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The *Streptomyces chromofuscus* Strain RFS-23 Induces Systemic Resistance and Activates Plant Defense Responses against *Tomato Yellow Leaf Curl Virus* Infection

Delai Chen [1,2,*], Mian Noor Hussain Asghar Ali [3], Muhammad Kamran [4], Manzoor Ali Magsi [5], Freddy Mora-Poblete [6], Carlos Maldonado [7,*], Muhammad Waris [8], Reem M. Aljowaie [9], Mohammad Yakoob Zehri [10] and Mohamed S. Elshikh [9]

1 College of Life Science and Technology, Longdong University, Qingyang 745000, China
2 Gansu Key Laboratory of Protection and Utilization for Biological Resources and Ecological Restoration, Qingyang 745000, China
3 Department of Farm Structures, Faculty of Agricultural Engineering, Sindh Agriculture University Tandojam, Sindh 70060, Pakistan
4 School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA 5005, Australia
5 Department of Farm Power and Machinery, Faculty of Agricultural Engineering, Sindh Agriculture University Tandojam, Sindh 70060, Pakistan
6 Institute of Biological Sciences, University of Talca, 2 Norte 685, Talca 3460000, Chile
7 Centro de Genómica y Bioinformática, Facultad de Ciencias, Universidad Mayor, Santiago 8580745, Chile
8 Department of Plant Pathology, Balochistan Agriculture College, Quetta 87100, Pakistan
9 Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
10 PARC-Horticulture Research Institute, Khuzdar 89100, Pakistan
* Correspondence: cdl829@126.com (D.C.); cmaldo1782@gmail.com (C.M.)

Abstract: Insect-vectored plant viruses pose a serious threat to sustainable production of economically important crops worldwide. This demands a continuous search for environmentally-friendly, sustainable and efficient approaches based on biological agents to address the mounting challenges of viral disease management. To date, the efficacy of actinomycetes bacteria against DNA plant viruses remains unknown. Here, through comparative analyses, we demonstrate that the RFS-23 strain of *Streptomyces cellulase* possesses protective activity as it positively regulated the plant growth and development. and diminished the severity, of disease symptoms, together with reduced accumulation of *Tomato yellow leaf curl virus* (TYLCV) DNA. The RFS-23 strain maintained relative chlorophyll contents by promoting the expression of genes (*CLH1*, *HEMA1* and *PORA*) associated with chlorophyll biogenesis. As compared to another strain, CTF-20, the RSF-23 induced a significantly higher expression of plant defense-related genes (*NbCIS* and *NbNCED*) associated with biogenesis and accumulation of salicylic acid and abscisic acid. Additionally, the activity of antioxidant enzymes (SOD, CAT, POD and MDA) was significantly enhanced by RSF-23 treatment, despite the presence of viral infection. These findings suggest that RSF-23 is a novel biocontrol agent with protective activity, and it could be a potential candidate for the management of plant viral infections.

Keywords: *Begomovirus*; biocontrol agent; disease resistance; molecular plant-virus interaction; phytohormones; chlorophyll contents; disease management

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1. Introduction

Plant diseases pose a continuous threat to sustainable crop production and raise concerns regarding global food security. Among others, phytoviruses represent the most important group of plant pathogens, causing devastating crop losses in different regions of the world [1–3]. The *Tomato yellow leaf curl virus* (TYLCV), from the *Begomovirus* genus of the *Geminiviridae* family, is a plant virus transmitted by a whitefly (*Bemisia tabaci*), that

is mainly known to infect tomatoes (*Solanum lycopersicum*) [4,5]. The virions of TYLCV consist of a single-stranded DNA (ssDNA) genome of ~2.8 kb in size. The viral nucleic acid is encapsulated in a twinned, icosahedral shaped structure [6]. Upon TYLCV infection, the host plants display typical symptoms of viral infection, including yellowing, leaf curling and stunting, which leads to substantial crop losses in tomatoes [7]. In addition to tomatoes, several species of cultivated plants, including cucurbits (*Cucumis* species), pepper (*Capsicum* species), eustoma (*Eustoma grandiflora*) and common bean (*Phaseolus vulgaris*), have been documented to be infected by TYLCV [6,8–11]. The first record of TYLCV was reported from the Middle East in 1931 and, since then, the virus has continuously spread across tropical and sub-tropical regions of the world [4].

The application of biocontrol agents/microbes is considered a sustainable technique to control plant pathogens. These microorganisms can confer long-term disease protection to plants in addition to their known roles in plant growth, development and overall health [12,13]. The control of plant viral diseases by using biocontrol agents has gained much attention as it is considered to be environmentally-friendly and safe disease management approach [14]. The majority of these biocontrol microbes aew bacterial, such as *Bacillus* and *Pseudomonas* spp., and fungi, such as *Trichoderma* spp. [15–17]. In addition to these, *Streptomyces* spp. represents the largest group (containing 780 species, 30 sub-species) associated with actinobacteria [18]. The actinomycetes are known to produce a wide variety of secondary metabolites, including extracellular enzymes and antibiotic compounds [19]. These secondary metabolites are capable of inhibiting the growth of several bacterial and fungal plant pathogens [20]. Importantly, *Streptomyces* spp. play vital roles in pathogen inhibition during plant–pathogen interactions, as well as during biocontrol of bacterial and fungal plant diseases [21]. Nevertheless, the implications of *Streptomyces* spp. for the bio-management of plant viral pathogens are still limited and the potential underlying mechanisms mostly remain in the shadows.

Several bioactive substances derived from different strains of *Streptomyces* spp. have been effectively used against RNA plant virus (for example *Tobacco mosaic virus*, TMV) to reduce the severity of local lesions on the leaves of the host plant, *Datura metel* [22]. Another bioactive compound known as ε-poly L-lysine, derived from *S. ahygroscopicus* species, was shown to display protective, as well as curative, activities against the same (TMV) pathogen [23]. In a different study, it was demonstrated that a foliar spray with *S. sparsogenes* and *S. albovinaceus* species reduced the severity of mosaic symptoms induced by *Zucchini yellow mosaic virus* (ZYMV) by 95–100% [24].

Viral infection can induce resistance among host plants via two mechanisms; induced systemic resistance (ISR) or systemic acquired resistance (SAR). Researchers have documented that in response to viral infection, induced plant resistance might be associated with both pathways [25]. Ethylene and jasmonic acid (JA) are known to regulate ISR which is induced by microbes. The ISR promotes plant growth and activates rapid defense responses, coupled with higher capabilities of the plants to defend against/resist the diseases [26–28].

