, 1976). The serious losses are impacting tomato fruit production, size, and quality across the globe (Bhandari, 2018).

The component of the ToMV genome includes single-stranded positive-sense RNA (ssRNA), which is roughly 6.4 kb in length and encodes at least four proteins, including the coat protein (CP) (Nishikori et al., 2006). These different proteins are the 183 kDa, 126 kDa, and 30 kDa proteins. The two proteins of 126 kDa and 183 kDa are expressed from the genomic RNA, the latter of which is translated as a read-through product. Together, they can be responsible for the replication of the virus, whereas the 30 kDa protein and the CP are individually expressed from a different subgenomic RNA. In contrast to the CP, the virus's only structural protein, the 30 kDa protein encourages cell-to-cell migration (Li et al., 2005; Xu et al., 2021).

Trichoderma's mycoparasitic nature and its ability to be used as a biocontrol agent for fungi that cause plant damage have been recognized for over 60 years (Elnhas et al., 2020; Sadik et al., 2022). The benefits of utilizing this biocontrol agent in agriculture depend on the strain, and the advantages of plant association include the following: 1) competence in the rhizosphere, which enables rapid establishment of a stable microbial community there; 2) pathogen suppression through a range of mechanisms; 3) general plant health improvement; 4) promotion of plant growth; 5) improved nutrient availability and uptake; and 6) induction of host resistance identical to that induced by beneficial rhizobacteria (Harman et al., 2004; Vinale et al., 2012). Trichodermal fungi are renowned for producing secondary metabolites with multiple biological functions (Ghisalberti et al., 2005; Reino et al., 2007; Vinale et al., 2012).

Reactive oxygen species (ROS) generation is the primary defense mechanism used by host plants against many illnesses (Vitti et al., 2015; Keswani et

al., 2019). Superoxide radicals (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-) are all involved in ROS generation, which indicates early plant protection against pathogen invasion (Chen et al., 2009; Lehmann et al., 2015). Systems heavily rely on biological and physiological capabilities (Baxter et al., 2014).

The infected plants can accumulate defensive compounds; these compounds are called secondary metabolites. Plants have a highly evolved chemical defense system that relies on secondary metabolites to protect them (Pagare et al., 2015). *Trichoderma* interacts with plants via structural, secretor, and secondary metabolite proteins and metabolites that support defense responses and activate a network of phytohormones that enhance defense responses. Besides aiding in growth signaling pathways, this can be employed to defend against pathogen assault and environmental stress (Carrero-Carrón et al., 2018; Morán-Diez et al., 2020; Aldaby et al., 2021). Furthermore, in addition to supporting plant growth and development, secondary metabolites like polyphenolic compounds also act as a buffer against biotic and abiotic stressors like viruses (Akyol et al. 2016). Furthermore, secondary metabolites are useful for enhancing plant health (El-Bilawy et al., 2022).

Two pathways contribute to plant virus resistance: systemic acquired resistance (SAR), which is primarily controlled through salicylic acid and induced by pathogens in pneumatic parts of plants (Abo-Zaid et al., 2020; Vlot et al., 2021). The second mechanism is induced systemic resistance (ISR), modulated by jasmonic acid and ethylene (Vlot et al., 2021; Pieterse et al., 2014). SAR and ISR cause various partially overlapping biological reactions, such as activating antioxidant enzymes and defense genes (Vlot et al., 2021; Abo-Zaid et al., 2021; Veselova et al., 2022). Pathogenesis-related proteins (*PR-2* and *POD*) are examples of SAR pathways. The polyphenol metabolism pathways (*PAL* and *CHS*) are considered polyphenolic substances that are secondary metabolites involved in developing, growing, and fighting plants against biotic and abiotic stressors (Akyol et al. 2016). The jasmonic pathway (*JERF3* and *WRKY19*) is also a defense signaling pathway implicated in resistance to pathogens and increasing secondary metabolite yields (Krisa et al., 1999; Ruan et al., 2019).

The main objective of this research was to evaluate the protective and curative activities of *Trichoderma viride* and *Trichoderma harzianum* filtrates against ToMV under greenhouse conditions. This research analyzed the impact of *T. viride* and *T. harzianum* on plant outgrowth parameters, chlorophyll content, total phenolic content, flavonoid content, and DPPH. Antioxidant enzymes (polyphenol oxidase, peroxidase, superoxide, and catalase) that participate in a part of the metabolism of ROS and oxidative markers (malonaldehyde and hydrogen peroxide) were estimated. Transcriptional expression levels of six genes—two genes of the polyphenol biosynthetic pathway (*PAL-1* and *CHS*), two genes of pathogenesis-related protein-encoding (*PR-2* and *POD*), two genes of the jasmonic pathway (*JERF3* and *WRKY19*)—and the accumulation of the ToMV-CP protein were evaluated.

MATERIALS AND METHODS

Plant material, virus isolation, and identification

In this study, the tomato (*Solanum lycopersicum* L.) cultivar GSS-12 was used in the greenhouse experiments. The virus isolate was obtained from samples of tomatoes *(Solanum lycopersicum* L.) naturally infected with ToMV that were collected from Borg El-Arab, Alexandria, Egypt, and continuously maintained as a source of virus inoculums on tomato plants for virus propagation under greenhouse conditions (SRTA-City, Alexandria). Furthermore, the CP gene was amplified by specific primers to identify the isolated ToMV at the molecular level (Table 1). The PCR assay reaction included 1 µL of cDNA (30 ng), 1 µL from each primer (10 pmol/µL), 12.5 µL of 2x Taq PCR Mix, and the dH_2O was added to reach the total volume of 25 µL. The CP gene was amplified by employing the following: 94°C for 3 min, followed by 35 cycles at 94°C, 60°C, and 72°C for 45 s each, and final elongation at 72°C for 5 min (Aseel et al., 2023a). The PCR products were separated by agarose gel electrophoresis, as described by Shaikhaldein et al. (2018).

Isolation and identification of *Trichoderma* spp.

Trichoderma spp. was isolated from soil rhizosphere zone samples collected from healthy tomato-growing areas in Borg El-Arab, Alexandria, Egypt. The hyphal tip isolation technique was applied to purify the culture, which was then kept on potato dextrose agar (PDA) slants for further identification procedures. Their morphological and molecular characteristics were used for fungal identification (Heflish et al., 2021; Samuels et al., 2002). Specific primers for the internal transcribed spacer (ITS) gene were used to

identify the *Trichoderma* isolates (Table 1). The *Trichoderma* ITS region was amplified by PCR with ITS1 and ITS4 primers (Murugan et al., 2020). The PCR reaction mix was done as previously described except for annealing at 55°C for 1 min. After PCR amplification, the PCR fragments were purified using a PCR clean-up column kit (Maxim Biotech Inc., USA) according to the manufacturer's instructions and then sequenced by Macrogen Company Ltd. (Korea). Nucleotide sequences were compared using the NCBI-BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which is available on the GenBank homepage, to compare the newly obtained sequences with others in the nucleotide databases. The MEGA 11 program was employed to construct the phylogenetic tree, which was created using the maximum likelihood method (Tamura et al., 2021).

HPLC analysis of active biomolecules in *Trichoderma* spp. filtrates

HPLC analysis was conducted to detect and determine the active biomolecules in fungal filtrates using the Agilent 1100 HPLC System (Colorado, USA). The system is equipped with a quaternary gradient pump, an in-line degasser, an automatic injection, and a dual-wavelength UV/Vis detector. Moreover, HPLC is accompanied by a Zorbax Eclipse Plus C18 (RP18, ODS, Octadecyl) column with a particle size of 5 mm and a dimension of 25 × 0.4 cm. A column temperature was determined at 40°C, the injection volume was 10 µL, and the flow rate was set to 1 ml/min. Each chromatogram can be identified according to the highest value of similarity.

Greenhouse experiments and plant growth-enhancing capacities of *Trichoderma* spp.

The four-week-old tomato seedlings (cv. GS-12) were developed and grown in plastic pots (15 cm). Each pot has been filled with autoclave-sterilized clay and sand (1:1). Under greenhouse conditions, at 28°C and 16°C, day and night temperatures were used, with a relative humidity of 70%, for the incubation of tomato seedlings. On the 28th day after planting, each tomato seedling's two uppermost true leaves were automatically inoculated with the purified ToMV, according to Aseel et al. (2019a). The Tvd44 or Th-DA66 spraying solution was prepared as described by Yedidia et al. (1999) and Aseel et al. (2023a). Eight treatments were used in the experiment, and each tomato leaf (three leaves per plant) treatment had five biological replicates as follows: C: tomato plant control; V: tomato plants infected with ToMV only; T.v: tomato plants treated with *T. viride* alone; V + T.v: tomato plants inoculated with ToMV and after 24 hours of treatment with *T. viride* (curative application); T.v + V: tomato plants treated with *T. viride* and after 24 hours of inoculation by ToMV (protective application); T.h: the tomato plants treated by *T. harzianum* only; V + T.h: tomato plants inoculated with ToMV and after 24 hours of treatment with *T. harzianum* (curative application); T.h + V: tomato plants which were treated with *T. harzianum* and after 24 hours of inoculation with ToMV (protective application). All plants were conserved in the greenhouses for three weeks, and the development of mosaic symptoms was examined daily. Furthermore, plants were washed under water to estimate the effect of *Trichoderma* spp. on the following growth parameters: plant length, shoot and root lengths (cm), fresh and dry weights of shoot and root (g), and leaf number.

