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Induction of Plant Resistance against Tobacco Mosaic Virus Using the Biocontrol Agent *Streptomyces cellulosae* Isolate Actino 48

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Abstract: Viral plant diseases represent a serious problem in agricultural production, causing large shortages in the production of food crops. Eco-friendly approaches are used in controlling viral plant infections, such as biocontrol agents. In the current study, *Streptomyces cellulosae* isolate Actino 48 is tested as a biocontrol agent for the management of tobacco mosaic virus (TMV) and inducing tomato plant systemic resistance under greenhouse conditions. Foliar application of a cell pellet suspension of Actino 48 (2×10^7 cfu. mL^{-1}) is performed at 48 h before inoculation with TMV. Peroxidase activity, chitinase activity, protein content, and the total phenolic compounds are measured in tomato leaves at 21 dpi. On the other hand, the TMV accumulation level and the transcriptional changes of five tomato defense-related genes (PAL, PR-1, CHS, PR-3, and PR-2) are studied. Treatment with Actino 48 before TMV inoculation (48 h) induced tomato plants to increase their levels of peroxidase and chitinase enzymes. Furthermore, a significant increase in the concentration of total phenolic compounds was observed in Actino 48 and TMV-treated tomato plants compared to TMV-treated tomato plants alone. Treatment with Actino 48 reduced the TMV accumulation level (53.8%) compared to treatment with the virus alone. Actino 48 induced plant growth, where the fresh and dry weights of tomato plants increased. Additionally, significant increases of the PAL, PR-1, CHS, and PR-3 transcripts were observed. On the other hand, a higher induction of PR-2 was only observed in TMV-treated tomato plants. In conclusion, *S. cellulosae* isolate Actino 48 can be used as a biocontrol agent for the reduction of symptoms and severity of TMV.

Keywords: tobacco mosaic virus; *Streptomyces cellulosae*; biocontrol; gene expression

1. Introduction

Tobacco mosaic virus (TMV, genus *Tobamovirus*) comprises positive-sense single-stranded RNA and infects over 885 plant species in 65 families, especially tobacco and tomato plants, as well as other members of the family Solanaceae [1,2]. Infection with TMV causes mosaic symptoms on leaves and the yellowing of plant tissue. The virus causes severe economic losses worldwide [3]. The management of TMV is very difficult because it is easily dispersed wherever it is transmitted mechanically, and symptoms show at 7 to 14 days past infection (dpi) once a susceptible plant is infected [4].

The management of viral plant diseases using beneficial microbes has received much interest, because it is a safe and friendly approach for controlling viruses [5]. Most of the biocontrol agents used for controlling viral plant diseases are bacteria, for example, *Bacillus* spp. [6] and *Pseudomonas fluorescens* [7], besides some fungi, such as *Trichoderma* spp. [8]. *Streptomyces* spp. is characterized as a large group of actiobacteria that contains more than 780 species and 30 subspecies [9]. Additionally, *Streptomyces* spp. has a great role in inhibiting the interactions between plant and pathogens, as well as in the biocontrol of plant fungal and bacterial diseases [10]; however, even now, the utilization of *Streptomyces* spp. for the biocontrol of viral plant diseases is limited and its prospective mechanisms against viral diseases are not clear [11].

Induced resistance (IR) can be divided into two major mechanisms. The first is systemic acquired resistance (SAR), which is induced by plant pathogens or chemical compounds, and its regulation is based on salicylic acid (SA). This resistance pathway mainly features pathogenesis-related proteins and other disease resistance proteins. A number of the pathogenesis-related proteins (PRs) play important roles as anti-pathogenic agents [12]. The induction of plants to synthesize PRs is accomplished by infection with viruses, bacteria, fungi, or viroids [13–15]. Additionally, Neetu et al. [16] documented that the level of various PR proteins, such as isozymes of peroxidase and chitinase, are increased by biotic inducers. Defense enzyme activities can be induced by *Streptomyces* spp., which represents an important group of plant-associated microorganisms [17]. Defense-related proteins have been shown to be induced in tomato plants treated with *P. fluorescens* that have been infected with the viral tomato spotted wilt virus (TSWV) pathogen [18]. The second major mechanism is induced systemic resistance (ISR), which is induced by plant microbes that promote growth, and its regulation is based on jasmonic acid and ethylene, which enables faster defense responses and greater defensive capabilities in plants when resisting disease [19–21]. Alazem and Lin [22] confirmed that plant resistance for viral infection may be related to both mechanisms.

Among the secondary metabolites, polyphenolic compounds play many crucial roles in plant growth, development, and resistance against various biotic and abiotic stresses [23]. Beside it being the first enzyme in the phenylpropanoid pathway, PAL is involved in the biosynthesis of salicylic acid [24]. Upon pathogen infection, the activation of SA is usually correlated with the accumulation of PR-1 as a SA marker gene [25]. Peroxidase, which is considered an imperative pathogen-related protein or defense protein, is implicated in various physiological responses in plants to biotic stresses, as well as for studying pollutant degradation and management [26,27]. Galal [28] reported that treatment with certain *Streptomyces* strains induced systemic acquired resistance (SAR) for virus infections, while *P. aeruginosa* was effective at enhancing the resistance of tobacco plants to TMV [29]. In addition, an antiviral agent from *S. noursei* var *xichangensisn* has been shown to induce systemic resistance to TMV [30]. Li et al. [11] documented that *S. pactum* Act12 induced systemic resistance in tomato plants against tomato yellow leaf curl virus (TYLCV), where the levels of salicylic and jasmonic acids increased in tomato plants. Induction of resistance was accomplished in tomato plants against tomato mottle virus by the application of PGPR isolates, *B. amyloliquefaciens* 937b, and *B. pumilus* SE-34 [31].