In the present study, we evaluated and compared the protective activities of two strains (RSF-23 and CTF-20) of *Streptomyces* spp. by using *N. benthamiana* and the TYLCV host–virus system. We investigated the effects of 24 h early spray, using RSF-23 and CTF-20 pellets, prior to TYLCV inoculation and analyzed the leaf area, fresh weight, and relative chlorophyll content data. Additionally, we also measured the transcriptional changes associated with genes responsible for biosynthesis and accumulation of chlorophyll and those related to the mediated defense signaling of salicylic acid (SA) and abscisic acid (ABA). To gain a better understanding of the actinomycetes-induced defenses, we also measured the relative concentrations of key enzymes involved in the antioxidant pathways post-viral infection.

2. Materials and Methods

2.1. Maintenance of Plants, Viruses and Bacterial Isolates

The wild-type *Nicotiana benthamiana* seeds were grown in a substrate mixture containing black soil, perlite, vermiculite and artificial soil (2:1:2:2 ratio) for one week at a temperature of 25 ± 1 °C. Subsequently, seedlings with uniform size were transplanted into plastic pots (8 cm diameter, 10 cm height) using the aforementioned growth substrate. The growth and development of *N. benthamiana* plants was maintained in a controlled growth chamber at 25–27 °C temperature and 60–64% relative humidity. The *Agrobacterium* strain EHA105, containing full-length TYLCV, was inoculated to *N. benthamiana* plants at the 5–7 fully-expanded leaf stage, following a previously described protocol [29]. The *S. chromofuscus* strain RFS-23 (GenBank Accession: EU301837) and CTF-20 strain (GenBank Accession: EU294136) of *S. rochei* were maintained by streaking on the starch nitrate agar (SNA) medium supplemented with ingredients (g/L): $CaCO_3$ (3), starch (20), KNO_3 (1), NaCL (2), $MgSO_4.7H_2O$ (0.5) and $FeSO_4.7H_2O$ (0.01). The colonies were sub-cultured on an ISP-2 medium supplemented with malt extract (10 g/L, yeast extract (4 g/L) and dextrose (4 g/L; pH = 7) [30]. The bacterial culture was incubated at 30 °C and 200 rpm for one week until it reached a concentration of 2×10^7 cfu/mL. Then the bacteria were pelleted by centrifugation at 5590× *g* for 20 min followed by collection, washing and centrifugation of the cell pellet. Finally, the cell pellets of both strains were suspended in double distilled water and applied as foliar spray.

2.2. Bacterial Treatment of N. benthamiana Plants and Viral Inoculation

The cell pellets of RFS-23 and CTF-20 strains (2×10^7 cfu/mL) were dissolved in double distilled water and foliar sprayed on to the *N. benthamiana* leaves at 24 h before virus inoculation. Following bacterial suspension spray, the TYLCV was introduced into plants by agrobacterium-mediated inoculation, following the aforementioned described method. For this experiment, a total of four treatments were included, with each containing 3 replications. Each replication contained 12 *N. benthamiana* plants. The first treatment consisted of control (untreated) plants that we sprayed with distilled water only. The second treatment was inoculated with virus (TYLCV) only. The third treatment was exposed to the TYLCV and RFS-23 strain, while the fourth treatment included a combination of TYLCV and CTF-20 strain. All plants were maintained in an insect-free environment under controlled growth conditions, as mentioned earlier.

2.3. Monitoring of Disease Symptoms and Samples Collection

The plants among all treatments were regularly monitored, irrigated and fertilized to ensure proper growth and development. The confirmation of viral infection was done by appearance of typical viral symptoms (yellowing, wrinkling, leaf curling, mosaic, stunting) at ~5 days post-inoculation (dpi). The data (viral symptoms, infection percentage and relative chlorophyll contents) were recorded on a daily basis and a few measurements were taken at 9, 18 and 28 dpi. For this purpose, either intact leaves or fresh leaf tissues were used and, additionally, the tissue samples were snap-frozen in liquid nitrogen and preserved at −80 °C for subsequent analyses.

2.4. Quantification of Relative Chlorophyll Contents

The in-situ measurement of relative chlorophyll contents (Chl*a* + Chl*b*) was performed by using a soil plant analysis development (SPAD) and a 502-Plus chlorophyll meter (Konica Minolta, Inc., Osaka, Japan). This hand-held device determines the leaf greenness level together with the interaction of thylakoid chlorophyll and the incident light 7. For each leaf, three points were selected (about 15–35 mm distance from the midrib) to take the SPAD readings at a transmittance ratio of 650/940 nm wavelength. For each treatment, 24 leaves were used to record SPAD values to estimate the net chlorophyll contents in the foliar tissues.

2.5. Nucleic acid Extractions cDNA Synthesis and RT-qPCR

The extraction of total RNA was performed for control, TYLCV-infected, TYLCV + RSF-23, and TYLCV + CTF-20-treated *N. benthamiana* leaves at 9, 18 and 28 dpi using TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD, USA), according to the manufacturer's protocol. The quantitative and qualitative assessment of RNA was done by measurement of the A260/A280 ratio using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Additionally, the RNA integrity was also analyzed by visualizing it in 0.8% agarose gel electrophoresis. The synthesis of cDNA from extracted total RNA was done by using the PrimeScript RT Master Mix reagent kit (Takara Bio. Tech. Co., Ltd., Beijing, China), according to the given instructions. The cDNA was either used immediately or preserved at −80 °C for downstream experiments. For the RT-qPCR assay, a real-time PCR system (LightCycler 96, Roche, Basel, Switzerland) was used. The reaction mixture was prepared by adding SYBR Premix Ex Taq II (1×), forward and reverse primers (0.4 µM each), cDNA (2 µL) and ddH_2O (8.5 µL). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene of *N. benthamiana* was used as a reference/internal control to calculate the relative mRNA expression of the target genes. In RT-qPCR experiments, three biological and nine technical repeats were used, followed by statistical analysis of data. The PrimerQuest tool (Integrated DNA Technologies, Coralville, IW, USA) was used to design the gene-specific primer pairs for RT-qPCR analysis.

Transcriptional changes in chlorophyll biosynthesis-related genes were measured by analyzing the transcriptional profiles of *CLH1*, *HEMA1* and *PORA*, while the expression of plant defense-related genes was measured by targeting *NbCIS* and *NbNCED*, that are known for biosynthesis of salicylic acid (SA) and abscisic acid (ABA), respectively. The information of all primers used in this study is provided in Table 1.

Table 1. Description of the nucleotide sequences, target genes and experimental purposes associated with primers used in this study.

