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RESEARCH ARTICLE

Anti-oxidative Response of *Bacillus thuringiensis*-Primed Tomato Plants to *Fusarium oxysporum* f. sp. *Lycopersici*

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ABSTRACT: Under global warmth conditions, it is expected that tomato yield will reduce due to insect pests and fungal diseases such as fusarium wilt. Using of biological control agents is effective in the control of both groups as regard as an ecofriendly and economically rational practice. Here, *Bacillus thuringiensis* (*Bt*) was used to study its capability to prime tomato resistance against fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Priming of tomato cv. Falat C.H. seedlings was performed at 4-5 leaf stage and leaf samples were analyzed 3, 18, 24, 48 and 72 hours after fungal treatment (hat). The rate of hydrogen peroxide (H_2O_2) and changes in the relative transcription of the antioxidant enzyme genes such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were evaluated using qRT-PCR. No significant change was observed in the relative transcription of the *CAT* gene. The relative transcription of *SOD*, and *GST* genes was increased with time in the treated plants compared to control plants. The highest rate of relative transcription of *SOD* was found at 18 and 24 hat, and for *GST* at 18 and 72 hat. The increment of genes transcripts was in agreement with the reduced level of H_2O_2 in *Bt*-primed plants. These results are in accordance with the effectiveness of *Bt* in the induction of tomato systemic resistance to the *F. oxysporum* f. sp. *lycopersici*.

KEYWORDS: Biocontrol, Catalase, qRT-PCR, Stress, Wilt.

INTRODUCTION

Tomato plants are invaded by a range of plant pests and diseases. One of the most important diseases of tomato plants is fusarium wilt, a vascular disease caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H. N. Hansen (*Fol*) [1]. The dormant chlamydospores of *Fol* can indefinitely survive in infested soils in the absence of host plants [2]. *Bacillus thuringiensis* (*Bt*) is a globally well-celebrated biological control agent (BCA) effective against insect pests [3], nematodes, and fungi such as *Fol* [4]. *Bt* produces long-lasting endospores resistant to extreme conditions [5]. It also promotes plant growth and increases crop yield via its effects on edaphic factors as well as plant itself [6]. Therefore, *Bt* can be considered as a key BCA and biofertilizer (BF) in global warmth conditions [7]. From a cellular and molecular viewpoint, the generation of reactive oxygen species (ROS) is a permanent plant response under abiotic and biotic stress conditions [8]. ROS are relevant signals involved in the activation of plant defense genes [9]. Under normal conditions, the level of generated ROS is in balance to cellular anti-oxidative capacity as the consequence of the equilibrium between oxidants versus enzymatic and non-enzymatic anti-oxidants [10]. The anti-oxidative enzymes super-

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oxide dismutase (SOD) and catalase (CAT) have a considerable share in plant defense against ROS generated in cell [11], where the superoxide radicle (O_2^-) is first converted to hydrogen peroxide (H_2O_2) and oxygen. The reaction catalyzed by SOD is then followed by further neutralization of the produced hydrogen peroxide, enzymatically catalyzed by CAT and ascorbate peroxidase (APX) [12]. Plant Glutathione-S-transferases (GST) are multifunctional enzymes [13] of important role in detoxification of toxic compounds such as 4-hydroxynonenal [14]. The activity of the enzyme reduces oxidative stress and cell damage and leads to increased tolerance to environmental stresses [15]. Considering the increasing importance of healthy organic crop production, the biological control of tomato fusarium wilt can help farmers to reduce their dependence on agrochemicals. With stable agriculture, it is attempted to apply to reduce the application of agrochemicals and to acquire healthy organic crops, so biological control agents provide one of the measures for agricultural production and are regarded as appropriate substitutes of chemical fungicides [16]. The bacteria of the genus *Bacillus* are known as one the best microbial biocontrol agents [17, 18] due to their potential for resistant spore formation [18], plant defensive system induction against environmental stresses, plant growth increase and improvement, as well as the production of antifungal substances such as surfactin and itidine [17, 18]. In addition to the above-mentioned superiorities, *B. thuringiensis* is mainly known as a bioinsecticide because of its entomotoxic proteins [3]. Considering the impact of fusarium wilt on economical yield of tomato fields, and that ROS are generated during pathogenic attack in plant [19], the aim of this study was to investigate the effect of *B. thuringiensis* as a biological control agent against the disease, and the changes in the expression of antioxidative enzymes such as SOD, CAT, and GST using qRT-PCR technique. Also, the rate of H_2O_2 was evaluated.