Different bioactive compounds extracted from various strains of *Streptomyces* have been effective at reducing the local lesions of TMV on *Datura metel* plant leaves [4].Additionally, a bioactive compound characterized as ε-poly-L-lysine, produced by *S. ahygroscopicus*, has exhibited a significant protective activity and curative activity against TMV [32].The reduction percentage of mosaic symptoms caused by zucchini yellow mosaic virus (ZYMV) has been shown to change to 95% and 100% with the foliar treatment of cucumbers with *S. albovinaceus* and *S. sparsogenes*, respectively [33]. Also, *T. harzianum* has reduced symptoms of TMV on tomato plants by inducing systemic resistance [34]. Li et al. [35] reported that *Enterobacte rasburiae* BQ9 induced resistance to TYLCV under greenhouse conditions and reduced disease severity, reaching 42%, even at 45 dpi.

In the current study, we investigate the controlling activity of *S. cellulosae* isolate Actino 48 against TMV when it is applied as a foliar treatment 48 h before inoculation with TMV. We also examine its efficiency for inducing tomato plant growth and systemic resistance under greenhouse conditions. The peroxidase activity,

chitinase activity, protein content, and total phenolic compounds are evaluated. In addition, we study the TMV accumulation level (TMV-coat protein gene), and the transcriptional levels of tomatodefense genes, such as phenylalanine ammonia-lyase (PAL), pathogen-related protein 1 (PR-1), chalcone synthase (CHS), pathogen-related protein 3 (PR-3), and pathogen-related protein 2 (PR-2).

2. Materials and Methods

2.1. Plant Materials and Source of Viral Isolate

Virus-free seeds of the GS 12 cultivar of the tomato (*Solanum lycopersicum* L.) plant were obtained from the Ministry of Agriculture, Agriculture Research Center, Egypt. The source of the tobacco mosaic virus (accession No., MG264131) was previously isolated from infected tomato plants [36] and continuously maintained on tobacco plants under greenhouse conditions.

2.2. Actinobacterial Isolate

Actinobacterial isolate Actino 48, registered in GenBank as *S. cellulosae* with the accession number of MT573878 (https://www.ncbi.nlm.nih.gov/nuccore/MT573878.1/ 8 June 2020), was provided by Dr. Gaber A. Abo-Zaid of the City of Scientific Research and Technological Applications (SRTA-City).

2.3. Cultivation of Actinobacteria

S. cellulosae isolate Actino 48 was streaked on a starch nitrate agar medium (SNA) containing the following (as g L^{-1}): starch, 20; KNO_3, 1; $MgSO_4{\cdot}7H_2O$, 0.5; NaCL, 2; $FeSO_4{\cdot}7H_2O$, 0.01. Colonies of *S. cellulosae* were inoculated into an ISP-2 medium containing the following (as g L^{-1}): yeast extract, 4; malt extract, 10; dextrose, 4; pH of 7 [37]. The culture was cultivated at 30 °C and shaken at 200 rpm for 7 days until reaching 2×10^7 cfu. mL^{-1}. The culture was centrifuged at 5590× *g* for 20 min and a cell pellet was collected, washed, and centrifuged as with the previous conditions. After that, the cell pellet of Actino 48 was suspended in sterile distilled water.

2.4. Greenhouse Experimental Design and Antiviral Activity Assay

The antiviral activity of the cell pellet suspension of *S. cellulosae* isolate Actino 48 (2×10^7 cfu. mL^{-1}) was firstly checked with *Datura stramonium* plants, as a local lesion host for TMV, calculated according to the inhibition percentage towards the number of local lesions developed on the leaves. After surface sterilization of the tomato seeds, the cultivation process occurred in plastic pots (20 cm in diameter) supplied with sterilized soil (clay and sand in a 1:1 ratio) ininsect-proof greenhouse conditions. After the 28 days of seed growth, the tomato seedlings were transplanted into new pots, and after one week, the two upper true leaves of each tomato plant were mechanically inoculated with 1 mL of semi-purified TMV using carborundum, as described previously [38,39]. We carried out the experiment with four treatments, each treatment comprised of three replicates and each pot featuring three tomato plants (Table 1). The first treatment featured mocktomato plants (control sample), in which plants were mechanically inoculated with a viral inoculation buffer, besides foliar spraying of sterile distilled water on the plants. The second treatment included tomato plants inoculated with TMV, besides the foliar spraying of sterile distilled water (infected). The third treatment featured tomato plants treated by foliar spraying of the cell pellet suspension of Actino 48 (2×10^7 cfu. mL^{-1}). The fourth treatment contained the tomato plants that underwent foliar spraying with the pellet suspension of bacteria 48 h before mechanical inoculation with TMV. All plants were kept inan insect-proof greenhouse under conditions of 28 °C/16 °C (day/night) and 70% relative humidity. The plants were observed daily for the recording of symptom development. For all treatments, three biological replicates of tomato leaves from three different plants were collected at 21 dpi and subjected for the determination of enzyme activity, protein content, total phenolic compounds, and RNA extraction. The fresh and dry weights of the shoots and root systems were recorded for all treatments.