Determination of chlorophyll pigment content

First, 0.1 g of fresh leaves was ground in a mortar in 5 mL of 80% acetone in the dark, according to the methods of Ahmad et al. (2015) and Arnon (1949). The chlorophyll a and b content were estimated using a SPECTROstar®*Nano* (BMG LABTECH, Germany) at 645 nm and 663 nm. The content was determined as follows:

Chl.a (mg/ml) is equal to $11.64 \times (A_{663}) - 2.16 \times (A_{645})$.
Chl.b (mg/ml) is equal to $20.97 \times (A_{645}) - 3.94 \times (A_{663})$.

Total phenolic content estimation

According to Dewanto et al. (2002), total phenolic content was determined using the Folin–Ciocalteu reagent (Hamrouni-Sellami et al., 2013). The Folin–Ciocalteu reagent (0.125 mL) and 0.5 mL of deionized water were mixed with an aliquot (0.125 mL) of a sufficiently diluted methanolic extract sample. This mixture was shaken and left to stand for 6 min before adding 1.25 ml of a 7% sodium carbonate (Na_2CO_3) solution. Then, 3 mL of deionized water was added to the mixture and mixed. After 90 min of incubation at 23°C, the absorbance was estimated at 760 nm using a SPECTROstar®*Nano* (BMG LABTECH, Germany). Using the standard curve of gallic acid, the total phenolic content of dried tomato leaves (three replicates for each drying process) was calculated as milligrams of gallic acid equivalent per gram of dry matter (mg of GAE/g of DM). The standard curve was in the range of 50–400 mg/mL (R2 = 0.99).

Total flavonoid content estimation

As described by Dewanto et al. (2002), the total flavonoid content was determined. About 250 µl of the properly diluted sample was mixed with 75 µL of 5% $NaNO_2$ (sodium nitrite). Then, after about 6 minutes, 150 µL of 10% aluminum chloride ($AlCl_3$) and 500 µL of NaOH (1 M) were added to the mixture. Finally, distilled water was utilized to dilute this mixture to 2.5 mL. The absorbance was measured at 510 nm using a SPECTROstar®*Nano* (BMG LABTECH, Germany).

Free radical scavenging (DPPH) estimation

Based on the method described by Hamrouni-Sellami et al. (2013), the antioxidant ability of methanolic leaf extracts was evaluated by bleaching the purple DPPH radical solution. Then, 0.5 mL of a 0.2 mmol l/l DPPH methanolic solution and 1 mL of various tomato leaf extract concentrations. After a vigorous shake, the combination was maintained at 20°C for 30 min. After that, the absorbance of the solution at 517 nm was measured using a SPECTROstar®*Nano* (BMG LABTECH, Germany). The concentration needed to block DPPH by 50%, or IC_{50} (micrograms per milliliter), was used to express the antiradical activity. The following equation was used to calculate the capacity to scavenge DPPH radicals:

$$\text{DPPH scavenging (\%)} = [(A_0 - A_1)\ A_0]\ 100.$$

A_0 refers to the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min.

Measurement of antioxidant enzymatic activities

Enzyme extraction: A fresh leaf sample of about 1 g was frozen and homogenized by adding 3 mL of 50 mM cold phosphate buffer (pH = 7.5). The homogenate was centrifuged for 30 min at 12,000 rpm at 4°C (*PrO-Research,* UK). The supernatant was used as a crude extract for enzymatic assays except for the peroxidase enzyme.

Determination of polyphenol oxidase (PPO) activity: Each microplate had 50 µL of a 150 mM catechol or guaiacol solution added to 100 µL of a 100 mM sodium phosphate buffer (pH = 7). To begin the reaction, 50 µL of crude extract was added to each well. The change in absorbance was measured using a microplate reader (Synergy-HT, BioTek, Winooski, VT, USA) at 404 nm and a temperature of 25°C.

Determination of peroxidase (POX) activity: Leaf tissue was ground into a solution containing 125 µL of sodium phosphate buffer (100 mM, pH=7), 25 µL of guaiacol (24 mM), 25 µL of enzyme extract, and 25 µL of 12 mM hydrogen peroxide solution and put in a microplate well to start the reaction. The absorbance was measured at 465 nm using a microplate reader (Synergy-HT, BioTek, Winooski, VT, USA).

Determination of superoxide dismutase (SOD) activity: The reaction was initiated by mixing 3 mL of the reaction mixture with 100 µL of crude extract. The reaction mixture contained 75 µM NBT, 13 mM methionine, 50 mM potassium phosphate buffer (pH = 7.8), 2 mM riboflavin, 2 µM riboflavin, and 0.1 mM EDTA. The reaction mixture without any NBT or enzyme was used to create a blank. The absorbance of the solution-filled tubes was immediately measured at 560 nm after 15 min of exposure to 400 W (4x100 W bulbs) (Moattar et al., 2016). Then, using the following equation, the percentage of scavenging the superoxide radical was determined:

$$\text{Scavenging percentage} = (1-A_e/A_0)\ /\ 100,$$

where A_e is the leaf extract absorbance of treated tomato plants, while A_0 is the leaf extract absorbance of healthy tomato control.

Determination of catalase (CAT) activity: The activity of CAT was estimated using the adjusted method of Aebi (1948). In each well of the microplate, 10 µL of crude extract and 250 µL of 50 mM phosphate buffer were mixed. Finally, 10 µL of H_2O_2 was added, the container was gently shaken, and then the absorbance was measured at 240 nm using a microplate reader (Synergy-HT, BioTek, Winooski, VT, USA). Furthermore, 10 µL of water was substituted for the enzymatic crude extract in the case of the blank. Then, 1 nmol of H_2O_2 dissociated min^{-1} was the unit of enzyme activity according to the following formula:

The activity of the CAT (mM/g FW) is equal to (activity A V/a) = (E × W).

where activity is the OD value, A is the total assay volume, V is the total volume of the buffer solution used to extract the enzyme, W is the fresh weight of the sample, a is the amount of enzyme extract utilized in reaction solution to test, and E is the extinction coefficient, i.e., 39.4 mM/cm.

Evaluation of oxidative stress markers

Determination of malonaldehyde (MDA): MDA was estimated by assaying the content of reactive compounds in thiobarbituric acid (TBARS), based on the method of Chappell and Cohn (2011), with minor modifications. In a prechilled mortar, 0.25 g of tissues was ground and then transferred to an Eppendorf

tube filled with 2 mL of precold TCA (1%, w/v). The samples were homogenized with a vortex and then centrifuged at 12.000 rpm for 15 min. Half mL of supernatants were mixed with 1 mL of TBA and TCA (added 0.01% butylated hydroxyl toluene) solutions in a 4 mL Eppendorf tube. The samples were heated at 95°C for 15 min, followed by putting them immediately in an ice bath. Then, it was centrifuged at 4800 rpm for 10 min. Finally, 250 µL of supernatants was added to each well of a microplate, and the change in absorbance was measured at 532 and 600 nm. The nonspecific turbidity correction was conducted by subtracting the absorbance at 600 nm. Three practical replicates for each sample and for the blank were employed using distilled water. The TBARS was evaluated according to this equation:

TBARS (nmol/g FW) equal to $[(OD_{532}-OD_{600})\times A\times V]/(a\times E\times W)$,

where A is the total assay volume, V is the total volume of phosphate buffer employed for enzyme extraction, a is the volume of supernatant utilized, W is the fresh weight of the sample, and E is the extinction coefficient (1.55×10^2) 155 Mm^{-1} cm^{-1}.

Determination of hydrogen peroxide (H_2O_2): To estimate the concentration of hydrogen peroxide (H_2O_2), 0.2 g of leaf tissues was taken into a prechilled mortar and powdered. Then, they were transferred into a tube of 5 mL containing 2 mL of precooled TCA (0.1% w/v). The sample was vortexed and centrifuged for 15 min at 12,000 rpm (Velikova et al., 2000). In each well, 50 µL of 10 mM potassium phosphate buffer (pH 7.0), 50 µL of supernatant, and 100 µL of 1 M KI were mixed. The absorbance was recorded at 390 nm. The H_2O_2 concentration was calculated using a standard curve with known concentrations of H_2O_2, and the quantity of H_2O_2 was expressed as µmol/g FW.