MATERIALS AND METHODS

Bacterial inoculum preparation

The lyophilized *Bt* strain IBRC-M11096 was purchased from National Genetic Engineering Institute in Tehran. The bacterial suspension (10^7 CFUs mL^{-1}) was prepared following the previously described method [20]. Briefly, the bacterial strain (50 µL) was inoculated to nutrient broth (500 mL) and incubated on a shaker (150 rpm) at 25˚C for 48 h. When the optical density of the bacterial suspension reached 0.9 (almost equal to 10^9 CFUs mL^{-1}; read at 600 nm), the culture was diluted with the ratio of 1: 100 (V/V) to adjust the final density of bacterial cells to ca. 10^7 CFUs mL^{-1}. The suspension was immediately applied.

Pathogen inoculum preparation

The isolate of *Fol* was grown on corn meal agar plates for fungal sporulation. The cultures were incubated at 25°C under photoperiodic conditions of 16h light: 8 h dark for 14 days. The required spore suspension (10^5 spores mL^{-1}) was prepared following the already described method [21]. Briefly, spores (microconidia) were gently harvested from 14-day-old culture plates using a sterile loop. A 30 mL spore suspension was prepared in sterile aqueous solution of Tween 80 (0.1%) in a 50 mL falcon tube. The suspension was mixed using a vortex mixer. Spore suspension was quantified in a hemocytometer and its density was adjusted to 10^5 spores mL^{-1}.

Tomato seedling preparation

Seed of tomato cv. C. H. were grown in plastic pots 10 cm in diameter and 10 cm in depth. The pots were kept at 25°C under conditions of 16h light: 8h dark, and relative humidity (RH) of 50%. The pots were irrigated in 48 h intervals. A seedling was grown per pot. 3 pots were considered for each time point.

Priming of tomato seedling

The tomato seedlings in 4-5 leaf stage were primed via pretreatment with *Bt*. Priming was performed via addition of 30 mL fresh bacterial suspension (10^7 CFUs mL^{-1}) to each pot.

Fungal treatment of tomato seedlings

Treatment of tomato seedlings was carried out by addition of 20 mL *Fol* spore suspension (10^6 spores mL^{-1}) 48 h after priming.

Sampling and storage of leaf samples

The middle leaves of seedlings were sampled from three control primed seedlings just before fungal treatment, and from treated primed seedlings 3, 18, 24, 48, and 72 h after treatment (hat) of primed seedlings with the pathogen *Fol*. With each time point, a middle leaf was taken as a biological sample from each of three seedlings. The foliar samples were instantly wrapped in aluminum foil pieces,

frozen in liquid nitrogen, and stored at -80°C in a freezer for next uses.

Extraction of total RNA and synthesis of cDNA

Total RNA extraction was carried out using the RNA extraction kit (Cat. No. T2010S) produced by NEB Co., and following the manufacturer's protocol. The quantity and quality of the extracted RNA was analyzed via the use of Nanodrop apparatus (Thermo Scientific NanoDrop 2000, USA) as well as electrophoresis with 1% agarose gel. The first cDNA strand was synthesized using cDNA synthesis kit (Cat. No. E6300S) and following the protocol presented by NEB Co. 500 ng of each RNA sample was separately applied for each 20 µL cDNA biosynthesis reaction using Oligo dT primer. The synthesized cDNA samples were stored at -20°C till next uses.

Designing of primers

Primers for quantitative Real Time-PCR (qRT-PCR) were designed for the genes coding for the enzymes CAT, SOD, GST, and *Lycopersicum* elongation factor-1 alpha taking advantage of the online software Primer Quest in the website of Integrated DNA Technologies, https://www.idtdna.com (Table 1). The latter gene (*loc*) was applied as a reference gene.

Optimization of thermal conditions for qRT-PCR

To optimize thermal conditions for annealing of primers, gradient PCRs were separately developed for each set of the primers, where the thermal range varied between 54 to 62°C. Each reaction was of 20µL final volume including: Taq polymerase PCR kit (NEB Co., Cat. No. M0273S) The thermal conditions required for the promotion of PCRs were provided using PEQLAB thermocycler (peqSTAR, Germany). The PCR products were analyzed via horizontal electrophoresis (90 v, TBE buffer, 1 % agarose gel, running time 50 min) of 5 µL. samples stained with 1 µL loading dye.