Table 1. Greenhouse experiment scheme.

Treatments	Replicates	No. of Plants in Each Pot	No. of Plants in Each Treatment	Total Plants in Greenhouse Experiment
1. Control	3 pots	3 plants	9 plants	36 plants
2. Virus	3 pots	3 plants	9 plants	
3. Actino 48	3 pots	3 plants	9 plants	
4. Actino 48 + virus	3 pots	3 plants	9 plants	

2.5. Determination of Enzymes Activity and Protein Content

2.5.1. Sample Extraction

One gram of macerated leaf tissue, using liquid nitrogen, was homogenized with 4 mL of a 0.1 M phosphate buffer solution at a pH of 7. Filtration of the extracts was accomplished by a nylon cloth. After that, the extracts were centrifuged at 10,000× *g* for 20 min at 4 °C [40]. The supernatants were stored at −80 °C and used to estimate the activity of peroxidase, chitinase, and for determination of protein content.

2.5.2. Peroxidase Assay (POD)

The determination of the peroxidase (POD) enzyme activity was performed according to Angelinai et al. [41] by adding 80 µL of the crude extract to 500 µL of a 0.1 M phosphate buffer at pH 7, 500 µL of 5 mM of guaiacol, and 60 µL of 2 mM of hydrogen peroxide (H_2O_2). The total solution was incubated at 30 °C for 10 min (tetraguaiacol will thus be formed). After that, the absorbance was measured at 480 nm, where ε = 26,600 M^{-1} cm^{-1}.

2.5.3. Chitinase Assay

The determination of the chitinase enzyme activity was performed according to Reissig et al. [42]. One hundred µL of the crude extract was incubated with 400 µL of colloidal chitin (1%) suspended in a citrate phosphate buffer (0.1 M at pH 6.5) at 30 °C for 2 h in shaking conditions. For stopping the reaction, 1 mL of the DNS (3,5-Dinitrosalicylic acid) reagent was added and kept in a boiling water bath for 5 min to improve the color. The tubes were cooled, centrifuged at 5000× *g* for 10 min, and the absorbance was measured at 575 nm. One unit of chitinase was defined as the amount of enzyme which releases 1 µmol of N-acetylglucosamine per min under the reaction condition.

2.5.4. Protein Content Determination

The Bradford method was used for the determination of protein concentrations with bovine serum albumin as the standard [43].

2.6. Determination of Total Phenolic Compounds (TCP)

The extraction from dried leaves was carried out using 80% methanol (v/v) and distilled water. Each sample of 0.5 g was weighed into conical flask that was covered with aluminum foil, and a 25 mL 80% methanol solution was added. The mixtures were then incubated and shaken at the temperature of 30 °C and at 150 rpm for 24 h. The samples were then centrifuged at 3200× *g* for 20 minto obtain a clean solution.

The Folin–Ciocalteau (FC) assay, described by Singleton and Rossi [44], was used for the estimation of total phenolic compounds (TPC) of samples with faint changes. Four hundred µL of the extracted samples was added into test tubes, followed by adding 2 mL of the FC reagent, then the mixtures were vortexed. After that, the mixtures were kept for 5 minat room temperature. A volume of 1.6 mL 7.5% Na_2CO_3 was added into the mixture and vortexed again. The mixtures were allowed to stand for 1h in the dark at room temperature (20 ± 5 °C). The absorbance was measured at 765 nm and

the calibration curve was prepared using gallic acid. The results were presented as mg gallic acid equivalents (GAE)/0.5 g of the dried samples.

2.7. Quantitative Real-Time PCR Analysis of TMV and Defense-Related Genes

2.7.1. Plant Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from tomato leaves (0.1 g, fresh weight) using the RNeasyPlant Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The concentration and purity of the extracted RNA was determined using the SPECTRO star Nano instrument (BMG Labtech, Ortenberg, Germany), while the integrity was assessed by agarose gel electrophoresis. One microgram of DNase-treated RNA was used to synthesize cDNA in a reverse transcription reaction, as described previously [45]. The RT-PCR reaction mixture was stored at −20 °C until used.

2.7.2. qRT-PCR Assay and Data Analysis

The TMVcoat protein (TMV-CP) accumulation level and transcriptional levels of five tomato defense genes (PAL, PR-1, CHS, PR-3, and PR-2, Table 2) were assayed and evaluated through the qRT-PCR technique, as performed previously [46,47]. The housekeeping gene, β-actin (Table 2), was used as a reference gene for the normalization of the expression levels of different genes [48]. By using the SYBR Green PCR Master Mix (Thermo, Applied Biosystems, Foster, CA, USA), each biological treatment was performed with three technical replicates and run on a Rotor-Gene 6000 instrument (QIAGEN, Germantown, AL, USA). The qRT-PCR was composed of 20 µL containing 1 µL of 10 pmol μL^{-1} of each primer, 10 µL of 2 × SYBR Green PCR Master Mix, 1 µL of the cDNA template, and 7 µL of nuclease-free water. The relative transcriptional level was precisely determined according to Livak and Schmittgen [49].

Table 2. Nucleotide sequences of qRT-PCR primers used in this study.