Primer Name	Direction	Nucleotide Sequence (5′-3′)	Target Gene	Amplicon Size	Purpose	Reference
TYLCV-CP-F	Forward	ATCATGGACGTACAGGCC	*CP*	200 bp	qPCR	this study
TYLCV-CP-R	Reverse	ACCTCTTACCAACTCTGTGA			qPCR	
qGPDH-F	Forward	TCGACAGAGAAGGTGCCGGA	*NbGAPDH*	190 bp	qPCR	this study
qGPDH-R	Reverse	TCAAGAACCTTGACAAAAGG			qPCR	
qSA-F	Forward	GGCTCTGCTGTCTTCTTTACT	*NbCIS*	200 bp	qPCR	[31]
qSA-R	Reverse	AGCTCATCGAACTCAACCTG			qPCR	
qABA-F	Forward	CGTGGACTCTTTGGACTTGTT	*NbNCED*	200 bp	qPCR	[31]
qABA-R	Reverse	GGGTGAGCTATCATTGTGGATT			qPCR	
qChbio1-F	Forward	GTTCCAATTGGGGTTGGAA	*CHL1*	195 bp	qPCR	this study
qChbio1-R	Reverse	GAGATGTTGATTCTTATCT			qPCR	
qChbio2-F	Forward	GGTGCGGTTTCGGTTAGCTCA	*HEMA1*	206 bp	qPCR	this study
qChbio2-R	Reverse	GGCATCTCCTCACGGATAGC			qPCR	
qChbio3-F	Forward	GACTTGAAGAACTCCGAT	*PORA*	200 bp	qPCR	this study
qChbio3-R	Reverse	TCTTTATACGCCTTTGCGCCA			qPCR	

2.6. Relative Accumulation of TYLCV Coat Protein

For estimation of the relative viral DNA accumulation in plants, the total DNA was extracted from apical leaf tissues using a cetyltrimethyl ammonium bromide (CTAB)-based protocol. The qualitative and quantitative assessment of the extracted DNA was performed by gel electrophoresis and by using a NanoDrop 2000 Spectrophotometer, as described above. An approximately 50 ng/sample DNA was used to perform qPCR. The relative quantification of the viral DNA was done by comparing the coat protein (CP) expression levels to that of GAPDH. The information of all primers used in this experiment is provided in Table 2.

Table 2. Comparative analysis of statistical significance between different treatments at 9, 18 and 28 days post-viral infection.

Analyzed Combination of Treatments	Time Post-Inoculation (days)	Morphological Parameter			
		Leaf Area		Fresh Weight	
		Significant?	*p*-Value *	Significant?	*p*-Value *
CK vs. TYLCV	9	Yes	0.009094	Yes	0.041102
	18	Yes	0.000150	Yes	0.003412
	28	Yes	0.000141	Yes	0.003676
CK vs. RSF-23 + TYLCV	9	No	0.297866	No	0.142864
	18	No	0.039482	No	0.709585
	28	Yes	0.011114	yes	0.043202
CK vs. CTF-20 + TYLCV	9	Yes	0.028906	No	0.241886
	18	Yes	0.001175	No	0.025486
	28	Yes	0.001723	Yes	0.006770
TYLCV vs. RSF-23 + TYLCV	9	Yes	0.020122	No	0.308886
	18	Yes	0.002545	Yes	0.002980
	28	Yes	0.043614	Yes	0.007267
TYLCV vs. CTF-20 + TYLCV	9	No	0.309201	No	0.110748
	18	No	0.180613	No	0.083647
	28	No	0.121887	No	0.278941

* Statistical significance for pairwise comparisons was determined using the Holm-Sidak method, with alpha = 0.05.

2.7. Biochemical Analysis of Antioxidant Enzymes

The biochemical analysis of key antioxidant enzymes was performed using fresh *N. benthamiana* leaf tissues collected from all treatments. To analyze the concentrations of key antioxidant enzymes, including superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and peroxidase (POD), leaf samples from all treatments were collected at 28 dpi and ground into fine powder using liquid nitrogen. The commercially purchased kits of SOD (SOD-1-W), POD(BC0090), MDA (A003-1) and CAT (CAT-1-W) (Comin Biotechnology Co., Ltd. Jiangsu, China) were then used to quantify the concentrations of these key enzymes. Approximately 0.1 g of tissue sample was used for this purpose and three biological replicates were considered for each measurement. The activities of SOD, MDA, POD and CAT were checked by measuring the sample absorbance at 405, 532, 40 and 550 nm, respectively, using a spectrophotometer (UV-1780, Kyoto, Japan). The relative concentrations of each target enzyme were expressed as unit (U)/mg protein.

2.8. Statistical Analysis

The statistical significance associated with leaf area and fresh weight among pairwise comparisons was determined using the Holm-Sidak method, with alpha = 0.05. The qPCR data included three biological and nine technical repeats and was subjected to one-way analysis of variance (ANOVA) using Tukey's hones significant differences (HSD) method at the probabilities of * $p \leq 0.05$ and ** $p \leq 0.01$. The relative expression levels of the target genes were normalized against GAPDH and the values higher or lower than 1 were regarded as higher or lower expressions, respectively. The vertical bar at each column represented standard deviation (±SD).

3. Results

3.1. Comparative Effect of Streptomyces Strains on Development of Viral Disease

The pattern of TYLCV symptom development was continuously observed up to 28 dpi to compare the protective role of RSF-23 and CTF-20. The results showed that control plants (untreated) continued normal growth and development without exhibiting any disease symptoms (Figure 1A) while TYLCV-inoculated *N. benthamiana* plants displayed a range of viral symptoms, including leaf yellowing, crinkling, downward curling, mosaic and stunting (Figure 1B). Interestingly, the TYLCV-infected plants treated with RSF-23 developed

mild disease symptoms (Figure 1C). Likewise, effects of CTF-20 were observed among TYLCV-infected plants at 28 dpi (Figure 1D), though the intensity of disease symptoms was higher than that of RSF-23-treated group. The time-based observation of disease development indicated that RSF-23 had a clear effect on the appearance and progression of viral symptoms, as compared to CTF-20-treated or TYLCV-infected plants (Figure 1E). Overall, the infection percentage among RSF-23-treated plants was lower than that of the plants treated with CTF-20, indicating the ability of the RSF-23 strain to lessen the intensity and development of viral symptoms (Figure 1F).