Effect of *Trichoderma* spp. on ToMV using defense-related gene expression levels

Extraction of total RNA and cDNA synthesis: The total RNA was extracted according to the manufacturer's instructions using the TRIZOL reagent kit (Life Technologies Invitrogen, CA, USA). The extraction buffer employed in this method includes guanidium thiocyanate, and RNA was precipitated using isopropanol. RNA concentration was measured by UV-Vis spectrophotometer (DU730, Beckman Coulter Inc., Brea, CA, USA). The reverse transcription reaction (M-MuLV Reverse Transcriptase, Biolabs, New England) was used to transform the RNA into cDNA in a thermal cycler (Eppendorf, Hamburg, Germany). Our prior investigations described the cDNA synthesis components and program (Rashad et al., 2020; Aseel et al., 2019b). The cDNA was kept at -20 °C until used in the q-PCR reactions.

Quantitative PCR (q-PCR): The effect of *Trichoderma* spp. treatment on the expression level of the ToMV-CP gene and defense-related genes in tomato plants was estimated by q-PCR. Specific primer sequences utilized in this study are shown in Table 1**.** The SYBR® green kit (Bioloine, Luckenwalde, Germany) was used. The q-PCR reaction components and program were as described by Aseel et al. (2022), Aseel et al. (2023b), and Abdelkhalek et al. (2022). The specific primers for tomato defense genes, two polyphenol biosynthetic pathway genes (*PAL-1* and *CHS*), two pathogenesis-related protein-encoding genes (*PR-2* and *POD*), and two jasmonic pathway genes (*JERF3* and *WRKY19*), were used in this study. Two housekeeping genes (*β-actin*) and (*EFα-1*) were employed to standardize the gene transcriptional levels (Table 1).

Statistical analysis

Statistical analysis was performed on the data by using one-way ANOVA using CoStat software version 6.311 (Monterey, CA, USA). The standard deviation (±SD) is displayed as a column bar after the statistical differences in probability at the mean level at $p \leq 0.05$ are determined using the least significant difference (LSD) method. Gene expression levels were calculated as fold changes using the C_T method ($2^{-\Delta\Delta CT}$) of Schmittgen and Livak (2008).

RESULTS

ToMV isolation and identification

ToMV collected from naturally occurring tomato plants showed symptoms like mosaic, yellowish, and spotty light and dark green leaves (Figure 1A)**.** The PCR technique was employed to identify the ToMV isolation using the CP gene sequence. The PCR fragment of 450 bp separated on the agarose gel **(**Figure 1D) indicates the presence of a plant viral infection**.**

Isolation and species identification of *Trichoderma* spp.

T. viride and *T. harzianum* were isolated from the soil rhizosphere zone samples that were collected from healthy tomato-growing areas in Borg El-Arab, Alexandria, Egypt. Meanwhile, colonies of both *T. viride* and *T. harzianum* were observed on PDA medium after 3-5 days. The colony morphology of *T. viride* is a circular ring, aerial, dark green and white as cotton, and powdery, as shown in Figure 1B. The morphological analysis of *T. harzianum* showed that it

has a single green and yellowish concentric ring, is highly branched, and its main branches produce lateral side branches (Figure 1C). The PCR amplicons of the ITS gene region were utilized in the detection and molecular identification of the isolated *Trichoderma* spp. (Figure 1E). The NCBI-BLAST search of nucleotide sequences for an ITS gene sequence (~550 bp) displayed 97% homology with *T. viride* (Acc#OL546802.1, Nigeria). Therefore, the isolated strain Tvd44 was identified as *T. viride*. Conversely, the isolated strain Th-DA66 was identified as *T. harzianum* and showed 98% homology with *T. harzianum* (Acc#MZ423061.1, Taiwan). The sequences of both isolates were submitted to the GenBank (NCBI database). The *T. viride* isolate obtained the accession number (OQ991378), while the *T. harzianum* isolate obtained the accession number (PP273158). A phylogenetic tree analysis demonstrated that the strain Tvd44 was highly connected to other *T. viride* strains and had a similar evolutionary relationship to other *T. viride* strains (supplementary material Figure 1). Concurrently, the phylogenetic tree analysis revealed that strain Th-DA66 was closely related to other *T. harzianum* available in the GenBank nucleotide database (supplementary Figure 2).

HPLC analysis of active biomolecules in *Trichoderma* spp. filtrates

The HPLC chromatograms of the active biomolecules of *T. viride* isolate Tvd44 and *T. harzanium* isolate ThDA66 filtrate extracts are shown in Supplementary Material Figure 3. The characterization of Tvd44 and ThDA66 filtrates exhibited the common six chemical components: gallic acid, protocatechuic acid, catechin, esculetin, vanillic acid, and pyrocatechol, but in ThDA66, the other four biomolecules were noticed, like coumarin, 4.3-indul butyl acetic acid, cinnamic acid, and naphthyl acetic acid. Tvd44 had the highest concentration reported by protochateuic acid (9.46 µg/mL), while the highest concentration of the chemical compounds in ThDA66 extract was 4,3-indul butyl acetic acid (8.79 µg/mL), which was the most common among 10 different compounds (Table 2).

Effects of *Trichoderma* inoculation on the growth and development of ToMV-infected tomato plants

In greenhouse experiments, the result of inoculation with ToMV showed characteristic symptoms like mosaic, yellowish, and dark green leaves, while reduced disease symptoms were observed in *Trichoderma* spp.-treated plants. These results suggest that *Trichoderma* may strengthen plant protection against ToMV replication in tomato tissues (Figure 2). The results of tomato plant growth parameters indicated a significant reduction in plant length, shoot and root length, shoot and root dry weight, shoot and root fresh weight, and also leaf number of tomato plants that were infected with ToMV, which recorded 14.33 ± 1.04 cm, 8.16 ± 0.13 cm, 6.17 ± 0.06 cm, 0.79 ± 0.31 g, 0.18 ± 0.02 g, 0.16 ± 0.01 g, 0.08 ± 0.01 g, and 11.67 ± 2.08, respectively (Table 3), compared to the control plants that had values of 25.30 ± 1.61cm, 13.70 ± 0.10cm, 11.60 ± 0.02 cm, 1.93 ± 0.59 g, 0.63 ± 0.06g, 0.33 ± 0.01g, 0.12 ± 0.01g and 22.67 ± 4.51, respectively (Table 3). Therefore, it was found that ToMV reduced all growth parameters in tomato plants. Conversely, the results of treatment with *Trichoderma* spp. revealed that plants treated with *T. viride* or *T. harzianum* showed significantly higher growth parameters than those inoculated with ToMV alone. Moreover, when T.v. or T.h. was applied to the tomato plants after or before the infection, *Trichoderma* spp. decreased the disease severity and the symptoms of the infection by ToMV.

Effect of *Trichoderma* inoculation on chlorophyll content

The final results of both chlorophylls a and b revealed that the control tomato plants exhibited the highest level of chlorophyll a and b recorded (21.13 mg/g f.wt. and 29.79 mg/g f.wt., respectively), followed by plants treated with *Trichoderma* spp., where the plants of tomato treated with each T.v and T.h alone reported chlorophylls a and b of 19.57 mg/g f.wt. and 28.40 mg/g f.wt., 13.69 mg/g f.wt., and 21.18 mg/g f.wt., respectively (Figure 3). Similarly, tomato plants treated by T. v+V and T. h+V recorded 15.03 mg/g f. wt. and 14.05 mg/g f. wt. of chlorophyll a and 21.43 mg/g f.wt. and 16.38 mg/g f.wt. of chlorophyll b, respectively. Additionally, the plants subjected to treatments V+T.v and V+T.h, after 24 hours of being inoculated with ToMV, shows amount of chlorophyll lower than those protected first by *Trichoderma* spp., which was recorded at 12.00 mg/g f.wt. and 11.73 mg/g f.wt., respectively. Conversely, the tomato plants infected with ToMV observed the lowest level of chlorophyll a (8.72 mg/g f.wt.) (Figure 3).

Impact of *Trichoderma* inoculation on total phenolic, flavonoid, and DPPH content

In this study, the control tomato plants revealed the highest value of phenolic content (345.39 mg/g d. wt.), while tomato plants infected with ToMV and then treated with *Trichoderma* spp. (V+T.h and V+T.v) recorded 344.79 mg/g d. wt. and 330.74 mg/g d. wt., respectively (Figure 4).