Primer Name	Abbreviation	Direction	Sequence (5′–3′)
Phenylalanine ammonia-lyase	*PAL*	Forward	ACGGGTTGCCATCTAATCTGACA
		Reverse	CGAGCAATAAGAAGCCATCGCAAT
Pathogenesis related protein-1	*PR-1*	Forward	CCAAGACTATCTTGCGGTTC
		Reverse	GAACCTAAGCCACGATACCA
β-1,3-glucanases	*PR-2*	Forward	TATAGCCGTTGGAAACGAAG
		Reverse	CAACTTGCCATCACATTCTG
Chalcone synthase	*CHS*	Forward	CACCGTGGAGGAGTATCGTAAGGC
		Reverse	TGATCAACACAGTTGGAAGGCG
Chitinase	*PR-3*	Forward	CAACTTGCCATCACATTCTG
		Reverse	CCAAAATGCTTCTCAAGCTC
Tobacco mosaic virus-coat protein	*TMV-CP*	Forward	ACGACTGCCGAAACGTTAGA
		Reverse	CAAGTTGCAGGACCAGAGGT
Beta-actin	*β-actin*	Forward	ATGCCATTCTCCGTCTTGACTTG
		Reverse	GAGTTGTATGTAGTCTCGTGGATT

2.8. Statistical Analysis

All data and the relative expression levels were analyzed by a one-way ANOVA using the CoStat software package, while the significant differences were calculated according to Tukey's honest significant differences method (H.S.D.) ata $p \leq 0.05$ level of probability. Standard deviation (±SD) is shown as a column bar here. Compared to the control, relative expression levels higher than 1 were displayed as an increase in gene expression (upregulation), while values lower than 1 denoted a decrease in the expression levels (downregulation).

3. Results

3.1. Effect of Actino 48 on TMV Symptoms Development

Under greenhouse conditions, the protective antiviral activity of *S. cellulosae* isolate Actino 48 against TMV was evaluated with tomato plants. The results show that the foliar spraying of a cell pellet suspension of Actino 48 (48 h before viral inoculation) significantly reduced disease symptoms, increased plant growth, and decreased TMV accumulation levels of treated tomato tissues when compared to non-treated plants (Figure 1). The symptoms of TMV, including the mosaic pattern and chlorosis symptoms, were clearly observed for the infected tomato plants at 14 dpi whenever a delay in symptom development for three days in the Actino 48 + virus-treated plants was observed (Figure 1). No symptoms were observed for either the mock (control) or Actino 48-treated plants (Figure 1).

Figure 1. Disease symptoms on tomato leaves infected with tobacco mosaic virus (TMV) at 21 days post-inoculation. (**A**) Mock-treated plants. (**B**) Plants inoculated with TMV only. (**C**) Plants treated with *Streptomyces cellulosae* isolate Actino 48 only. (**D**) Plants treated with Actino 48 before inoculation with TMV (48 h).

3.2. Determination of Enzymes Activity and Protein Content

3.2.1. Peroxidase (POD) Activity

Treatment that included tomato plants inoculated with TMV was more significant in terms of increasing the activity of peroxidase enzyme than other treatments followed by the treatment of tomato plants with a cell pellet suspension of Actino 48 and inoculated with TMV. The peroxidase activities reached 100.7 and 79.3 U L^{-1} min^{-1}, respectively. Treatment by a cell pellet suspension of Actino 48 achieved the lowest activity of peroxidase, reaching 35 U L^{-1} min^{-1} (Figure 2A).

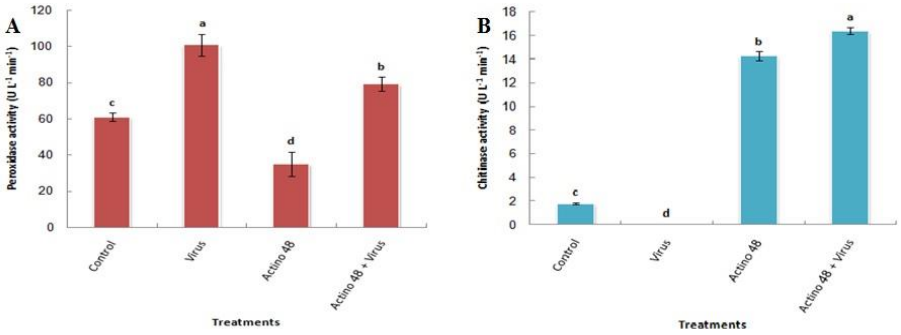

Figure 2. Effect of *Streptomyces cellulosae* isolate Actino 48 on the induction of (**A**) peroxidase (POD) activity and (**B**) chitinase activity in tomato leaves at 21 days post-inoculation. Columns with the same letter denote an insignificant difference.

3.2.2. Chitinase Activity

In this study, cell pellet suspension of Actino 48 in the presence of TMV showed significant increases in chitinase activity, followed by cell pellet suspension alone when compared to the control and TMV treatments. Chitinase activity was recorded as 16.4 U L^{-1} min^{-1} in tomato plants treated with the cell pellet suspension and inoculated with TMV, while it reached 14.3 U L^{-1} min^{-1} in tomato plants treated with the cell pellet suspension alone. No response was observed for chitinase activity in tomato plants inoculated with TMV (Figure 2B).

