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Research Article

Controlling Tomato Fusarium Wilt Disease through *Bacillus thuringiensis*-Mediated Defense Priming

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Background: Fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) (Sacc.) W.C. Snyder and H.N. Hans is one of the most prevalent and devastating diseases of tomato plants (*Solanum lycoprsicum* L.) that leads to a severe reduction in crop yield almost worldwide.
Objective: Evaluation of biocontrol potential of *Bacillus thuringiensis* (*Bt*) isolate IBRC-M11096, against *Fol* in tomato through priming
Materials and Methods: qRT-PCR technique was applied to analyze the effect of the strain on the hormonal defensive pathways; transcriptional responses of jasmonic acid (*COI1*, *Pin2*) and salicylic acid (*NRP1* and *PR1*) pathway genes in *Bt*-treated plants following inoculation of *Fol* as compared to the plants only challenged with *Fol*. Also, the potential of the bacterial strain as a biocontrol agent was studied by evaluating growth indices and area under disease progress curve (AUDPC).
Results: The transcription of both defensive hormonal pathway genes (*COI1, Pin2, NPR1, PR1*) increased due to bacterial priming. The bacterial priming reduced the AUDPC compared to the inoculation with only *Fol*. The strain reduced the disease symptoms, and compared to the plants only challenged with the fungus, the bacterial strain significantly raised shoot dry and fresh weights and root dry weight.
Conclusion: Priming with the *Bt* strain led to improved shoot and root growth indices, reduced AUDPC, and fortified responses of both JA and SA hormonal pathways. However, further full-span studies are required to judge the efficacy of the bacterial strain in the biological control of tomato fusarium wilt under field conditions.

*Keywords***:** AUDPC, Gene expression, Hormone-regulated defense, Priming, Resistance.

1. Background

Tomato (*Solanum lycopersicum*) is considered one of the world's most important crops due to its high levels of production and consumption. In 2018, the global area of the cultivated tomato was ca. 4.8 million hectares with a production of more than 180 million tons (1). Also, tomato is a model plant to investigate plant-pathogen interactions (2). Fusarium wilt is recognized as one of the most destructive diseases affecting tomato plants. The causal fungus, *Fusarium oxysporum* f. sp.

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lycopersici (*Fol*), is ranked as the fifth important plant pathogenic fungus, resulting in a yield loss of ca. 14% (3). The hyphae of the vascular fungus penetrate tomato plants through wounds or directly via the root cortex and develop in intra- as well as inter-cellular manners in root tissue to reach and colonize vascular vessels (4). To restrict fungal colonization, the plant reacts through various defensive responses, including the production of substances like gum and tylose (5). However, these reactions lead to vascular occlusion, endangered water transfer and wilt symptoms (6). The mode of parasitism of the fungus is controversial. While some researchers believe it is necrotrophic (7), others know it as a hemibiotrophic parasite (8, 9). Gordon (10) categorized the isolates of *F. oxysporum* into necrotrophic and biotrophic groups based on the death or survival of host cells near the infection site. The defensive mechanisms of tomato plants against *Fol* require the recognition of the pathogen through cell surface receptors that recognize pathogen-associated molecular patterns (PAMPs) and induce microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) (11).

Because of the soil-born nature of the fungus, its capability to colonize vascular tissues of infected plants and the high saprobic competitiveness, the control of the disease is difficult. Furthermore, the high rate of genetic mutation and the development of new, more invasive physiological races of *Fusarium* species lead to the inefficacy of host resistance. While chemical control has already lost its reputation due to the unwanted impacts of chemicals on the environment and soil microbiology, genetic diversity can also lead to fungicide resistance (12). So, it seems that the integrated management of the disease is the only choice to reduce yield loss. Besides the host plant resistance, biological control is a fundamental part of most integrated disease management programs (13).

B. thuringiensis (*Bt*) Berliner is known as the most abundantly applied *Bacillus* species throughout the world (14), and its importance as an effective biological control agent in the integrated management of plant diseases and pests has recently been reviewed (13). *In vitro* studies have shown the potential of some *Bt* isolates in the effective and long-lasting inhibition of *Fol* (15). previously, we reported that the *Bt* strain IBRC-M11096 alone induced the expression of jasmonic acid/ethylene (JA/ET) and salicylic acid (SA)-related marker genes in tomato cultivar Early Urbana prior to *Fol* inoculation (16). Furthermore, the study on the effect of the same *Bt* strain on the transcription of antioxidant genes indicated the increase of superoxide dismutase (*SOD*) as well as glutathione-*S*-transferase (*GST*) genes accompanied by the reduced rate of hydrogen peroxide (H_2O_2) in tomato cultivar Falat C.H. plants challenged with *Fol* (17).

