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Research Article

Molecular and Phenotypic Responses of Rhizobacteria-Treated Tomato Plants to *Tomato Mosaic Virus* Under Greenhouse Conditions

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Background: Tomato mosaic disease, mainly caused by *Tomato mosaic virus* (ToMV), is one of the devastating viral diseases which adversely affects tomato yield, globally. Plant growth-promoting rhizobacteria (PGPR) have been recently used as bio-elicitors to induce resistance against plant viruses.

Objectives: The goal of this research was to apply PGPR in the tomato rhizosphere and to determine the response of plants challenged with ToMV infection, under greenhouse conditions.

Materials and Methods: Two different strains of PGPR, *Pseudomonas fluorescens* SM90 and *Bacillus subtilis* DR06, in single- and double-application methods applied to evaluate their effectiveness in inducing defense-related genes, viz., *NPR1*, *COI1,* and *PR1-a* before (induced systemic resistance [ISR]-prime) and after (ISR-boost) ToMV challenge. Additionally, to investigate the biocontrol potential of PGPR-treated plants against viral infection, plant growth indices, ToMV accumulation, and disease severity were compared in primed and non-primed plants.

Results: Analysis of expression patterns of putative defense-related genes before and after ToMV infection indicated that studied PGPR trigger defense priming through different signaling pathways acting at the transcriptional level and in a species-dependent manner. Moreover, the biocontrol efficacy of consortium treatment did not differ significantly from the single bacteria treatments, even though their mode of action differed in transcriptional changes of ISR-induced genes. Instead, simultaneous application of *Pseudomonas fluorescens* SM90 and *Bacillus subtilis* DR06 led to more significant growth indices than the single treatments suggesting that integrated application of the PGPR could additively reduce the disease severity and virus titer and promote the growth of the tomato plant.

Conclusions: These results suggested that enhanced defense priming via activation of the expression pattern of defense-related genes is responsible for biocontrol activity and growth promotion in PGPR-treated tomato plants challenged with ToMV compared to non-primed plants, under greenhouse conditions.

Keywords: Defense-related gene, Defense priming, Mosaic disease, Transcriptional changes, Virus accumulation

1. Background

Mosaic diseases are one of the worldwide causes for a significantly reduced yield of cultivated tomato (*Solanum lycopersicum* L.) (1). The disease is caused mainly by Tomato mosaic virus (ToMV), which induces leaf mosaic or mottle with alternating yellowish and darker green areas, leading to malformation of leaves in the virus-infected tomato plants (2). ToMV has taxonomically placed in the genus *Tobamovirus* and family *Virgaviridae* (3). The yield of ToMV-susceptible cultivars may be reduced up to 25% by ToMV infection (4).

Viral disease incidence can be minimized by applying chemical-based insecticides that are used to control the vector population transmitting the viral disease (5). However, public concerns about emerging pesticide -resistant phytopathogen and environmental contaminants have limited their application (6). In addition, conventional breeding programs and genetic manipulation to develop virus-tolerant cultivars are alternative methods for controlling the viral disease. However, the time-consuming crossing process, limited availability of natural resistance sources at the commercial level, and environmental safety issues on genetically modified crops have limited success in these techniques (7). It has been demonstrated that virus-mediated systemic acquired resistance (SAR) is effective for managing the viral diseases (8). Besides plant resistance responses that are triggered by a localized virulent strain of virus, the development of biological control methods based on enhanced defensive capacity using plant growth-promoting rhizobacteria (PGPR) has been reported to reduce the deteriorative effects of tomato viruses (7, 9). In recent years, rhizobacteria has been introduced as a suitable and alternative strategy to control plant diseases (10-13). Among them, *P. fluorescens* and *Bacillus subtilis* are well-known as probiotic agents that have a high ability to biologically control plant pathogens (10, 14, 12). Notably, Megahed *et al*. (15) showed that treatment of *Datura metel* L. seed with *P. fluorescens*, and *B. circulans* reduced ToMV symptoms and local lesions. However, their mechanism for symptom remission has not been determined.

Rhizobacteria-mediated induced systemic resistance (ISR) was found to be effective against a wide variety of plant pathogens (16). It has been shown that ISR predominantly functions through jasmonate (JA) and ethylene (ET) signaling pathways in an SA-independent manner (17). Although elicitor type and signaling pathways involved in ISR and SAR are different (18), several PGPR have been demonstrated to elicit SA-dependent ISR resembling pathogen-induced SAR (19). Moreover, both SAR and ISR require redox-regulator protein Nonexpressor of *PR* genes 1 (NPR1). The monomeric form of NPR1 nuclear localization acts as a critical transcription co-regulator of SA-responsive pathogenesis-related (PR) genes. It is, however, interesting to note that the function of cytoplasmic NPR1 mediating JA/ET- dependent ISR seems to be clearly distinguished from that in SAR (reviewed in 20). Moreover, it has been found that F-box protein coronatine insensitive1 (COI1) as jasmonate receptor plays an essential role in the activation of JA-mediated resistance responses (21). Surprisingly, simultaneous expression of both types of induced resistance, JA and SA-dependent pathways, leads to an increasingly enhanced defensive response against a broader spectrum of pathogens than ISR and SAR alone (22). Induced resistance is accompanied by a potentiated expression pattern of defense-related genes upon colonization of plants with beneficial microbes that invisibly prepares the whole plant to better activate systemic immune responses when subsequently challenged with pathogens (20). This alert state of gene activation induced by biocontrol agents (BCAs) is called "priming". Accordingly, two distinct definitions are observed in plant systemic immunity: "ISR-boost" is the phenomenon that explains the more protective potential of the defense system upon pathogen attack. However, "ISR-prime" refers to ISR that is stimulated by BCAs before additional inoculation with a pathogen (23).