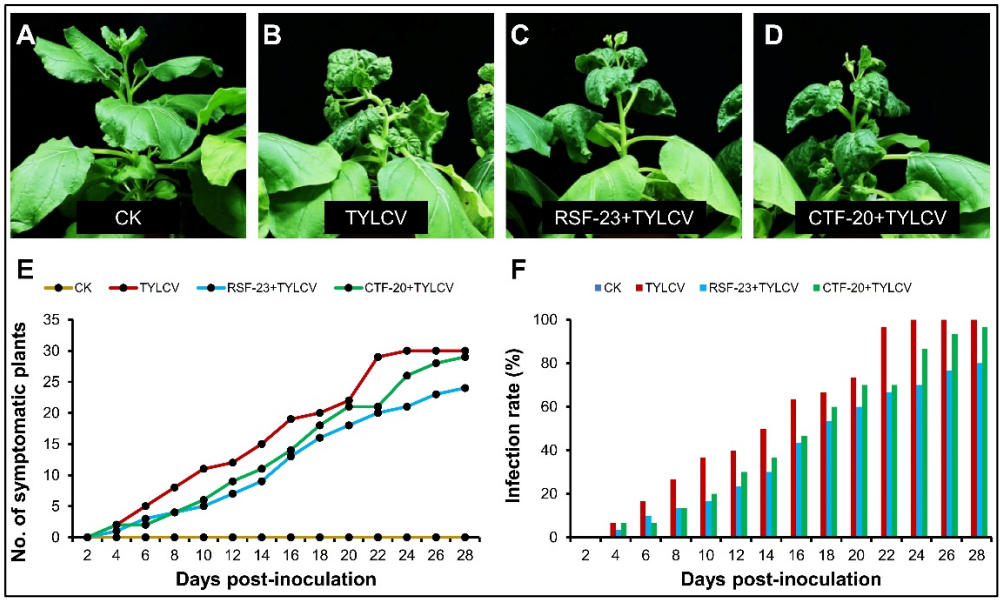

Figure 1. Comparison of viral disease symptoms in response to different treatments. (**A**) untreated (control) *Nicotiana benthamiana*; (**B**) TYLCV-infected plants; (**C**) TYLCV-infected and RSF-23-treated plants; (**D**) TYLCV-infected and CTF-20-treated plants at 28 dpi; (**E**) number of TYLCV-infected *N. benthamiana* with disease symptoms and (**F**) percentage of TYLCV-infected plants from 0–28 dpi.

3.2. Estimation of Plant Growth Parameters

To further expand the understanding of the plant's morphological responses towards bacterial treatment, we measured and compared the leaf area and fresh weight among all treatments. We observed that at 9, 18 and 28 dpi, the leaf area of TYLCV-infected plants was significantly lower than untreated (control) plants (Figure 2A and Table 2). The treatment with RSF-23 strain appeared to improve the leaf area among TYLCV-infected plants and the impact was significant at 9 dpi (p = 0.020122), 18 dpi (p = 0.002545) and 28 dpi (p = 0.043614), as compared to TYLCV-infected plants without bacterial treatment (Figure 2A and Table 2). On the contrary, although CTF-20 treatment apparently maintained the leaf area among virus-infected plants, the difference was slightly higher as compared to the TYLCV-infected plants (Figure 2A and Table 2).

Furthermore, the RSF-23 treatment was observed to maintain the fresh weight among TYLCV-infected plants, as compared to those infected with TYLCV only (Figure 2B and Table 2). Notably, this observed difference was slightly higher at 9 dpi. However, the fresh weight of RSF-23-treated plants was significantly higher at 18 dpi (p = 0.002980) and 28 dpi (p = 0.007267), as compared to the virus-infected plants without receiving bacterial treatment (Figure 2B and Table 2). On the other hand, despite the observed higher fresh weight among CTF-20-treated plants, the values were statistically not significant, as compared to those of TYLCV-treated plants (Table 2). Overall, these results indicated that, as compared to CTF-20, pre-treatment of plants with the RSF-23 strain maintained normal morphological development among virus-infected plants by improving leaf area and fresh weight.

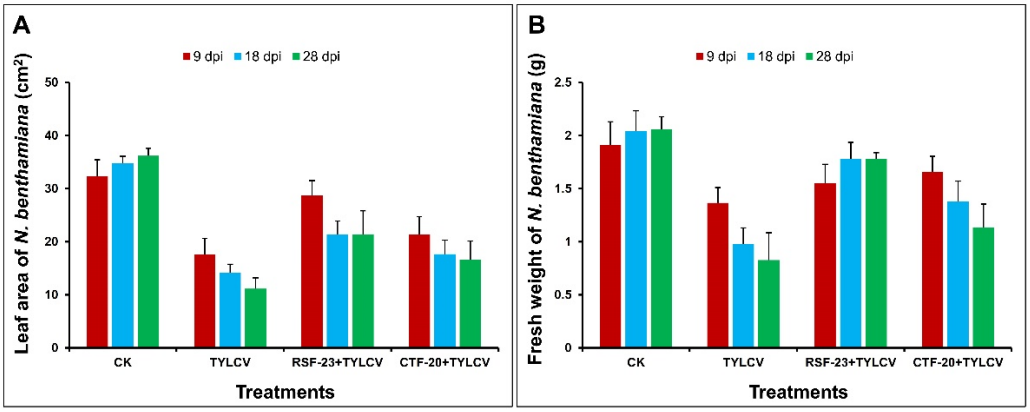

Figure 2. Measurement of *N. benthamiana* morphological parameters in response to different treatments. (**A**) leaf area and (**B**) fresh weight of *N. benthamiana* plants among control (CK), TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatments at 9, 18 and 28 dpi.

3.3. The Relative Accumulation of TYLCV Coat Protein

In addition to the development of viral symptoms, changes in the leaf area and fresh weight, the success of viral infection was also analyzed by quantifying the accumulation of viral nucleic acid. The results indicated that among virus-infected plants that did not receive any bacterial treatment, the DNA of TYLCV tended to gradually increase at 9, 18 and 28 dpi, denoting the establishment and progress of viral infection (Figure 3). However, the quantity of TYLCV-DNA was significantly lower among plants treated with the RSF-23 strain prior to virus inoculation (Figure 3). Furthermore, a similar trend was observed among CTF-20-treated plants at 9, 18 and 28 dpi, where the accumulation of viral DNA gradually increased but significantly remained lower than that of TYLCV-infected plants (Figure 3). These results indicated that early treatment of plants with RSF-23 could significantly reduce the accumulation of viral DNA with an efficacy higher than that of the CTF-20 strain.

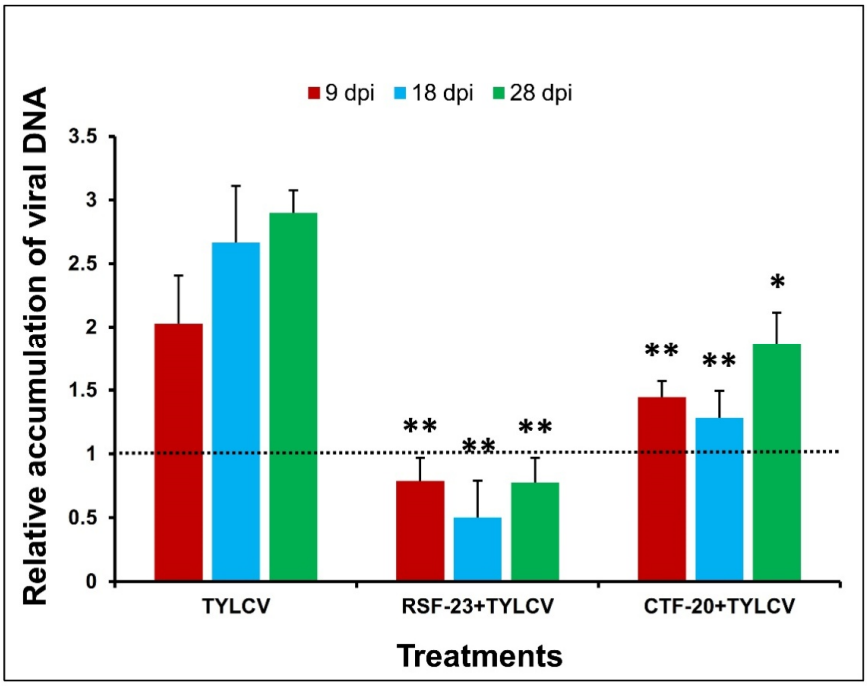

Figure 3. Relative accumulation of TYLCV coat protein (CP) among TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatmented *N. benthamiana* plants at 9, 18 and 28 dpi. The dotted horizontal line denotes that gene expression was normalized to 1. Asterisks indicate the levels of significance at * $p \leq 0.05$ and ** $p \leq 0.01$.