2. Objective

The potential of the *Bt* strain IBRC-M11096 in inducing defensive pathways in tomato plants against *Fol* was investigated. Also, the effects of the bacterial strain on fusarium wilt severity and plant growth indices under pot conditions were studied.

3. Materials and Methods

3.1. Plant Material

The tomato seeds of the cultivar Falat C.H. were superficially sterilized by soaking in a 2% aqueous solution of sodium hypochlorite for 5 minutes, followed by rinsing twice with sterile deionized water. The seeds were then planted in a culture tray containing pre-autoclaved cocopeat and perlite (in a ratio of 3: 1). When the seedlings reached the 4-leaf stage, they were transferred into 1-liter plastic vases containing sterilized vermiculite and soil (in a 1:1 ratio, W/W). The vases were kept at 25 °C under photoperiod conditions of 16 h light: 8 h dark in a growth chamber.

3.2. Preparation of Bacterial Suspension

The bacterial strain IBRC-M11096 of *Bt* was purchased from the National Genetic Engineering Institute in Tehran. The bacterial suspension was prepared following the method used by Lacy and Lukezic (18): a 50 µL bacterial aliquot was transferred into a nutrient broth (1.5%) medium in a 1000 mL flask. The culture was incubated at 25 °C in a shaker-incubator adjusted to a velocity of 150 rounds per minute (rpm) for 48 h. The bacterial suspension was incubated until its optical density reached 0.9, measured at a 600 nm wavelength (almost equal to 10^9 colony forming units (CFU. mL^{-1})); then, it was diluted to a volumetric ratio of 1: 100 (V/V) to achieve a final bacterial cell density of 10^7 CFU. mL^{-1}. The suspension was immediately applied.

3.3. Preparation of Fungal Suspension

The fungus *Fol* was obtained from the fungal collection

at the Department of Plant Protection, Agricultural Sciences and Natural Resources University of Khuzestan. The fungus was cultured on corn meal agar (CMA) plates and incubated at 25 °C under 16 h light: 8 h dark photoperiod conditions for 14 days. The fungal spore suspension (10^6 spores. mL^{-1}) was prepared following the methodology previously described (19): Briefly, spores (microconidia) were gently collected from 14-day-old culture plates using a sterile loop. The harvested spores were suspended in a 30 mL sterile aqueous solution containing 0.1% Tween 80. The suspension was agitated by vortexing to distribute the spores evenly. Finally, the concentration of microconidia was measured using a hemocytometry lamella (Neubauer Improved, HBG, Germany) under a light microscope (Olympus, Japan) and adjusted to 10^6 spores. mL^{-1}.

3.4. Treatments

Seven days after transferring the seedlings to the plastic vases, experimental treatments, including untreated control, bacterial treatment (T_{Bt}), fungal treatment (T_{Fol}), and a combination of both bacterial and fungal treatment (T_{Bt+Fol}) were performed on 4-5 leaf stage tomato seedlings. Bacterial treatment (T_{Bt}) involved adding a 30 mL volume of *Bt* suspension (10^7 CFU. mL^{-1}) to the soil inside each pot. The treatment with the pathogenic fungus, *Fol* (T_{Fol}), was performed by adding a 20 mL volume of microconidial suspension (10^6 spores. mL^{-1}). In the treatment including both microorganisms (T_{Bt+Fol}), the microconidial suspension of the pathogenic fungus (*Fol*) was added to each vase's soil 48 h after the amendment of the bacterial suspension, following the method mentioned above. The effect of bacterial pre-treatment on the induction of defensive genes was evaluated by investigating the transcriptional rates of the selected genes 3, 24, 48, and 72 h after treatment with *Fol* conidial suspension using the qRT-PCR technique, and the results were compared with those from the plants only treated with the pathogenic *Fol* conidial suspension.

3.5. Preparation of Foliar Samples

Samples were collected from the middle leaves of seedlings at the 4-5 leaf stage. Three biological replicates were conducted for each temporal treatment, with only one seedling per replicate. The foliar samples were wrapped in pieces of aluminum foil sheet, rapidly frozen in liquid nitrogen, and immediately stored at -80 °C until the next steps.