3.2.3. Protein Content

Protein content significantly increased with the treatment with TMV compared to the control and other treatments, reaching a maximum value of 558.7 mg mL^{-1}. Tomato plants that were treated with the cell pellet suspension of Actino 48 and inoculated with TMV recorded a protein content of 537.7 mg mL^{-1}, with no significant differences between this group and the control treatment, which observed a protein content of 532.6 mg mL^{-1} (Figure 3A).

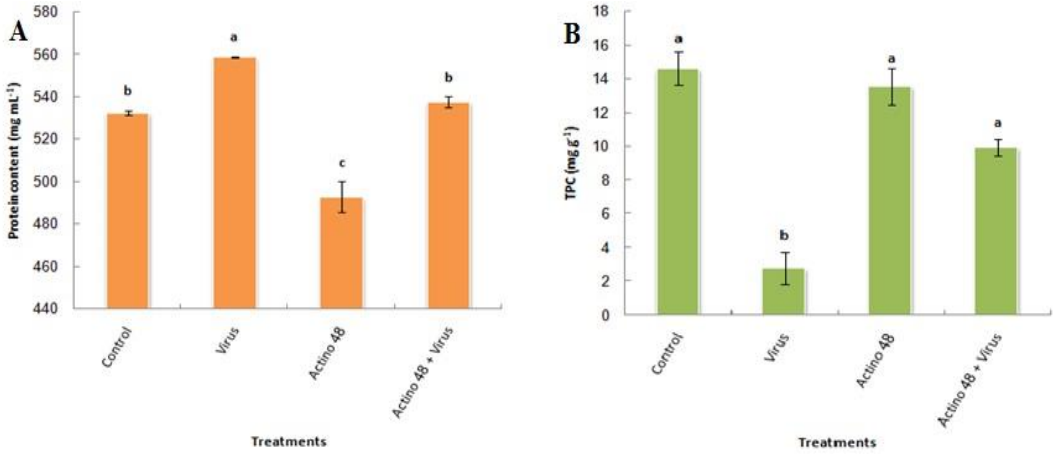

Figure 3. Effect of *Streptomyces cellulosae* isolate Actino 48 on the (**A**) protein content and (**B**) total phenolic compounds in tomato leaves at 21 days post-inoculation. Columns with the same letter denote insignificant differences.

3.3. Determination of Total Phenolic Compounds

TMV infection in tomato plants significantly decreased the total phenolic compounds concentration to reach the lowest value of 2.8 mg g^{-1}. On the other hand, the total phenolic compounds recorded significantly increasing with the control treatment, cell pellet suspension of Actino 48, and in tomato plants treated with the cell pellet suspension that were inoculated with TMV, reaching 14.6, 13.6, and 9.9 mg g^{-1}, respectively, with no significant differences between each group (Figure 3B).

3.4. Effect of Actino 48 on TMV Accumulation Level and Growth Parameters

Compared to the control, the highest accumulation level of TMV-CP with a relative expression level of 133.4-fold was reported in the virus-treated plants (Figure 4). On the other hand, a significant decrease in the viral accumulation level was observed, with a relative transcriptional level of 61.7-fold in the Actino 48 + virus-treated plants (Figure 4).

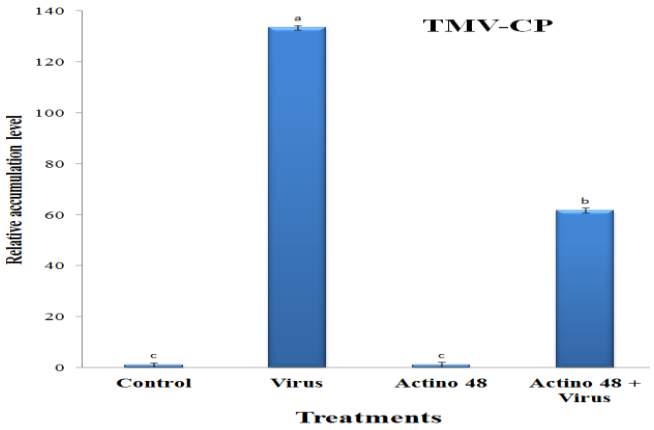

Figure 4. The relative expression level of the TMV-CP (coat protein) gene in TMV-infected tomato plants at 21 days post-inoculation. Columns with the same letter denote insignificant differences.

Treatment with a cell pellet suspension of Actino 48 before TMV-inoculation was sufficient in terms of increasing the fresh and dry weights of the shoot and root systems of tomato plants. Actino 48 significantly increased the fresh weights of the shoot and root systems of tomato plants inoculated with TMV, recorded as 33.7 and 4.5 g, i.e., increase percentages of 28.3% and 19.7%, respectively. On the other hand, the tomato plants treated with TMV had lower fresh weights for their shoot systems (24.2 g) and root systems (3.6 g) compared to the control plants (Table 3). Moreover, treatment with Actino 48 before TMV inoculation revealed significant increases in the dry weights of shoot systems (3.4 g) and root systems (0.5 g) of tomato plants, with increase percentages of 28% and 35.6%, respectively. In contrast, significant decreases were observed in the dry weights of the shoot and root systems of tomato plants treated with TMV alone (Table 4).

Table 3. Effect of treatment with a cell pellet suspension of *Streptomyces cellulosae* Actino 48 on the fresh weights of shoot and root systems in tomato plants inoculated with tobacco mosaic virus.