3.4. Determination of Relative Chlorophyll Contents

The symptoms of TYLCV infection include yellowing accompanied by lower levels of chlorophyll contents. To evaluate the effect of RSF-23 and CTF-20 treatments on viral infection, we measured and compared the relative chlorophyll contents among all treatments in a time-dependent manner. Our findings indicated that until 6 dpi, the total chlorophyll contents did not show a visible difference among untreated (control), TYLCV-infected or bacteria (RSF-23 and CTF-20)-treated plants (Figure 4). However, the impact of TYLCV infection on the total chlorophyll contents became obvious at 8 dpi where untreated and virus-infected groups of plants were clearly separated. Notably, the effect of bacterial treatment was higher at 10 dpi, which remained distinct until 28 dpi (Figure 4). Remarkably, the plants treated with RSF-23 clearly maintained chlorophyll contents, despite viral infection. A similar accumulation pattern of chlorophyll contents was observed in response to CTF-20 treatment, as the relative chlorophyll contents were higher than that of TYLCV-infected plants; however, the trend line remained lower than that of RSF-23 (Figure 4). These results indicated that post-viral infection, the RSF-23 treatment exhibited better ability to maintain the relative chlorophyll contents among virus-infected plants.

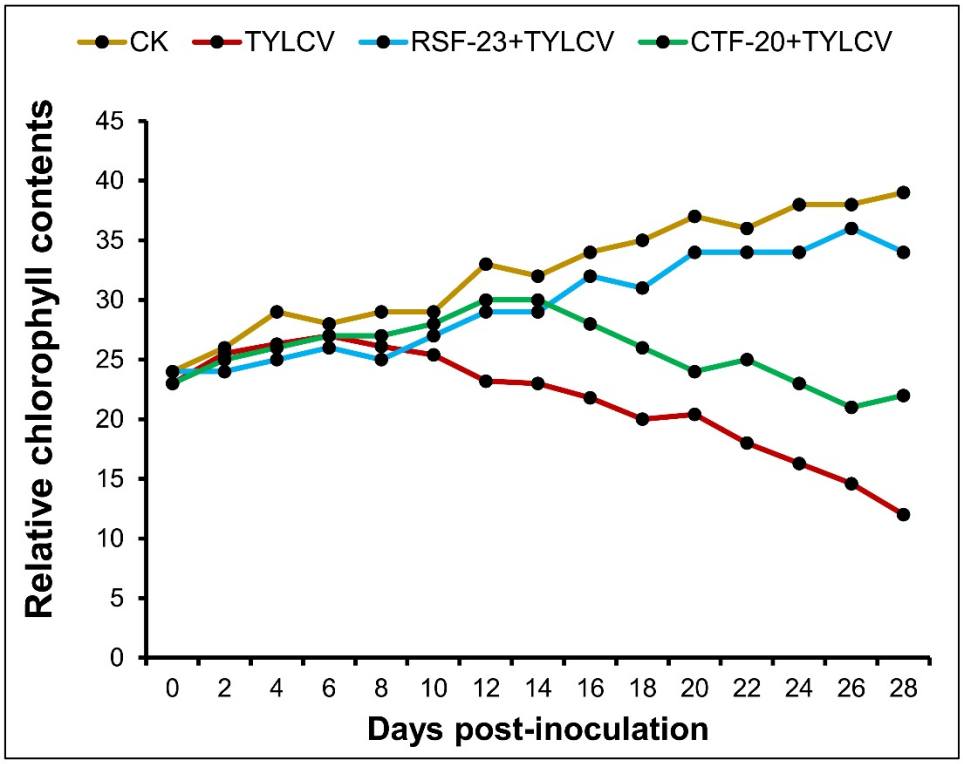

Figure 4. Estimation of total chlorophyll contents among CK (control), TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatmented *N. benthamiana* plants at 0–28 dpi.

3.5. Comparative Effect of Bacterial (RSF-23 and CTF-20) Treatment on the Chlorophyl Biosynthesis Pathway

To further expand the mechanistic understanding behind the effect of bacterial strains treatment on chlorophyll biogenesis, we estimated the relative transcription levels of three structural genes (chlorophyllase 1, *CLH1*; glutamyl tRNA reductase, HEMA1 and protochlorophyllide oxidoreductase A, PORA) associated with the chlorophyll biogenesis pathway. The results revealed that the expression of *CLH1*, *HEMA1* and *PORA* was significantly reduced among TYLCV-infected plants at 28 dpi, as compared to that of untreated (control) plants (Figure 5). However, the relative transcriptional levels of these three genes were significantly higher among TYLCV-infected plants treated with RSF-23; although their expression remained lower than that of untreated (control) plants (Figure 5). Moreover, the transcriptional changes associated with *CLH1*, *HEMA1* and *PORA* were

either lower than, or remained non-significantly different among, the plants treated with the CTF-20 strain (Figure 5). These findings implied that the treatment of RSF-23 exhibited potential to improve/maintain the biosynthesis of chlorophyll, despite the onset of viral infection, and this effect was higher, as compared to CTF-20 treatment.