3.6. RNA Extraction, cDNA Synthesis, and Designing Primers for qRT-PCR

RNA extraction was made using a RNA extraction kit (Access No. # S1020, manufactured in Dena Zist Asia, Mashhad, Iran) following the manufacturer's recommendations. The extracted RNA samples were

Table 1. The sequence of primers applied in qRT-PCR reactions

Gene	Oligonucleotide	Sequence (5'-3')	Ta (°C)*	Expected product size (bp)
COI1	F	A G T G A G A G G C T G C T G T T T C	57.86	125
	R	CCATCGCTAAGAGATCACGAC		
PR1	F	TCTTGCGGTTCATAACGATG	57	104
	R	CAGCTCTTGAGTTGGCATAG		
NPR1	F	CCAAGTCTACAGAGGAAGGA	56.3	131
	R	CAAATCATCGCCTGCCATAG		
PIN2	F	GCACTGGTTACAAGGGTTG	57	145
	R	TTTGCCAATCCAGAAGATGG		
Reference gene (*LOC544055*)	F	GTTGTTGAGACCTTTGCTGA	56.8	112
	R	CAGTTGGGTCCTTCTTGTCA		

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

quantified by their optical absorbance determined with a Nanodrop apparatus (Spectrophotometer 2000c, Thermo Scientific, USA). Electrophoresis of the RNA on 1% agarose gel was performed for the qualitative analysis of the RNA integrity. The first stranded cDNA was synthesized using a cDNA synthesis kit (Access No. # E6300S, New England BioLabs, England) and Oligo dT primer following the manufacturer's guidelines. To study changes in the transcription rate of plant defensive pathway genes (*COI1*, *PR1*, *NPR1*, and *PIN2*), primers were designed using the online software Primer Quest freely available at IDT website (www.idtdna.com). The synthesis of the designed primers (**Table 1**) was carried out by Takapoozist Company, Tehran, Iran.

Quantitative real time-PCR (qRT-PCR) was performed using Master Mix SYBR Green (High ROX) kit (Cat. No. M3003S) and gene-specific primers and Step One Plus® Real-Time PCR System (ABI Company, USA). The protocol recommended by the kit manufacturer NEB Co., was followed. The thermal cycle's conditions applied were as follows: one cycle pre-denaturation of 10 min at 95 °C; 40 cycles of denaturation at 95 °C for 15 s; annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. Three seedlings were used as biological replicates, while two technical replicates (cDNA samples) were considered for each biological replicate. The relative expression level (fold change) of target genes in T_{Bt+Fol} relative to T_{Fol} was calculated by the $2^{-\Delta\Delta CT}$ comparative method (20). *Lycopersicum* elongation factor-1 alpha (*LOC544055*) has been reported as a reference (internal) gene in tomato plants (21). The data acquired from various samples were first normalized using the difference in cycle threshold (Ct) values against the internal gene *LOC544055*. Then, the relative expression rate of target genes was calculated using the Relative Expression Software Tool (REST) software (22).

3.7. Determination of Growth Indices

The experiment was performed based on a completely randomized design with four treatments (control, T_{Bt}, T_{Fol}, and T_{Bt+Fol}), each with 4 replicates. The measurement of growth indices was conducted 40 days after treatment with the pathogen. The weights of fresh shoots and roots were measured using a laboratory balance and recorded. To determine dry weights, the samples were kept in an oven at 72 °C until their dry weights no longer changed after 72 h. The experimental data were analyzed using SAS software (Version 9.1), and the means of treatments were compared using Duncan's multiple-range test.

3.8. Evaluation of Disease Severity

To investigate the progression of the disease over time, the percentage of leaves displaying symptoms at the whole plant level was monitored every three days, starting from the 10th day post-inoculation (dpi) and extending for 30 days, as described previously (23). For this, the number of symptomatic leaves of 4-leaf stage seedlings was recorded on the 10th, 13th, 16th, 19th, 22nd, 25th, 28th, 31th, 34th, 37th, and 40th days after fungal treatment. Three seedlings were considered for each treatment (T_{Bt}, and T_{Bt+Fol}). The experiment was designed as a random complete block. Disease progress curves were drawn by plotting the disease incidence percentage versus time, and their regression lines were derived using Excel software. The AUDPCs of both treatments were calculated following the published methodology (24). The ANOVA analyses of total AUDPCs and interval AUDPCs data were performed using IBM® SPSS® Statistics 26 software (IBM Corp., USA).