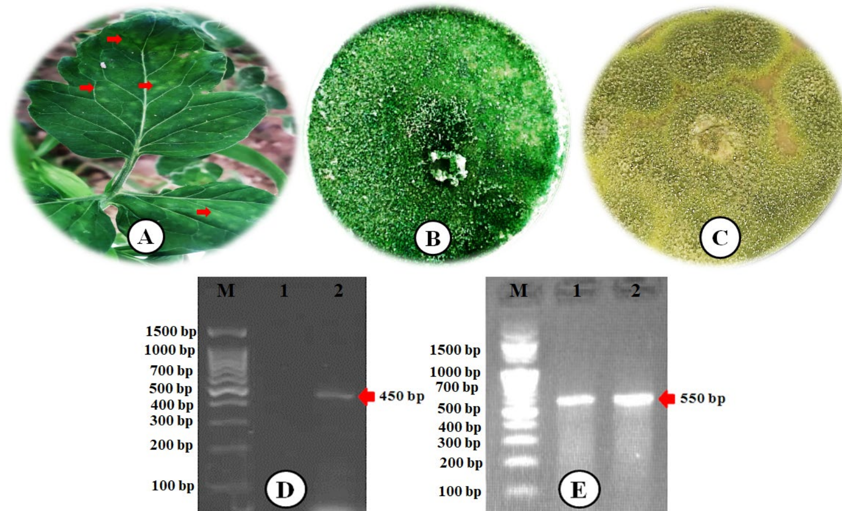

Figure 1. Naturally occurring symptoms of tomato plants infected with ToMV (A); morphology of the isolates used in this study: *T. viride* (B) and *T. harzanium* (C) on PDA. Agarose gel of PCR products of the ToMV-CP gene: M, 1.5 Kbp DNA ladder marker, Lane 1: Healthy tomato plant (Control), Lane 2: Tomato plant infected with ToMV (D); ITS gene: Lane 1: *T. viride*: Lane 2: *T. harzanium* (E).

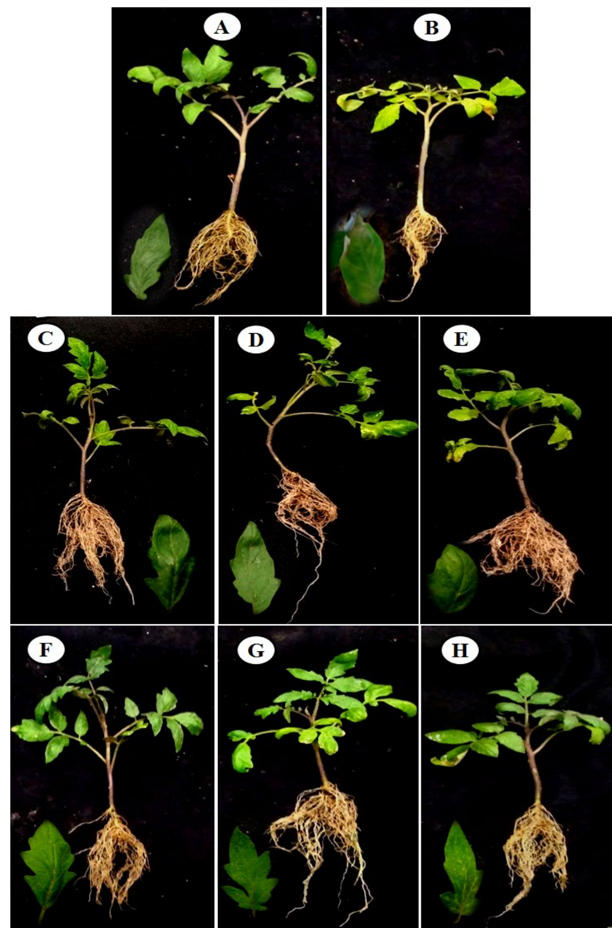

Figure 2. Effect of *Trichoderma* spp. on ToMV-infected tomato plants under greenhouse conditions. A: tomato plants control; B: tomato plants inoculated with ToMV only; C: tomato plants treated with *T. viride*; D: tomato plants inoculated with *T. viride* 48 h before inoculation with ToMV (protective application); E: tomato plants inoculated with ToMV48 h before treated with *T. viride* (curative application); F: tomato plants treated with *T. harzanium*; G: tomato plants inoculated with *T. harzanium* 48 h before inoculation with ToMV (protective application); H: tomato plants inoculated with ToMV48 h before treated with *T. viride* (curative application).

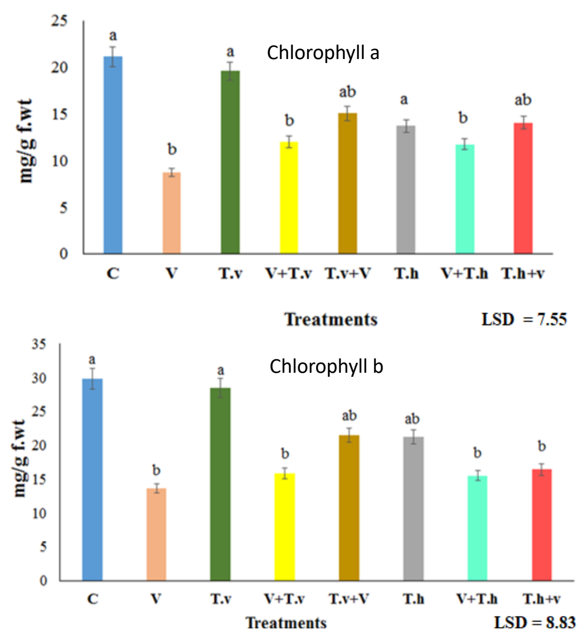

Figure 3. Effect of treatment with *Trichoderma* spp. on chlorophyll a and chlorophyll b of tomato plants. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T. viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T. viride*; T.v+V: tomato plants treated by *T.viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T. harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T. harzianum*; T.h+V: tomato plants treated by *T. harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

There was no significant difference between the treatments with both *Trichoderma* spp., either T.v (313.42 mg/g d. wt.) or T.h (307.14 mg/g d. wt.). However, the lowest value was observed in the case of the treatment with T.v+V (305.10 mg/g d. wt.). The tomato plants infected with ToMV were reported at 274.31 mg/g d. wt., followed by treatment with T.h+V (247.12 mg/g d. wt.).

Regarding the flavonoid content (Figure 4), the treatment with T.h+V and T.v+V recorded the highest level of flavonoid content (225.73 mg/g d. wt., 216.87 mg/g d. wt., respectively). It showed a significant change in the case of the four treatments: V+T.v, 213.35 mg/g d. wt.; V+T.h, 211 mg/g d. wt.; T.v, 201.24 mg/g d. wt.; T.h, 200.17 mg/g d. wt. The tomato plants infected with ToMV only reported the lowest flavonoid content (195.41 mg/g d. wt.).

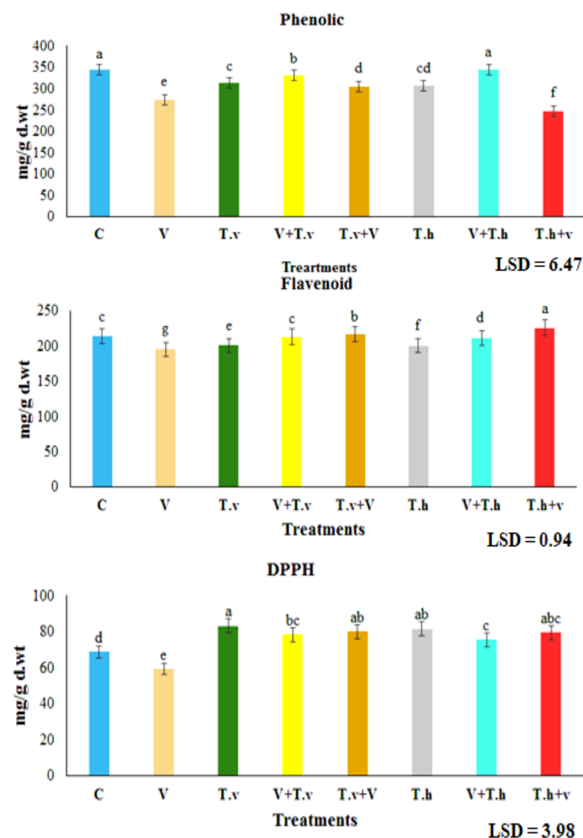

Figure 4. Effect of *Trichoderma* spp. treatments on phenolic content, flavonoids, and DPPH in tomato plants. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T.viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T.viride*; T.v+V: tomato plants treated by *T.viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T.harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T.harzianum*; T.h+V: tomato plants treated by *T.harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

The DPPH method detected free radical scavenging activities in eight tomato plant treatments. The results revealed that the *Trichoderma* spp. treatments significantly increased free radical scavenging activities (Figure 4). The T.v-treated tomato plants alone showed the highest DPPH levels at 83.35 mg/g, followed by those treated with T.h. (81.64 mg/g d. wt.), T.v+V (80.19 mg/g d. wt.), and T.h+V (79.48 mg/g d. wt.). No significant difference was observed between V+T.v (78.50 mg/g d. wt.) and V+T.h (75.63 mg/g d. wt.). Furthermore, the tomato plants infected with ToMV only recorded the lowest DPPH level (59.37 mg/g d. wt.), as demonstrated in Figure 4.