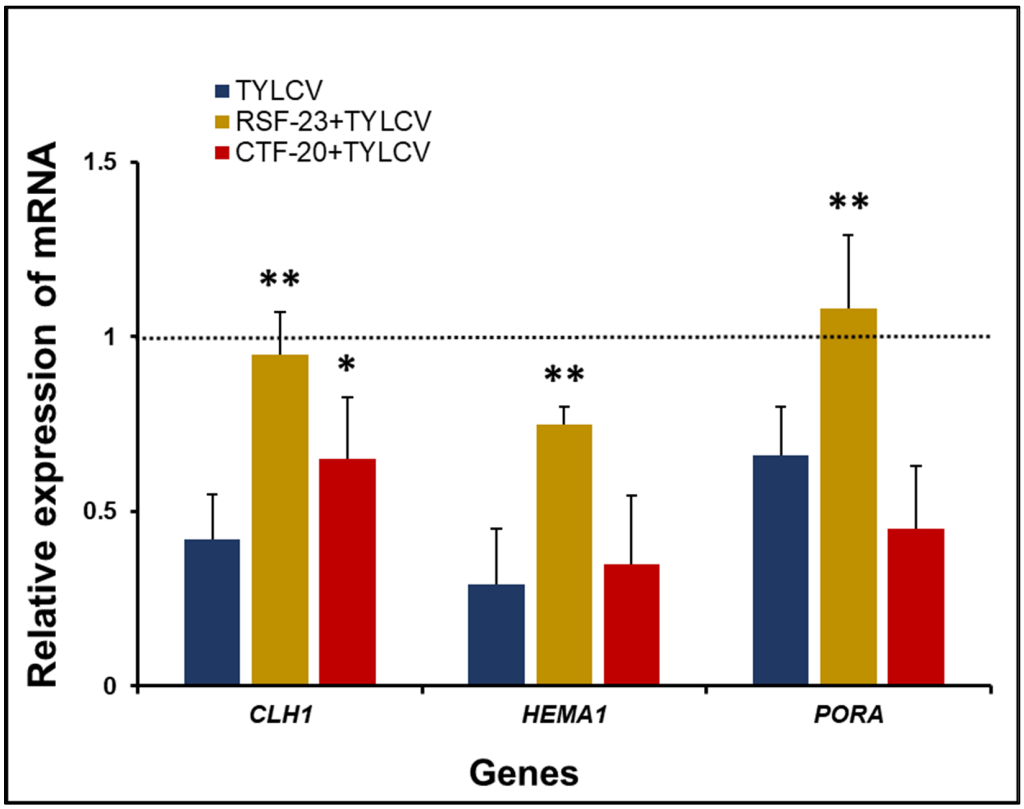

Figure 5. Relative expression of different genes (*CLH1*, *HEMA1* and *PORA*) associated with biogenesis of chlorophyll. The comparison was made among CK (control), TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatmented *N. benthamiana* plants at 28 dpi. The dotted horizontal line denotes that gene expression was normalized to 1 (CK). Asterisks indicate the levels of significance at * $p \leq 0.05$ and ** $p \leq 0.01$.

3.6. Activation of Defense-Related Genes in Response to Bacterial Treatment

Since the salicylic acid (SA) and abscisic acid (ABA) pathways are well-known to be associated with defense-related signaling, we opted to quantify the transcriptional changes of two genes (*NbCIS* and *NbNCED*) associated with the biogenesis of SA and ABA, respectively. Our results indicated that at 9, 18 and 28 dpi, the expression of *NbCIS* gradually increased among the plants infected with TYLCV, indicating the activation of plant defense-related SA signaling. However, at 9 and 18 dpi, the expression of *NbCIS* was significantly higher among virus-infected plants that were treated with the RSF-23 strain (Figure 6A). At 28 dpi, although the expression of *NbCIS* was still higher as compared to that of TYLCV-infected plants, the difference was statistically not significant. On the other hand, the CTF-20-mediated effect on the mRNA expression of *NbCIS* was higher at 9 dpi (non-significant) and 18 dpi (significant), while the *NbCIS* expression remained lower than TYLCV-infected and RSF-23-treated plants at 28 dpi (Figure 6A).

Furthermore, the expression of *NbNCED* among TYLCV-infected plants increased at 9 and 18 dpi, while it decreased at 28 dpi, as compared to that of untreated (control) plants (Figure 6B). Interestingly, at all of the time points, the mRNA expression levels of *NbCIS* were significantly higher among virus-infected plants treated with RSF-23. On the contrary, the expression of *NbCIS* among TYLCV-infected plants treated with CTF-20 was slightly higher at 18 dpi, as compared to the TYLCV-infected group (Figure 6B); although,

this difference remained lower (at 9 dpi), or slightly higher (at 28 dpi), implying a mild or non-significant effect of the CTF-20 strain in the induction of plant defense responses.

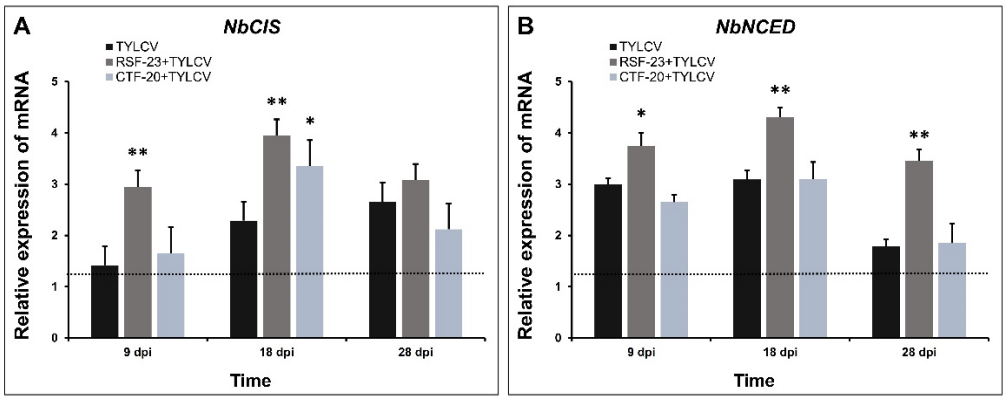

Figure 6. Relative expression of different genes such as *NbCIS* (**A**) and *NbNCED* (**B**) associated with biogenesis and accumulation of salicylic acid and abscisic acid. The comparison was made among CK (control), TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatmented *N. benthamiana* plants at 9, 18 and 28 dpi. The dotted horizontal line denotes that gene expression was normalized to 1 (CK). Asterisks indicate the levels of significance at * $p \leq 0.05$ and ** $p \leq 0.01$.

3.7. Activation of Antioxidant Enzymes by RSF-23 Strain in Response to TYLCV Infection

The combinatory effect of TYLCV infection and bacterial treatment on the induction of antioxidant enzymes is poorly understood. We, therefore, analyzed the enzymes associated with reactive oxygen species (ROS), including superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and peroxidase (POD), at 28 dpi. The results indicated that at 28 dpi, the activity of SOD increased by 26.31%, due to TYLCV infection, as compared to that of untreated (control) plants. While the SOD activity in response to RSF-23 treatment was significantly higher (34%), as compared to that of TYLCV, followed by a (non-significantly) higher (11%) activity of SOD among CTF-20-treated plants (Figure 7A). A similar pattern of changes in the activity of MDA enzyme was observed, where the MDA activity was significantly higher ($p < 0.01$ and $p < 0.05$) among RSF-23 (33.63%) and CTF-20 (27.27%)-treated plants as compared to that of the TYLCV-infected group of plants (Figure 7B). Further, a contrasting pattern in the change of CAT activity was observed where RSF-23 and CTF-20 successfully induced enzymatic activity by 47.3 and 17.36% as compared to the control group, but it was not significantly higher than that induced by the viral infection (Figure 7C). Finally, the POD enzyme was significantly ($p < 0.01$) induced both by RSF-23 (35.71%) and CTF-20 (128.57%) strains, as compared to the changes induced by TYLCV infection (Figure 7D). Overall, these results indicated that RSF-23 treatment prior to TYLCV infection could successfully induce antioxidant responses, mediated by POD, MDA and SOD, followed by a non-significantly higher activity of CAT.