4. Results

4.1. Effect of Bt on Growth Indices of Tomato Plant

Except for root fresh weight, the biological treatments of tomato seedlings led to highly significant differences in shoot fresh weight, shoot dry weight and root dry weight. *Bt* could significantly increase shoot fresh and dry weights compared to untreated control and *Fol*-treated plants (**Fig. 1A, 1B**). Also, compared to the plants treated with *Fol* (T_{Fol}), the dry weight of root significantly increased in the plants only treated with *Bt* (T_{Bt}) and also in *Bt*-primed plants challenged with *Fol* (T_{Bt+Fol}) (**Fig. 1C**).

4.2. Transcriptional Responses of Plant Defense Hormonal Signaling Pathway Genes

The study on the transcription rate of plant defense hormonal signaling pathway genes indicated that compared to the plants only treated with *Fol*, the transcription rate of the *NPR1* gene significantly increased in the *Bt*-primed plants subsequently treated with *Fol* in the third hour after fungal treatment. Although no

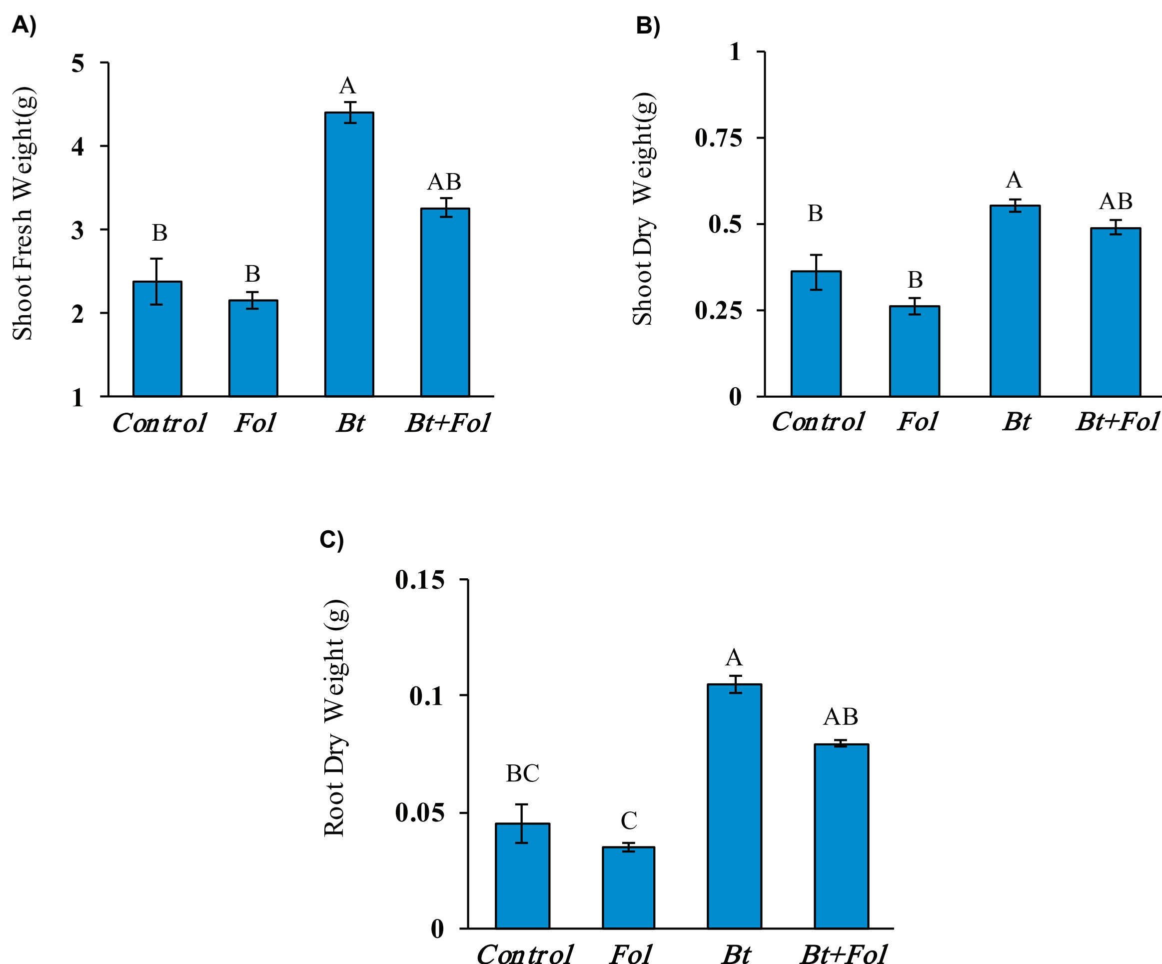

Figure 1. **A**) Shoot fresh weight, **B**) shoot dry weight and **C**) root dry weight in various treatments. The columns with only a common letter are not statistically of significant difference. The mean of treatments has been compared by Duncan's multiple-range test (P≤ 0.05). Each mean is the average of four repeats.

significant change was found in the transcriptional rate of the *NPR1* gene in the T_{Bt+Fol} plants in the 24th h after treatment with *Fol* compared to T_{Fol} plants at that time point, a significant 12.2-fold increase of *NPR1* gene transcription was found 48 h after *Fol* treatment. 