Treatments	Fresh Weight of Shoot System (g/pot)	Increase ^{Z} (%)	Fresh Weight of Root System (g/pot)	Increase ^{Z} (%)
Control	* 33.81 ^{a,**}	28.45	5.53 ^{b}	34.18
Virus	24.19 ^{b}	-	3.64 ^{d}	-
Actino 48	36.77 ^{a}	34.21	7.21 ^{a}	49.51
Actino 48 + virus	33.74 ^{a}	28.30	4.53 ^{c}	19.65
Tukey's H.S.D. ***	4.2		0.47	

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters; ^{Z} increase % = (treatment—check)/treatment × 100; *** H.S.D.: honest significant differences.

Table 4. Effect of treatment with cell pellet suspension of *Streptomyces cellulosae* Actino 48 on dry weight of shoot and root systems in tomato plants inoculated with tobacco mosaic virus.

Treatments	Dry Weight of Shoot System (g/pot)	Increase ^{Z} (%)	Dry Weight of Root System (g/pot)	Increase ^{Z} (%)
Control	* 3.04 ^{b,c,**}	19.41	0.48 ^{b}	65.52
Virus	2.45 ^{c}	-	0.29 ^{c}	-
Actino 48	3.81 ^{a}	35.70	0.65 ^{a}	55.38
Actino 48 + virus	3.40 ^{a,b}	27.94	0.45 ^{b}	35.56
Tukey's H.S.D. ***	0.73		0.07	

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters; ^{Z} increase % = (treatment—check)/treatment × 100; *** H.S.D.: honest significant differences.

3.5. Effect of Actino 48 on Defense-Related Gene Expression in Tomato

Significant increases in the relative expression levels of PAL, PR-1, CHS, PR-2, and PR-3 were observed in the treated plants when compared with the non-treatment plants ($p \leq 0.05$) at 21 dpi. Compared to the controls, a significant upregulation of PAL, with relative expression levels representing 1.6- and 1.5-fold changes, were observed in the Actino 48 and Actino 48 + virus-treated plants, respectively. On the other hand, virus-treated plants showed a downregulation of PAL, with a relative expression level representing a 0.7-fold change lower than the control (Figure 5). Like the PAL transcript, PR-1 was significantly upregulated, with relative transcription levels representing 2.1- and 11.2-fold changes higher than the control in the Actino 48 and Actino 48 + virus-treated tomato plants, respectively. On the other hand, the virus-treated plants showed a reduction of PR-1 with relative expression levels representing a 0.8-fold change, where no significant changes were reported when compared with the control (Figure 5).

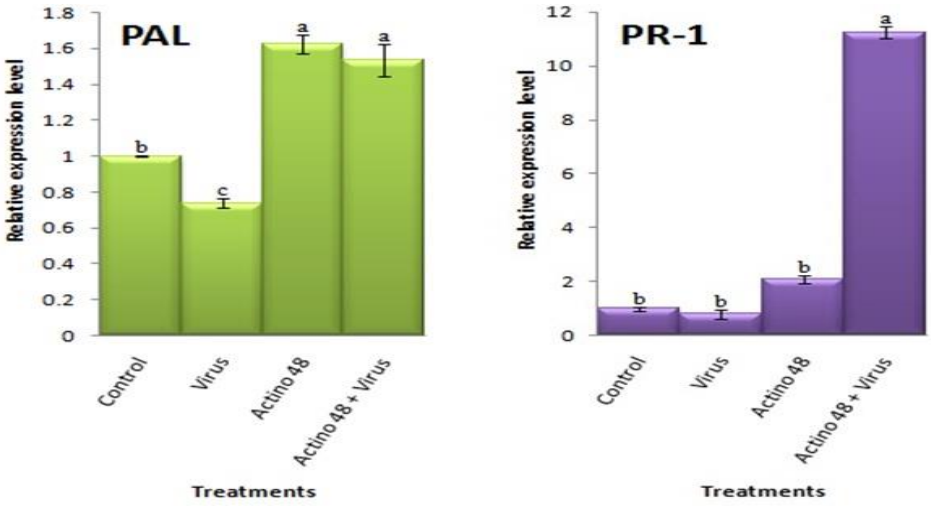

Figure 5. The relative expression level of the phenylalanine ammonia-lyase (PAL) and pathogen-related protein 1 (PR-1) genes in TMV-infected tomato plants at 21 days post-inoculation. Columns with the same letter do not differ significantly.

Regarding the CHS transcript profiles, the induction of CHS was reported in all treatments when compared to the control plants. Both treatments of the virus and Actino 48 were triggered CHS transcriptions with relative expression levels representing 1.4- and 1.5-fold changes, respectively, higher than control. The highest CHS transcript level was observed in Actino 48 + virus-treated plants, with a relative expression level representing a 2.3-fold change over the control (Figure 6). For the PR-3 transcripts levels, only the treatment of Actino 48 + virus exhibited an induction of PR-3 with a relative expression level representing a 1.2-fold change higher than the control. Both treatments by the virus and Actino 48 showed CHS transcriptions with relative expression levels representing 0.95- and 0.96-fold changes, respectively (Figure 6). On the other hand, the induction of PR-2 with a relative expression level representing a 7-fold change higher than the control was only reported in the virus-treated tomato plants. Although the Actino 48 + virus-treated plants showed a slight induction of PR-2, with relative expression levels representing a 1.3-fold change, no significant changes were reported when compared with the controls (Figure 6).