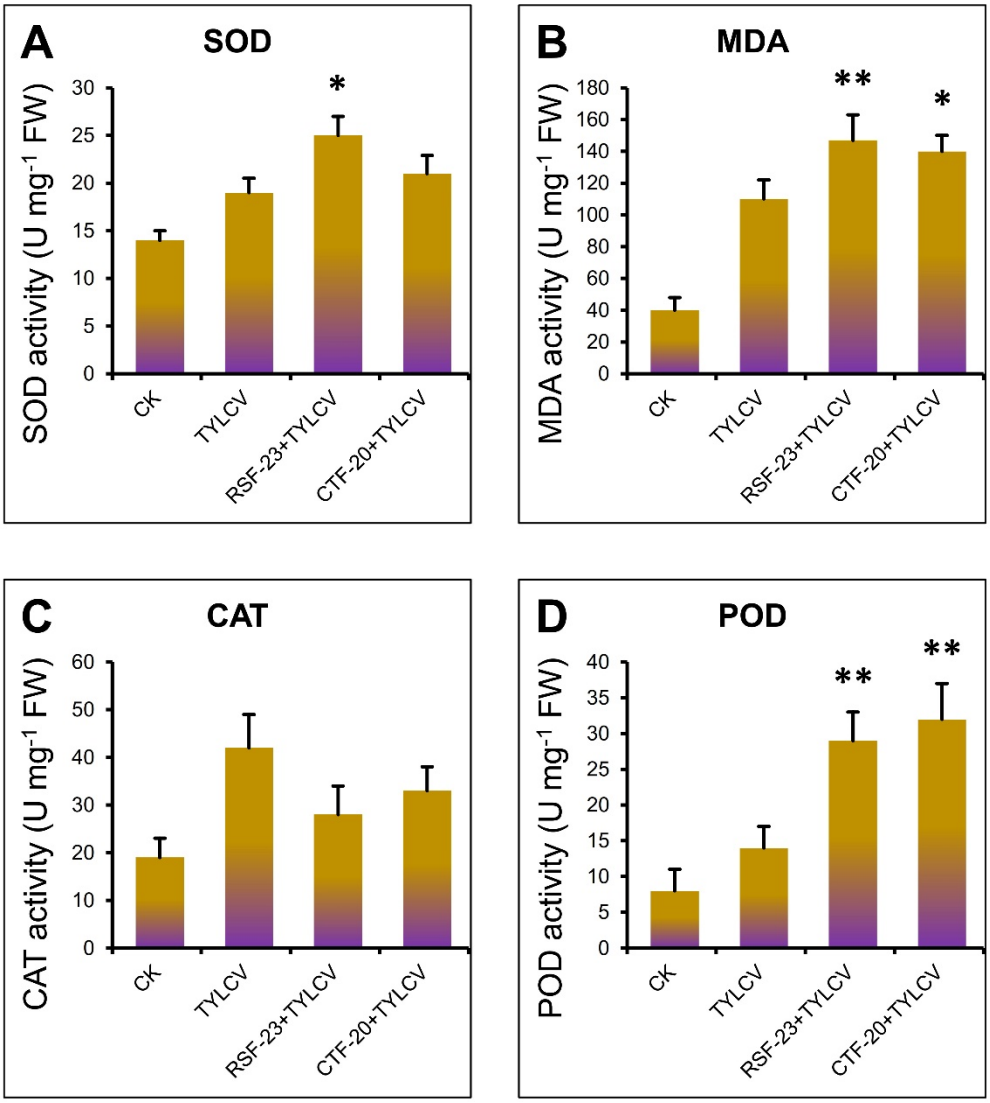

Figure 7. Analysis of key enzymes involved in the antioxidant pathway. (**A**) superoxide dismutase (SOD); (**B**) malondialdehyde (MDA); (**C**) catalase (CAT) and (**D**) peroxidase (POD). The enzyme concentrations were estimated at 28 dpi and comparisons were made between CK (control), TYLCV-infected, RSF-23 + TYLCV and CTF-20 + TYLCV-treatmented *N. benthamiana* plants. Asterisks indicate the levels of significance at * $p \leq 0.05$ and ** $p \leq 0.01$.

4. Discussion

Plant pathogenic bacteria, viruses, nematodes and fungi cause several devastating diseases of wheat, rice, potato, soybean, maize and other economically important crops worldwide [32]. These diseases are capable of causing crop losses up to 40%, which, on average, could cause annual losses of ~220 billion USD [32]. To date, several integrated, environmentally safe and efficient management strategies (based on the use of biological and/or non-biological disease controlling agents) have been proposed and implicated in tackling these plant pathogens. These agents not only function to control the diseases but also improve the overall plant growth and development and resistance to plant pathogens [33–37]. TYLCV is a whitefly (*Bemisia tabaci*)-vectored, DNA plant virus that induces severe disease symptoms accompanied by physiological abnormalities in the infected plants. and which has the capability to cause up to 100% yield losses in tomato/ It, thus, poses an increasing challenge to crop management strategies worldwide [38–41]. It is currently unknown how actinomycetes bacterial strains can be employed as disease protective agents, especially against DNA plant viruses. In the current study, we employed

two strains (RSF-23 and CTF-20) of actinobacterial streptomyces spp. and compared their effects on the morphology, physiology, gene expression and enzymatic activity of TYLCV-infected plants. We demonstrated that both strains (RSF-23 and CTF-20) could successfully hinder the development of viral symptoms, maintain the leaf area, fresh weight and relative chlorophyll content. Moreover, these actinobacterial strains were able to induce plant defense responses and antioxidant systems resulting in reduced virus accumulation in the TYLCV-infected plants. Importantly, the intensity of protective effects was significantly higher among RSF-23-treated plants, as compared to those treated with the CTF-20 strain. To our knowledge, this is the first study that has evaluated the protective role of actinobacterial strains against DNA plant viruses.