72 h after treatment with *Fol*, no significant difference was detected in the transcription of the *NPR1* gene between T_{Bt+Fol} plants and T_{Fol} plants. A 7.821-fold increase in the *COI1* gene transcription occurred in T_{Bt+Fol} plants compared to T_{Fol} plants 3 h after treatment with *Fol*. 48 h after treatment with *Fol*, the *COI1* gene transcription attained its maximal rate, 16.7 folds that of its transcription rate in T_{Fol} plants. The transcription rate of the *COI1* gene in T_{Bt+Fol} plants was significantly higher 72 h after treatment with *Fol* (11.75 times higher than that in T_{Fol} plants). A significant increase in *PIN2* gene expression was found in T_{Bt+Fol} plants up to 48 h after the subsequent treatment with *Fol*. At this time point, the transcription rate of the gene reached its maximal rate, which was 38.5 times higher than that observed in T_{Fol} plants. The transcription rate of the *PR1* gene in T_{Bt+Fol} plants was significantly higher than in T_{Fol} plants at all time points, attaining its highest rate, 66.13 folds, 48 h after treatment with *Fol* (**Fig. 2**).

4.3. Disease Severity

Study on the AUDPC in the T_{Fol} as well as T_{Bt+Fol} plants indicated that pre-treatment with *Bt* led to a highly

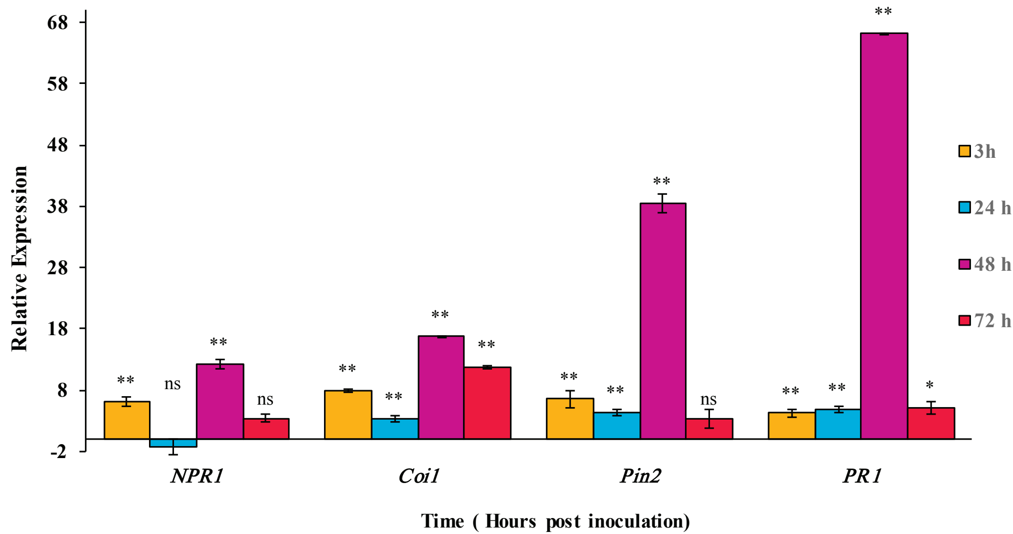

Figure 2. The Effect of pre-treatment of tomato with *Bacillus thuringiensis* on the relative transcriptional rate of *NPR1*, *COI1*, *PIN2*, and *PR1* genes in different time points after treatment with the pathogenic fungus *Fusarium oxysporum* f. sp. *Lycopersici*. ns: not significant, *: significantly different ($P \leq 0.05$), and **: highly significant different ($P \leq 0.01$) from that in the plants only treated with the fungus.