Many *Bacillus* and *Pseudomonas* species can improve the growth condition and induce resistance against the invading virus (24). PGPR have been used to control several tomato-infecting viruses such as *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato chlorotic spot virus* (TCSV), *Potato virus Y* (PVY) and *Papaya ringspot virus* (PRSV) and ToMV (reviewed in 9). However, their application on ToMV-infected tomato host and the mechanism by which PGPR induce resistance against the virus has not been investigated.

2. Objectives

This study was aimed to assess the effectiveness of two different PGPR, including *Pseudomonas fluorescens* SM90 and *Bacillus subtilis* DR06, in inducing defense-related genes, viz., *NPR1*, *COI1,* and *PR1-b* before (ISR-Prime) and after (ISR-boost) ToMV challenge. Also, to investigate the biocontrol potential of PGPR-treated

plants against viral infection, growth indices and disease severity in primed and non-primed tomatoes were evaluated.

3. Materials and Methods

3.1. Plant Material

Seed of tomato (*Solanum lycopersicum* L. cv. Early Urbana Y) was sown into seed tray containing pre-autoclaved perlite-cocopeat (the volume ratio of 1:1). The seed was irrigated daily and kept in the dark chamber at room temperature for three days. The emerged seedling was then transferred into 1-liter plastic pots containing sterilized peat moss and soil (1:1) and grown in a growth chamber with conditions of 24-28 °C, 40% RH, and 16/8 photoperiod.

3.2. Bacterium Source

Two bacterial strains, including *P. fluorescens* SM90 and *B. subtilis* DR06, were provided by Persian Type Culture Collection at the Iranian Research Organization for Science and Technology (Tehran, Iran). Nutrient Broth (NB, Merck, Germany) medium containing 12.5 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$ of chloramphenicol (Sigma-Aldrich, USA) was used to grow *P. fluorescens* SM90 and *B. subtilis* DR06, respectively. The cultured media were incubated at 25 °C for 24 h. To produce bacterial suspensions, Luria Broth (LB, Merck, Germany) was inoculated with the resulting colonies of each strain and kept at 25 °C for 48 h while shaking (150 rpm). The optical density (OD) of bacterial suspensions was measured at 600 nm wavelength using Cary 100 UV-Vis (Agilent, USA) and then adjusted to OD: 1 (10^8 $cell.mL^{-1}$) (25).

3.3. PGPR Treatment

An equal volume of 10 mL from each bacterial suspension (10^8 cfu. mL^{-1}) was added to the rhizosphere of tomato seedlings at the four-leaf stage. The double application included 5 mL of each bacterial suspension. Treatments included two single-applications of *P. fluorescens* (Pf) and *B. subtilis* (Bs), as well as one double-application of *P. fluorescens*+*B. subtilis* (Pf+Bs). Plants treated with a bacterium-free LB medium were used as control. Two sets of control, including negative and positive (non-treated and ToMV-infected, respectively) controls, were considered in the experiment. The plants were sampled 24, 72, and 110 hours post treatment (hpt), and sample tissues were immediately immersed into liquid nitrogen and stored at -80 °C.

3.4. Virus Source

An Iran-originated isolate of ToMV (AWZHR-S95) which had been partially characterized (26) was used in this study.

3.5. Virus Inoculation

ToMV-infected tomato leaves showing mosaic symptoms were used as inoculum for mechanical inoculation of the tomato plant. The plant tissue of symptomatic leaves was first homogenized into 0.01 M sodium phosphate buffer pH 7.0 (1 g tissue to 1 mL buffer). The resulting extract was rubbed on carborundum-dusted leaves of rhizobacteria-treated tomato plants at the four-leaf stage, 24 days post treatment (dpt). At least three plants per inoculation were tested, and the whole experiment was repeated two times. The inoculated plants were kept in greenhouse conditions at 21-26 °C. The plants were sampled 48 hours post inoculation for gene expression analysis. Symptom development on inoculated plants was inspected weekly till 28 days post inoculation (dpi).

3.6. Determination of Biological Indices

Biological indices, including above- and under-ground fresh weight and plant height, were measured for rhizobacteria-treated plants challenged with ToMV 28 dpi. To determine the plant biomass, the tissues were incubated at 72 °C for 72 h. The mean value of each treatment was subjected to variance analysis (ANOVA) using SAS software (ver. 9.3). Then Duncan's multiple range test was applied to find any significant difference at two levels of $P = 0.01$ and $P = 0.05$.