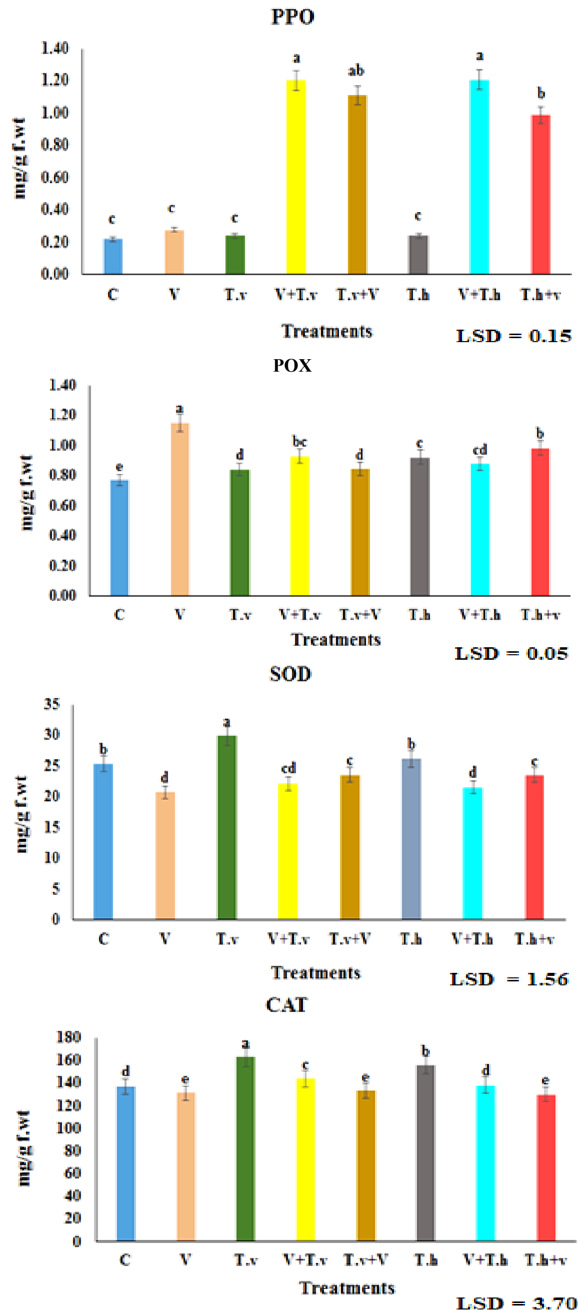

Figure 5. Effect of *Trichoderma* spp. treatments on polyphenol oxidase (PPO), peroxidase (POX), superoxide dismutase (SOD), and catalase (CAT) activities in tomato plants. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T. viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T. viride*; T.v+V: tomato plants treated by *T. viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T. harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T. harzianum*; T.h+V: tomato plants treated by *T. harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

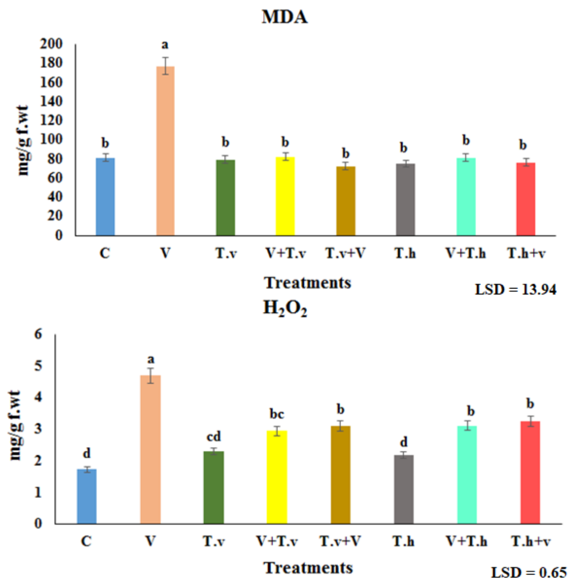

Figure 6. The effects of *Trichoderma* spp. treatments on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in tomato plants. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T. viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T. viride*; T.v+V: tomato plants treated by *T. viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T. harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T. harzianum*; T.h+V: tomato plants treated by *T. harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

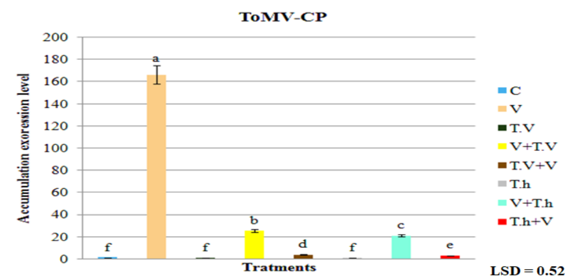

Figure 7. Effect of *Trichoderma* spp. on accumulation level of the ToMV-CP gene. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T. viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T. viride*; T.v+V: tomato plants treated by *T. viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T. harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T. harzianum*; T.h+V: tomato plants treated by *T. harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

Effect of *Trichoderma* inoculation on antioxidant enzymatic activities

The evaluated four antioxidant enzyme activities (PPO, POX, SOD, and CAT) were variable (Figure 5). The results indicate that PPO showed elevated activity in tomato plants treated after the infection with ToMV, where V+T.v and V+T.h recorded 1.20 mg/g f. wt. and 1.21 mg/g f. wt., respectively, followed by the T.v+V (1.11 mg/g) and T.h+V (0.99 mg/g) treatments. The tomato plants infected with ToMV alone recorded 0.28 mg/g f. wt. No significant difference was noticed between the treatments of T.v. (0.24 mg/g f. wt.) and T.h. (0.24 mg/g f. wt.). For POX activity, the tomato plants that were infected with ToMV showed the most significant degree of peroxidase activity (1.15 mg/g f. wt.). In comparison, the treatment of T.h+V recorded 0.98 mg/g f. wt., followed by V+T.v (0.93 mg/g f. wt.) and T.h (0.92 mg/g f. wt.). No significant change was found between T.v+V and T.v (0.85 mg/g f. wt. and 0.84 mg/g f. wt., respectively), as shown in Figure 5.

In the case of SOD and CAT, it was reported that plants treated with *T. viride* or *T. harzianum* revealed the highest levels of SOD and catalase activity (Figure 5). Conversely, in SOD, T.v. and T.h. treatments reported 29.88 mg/g f. wt. and 26.13 mg/g f. wt., respectively. Moreover, the treatments with T.v+V (23.57 mg/g f. wt.) and T.h+V (23.50 mg/g f. wt.) recorded higher SOD activity than those infected initially with ToMV: V+T.v (22.20 mg/g f. wt.) and V+T.h (21.53 mg/g f. wt.) and tomato plants infected with ToMV only (20.47 mg/g f. wt).

Likewise, in CAT activity, tomato plants treated with T.v. or T.h. only showed a significant increase of 162.84 mg/g f. wt. and 156.01 mg/g f. wt., respectively (Figure 5). Moreover, the treatments with V+T.v (144.13 mg/g f. wt.) and V+T.h (138.36 mg/g f. wt.) resulted in increased levels higher than T.v+V (133.12 mg/g f. wt.) and T.h+. V (130.14 mg/g f. wt.). In contrast, the tomato plants infected with ToMV recorded the lowest level of catalase activity (131.35 mg/g f. wt.).

Effect of *Trichoderma* inoculation on oxidative stress markers

The oxidative stress markers, including MDA and H_2O_2, were measured in eight treatments of tomato plants (Figure 6). Compared to the *Trichoderma* treatments, tomato plants inoculated with ToMV noted a crucial increase in the contents of MDA and H_2O_2. For MDA, the treatment of ToMV alone recorded the highest level (177.13 mg/g). Additionally, the treatments of V+T.v (82.30 mg/g) and V+T.h (81.10 mg/g) showed higher levels of increase than T.h+V (76.46 mg/g) and T.v+V (72.43 mg/g). There was no noticeable difference between the treatments of T.v or T.h, which showed the lowest levels (79.45 mg/g and 74.99 mg/g, respectively). In H_2O_2, similar to MDA, the treatment of ToMV recorded the highest level (4.69 mg/g). Like MDA, the treatments with T.v (2.30 mg/g) and T.h (2.19 mg/g) resulted in the lowest levels of H_2O_2 (Figure 6).

Effect of *Trichoderma* inoculation on the ToMV-CP accumulation level

In the greenhouse experiment, treatment with *T. viride* or *T. harzianum*, either before or after 24 hours of infection with ToMV in tomato plants, decreased the amount of accumulation of ToMV-CP (CP gene expression level) when compared with the ToMV-infected tomato plants alone (166.17-fold). This means that the application of *T. viride* and *T. harzianum* significantly decreased the accumulation levels of the viral CP in the *Trichoderma*-treated tomato plants (Figure 7).