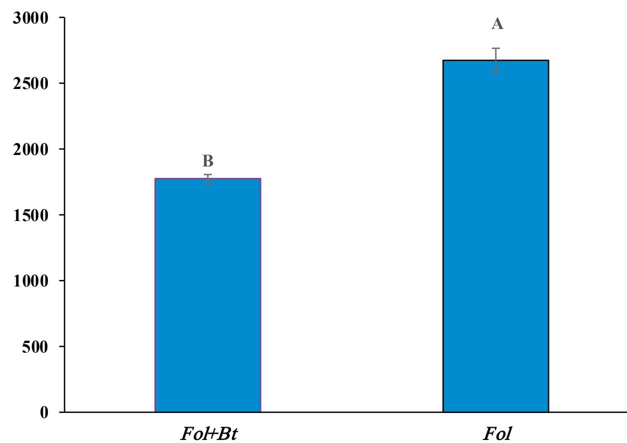

Figure 3. Comparison of area under disease progress curves (AUDPCs) calculated for tomato (cv. Falat C.H.) plants primed and non-primed with *Bacillus thuringiensis* (*Bt*) IBRC-M11096 and challenged by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). The mean of treatments has been compared by Duncan's multiple-range test ($P \leq 0.01$). Each mean is the average of three repeats.

significant reduction of fusarium wilt disease in T_{Bt+Fol} plants compared to T_{Fol} plants ($F_{1, 64} = 50.681$***; **Fig. 3**). Highly significant differences were found in the disease incidence percentage of both treatments in the time points after treatment with *Fol* ($F_{21, 44} = 16.101$***). These differences were also well reflected in the AUDPC between time intervals ($F_{21, 44} = 16.628$***). Also, the comparison of linear regressions of disease progress curves of the bacterial primed and *Fol*-treated plants at different time points was illustrated in **Figure 4**.