3.7. Disease Severity Assay

Disease severity of the ToMV-inoculated plants was measured by visual scoring based on their symptoms [0, no symptom; 1, mild mosaic; 2, mosaic and malformation; 3, severe mosaic and malformation; 4, death]. The scoring was carried out 22- and 30-days post inoculation and the data was analyzed as described above.

3.8. Virus Detection

Plant tissues collected from inoculated plants 28 dpi were subjected to Indirect-ELISA with a polyclonal

antibody of ToMV (BIOREBA, Switzerland) using the method described by Clark and Adams (27). To perform a semi-quantitative ELISA, plant tissue was homogenized in extraction buffer in a ratio of 1:10, 1:20, 1:40, 1:50, and 1:60 (plant material: buffer). The data were statistically analyzed and the results of 1:40 ratio, in which the OD measured at 405 nm wavelength (OD_{405nm}) was correlated with the extract concentration, were selected. ToMV-infected tobacco (*N. tabacum* var. Turkish) sample was used as a positive control. Plant tissue of a healthy tomato plant was also used as a negative control. Three replicates were considered for each type of control. ELISA plate was analyzed using Thermo Labsystems microplate reader (Thermo Scientific, Germany) and the OD_{405nm} was measured. The samples with an OD_{405nm} value more than *M*+3*SD* (*M*; mean OD value of negative controls*, SD;* mean standard deviation value of negative controls) were considered positive.

3.9. RNA Isolation and ART-PCR

Total RNAs were extracted from the plant tissues using Column RNA Isolation Kit (Denazist Asia, Iran) and were quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). First strand complementary DNA (cDNA) was synthesized using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, UK) according to the manufacturer's protocol. To this end, 1 µg of isolated RNA was mixed with 1 µL of the reverse primer (10 pmoL.µL^{-1}) (Oligo dT), 10 µL of DEPC ddH_2O, 2 µL of dNTP Mix, 2 µL of RT buffer, and 1 µL of M-MuLV RT (0.5 unit. µL^{-1}) (New England Biolabs, UK). The incubation program included two steps of 50 °C for 50 min and 37 °C for 15 min.

To determine the change in expression of genes involved in plant defense pathway including *COI1, PR1B,* and *NPR1* as well as *Lycopersicum* elongation factor-1 alpha (*LOC544055*) as a reference gene, the oligonucleotides (**Table 1**) were designed using online Primer Quest ™ software, Integrated DNA technology available at https://eu.idtdna.com/.

To confirm the specific amplification of the genes using designed oligonucleotides, PCR was performed. To this end, 25 µL reactions consisting of 2 µL (30 ng) of cDNA, 1 µL of each primer (10 pmoL.µL^{-1}), 2.5 µL of 10X PCR buffer and 1 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 1 U of *Taq* DNA polymerase (Ampliqon, Denmark) and 16.8 µL of PCR-grade H_2O were prepared. The reaction microtube was primarily heated at 95 °C for 5 min and then subjected to a 35-cycle PCR procedure of 0.5 min at 95 °C, 0.5 min at 56-60 °C, 45 s at 72 °C followed by an extension step at 72 °C for 10 min. The Bio-Rad iCycler Thermal Cycler (Bio-Rad, USA) was used for PCR performance. Electrophoresis was then performed by running approximately 5 µL of the PCR products onto agarose (1.0%) gel previously stained with DNA Safe Stain (CINACLONE, Iran) and visualized under UV light.

Quantitative real time PCR (qRT-PCR) assay was performed in 25 µL reactions containing 2 µL (100 ng) of cDNA, 1 µL of each primer (10 pmoL.µL^{-1}), 12.5 µL of 2X SYBR Green qPCR Master Mix (High ROX, Cat. No. M3003S) and 8.5 µL of PCR-grade H_2O were prepared. The reaction mixtures were run using StepOnePlus™ Real-Time PCR System (ABI, USA) by following thermal condition; an initial denaturation of 95 °C for 10 min followed by 40 cycles, with one cycle consisting of denaturation at 95 °C for 15 s,

Table 1. The oligonucleotides used in this research.

Gene	Oligonucleotide	Sequence (5'-3')	*Ta* (°C)*	Expected product size (bp)
COI1	F R	AGTGAGAGGCTGCTGTTTC CCATCGCTAAGAGATCACGAC	57.86	125
PR1B	F R	ACTACGCTACCAACCAATGTG AGAAATGAACCACCATCCGT	58	125
NPR1	F R	CCAAGTCTACAGAGGAAGGA CAAATCATCGCCTGCCATAG	56.3	131
LOC544055	F R	GTTGTTGAGACCTTTGCTGA CAGTTGGGTCCTTCTTGTCA	56.8	112

*According to Integrated DNA Technologies (available at https://eu.idtdna.com/).

Table 2. The results of ANOVA for PGPR effect on biological indices of tomato plants.