Expression analysis of defense-related genes

Polyphenol biosynthetic pathway: The expression levels, determined using q-PCR, of the two genes (*PAL-1* and *CHS*) belonging to the polyphenol biosynthetic pathway showed an increase in tomato plants treated with T.v, both before and after infection with ToMV. In the case of *PAL-1*, the treatment T.v+V recorded a transcriptional level that is 3.33-fold higher than that of V+T.v (2.61-fold). Similarly, the treatment with T.h+V recorded a transcriptional level (2.19-fold) higher than V+T.h (1.19-fold). No significant difference was observed between plants treated with T.v. (1.27-fold) and those treated with T.h. (1.21-fold). However, it is still higher than that recorded in ToMV-infected tomato plants (Figure 8). In the case of *CHS*, the treatment with V+T.v gave the highest expression level of 59.42-fold, while the T.v treatment showed a transcriptional level of 53.82-fold greater than that of ToMV-infected plants. In contrast, a significant downregulation with treatments (T.h+V, V+T.h, T.h, and T.h+V) was recorded at 51.77-, 47.18-, 46.47-, and 39.04-fold, respectively (Figure 8).

Pathogenesis-related genes: In this study, the expression of two pathogenesis-related genes (*POD* and *PR-2*) was determined in the treated tomato plants (Figure 8). For *POD*, V+T.h-treated plants reported the highest expression level of 32.83-fold, while tomato plants treated with T.h. recorded a transcriptional level (10.20-fold) more significant than

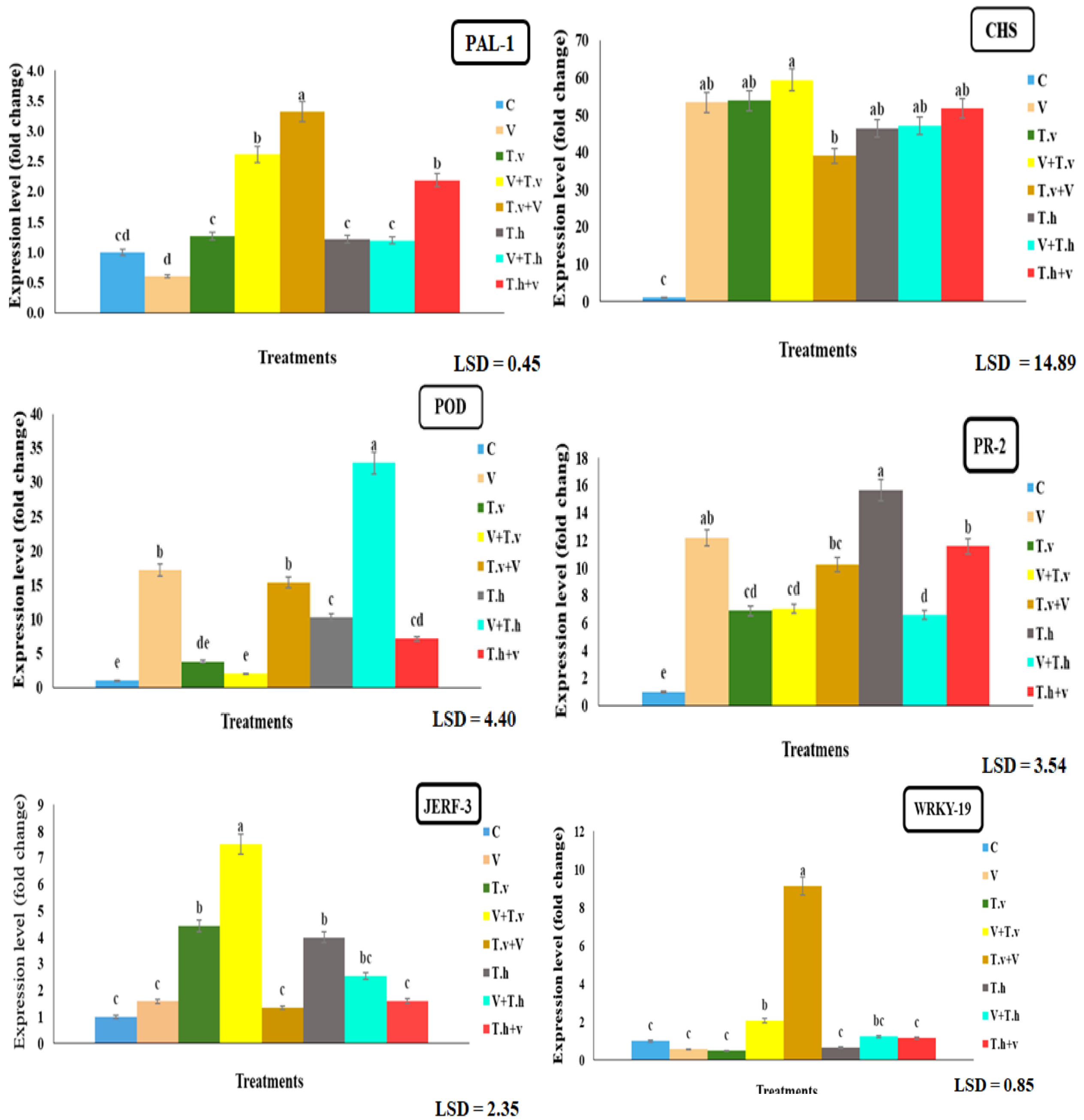

Figure 8. Effect of *Trichoderma* spp. treatments on expression levels for polyphenol biosynthetic pathway genes (*PAL-1* and *CHS*), pathogenesis-related protein genes (*POD* and *PR-2*), and Jasmonic pathway genes (*JERF3* and *WRKY19*) measured by qRT-PCR in tomato plants. C: control tomato plants; V: tomato plants infected by ToMV; T.v: tomato plants treated with *T. viride*; V+T.v: tomato plants inoculated with ToMV before 24 hours from treatment with *T. viride*; T.v+V: tomato plants treated by *T.viride* before 24 hours from infection by ToMV; T.h: tomato plants treated with *T. harzianum*; V+T.h: tomato plants inoculated with ToMV before 24 hours from treatment with *T. harzianum*; T.h+V: tomato plants treated by *T. harzianum* before 24 hours from infection with ToMV. The columns represent the mean values of five replicates, and the error bars represent the standard deviations. Significant differences were calculated using one-way ANOVA according to the least significant difference (LSD) method at a $p \leq 0.05$. The same letters are not significantly different.

Table 1. Sequences of all primers used in this study.

Genes name	Primers name	Direction	Primers Sequence (5`–3`)	Related pathway
Phenylalanine ammonia-lyase 1	PAL-1	Forward Reverse	ACGGGTTGCCATCTAATCTGACA CGAGCAATAAGAAGCCATCGCAAT	Phenylpropanoid biosynthetic
Chalcone synthase	CHS	Forward Reverse	CACCGTGGAGGAGTATCGTAAGG TGATCAACACAGTTGGAAGGCG	
β-1,3-glucanase	PR-2	Forward Reverse	TATAGCCGTTGGAAACGAAG CAACTTGCCATCACATTCTG	Pathogenesis-related proteins
Peroxidase	POX	Forward Reverse	CCTTGTTGGTGGGCACACAA GGCCACCAGTGGAGTTGAAA	
Jasmonate and ethylene-response factor 3	JERF3	Forward Reverse	GCCATTTGCCTTCTCTGCTTC GCAGCAGCATCCTTGTCTGA	Jasmonic pathway
WRKY transcription factor 19	WRKy19	Forward Reverse	CGTGCAGCAGCAAAGCAA GTCGCAGGTATGCTCGTTGA	
Beta actin	β-actin	Forward Reverse	ATGCCATTCTCCGTCTTGACTTG GAGTTGTATGTAGTCTCGTGGATT	Housekeeping gene
Elongation factor *1* alpha	EFα-1	Forward Reverse	GAACTGGGTGCTTGATAGGC AACCAAAATATCCGGAGTAAAAGA	
Internal transcribed spacer	ITS	Forward Reverse	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Eukaryotic identification
ToMV-Coat Protein	ToMV-CP	Forward Reverse	ACGACTGCCGAAACGTTAGA CAAGTTGCAGGACCAGAGGT	Viral replication

Table 2. The HPLC analysis for quantification of active biomolecules in *Trichoderma* extracts.

No.	Compounds	*Trichoderma viride*		*Trichoderma harzanium*	
		Retention time	Concentration (µg/mL)	Retention time	Concentration (µg/mL)
1	Gallic acid	3.0	8.38	3.0	4.87
2	Protochateuic acid	3.5	9.46	3.6	7.04
3	Catechin	3.8	2.74	3.8	1.65
4	Esculatin	4.4	6.15	4.4	7.08
5	Vanillic acid	4.9	3.04	4.9	5.17
6	Pyrochatechol	5.1	4.42	5.4	7.85
7	Coumarine	-	-	11.8	3.44
8	Cinnamic acid	-	-	12.3	1.32
9	4.3-indul butyl acetic acid	-	-	13.4	8.79
10	Naphthyl acetic acid	-	-	14.6	7.09

Table 3. Effect of treatment with *Trichoderma* spp. on the growth parameters of tomato plants infected with ToMV.