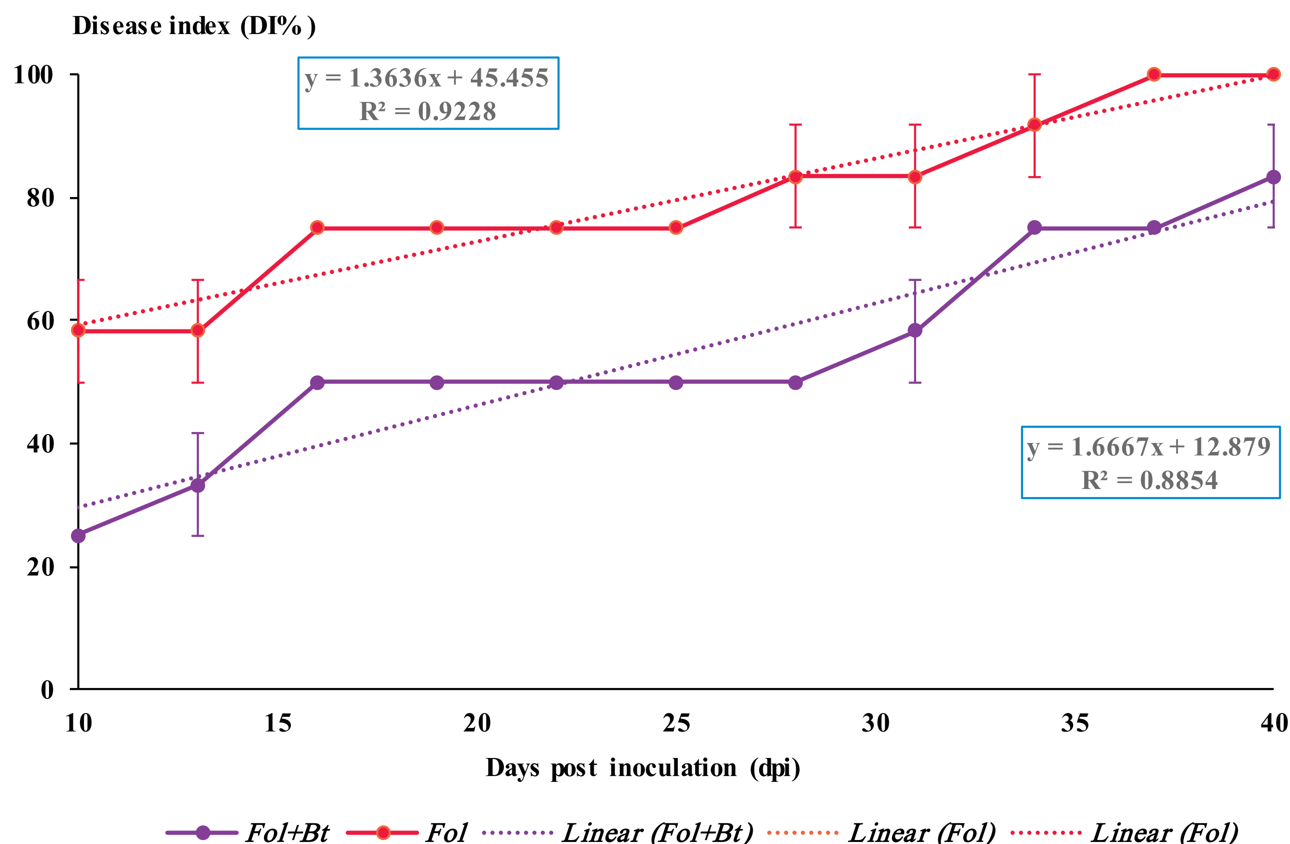

Figure 4. Effect of *Bacillus thuringiensis* (*Bt*) IBRC-M11096- based priming on fusarium wilt disease progress in tomatoes (cv. Falat C.H.) after inoculation with the causal pathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). The trendlines of both disease progress curves (DPCs) have been demonstrated.

5. Discussion

The ability of certain rhizobacteria to enhance plant growth through their interactions with plants and other PGPR (plant growth promoting rhizobacteria) has been formerly reported (25). The *Bt* strain, RZ2MS9, was able to improve the growth of maize and soybean under greenhouse conditions, significantly increasing their dry weights. These activities were attributed to atmospheric nitrogen fixation and the production of auxin, phosphate, and siderophores (26). The strain could endophytically colonize maize roots and leaves, and when co-inoculated with *Azospirillum brasilense* Ab-V5, it respectively increased maize root and shoot dry weights by approximately 50% and 80% compared to untreated controls (27). In the present study, the *Bt* strain significantly increased root and shoot dry weights and raised shoot fresh weight in *Bt*-primed tomato plants compared to those only treated with the pathogenic fungus, *Fol*.

Plant hormones act as crucial regulators of plant defensive responses that regulate various aspects of plant immunity against pathogens. The signaling role of JA/ET against necrotrophic pathogens is well known (28-30). To survey the involvement of the JA in the protection mediated by *Bt* against *Fol* in tomato, we monitored the transcript level of JA signaling marker genes in plants treated with *Bt* after *Fol* challenge. F-box protein coronative insensitive1 (COI1) is a JA receptor, the primary regulator of defensive responses in rhizobacteria-mediated induced systemic resistance (ISR) (31). Furthermore, the proteinase inhibitor gene is crucial for JA-dependent signaling and serves as a

marker gene for the related pathway in the *Fol*/tomato pathosystem (32). We showed that the transcription of *COI1* significantly increased in the plants pre-treated with *Bt* at all time points after treatment with the fungus. Furthermore, the level of *PIN2* gene (coding for a proteinase II inhibitor) transcription attained its highest rate in the plants pre-treated with *Bt* 48 h after the fungal treatment. Similarly, Pazarlar *et al.* (33) showed that the JA/ET-related marker genes, such as proteinase inhibitor I (I-1) and ethylene receptor4 (ETR4), were up-regulated following the *Fol* challenge in *Bacillus cereus* EC9 – treated plants, indicating that EC9 stimulated defense response by priming the JA/ET signaling pathway in tomato-*Fol* interaction.