Variation source	FD	MS				
		UFW	UB	AFW	AB	H
Treatment	4	10.91**	6.82**	55.52**	14.89**	30.63**
Error	10	0.79	0.54	1.25	0.58	0.97
Variation ratio		11.83	17.15	5.97	7.91	3.8

** represents the significance level of p=0.01.
FD: freedom degree, MS: mean square, UFW: under-ground fresh weight, UB: under-ground biomass, AFW: above-ground fresh weight, AB: above-ground biomass, H: height.

annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Two technical replications (cDNA) and two biological replications (plants) were considered per genes of interest. A melting curve was programmed after each run to verify the lack of dimerization or nonspecific amplification.

The analysis of obtained data was conducted according to the method described previously by Livak and Schmittgen (28). The data were first normalized using the difference in cycle threshold for different samples against the reference gene ($\Delta\Delta C_T$). Then the relative difference in expression of target genes was calculated using the Relative Expression Software Tool (REST) (29).

4. Results

4.1. PGPR Effect on Biological Indices of Tomato Plant

Statistical analysis showed that plant height, fresh weight of above- and under-ground organs, as well as above- and under-ground biomass were significantly affected by PGPR application (**Table 2**). Also, the results of the Duncan test showed that the mean of plant height under combination treatment (*B. subtilis*+*P. fluorescens*) (27.38 cm) was significantly more than that of plants under single treatments, including *P. fluorescens* and *B. subtilis* (24.83 cm and 24.74 cm, respectively), and virus control (ToMV-inoculated plant control) (20.87 cm). Moreover, the mean of under-ground fresh weight and biomass was recorded for *B. subtilis*+*P. fluorescens* treatment (9.06 and 5.66 g, respectively) was significantly more than those recorded for virus control (ToMV-inoculated plant) (4.18 g and 2.43 g, respectively). The results showed that the mean of above-ground fresh weight and biomass of plants under combination treatment (22.45 g and 11.05 g, respectively) was significantly more than those of plants under single treatments and virus control (12.89 g and 6.73 g, respectively). These results demonstrated the significant effect of PGPR on biological indices of ToMV-challenged plants (**Fig. 1, 2**).

4.2. Disease Severity

Investigation of PGPR effects on disease severity of ToMV showed that tomato plants treated with single treatments including *B. subtilis* and *P. fluorescens*, and combination treatment (*B. subtilis*+*P. fluorescens*) had a mean index of 1.41, 1.08, and 0.91, respectively, compared to the positive control (ToMV-infected plant) (2.75) exhibiting a significant reduction in disease severity index ($p \leq 0.05$) **(Fig. 3)**.

4.3. ToMV Coat Protein Titration

ELISA-based assessment of viral infection in plants showed that the viral coat protein titer found in tomato seedlings treated with rhizobacteria was significantly low compared to the positive control (ToMV-infected plant) (**Fig. 4**). These results showed that combination and, or single treatment of *B. subtilis* and *P. fluorescens* can reduce the ToMV coat protein titer within tomato plants.

4.4. Analysis of Defense-Related Genes Expression Level

Application of the PGPR strains to the rhizosphere of tomato plants resulted in significant changes in the expression level of defense-related genes, *COI1, NPR1,* and *PR1b,* 24, 72, and 110 hpt (**Fig. 5**).

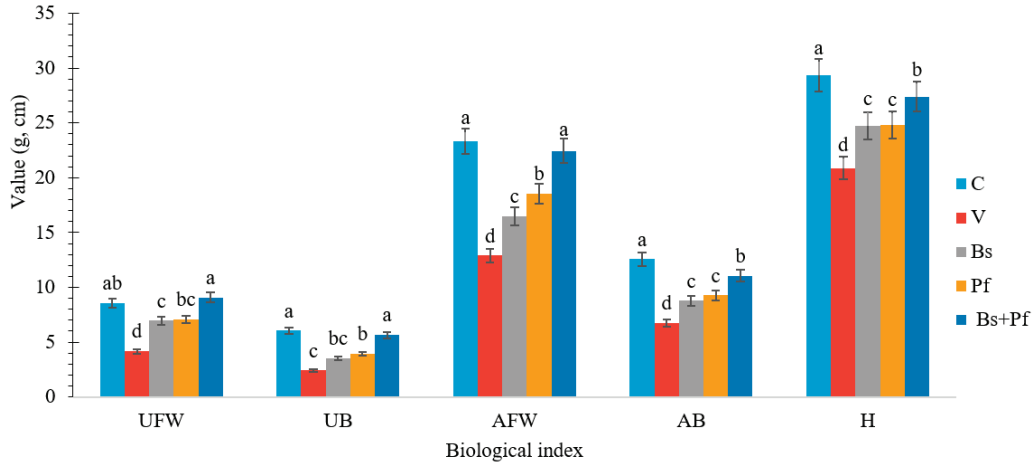

Figure 1. Effect of PGPR application on biological indices of ToMV-challenged tomato plant. Letters on bars show the significant difference between the treatments according to the results of Duncan's multiple range test. C: negative control (non-treated), V: virus control (ToMV-infected), Bs (*B. subtilis*-treated), Pf (*P. fluorescens*-treated), Bs+Pf (*B. subtilis*+*P. fluorescens*-treated). UFW: under-ground fresh weight, UB: under-ground biomass, AFW: above-ground fresh weight, AB: above-ground biomass, H: height. Error bars refer to the standard error of the mean values.