In this study, we observed that the RSF-23 strain significantly promoted plant growth and development and reduced the accumulation of TYLCV DNA in the infected plants, resulting in the mild disease symptoms, as compared to the plants that were untreated. This observation was supported by the fact that actinobacterial strains are well-known to produce metabolites that can not only promote plant growth [42], but also exhibit many other properties, including antiviral [43] and antitumor activities [44], stimulation of immunity [45] and antibiotic activities [46]. These actinobacterial strains can directly or indirectly increase plant growth and yield [42]. The antiviral activity demonstrated by RSF-23 is presumably due to the production of biologically active substances by these strains that could block the replication/proliferation of the viruses. For instance, the antiviral/inhibitory activities associated with biologically active molecules produced by the actinobacterial strains have been documented by researchers [47]. We also found that actinomycetes strains (RSF-23 and CTF-20) maintained the relative chlorophyll contents, despite the status of the plants having been infected with virus. These results are supported by a recent study which evaluated 14 actinomycetes strains of plant growth-promoting bacteria (PGPB) for their antibiotic activities. Reportedly, these strains were able to maintain normal plant growth and development, morphological and biochemical changes, and also the chlorophyll contents remained less affected [48].

Our findings also demonstrated that the defense responses among TYLCV-infected plants were significantly upregulated by RSF-23 strain. A recent study has reported that despite the presence of fungal (*Fusarium verticillioides*) infection, the actinomycetes bacterial strain ST03 was able to promote plant growth and induced the transcription of several genes involved in the ABA, auxins, SA, jasmonic acid (JA) and gibberellic acid-mediated signaling pathways [49]. Similarly, several studies have documented the ABA- and SA-mediated activation of systemic acquired resistance against plant viruses resulting in the establishment/development of resistance/antiviral signaling pathways and reduced virus accumulation [50–53]. Likewise, findings of a recent study demonstrated that *S. cellulosae* (isolate Actino-48) could efficiently induce the SAR among plants infected by an RNA virus (Tobacco mosaic virus, TMV) [24]. The SAR-associated activity of Actino-48 isolate was assessed by the activation of defense-related genes (*CHS*, *PAL*, *PR-1*, *PR-2* and *PR-3*) in the TMV-infected tomatoes [24].

In addition to the activation of defense-related genes, our findings revealed that the RSF-23 strain successfully enhanced the activities of defense-related enzymes, including MDA, DOD, CAT and POD. Notably, the incline in the measured enzymatic activities was significantly higher among RSF-23-treated plants, as compared to those observed in the plants treated with CTF-20 strain. These results are in accordance with findings that DH-16 strain of *S. hydrogenans* successfully activated several enzymes, including CAT, MDA, SOD, POD and GPOX, in response to pathogenic infection of tomato seedlings [54]. Similarly, another study, involving the SPS-33 strain of *S. lavendulae*, investigated the role of volatile organic compounds (VOCs) inducing changes in antioxidant enzymes, and concluded that VOCs from SPS-33 enhanced the activities of SOD, CAT, and POD, while decreasing the activity of MDA in *Ipomoea batatas* (L.) Lam. infected with *Ceratocystis fimbriata* [55]. We hypothesize that the disease protective activity demonstrated by RSF-23 might be associated with VOCs induced by this strain; however, this requires further extensive

studies for validation. To date, numerous studies have documented that VOCs from actinomycetes exhibit a variety of functions, including antiviral, antifungal, antibacterial, antitumor, cytotoxic and antioxidant activities [44,54,56–60]. The results of our studies enrich the current knowledge of novel biocontrol agents that exhibit potential protective activities and pave the way to plan and establish sustainable disease management strategies against plant viral pathogens.

5. Conclusions

Our findings demonstrate that the RSF-23 strain of *Streptomyces* spp. efficiently limited viral infection among TYLCV-infected plants and limited disease development, by dampening the disease symptoms and virus accumulation. The RSF-23 strain also maintained the relative chlorophyll content and promoted the mRNA expression of the genes (*CLH1*, *HEMA1* and *PORA*) associated with chlorophyll biogenesis. This was accompanied by higher expression of plant defense-related genes (*NbCIS* and *NbNCED*) and activation of significantly high defense responses, representing elevated SR via higher expression of antioxidant enzymes (SOD, CAT, POD and MDA), among virus-infected plants. A mechanistic explanation of the protective activity displayed by RSF-23 is shown in Figure 8. Taken together, this is the first study that provides clues on the protective activity of the RSF-23 strain against a DNA plant virus.

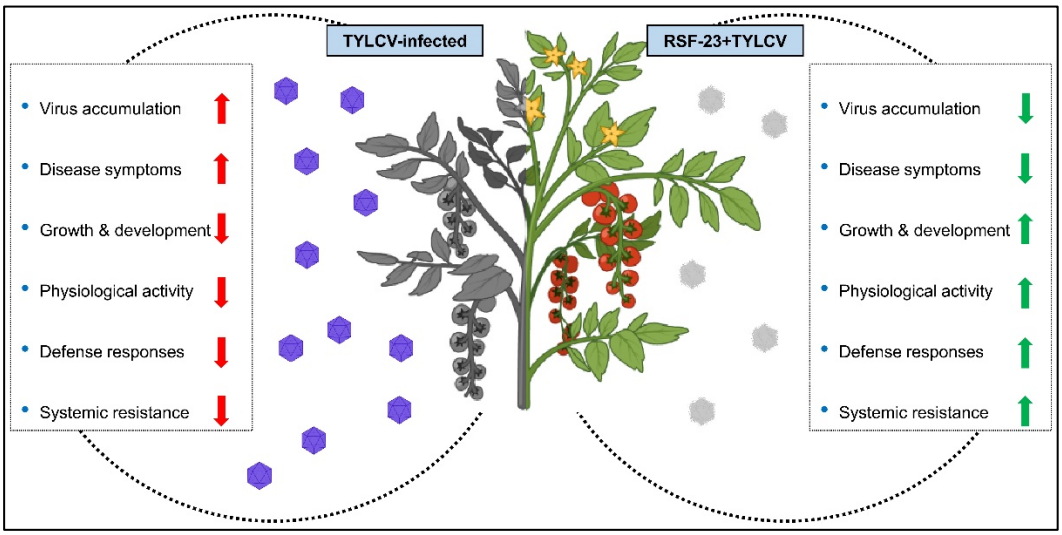

Figure 8. A proposed mechanism of protective activity from RSF-23 strain.

Author Contributions: Conceptualization, D.C., M.S.E., C.M. and M.N.H.A.A.; methodology and software, M.K., D.C., M.N.H.A.A. and M.A.M.; Investigation, D.C., F.M.-P., M.W., M.A.M. and M.N.H.A.A.; validation, M.W., M.K., M.S.E., F.M.-P. and M.Y.Z.; formal analysis and investigation, R.M.A. and D.C.; writing & original draft preparation, D.C., M.N.H.A.A., C.M. and M.K.; writing—review and editing, R.M.A., M.A.M., F.M-P., M.S.E. and M.W.; visualization, D.C., M.Y.Z. and M.K.; supervision, project administration, and funding acquisition, D.C., M.S.E., R.M.A. and C.M. All authors have read and agreed to the published version of the manuscript.

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