Although the efficacy of the external application of SA in the induction of *PR1* and its contribution to the development of defensive responses has been illustrated, some studies indicate that the accumulation of internal SA inhibits the development of defensive responses against necrotrophic pathogens (such as *Fo*). In contrast, it induces an efficient defensive response against biotrophic pathogens (28, 34). For instance, some investigations have shown that the necrotrophic pathogen takes advantage of the SA signaling pathway to strengthen the disease development in tomato (35). However, some reports indicate the effect of SA in the defensive responses against necrotrophy. Despite the proven role of JA and ET in the immune responses of plants against *Fo,* the role of SA in immune responses is complicated and seemingly dependent on the nature of the host and pathogen (36,37). Hence, we aimed to investigate whether the SA signaling is also implicated in boosting plant immunity by *Bt* against *Fol.* This was assessed using the qRT-PCR to measure the expression level of the SA signaling marker genes. As one of the positive regulators of *PR* gene expression and a SA receptor, the nonexpressor of pathogenesis-related 1 (NPR1) protein plays a vital role in the induction of systemic acquired resistance (SAR) (38). We observed that *Bt* pre-treatment elevated the transcript level of *NPR1* and *PR1* following the *Fol* challenge; it can be speculated that the SA signaling also functions in *Bt*-stimulated immune response against *Fol* in tomato. Former studies have indicated that ISR is more dependent on JA/ET signaling pathway and is regulated through a pathway other than that of SA (39). However, some PGPR strains induce ISR through a SA-dependent pathway, exactly like pathogen-induced SAR (reviewed in 40). Multiple reports, mentioned subsequently, illustrated that certain PGPR strains could trigger host ISR through the synergistic activation of both JA/ET and SA signaling pathways. *Bacillus subtilis* MBI600 activated JA and SA signaling pathways simultaneously in tomato plants to control soil-borne pathogens (41). Similarly, *B. cereus* AR156 can trigger immune responses against *Pseudomonas syringae* pv. tomato DC3000 *via* ISR stimulated by both the JA/ET and SA signaling pathways (42). It has also been shown that both the SA and JA signaling were involved in endophyte-mediated resistance (EMR) against *Fol* in tomato plants (43). In this study, considering increased growth indices and reduced AUDPC obtained with T_{Bt+Fol} plants, it appears that *Bt* stimulates an immune response against *Fol* in tomato plants, possibly by priming both the JA and SA signaling pathways. Such a biological treatment with double effects is preferred to the treatments made only with chemical plant resistance inducers, where the induced resistance is acquired at the cost of reduced crop yield (44).

Considering the soil-borne nature of fusarium wilt disease, eliminating the potential inoculum of soil-borne pathogens is regarded as one of the most effective methods for disease control (45). A look at the disease progress curves of both treatments as well as their regression lines, indicates that y-intercept (the point where the regression line meets the axis of disease severity percentage, “y”) in T_{Bt+Fol} stands lower than T_{Fol}, indicating the effect of *Bt* on reducing the pathogenic fungus primary inoculum in tomato soil (**Fig. 4**). Despite these promising results, the greater slope of the regression line of the disease severity in T_{Bt+Fol} plants (**Fig. 4**) illustrates that the disease severity can even precede that in T_{Fol} plants over time if the conditions are conducive for disease development. Such a prediction does not seem improbable because the tomato plants reach their highest susceptibility in the fruit maturity step under field conditions. These findings indicated that such an approach may not be sufficiently effective in eradicating the whole soil-borne inoculum of the fungus. Even the volatile compounds used in soil fumigation have not led to the complete eradication of the pathogens in the treated soil and have exhibited only short-term effects (46). Thus, the survived pathogen inoculum can infect the host, and if the plant is not resistant or the applied control method leads to changes in plant defensive hormonal pathways

that favor the pathogen, it can lead to a severe disease in the infected plant. Hence, the final confirmation of the practical profitability of the studied *Bt* strain still requires field studies, where tomato plants are tested in their whole life span, and the effect of *Bt*-priming can be studied during fruiting stages till crop harvesting.

6. Conclusion

The findings of this research showed that the expression of defensive genes in the *Bt*-primed plants was detectable from 3 h after plant treatment with the pathogenic fungus (*Fol*) and continued at least till 48 h or 72 h after fungal treatment, indicating the induction of plant defensive genes of both hormonal pathways, JA as well as SA, following bacterial pre-treatment. We also found that *B. thuringiensis* acts as PGPR, positively affecting plant shoot and root growth indices, and reduced AUDPC in *Bt*-primed tomato plants, challenged with *Fol* (T_{Bt+Fol} plants). However, since tomato plants become most susceptible during the fruit maturity stage, conducting field experiments is necessary to obtain convincing results.

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