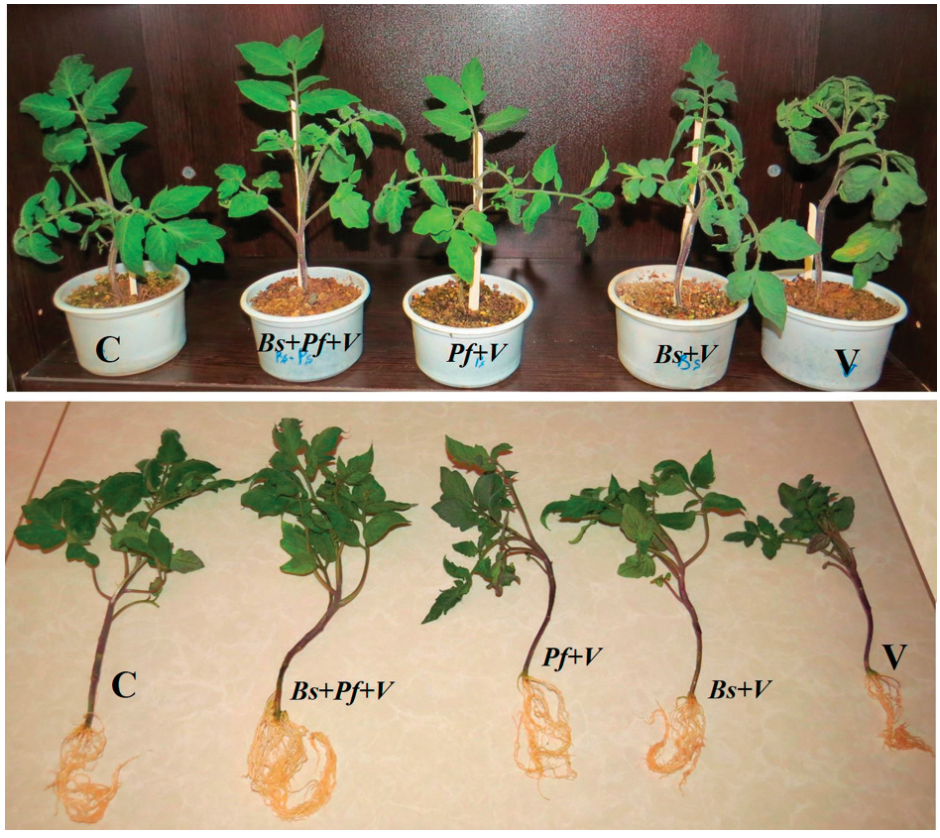

Figure 2. Effect of PGPR application on biological indices of ToMV-challenged tomato plant. Letters on bars show the significant difference between the treatments according to the results of Duncan's multiple range test. C: negative control (non-treated), V: virus control (ToMV-infected), Bs (*B. subtilis*-treated), Pf (*P. fluorescens*-treated), Bs+Pf (*B. subtilis*+*P. fluorescens*-treated).

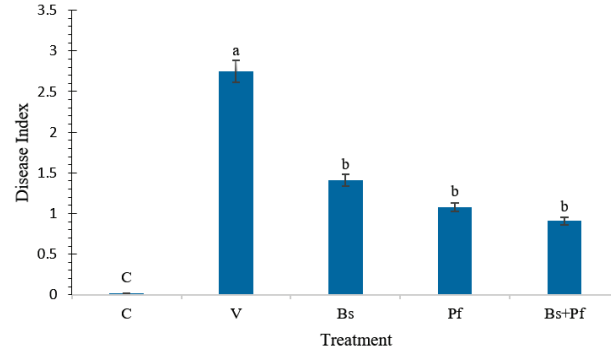

Figure 3. Mean of disease severity of ToMV in tomato plant treated with PGPR. C: negative control (non-treated), V: virus control (ToMV-infected), Bs (*B. subtilis*-treated), Pf (*P. fluorescens*-treated), Bs+Pf (*B. subtilis*+*P. fluorescens*-treated). Error bars refer to the standard error of the mean values.

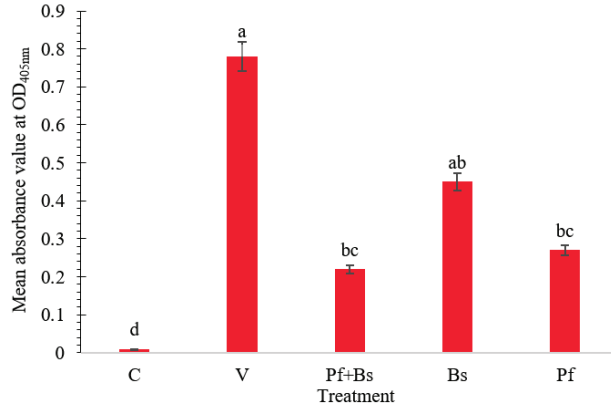

Figure 4. ELISA extinction values from ToMV-inoculated plants treated with different rhizobacteria including. C: negative control (non-treated), *Pseudomonads fluorescens* (Pf), *Bacillus subtilis* (Bs), and *Pseudomonads fluorescens*+*Bacillus subtilis* (Pf +Bs) compared to positive control (V). Error bars refer to the standard error of the mean values.