Treatments	Plant length ± SD	Length ± SD		Fresh weight ± SD		Dry weight ± SD		Leaves number ± SD
		Shoot	Root	Shoot	Root	Shoot	Root	
C	25.30±1.61^{b}	13.70±0.10^{a}	11.60±0.02^{f}	1.93±0.59^{a}	0.63±0.06^{d}	0.33±0.01^{b}	0.12±0.01^{f}	22.67±4.51^{c}
V	14.33±1.04^{c}	8.16±0.13^{f}	6.17±0.06^{g}	0.79±0.31^{b}	0.18±0.02^{e}	0.16±0.01^{e}	0.08±0.01^{g}	11.67±2.08^{d}
T.v	27.07±0.40^{b}	12.57±0.26^{c}	14.50±0.02^{e}	2.07±0.15^{a}	1.00±0.10^{cd}	0.32±0.01^{b}	0.32±0.01^{a}	25.67±2.08^{abc}
V+T.v	27.17±1.76^{b}	11.67±0.01^{d}	15.50±0.03^{c}	2.26±0.51^{a}	1.45±0.18^{ab}	0.40±0.02^{a}	0.18±0.01^{cd}	31.00±2.65^{a}
T.v+V	31.67±4.51^{a}	13.00±0.01^{b}	18.67±0.03^{a}	2.02±0.15^{a}	1.38±0.45^{abc}	0.29±0.01^{c}	0.19±0.01^{c}	29.33±1.53^{ab}
T.h	28.17±0.58^{b}	12.90±0.03^{b}	15.27±0.02^{d}	2.04±0.24^{a}	1.11±0.34^{bc}	0.25±0.01^{d}	0.18±0.01^{d}	25.67±1.53^{abc}
V+T.h	26.83±0.76^{b}	10.50±0.15^{e}	16.33±0.02^{b}	2.11±0.36^{a}	1.28±0.28^{abc}	0.25±0.02^{d}	0.15±0.01^{e}	24.00±1.00^{bc}
T.h+V	27.83±0.29^{b}	11.50±0.04^{d}	16.33±0.02^{b}	2.41±0.47^{a}	1.69±0.10^{a}	0.38±0.02^{a}	0.25±0.01^{b}	28.00±6.08^{abc}

*Values of each column followed by the same letter are not significantly different according to Tukey's multiple range test ($p \leq 0.05$), each value represents the mean of five replicates± SD.

those treated with T.v. Significantly, the treatment with T.v+V and T.h+V showed transcriptional levels of 15.33 and 7.13-fold, respectively. In the case of tomato plants infected with only ToMV, the expression level of *POD* was 17.13-fold. Regarding *PR-2*, treatment with T.h. recorded the highest transcriptional level (15.66-fold). The plant infected with ToMV alone recorded a transcriptional level (12.19-fold) higher than the tomato plants treated with T.h+V, with a transcriptional level of 11.58-fold, while T.v+V recorded 10.26-fold. Tomato plants treated with T.v., whether before or following the infection with ToMV (T.v+V and V+T.v), recorded 7.05-fold and 6.89-fold, respectively, while T.h+V resulted in an expression level of 11.58-fold (Figure 8).

Jasmonic biosynthetic pathway: In the case of two jasmonic pathway genes (*JERF3* and *WRKY19*) (Figure 8), the results revealed that *JERF3* gene expression was downregulated in the three treatments, T.h., V+T.h, and T.h+V, which recorded transcriptional levels of 4-, 2.55-, and 1.60-fold, respectively. The plants treated with T.v. only and V+T.v recorded the highest expression levels of 1.58-, 4.44-, and 7.53-fold, respectively, while T.v+V recorded the lowest level of 1.35-fold (Figure 8). For *WRKY19*, T.v+V recorded an expression level of 9.13-fold, which was higher than the transcriptional level of V+T.v (2.07-fold). In contrast, the treatment with V+T.h showed a transcriptional level of 1.23-fold, which was more significant than the treatment with T.h+V (1.17-fold). The tomato plants infected with ToMV recorded a downregulation of expression level of 0.57-fold, while the treatment with T.h. or T.v. only recorded expression levels of 0.66- and 0.50-fold, respectively (Figure 8).

DISCUSSION

Several pathogens, including ToMV, significantly impact the tomato crop, resulting in significant losses for the agricultural economy (Alkowni et al., 2019). Therefore, early detection is vital for selecting the proper treatment and preventing the disease from spreading. *Trichoderma* species are currently employed as biofertilizers and biopesticides due to their capacity to protect plants, trigger development, and regulate phytopathogenic substances in diverse agricultural environments (Azarmi et al., 2011). Recently, *T. harzianum and T. viride* were used as biocontrol agents as they are good producers of secondary metabolites and have efficiently inhibited plant pathogens such as *Fusarium solani* or mycotoxin-producing fungi by competition, antibiosis, and inducing plant defense responses. Specifically, *T. harzianum* has been proven to degrade its host hyphae and compete for space and nutrients against a wide range of fungal plant pathogens (Guzmán-Guzmán et al. 2023). Our study identified T. viride and T. harzianum isolates by sequencing of ITS region after being amplified by PCR. It has been reported that a sequence of PCR amplicons of the ITS gene was used to confirm the morphological identification of *Trichoderma* isolates (Abdelkhalek et al., 2022). Similarly, Aseel et al. (2023) used specific primers from the ITS region for molecular identification of *Trichoderma* isolates based on phylogenetic analysis of this sequenced region.

In this study, the results of HPLC chromatogram characterization of Tvd44 and Th-DA66 filtrates showed six chemical components in common. Many of these detected compounds, including polyphenols like catechins and gallic acid, have extreme antioxidant activity and can play an important therapeutic role in antimicrobial, antiallergenic, and anti-inflammatory activities (Mani et al. 2020; Shaygannia et al. 2016; Tian et al. 2014). Moreover, another study suggested that the compounds of polyphenols can work as elicitor molecules and have roles in SAR signaling (Abdelkhalek et al. 2021). Additionally, coumarins and phenolic acids may be employed by inducing rupture of cell membranes, inhibition of the synthesis of cell walls, and dysfunction of the mitochondria (Raman and Muthukathan, 2015). Furthermore, protocatechuic acid can also inhibit the growth of pathogens (Zhang et al. 2022), and vanillic acid is considered a component that may as anti-inflammatory, anticancer, antiosteolytic, and anti-Alzheimer (Brimson et al. 2019).

In addition to above-mentioned compounds, some growth-regulating compounds have been found in *Trichoderma* filtrates, such as cinnamic acid, which is considered one of the constituents that claim growth-promoting activity (Steenackers et al., 2019); indole-3-butyric acid (IBA), which is used efficiently in developing plant regulators to motivate the germination of the plant and the formation of rooting in various plants (Dong et al., 2018); and naphthalene acetic acid (NAA), which is known as a plant hormone in the auxin family. Moreover, NAA is a component in commercial postharvest horticultural yields and a rooting mediator that can be utilized for vegetative propagation of plants from cuttings of stem and leaf (Khandaker et al. 2017).

In this study, tomato plants treated with *T. viride* or *T. harzianum* showed significantly increased growth parameters, including plant length, dry weight, fresh weight, and leaf number, compared to ToMV-infected tomato plants only. Additionally, treatment with *Trichoderma* species reduced the ToMV symptoms due to the suppression of the accumulation of the viral CP in tomato plant leaves. This means that *Trichoderma* spp. acted as a protecting agent before or after the infection of tomato plants with ToMV. Conversely, the tomato plants exposed only to ToMV infection exhibited a decrease in all growth parameters. Similarly, diverse reports have demonstrated that *Trichoderma* spp. triggered plant growth and induced root and shoot length and dry weight (Garnica-Vergara et al., 2016; Mahato et al., 2018; Sulaiman et al., 2020; Zin and Badaluddin, 2020). In another instance, plant growth parameters were induced by *Arbuscular mycorrhizal* fungi. It has been shown that *Arbuscular mycorrhizal* colonization reduces the passive effects of ToMV infection compared to ToMV-uninfected plants (Aseel et al. 2019b).

In this study, ToMV-*infected plants* treated with *T. viride* or *T. harzianum* contained the highest levels of chlorophylls a and b, followed by treatments T.v.+V and T.h.+V., compared to those infected only with ToMV. These results correspond to many investigations, demonstrating that using *Trichoderma* spp. increased chlorophyll content in the treated plants paralleled the duration of viral infection, which reduced chlorophyll content (Sulaiman et al., 2020; Abdelkhalek et al., 2022; Azarmi et al., 2011; Aseel et al., 2023). It has been reported that viral infection affected the chlorophyll content that controlled the photosynthesis process and caused morphological and physiological changes (Abdelkhalek et al. 2021; Tamandegani et al. 2021).