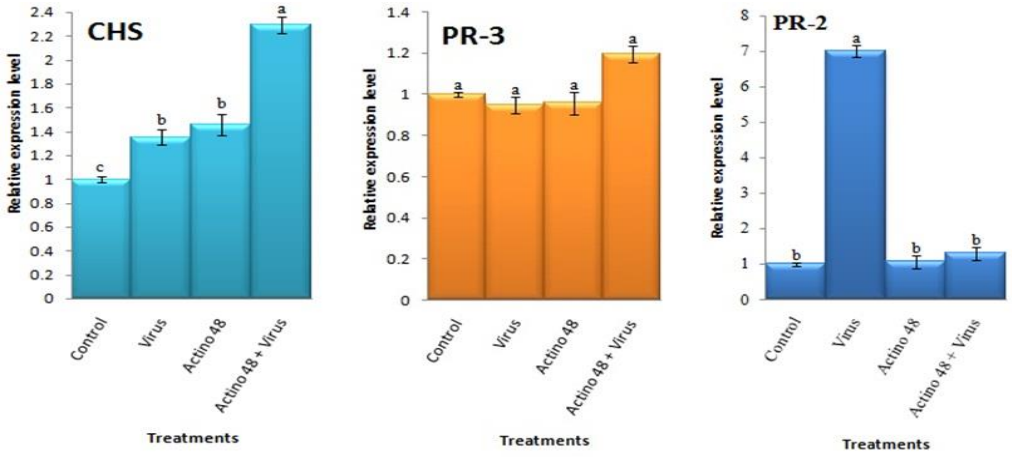

Figure 6. The relative expression levels of the chalcone synthase (CHS), protein 3 (PR-3), and pathogen-related protein 2 (PR-2) genes in TMV-infected tomato plants at 21 days post-inoculation. Columns with the same letter do not differ significantly.

4. Discussion

Plant diseases, especially viral plant infections, are responsible for severe crop losses in crop production worldwide, resulting in critical problems for food security [50]. The application of Actino bacteria as biocontrol agents for the management of viral plant diseases has been limited until now. In the current study, the antiviral activity of *S. cellulosae* isolate Actino 48 against TMV on tomato plants has been evaluated. Moreover, the peroxidase activity, chitinase activity, protein content and total phenolic compounds have been determined in leaves at 21 dpi. On the other hand, the effects of Actino 48 on the accumulation levels of TMV-CP and expression of five defense-related genes (PAL, PR-1, CHS, PR-3, and PR-2) at 21 dpi have been evaluated.

In the current study, peroxidase activity increased significantly in tomato plants infected with TMV followed by treatment with Actino 48 and TMV. *S. pactum* Act12 has enhanced peroxidase activity in tomato plants against tomato yellow leaf curl virus (TYLCV) [11]. Cucumber plants treated with *Streptomyces* as a biocontrol agent have shown an increasing peroxidase activity [51]. Also, the application of *Streptomyces* spp. has induced peroxidase activity as a defense enzyme in response to CMV [52]. Das and Roychoudhury [53] reported that peroxidase is supported by plant defense systems and is responsible for the reduction of the harmful effects of stresses via the scavenging of reactive oxygen species (ROS).

In our study, Actino 48 induced chitinase activity to reach its maximum value in tomato plants treated with Actino 48 and TMV compared to the control plants. These data are in accordance with the data obtained by Shafie et al. [52]. Shoman et al. [54] documented that *Streptomyces* spp. induces plant resistance against tobacco necrosis virus (TNV), with an increasing level of chitinase enzymes as one of the PR proteins. In addition, Kandan et al. [18] reported that the treatment with *P. fluorescens* increased chitinase activity in tomato plants in response to tomato spotted wilt virus (TSWV).

In the current study, protein content significantly increased in tomato plants infected with TMV followed by the treatment with Actino 48 and TMV, and the untreated tomato plants (control plants) showed no significant differences between each other. The obtained results may be related to the presence of the coat protein of TMV. Our results are similar to the results obtained by Barakat et al. [55], who reported an increasing protein content in watermelon plants infected with watermelon mosaic virus-2 (WMV-2) compared to other treatments.

Our study has revealed significant increases in the total phenolic compounds in untreated tomato plants (control), tomato plants treated with Actino 48 alone, and tomato plants treated with Actino 48 and TMV, with no significant differences between each other. On the other hand, treatment with TMV decreased the total phenolic compounds. Phenolic compounds play an important role in plant

resistance to viruses, and they are implicated in phytoalexin accumulation, the biosynthesis of lignin, and the formation of structural barriers [56].