In *Bs*-treated plants, the expression of *COI1* and *PR1b* was significantly increased 24 hpt while the expression *NPR1* was increased 72 hpt. Moreover, these plants exhibited a significant increase in expression of *COI1* at 110 hpt. Treatment of *Pf* resulted in a significant increase in *PR1b* at 24 hpt, while *COI1* did not show any significant expression until 72 hpt. *Pf*-treated plants exhibited a significant increase in *COI1* and *PR1b* expression 110 hpt. Double-application of the PGPR resulted in a significant increase in *COI1* and *NPR1* expression at 24 hpt, while the expression of *PR1b* was not significantly increased until 72 hpt. All three genes showed a significant increase up to at 110 hpt (**Fig. 5**). These results showed that the single and double-application of PGPR could significantly increase the expression of defense-related genes.

The qRT-PCR results showed significant differences in the expression of defense-related genes (*COI1, NPR1,* and *PR1b*) in ToMV-inoculated tomato seedlings. The transcription of *COI1* in ToMV-challenged plants under single treatments with *B. subtilis* and *P. fluorescens* showed a significant ($p \leq 0.05$) increase, 48 h post virus inoculation, and the highest level of expression was recorded for *P. fluorescens*+ToMV treatment (4.3 fold higher than that in the positive control) (**Fig. 6**). However, simultaneous application of the two bacterial isolates exhibited no significant increase in *COI1*. Also, the transcription of *NPR1* was significantly ($p \leq 0.01$)