In this study, the phenolic levels increased in the case of treatments of V+T.h and V+T.v, and then showed higher levels in *Trichoderma* spp. (*T. viride* or *T. harzianum*) regardless of whether they were inoculated with ToMV alone. This result is in agreement with Awad-Allah et al. (2022), who used both *T. viride* and *T. harzianum* treatments on cherry tomato plants grown in greenhouse conditions, with or without *F. solani* and found that various *Trichoderma* treatments significantly improved the content of total phenol either in presence or absence of *F. solani* infection. Our results are in line with the findings of Kumar et al. (2022), who used *T. viride* against *A. solani* and discovered that the extreme creation of total phenolic constituents was in *T. viride-treated* plants with *A. solani*. Polyphenolic chemicals such as flavonoids can change the peroxidation kinetics by reducing the fluidity of membranes, which is another mechanism supporting the antioxidative characteristics of phenolics (Karuppanapandian et al., 2011). Plants with higher concentrations of phenolic compounds have been linked to enhanced defense responses (Singh et al., 2013a, b). Similarly, our result exhibited enhanced flavonoid compounds for both *T. viride*- or *T. harzianum*-treated plants along with virus or alone, where T.h+V and T.v+V recorded the highest levels, followed by V+T.h, V+T.v, T.v., and T.h., respectively. This is contrary to Sobhy et al. (2022), who discovered higher flavonoid content in wheat seeds infected by *F. graminearum* compared to the healthy control.

DPPH is considered an indication of free radical scavenging activity. In our study, DPPH recorded higher levels in treatments with *Trichoderma* spp. than in the case of ToMV infection alone. In the same way, Abdelkhalek et al. (2022) discovered that the impact of *B. amyloliquefaciens* strain TBorg1CF had considerably higher DPPH activities than plants treated with the tobacco mosaic virus. Another report showed that DPPH activity demonstrated that the tomato cultivars Matina and Cochoro showed the most significant antioxidant activity improvements during water stress treatments (Klunklin and Savage, 2017). Conversely, PPO recorded high levels in the treatments of V+T.h and V+T.v, and the infection with ToMV showed an increase in PPO compared to treatments with *T. viride* or *T. harzianum*, which recorded the same value (0.24 mg/g f. wt.). In contrast, the PPO activity was drastically reduced in the *T. harzianum*-treated plants, or in both, where PPO activity was passively affected by all treatments of tomato plants with the pathogen *T. harzianum* (Patil et al. 2011; Mahmoud et al. 2021). However, our result agrees with that of Mei et al. (2019), who revealed an increase in the activity of PPO as a result of different *Trichoderma* spp. treatments. It was reported that PPO activity protects plant cells against a low level of oxidative stress (Boeckx et al. 2015b).

A crucial enzyme in cellular resistance against the ROS in living things is SOD. Consequently, it is a crucial antioxidant capability marker (Alici and Arabaci, 2016). In our study, CAT and SOD increased in the treatments with *T. viride* or *T. harzianum* while decreasing in ToMV-infected tomato plants compared to the untreated tomato plant control. T.v+V and T.h+V treatments are considered at the same level of

control as before treatment with ToMV. Similarly, an increase in catalase and SOD in plants treated with Th23 was reduced in plants treated with TMV compared to control plants (Abdelkhalek et al. 2022). Notably, CAT converts the components of ROS that damage plants to less toxic and more stable molecules (Abdelkhalek et al. 2021; Dumanović et al. 2021). The ROS, also known as oxidative stress, are produced by the host plant's first line of protection against most pathogens, and their main associated function is the alteration of physiological and biological mechanisms (Keswani et al., 2019; Kumar et al., 2022). Additionally, *T. viride* treatment increased total phenolics, total flavonoids, and the activities of CAT, PPO, and APX enzymes and decreased MDA and H_2O_2 contents in tomato seedlings (Metwally and Soliman, 2023).

The results of this study also indicated higher levels of MDA and H_2O_2 in the case of tomato plants infected with ToMV alone compared to those of plants treated with *T. viride* or *T. harzianum*. The same findings were reported on PVY in potato plants (Aseel et al. 2022) and Bean Yellow Mosaic Virus in faba bean plants (Yassin et al. 2024) were induced the levels of both MDA and H_2O_2 in only plants infected with viruses. On the other hand, ZnO-NPs were noticed as a treatment for ToMV and an increase in levels of oxidative markers (MDA and H_2O_2) when infected with ToMV (Sofy et al. 2021).

The pathway of phenylpropanoid plays a vital role in the plant defense system, and it showed increased expression after the pathogen infection, resulting in an increase in enzymatic activity and the building up of phenolic compounds (López-Gresa et al., 2012; Yadav et al. 2020). Similarly, in this study, *PAL-1* expression was upregulated in *T. viride*- or *T. harzianum*-treated tomato plants with ToMV. Likewise, in potato plants treated with nano-clay, higher expression of *PAL-1* was recorded compared to those inoculated with PVY only (Aseel et al., 2022). Moreover, the expression levels of the polyphenol biosynthetic pathway, *PAL-1*, and *CHS*, were upregulated in the *Trichoderma* spp.-treated plants, regardless of whether they were exposed to ToMV or not. In a recent study, *T. harzianum* has been used as a biocontrol agent for *Rhizoctonia solani*, where *T. harzianum* successfully recognizes and invades host cells and kills plant pathogens by regulating various differentially expressed genes at different culture periods. It is also upregulating the expression of carbohydrate activity enzyme-related genes, improving the activity of hydrolase to hydrolyze the host cell wall (Wang et al. 2024).

In this investigation, two genes of pathogenesis-related proteins, *PR-2* and *POD*, were studied. For *POD*, the treatment of V+T.h. recorded the highest expression level, followed by the ToMV infection. The result agrees with that of the study on *D. stramonium* leaves, which showed a higher expression level of *POD* in PEA1-CF-treated plants than that in control plants (Abdelkhalek et al., 2020). Regarding *PR-2*, treatment of plants with *T. harzianum* showed a higher expression level than ToMV alone. In contrast, using *Streptomyces cellulosae* Actino 48 as a biocontrol agent, the *PR-2* gene in ToMV-infected tomato plants showed a higher expression level than in the treatment with Actino 48 alone (Abo-Zaid et al. 2020). Based on various studies, *Trichoderma* spp. colonization causes metabolic changes in the root, likely the activation of PR-proteins, that strengthen the plant's defense against numerous microbial diseases. The synthesis of plant enzymes involved in defense, such as chitinases, β-1,3-glucanases, different peroxidases, and the lipoxygenase-pathway hydroperoxide lyase, can also be generally boosted by *Trichoderma* (Hanson and Howell, 2004; Harman, 2006).

In response to various stressors, a transcription factor known as jasmonate and ethylene-responsive factor 3 (*JERF3*) controls the expression of several genes linked to defense via the jasmonate and ethylene pathways in the plant (Müller and Munné-Bosch, 2015; Pérez-Llorca et al. 2023). In this study, the *JERF3* gene from the jasmonic biosynthetic pathway showed an increased expression level in V+T.v. -treated plants, followed by *Trichoderma* (*T. viride* or *T. harzianum*)-treated plants. Furthermore, a reduction was observed in plants infected with ToMV alone. Similarly, *WRKY19* had a higher expression level in T.v.+V -treated plants than in V+T.v.- treated plants but a lower expression level in ToMV-infected plants. In another study, Huang et al. (2016) found that the tomato *WRKY* genes have a unique pattern of spatial and temporal gene expression, control a variety of developmental processes and are also essential for defensive signals produced in response to tomato yellow leaf curl virus (TYLCV) infection in tomato. Furthermore, signal transduction pathways like ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) are primarily involved in plant-induced resistance. JA, SA, and ET contents increased in varying degrees in the interaction between *Trichoderma* and

Arabidopsis, tomato, and cucumber, indirectly enhancing plant resistance (Hinterdobler et al., 2021).

CONCLUSION

In this current study, the two species *T. viride* and *T. harzianum* were used as protective and curative agents against ToMV infection in tomato plants. Additionally, their effects on plant growth parameters, chlorophyll content, antioxidant enzymes, oxidative stress markers, and viral CP protein accumulation were studied. The results proved the positive role of *Trichoderma* spp. in enhancing ToMV viral resistance in tomato plants through upregulating pathogen-defense-related genes in different pathways and increasing antioxidant enzyme activities.

AUTHORS' CONTRIBUTIONS

Conceptualization, D.G.A.; methodology, D.G.A. and A.A.; software analysis, D.G.A. and A.A.; validation, D.G.A. S.E.E. and R.M.G.; data curation, D.G.A and A.A.; writing-original draft preparation, D.G.A. and A.A.; writing-review, S.E.E. and R.M.G.; writing-review and editing, D.G.A. and R.M.G.; visualization and supervision, D.G.A., S.E.E., and R.M.G. All authors have read and agreed to the publishing of the manuscript.

FUNDING

This research was not externally funded.

ACKNOWLEDGMENTS

The authors express their sincere thanks to the City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt, and the Faculty of Science, Tanta University, for providing the necessary research facilities.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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