Under greenhouse experiments, the foliar application of Actino 48 (48 h before viral inoculation) significantly increased the plant growth parameters, reduced disease symptoms, and decreased viral accumulation levels compared to infected tomato plants without any treatment. Treatment with Actino 48 induced the growth of tomato plants treated with TMV compared to virus-treated tomato plants alone. The fresh and dry weights of the shoot and root systems increased significantly in tomato plants treated with Actino 48 and TMV but showed a significant reduction in the fresh and dry weights of the shoot and root systems of tomato plants infected with TMV. The increase in plant growth may be related to the ability of Actino 48 to induce the synthesis of various phytohormones and the ability of Actino 48 to produce different hormones. For example, the synthesis of indole acetic acid (IAA) in plants was stimulated by *Streptomyces* sp. in greenhouse experiments [57,58]. Li et al. [35] reported that *S. pactum* Act12 promoted tomato plant growth, where plant biomass and fruit yield were increased, while *Streptomyces* spp. isolate GS-93-23 was more efficient in terms of enhancing the forage weight of alfalfa [59]. Additionally, the synthesis of plant hormones, such as cytokinins, IAA, and gibberellins, has been induced by *P. fluorescens* strains, leading to increased plant growth [60]. On the other hand, Zamoum et al. [61] reported that the *S. rochei* strain PTL2 enhanced the dry weight of tomato plants, where it had the ability to produce phytohormones of IAA and gibberellin (GA3). A significant decrease in the TMV accumulation levels in the Actino 48 + virus-treated tomato plants of 53.8% compared to the virus-treated tomato plants confirmed the efficiency of the biocontrol activity of Actino 48 against TMV infection. These results suggest that Actino 48 may produce secondary metabolites which can play a major role in SAR. Li et al. [11] reported that the pre-inoculation of tomato plants with Act12 reduced TYLCV by 37.9%. Thus, Actino 48 may activate induced systemic resistance (ISR) in tomato plants against TMV infection.

In the present study, non-treated tomato plants challenged with TMV showed decreasing PAL and PR-1 levels, with relative expression levels representing 0.7- and 0.8-fold changes, respectively, which is lower than control samples. Interestingly, both treatments, i.e., Actino 48-treated and Actino 48 + virus-treated plants, exhibited an induction and overexpression of PAL and PR-1. The Actino 48 + virus-treated plants showed the highest expression level of PR-1, with a relative transcriptional level representing a 11.2-fold change, while the Actino 48-treated plants exhibited the highest expression level of PAL, with a relative expression level representing a 1.6-fold change higher than the control plants. Consequently, we suggest that Actino 48 may work as an elicitor molecule that induces the immune defense system, which may result SAR activation. In this context, tomato plants pre-treated with Act12 exhibited an induction of PR-1 and enhanced POD and PAL activities in tomato leaves, resulting in the development of SAR against TYLCV [11].

Compared to the mock plants at 21 dpi, the transcripts of chalcone synthase (CHS) were induced after challenging the tomato plants with TMV and/or Actino 48. The greatest transcriptional levels of CHS were reported in the Actino 48 + virus-treated plants, with a relative expression level representing a 2.3-fold higher change than the control plants. Consequently, the application of Actino 48 showed the greatest induction of CHS, which is strictly required for the production of naringeninchalcones. CHS, the first enzyme in the flavonoid pathway, is responsible for the conversion of *p*-coumaroyl CoA to naringeninchalcones and is strictly required in various plant tissues for flavonoid production [62,63]. Naringeninchalconesare considered as the primary precursors and main intermediates for the biosynthesis of many flavonoids via the action other enzymes [64,65].

In the present study, only the Actino 48 + virus treatment showed a slight induction of PR-3, encodeschitinase activity [65], with a relative expression level representing a 1.2-fold higher change than the control plants. We assumed that PR-3 expression may not be specific to TMV infection in tomato plants [36]. In addition, the upregulation or downregulation of PR-3 may depend on the viral isolate, type of plant, and plant genotype [66–68].

It has been reported that TMV increases the activity of PR-2 to facilitate its movement through cells [36,69]. In the current study, the virus treatment only exhibited the induction of PR-2, with a relative transcriptional level representing a 7-fold increase over the controls. These results are similar to previous studies that have reported a clear induction of PR-2 during viral infections in *Arabidopsis* [70], tobacco [71–73], onion [74], potato [75], and tomato [36] plants. Moreover, a deficiency of tobacco PR-2 decreases susceptibility to viral infection [75], while overexpression results in expediting the spread of PVY (potato virus Y) infection between cells [76,77]. Interestingly, although both treatments of Actino 48 and Actino 48 + virus exhibited slight increases in PR-2, there were no significant changes reported with the controls. Thus, the application of Actino 48 may have diminished PR-2, resulting in inhibiting long distance viral infection between cells.

Overall, the present study has shown that *S. cellulosae* isolate Actino 48 enhances tomato plant growth, decreases the TMV accumulation level, induces systemic resistance, and increases the production of some defense enzymes. Consequently, Actino 48 could be introduced as a biocontrol agent against TMV infection. However, further examinations are needed for analyzing potential field and commercial applications.

5. Conclusions

S. cellulosae isolate Actino 48 is sufficient in terms of inducing plant systemic resistance and controlling TMV. Actino 48 induced TMV-inoculated tomato plants to increase the level of peroxidase, chitinase, and total phenolic compounds. Actino 48 was effective in reducing the symptoms and severity of TMV. Treatment with Actino 48 reduced the TMV accumulation level (53.8%) compared to treatment with the virus alone. In addition, Actino 48 increased the fresh and dry weights of the shoot and root systems of tomato plants here. Tomato plants treated with Actino 48 and TMV showed significant increases in upregulation of the relative expression levels of the PAL, PR-1, CHS, and PR-3 genes.

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