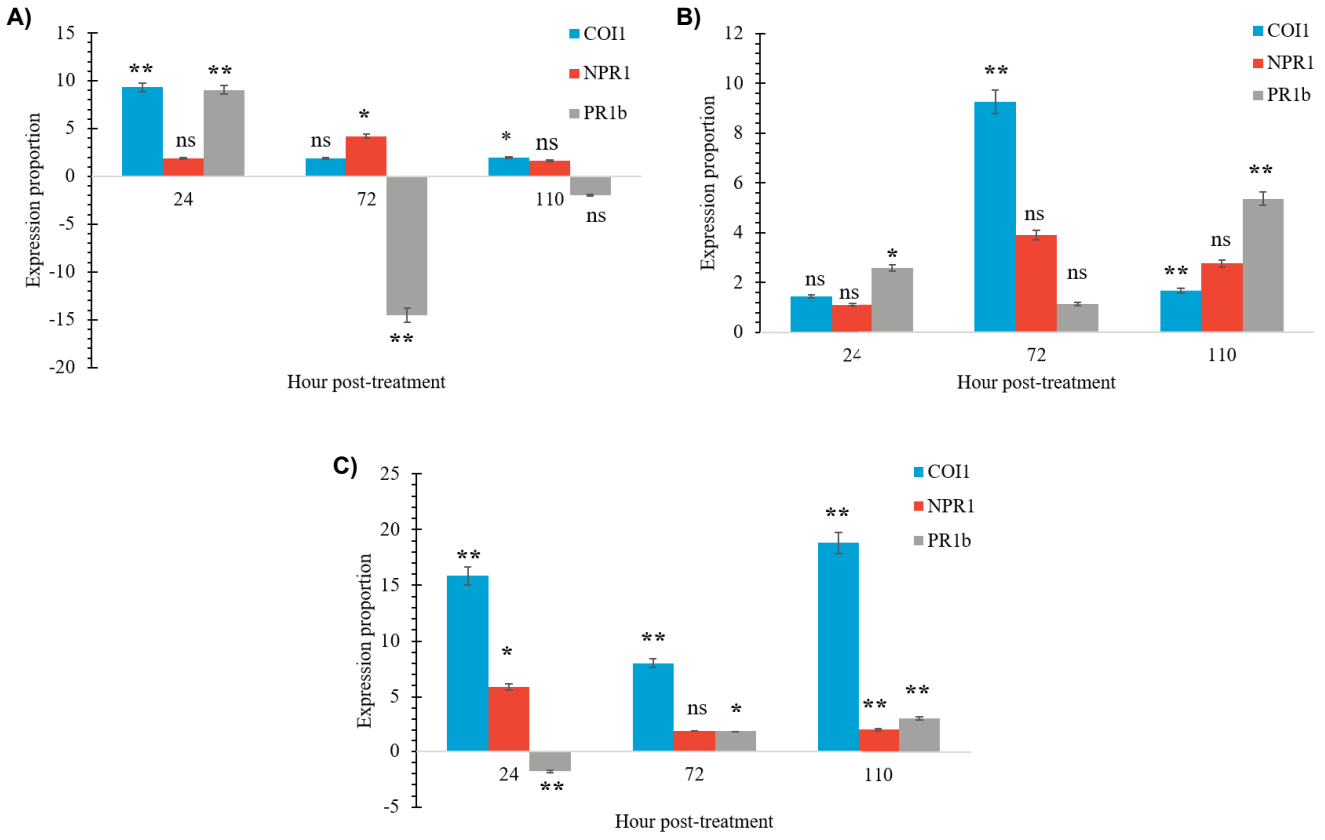

Figure 5. Expression of defense-related genes including *COI1, NPR1,* and *PR1b* in tomato plants treated with different rhizobacteria including A) *Bacillus subtilis*, **B)** *Pseudomonads fluorescens,* and **C)** *Pseudomonads fluorescens+Bacillus subtilis,* 24, 72, and 110 hour post-treatment. Fold change (Y-axis) represents the ratio of the expression of the target gene in PGPR-treated plants relative to that of negative control (non-primed plants). The relative expression of these genes was calculated by REST software described by Pfaffl (29). ** and * represent significant increases at p=0.01 and p=0.05 levels, respectively. ns: non-significant.

increased, 48 h post inoculation of ToMV in *B. subtilis*-treated tomato seedlings. The highest expression level of *NPR1* was found in *B. subtilis*+ToMV treatment (5.75 fold higher than that in the positive control), while ToMV-challenged plants pre-treated with *P. fluorescens* showed no significant increase. In the case of *PR1b*, however, the transcription was significantly increased when the two bacterial isolates were applied simultaneously and, or *B. subtilis* was treated 48 h post-ToMV inoculation. The highest expression level of *PR1b* was observed in the combined application of *B. subtilis+P. fluorescens* treatment (10.2 fold higher than that in the positive control). However, no significant change was observed in the expression level of *PR1b* within plants pre-treated with *P. fluorescens* (**Fig. 6**).

5. Discussion

To survey whether ISR is triggered by transcriptional reprogramming of defense-related genes, we analyzed the transcript abundance of *NPR1*, *COI1* (regulatory genes), and *PR1-b* (signaling defense gene) in PGPR-treated plants. The results showed that double application of DR60 and SM90 synergistically up-regulated *COI1* for all intervals. It has been shown that a mixture application of PGPR has an additive effect on improving the efficacy of ISR responses compared to treatment with either PGPR alone (30). *COI1* is a central up-regulator of the JA signaling pathway in PGPR-mediated ISR which enhances a large number of ISR-primed genes (21). In our experiment, *PR-1b* showed the highest relative expression ratio in *P. fluorescens*

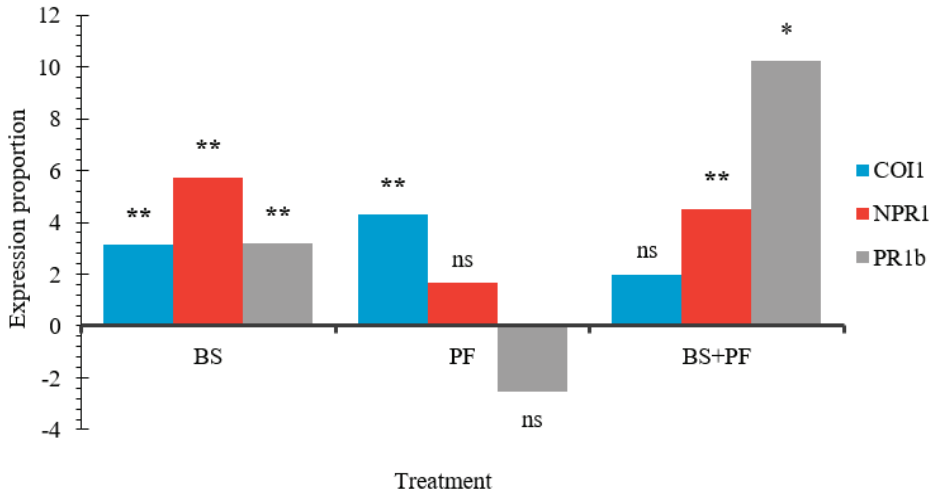

Figure 6. **Expression of defense-related genes including *COI1, NPR1,* and *PR1b* in ToMV-challenged tomato plants treated with different rhizobacteria including *Pseudomonads fluorescens* (Pf), *Bacillus subtilis* (Bs), and *Pseudomonads fluorescens*+*Bacillus subtilis* (Pf +Bs) 48 h post inoculation.** Fold change (Y-axis) represents the ratio of the expression of the target gene in PGPR-treated plants challenged with ToMV versus positive controls that are infected only with ToMV. The relative expression of these genes was calculated by REST software described by Pfaffl (29). ** and * represent significant increases at p=0.01 and p=0.05 levels, respectively. ns: non-significant.

SM90-treated plants at 110 hpt, while no significant change was detected in *NPR1* transcript accumulation within plants treated with the same bacterium at all intervals. In addition, mRNA accumulation of *COI1* and *PR1-b* reached a maximum level by treating *B. subtilis* DR06 at 24 hpt. The results showed that the interval of the maximum transcript accumulation of putative defense-related genes was different in plants inoculated with individual and combined strains. Indeed, systemically responsiveness by rhizobacteria was species-dependent. This suggests that differentially stimulated defense-related genes depend on the specificity of mutual recognition mechanisms in plant-PGPR interactions (31). Accordingly, in PGPR-mediated ISR, the explicit recognition and perception of microbe-associated molecular patterns (MAMPs) activate a variety of downstream signaling events mediating multiple expressions of the primed defense responses (31).

To gain an insight into underlying molecular responses of biocontrol protection conferred by PGPRs, we investigated the transcript profile of candidate genes in ISR-expressing plants 48 h after ToMV infection. Herein, *PR1-b* showed the highest expression in combined treatment of *B. subtilis* and *P. fluorescens* after ToMV inoculation. The induced expression of defense marker genes can be used as a molecular sign of incited disease resistance (32). Previous studies have illustrated that PGPR can enhance ISR through transcriptional activation of defense-related genes, especially *PRs,* upon viral infection (reviewed in 9). This enhancement has been reported for several PGPR-virus interactions such as *P. fluorescens* CHA0-TNV, *P. aeruginosa* 7NSK2-TMV, *Bacillus amyloliquefaciens* 5B6-CMV, *B. amyloliquefaciens* MBI600-*Potato virus Y* (PVY) and *B. amyloliquefaciens* MBI600-*Tomato spotted wilt virus* (TSWV) (reviewed in 9). In addition to antifungal and antibacterial properties, PR proteins including PR1-b are known to develop transgenic virus-resistant plants (reviewed in 33). It has been reported that the accumulation of PR proteins in non-infected organs inhibits the spread of viral infection between cells (reviewed in 33). As such, Abo-Zaid *et al*. (34) has reported that the systemic protection of tomato against TMV induced by *Streptomyces* was followed by the induction of the *PR1* gene. In addition, Wang *et al*. (35) proposed a significant relationship between enhanced defense responses

and the accumulation of PR proteins. Hence, our results supported the hypothesis that systemic defense response triggered by a combination of PGPR might be attributed to the antiviral activity of PR1-b protein. On the contrary, in single-PGPR treatment, *P. fluorescens* SM90, excluding *COI1*, no significant changes in the transcription of *NPR1* and *PR1-b* were observed after ToMV invasion. Moreover, disease protection conferred by *B. subtilis* DR06 against ToMV infection may be explained through co-expression of *PR1-b* and *COI1*. These results suggested that the PGPR trigger defense through manipulating transcriptional level in a species-dependent manner.

Also, it has been reported that PGPR application can decrease the viral disease symptom in plants (7, 9). The disease severity caused by TMV, for instance, has been decreased as a result of PGPR application in the tomato plant (36). Similarly, Wang *et al*. (35) showed that PGPR could decrease TMV symptoms in virus-infected tobacco plants. Moreover, virus accumulation within infected plants has been reported to decrease after PGPR treatment (35-36). These results are consistent with our observations in which PGPR-treated plants showed relatively low ToMV accumulation within their cells. Although integrated use of PGPR is expected to provide a synergistic effect on disease suppression, our results showed that biocontrol efficacy of consortium treatment did not differ significantly from the single bacteria treatments. Instead, simultaneous application of DR06 and SM90 strains led to more significant growth indices than their single treatment suggesting that integrated application of the bacteria could display additive effect in growth-promoting of the tomato plant. The positive effect of PGPR on growth indices of virus-challenged plants has been demonstrated in several studies. For instance, Li *et al*. (7) showed that the application of *Enterobacter asburiae* BQ9 could increase the fresh weight of tomato plants challenged by Tomato yellow leaf curl virus (TYLCV).

Accordingly, the outcome of the induced responses for ToMV invasion resulted in (1) decreased virus accumulation within infected cells, (2) symptom remission of the viral disease, and (3) improved growth indices. Hence, PGPR-induced defense priming was employed as an environmentally friendly strategy for biocontrol protection against ToMV. Moreover, single and double application of these PGPR showed similarly high efficacy against ToMV, even though their mode of action differed in transcriptional changes of ISR-induced genes before or after ToMV infection. The discrepancy between biocontrol protection and expression patterns of putative defense genes might be related to the specificity of mutual recognition mechanisms in plant-PGPR interactions when subsequently challenged with pathogens. However, more detailed molecular aspects about their interaction mechanisms are needed to recognize the differences.

6. Conclusion

Due to the considerable crop loss caused by ToMV, alternative control practices should be applied to increase the tomato yield. The PGPR have been described as bio-elicitors that can induce resistance against several plant viruses. Herein, *P. fluorescens* SM90 and *B. subtilis* DR06 could show the PGPR effects by increasing defense-related genes, including *NPR1*, *COI1,* and *PR1-a,* and, subsequently, improving plant growth indices in ToMV-infected tomato plants. Therefore, the application of the PGPR is recommended in the ToMV-tomato pathosystem to reduce the adverse effects of viral infection on cultivated plants.

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