qRT-PCR analysis

The qRT-PCR was performed using SYBR Green kit (Cat. No. M3003S) and following the protocol recommended by the manufacturer, NEB Co. The 25µL reactions were run using Step One plus Real-time PCR system, Applied Biosystems, USA. For each treatment, three biological repeats were applied for qRT-PCR analyses. For each biological replicate, three technical repeats (cDNA samples) were run. The threshold cycle (C_T) for each gene was normalized with *S. lycopersicum* elongation 1-alpha factor gene applied as the reference gene. Fold changes of target gene expression in *Fol*-challenged *Bt*-primed tomato seedlings (as treated sample) relative to non-primed seedlings only challenged by the pathogenic fungus (as untreated control) were calculated following the $2^{-\Delta\Delta CT}$ comparative method [22]. The rate of $-\Delta\Delta CT$ was calculated as $\Delta\Delta C_T = [(C_T \text{ of gene of interest} - C_T \text{ of reference gene})_{\text{treated sample}} - (C_T \text{ of gene of interest} - C_T \text{ of reference gene})_{\text{untreated control}}]$. Relative Expression Software Tool (REST) software was used to analyze the relative expression of target genes as described by Pfaffl [23].

Table 1. The characteristics of qRT-PCR primers designed and applied in this research

Gene	Primer sequence (5'→3')	Accession No.
Cat2	ctgcccttctattgtggttc agcacactttggagcattag	NM_001247257.2
BI-Gst/Gpx	cctttcctcccttctgatcc gctgtctcttgctcttctc	NM_001247450.2
Sod	acttgctcctggacttcac cgcatgacggatttcatctc	M37151.1
Loc	gttgttgagacctttgctga cagttgggtccttcttgtca	NM_001247106.2

Quantification of H_2O_2 concentration

A completely randomized design was applied to assess the measurement of H_2O_2 in plants pre-treated with bacteria at 3, 18, 24, 48 and 72 hours after treatment with the pathogen, at three replications in the experimental field of the University. The published methods [24, 25] were followed to quantify the concentration of H_2O_2 in tomato leaf samples. 1.8 mL trichloroacetic acid solution (1% w/v) was added to each fresh leaf sample (0.2 g) and the sample completely ground. Each of the homogenized samples was transferred into a separate 2 mL microtube and centrifuged at 12000 g for 15 min. 0.5 ml of supernatant was transferred into a 2 ml microtube, where 0.5 ml 10 mM potassium phosphate buffer (10 mM, pH 8.0) was added. 1 mL potassium iodide solution (1 M) was added into each of the same microtubes. The rate of absorbance of wavelength of 390 nm was measured using a visible-ultra violet spectrophotometer (Agilent Cary 100 UV-Vis, the USA). H_2O_2 solutions (2-10 mM) were applied to get the required standard graph used in the

determination of H_2O_2 concentration in the studied leaf samples. Statistical analysis was performed by using ANOVA of the SAS statistical program according to a RCD with three replications (version 9.1; SAS Institute Inc. Cary, NC, USA). LSD (least significant difference) test was employed to assess mean comparisons at confidence level of 95% (Table 1 and Fig. 2).

RESULTS

The transcription rate of *SOD* gene in *Fol*-challenged *Bt*-primed seedlings significantly increased 3, 18, 24, 48 hat compared to control *Fol* treated seedlings, however, it was not statistically significant at 72 hat (Fig. 1a). The highest expression of *SOD* gene was detected 18 and 24 hat compared to *Fol*-control plants, 13.44 and 11.6 folds, respectively. The *CAT* expression gene was not significantly changed in *Bt*-primed seedlings compared to *Fol*-controls (Fig. 1b). The highest relative *CAT* gene transcription (5.37 folds) was found 3 hat. The biotic stress imposed by *Fol* led to statistically highly significant increase of comparative rate of *GST* gene transcription 18 and 72 hat, 10.87 and 11.50 folds, respectively.

The analysis of variance (ANOVA) of data obtained from the measurement of hydrogen peroxide (H_2O_2) concentration (µM) in fresh foliar tissue of *Bt*-primed tomato seedlings challenged via post-priming treatment with *Fol* indicated that there was at least one-time point when the concentration of H_2O_2 was highly significantly different from others ($F_{6, 14} = 9.15^{**}$, $P < 0.01$). The LSD-based comparison of the means of the treatments has been presented in the Fig. 2. Result showed that a decrease in the amount of H_2O_2 in leaf tissue at the time of sampling at 72 hours was associated to an increase the genes expression at the time of sampling before 72 hours. In fact, by increasing the genes expression and producing the enzyme encoded by them during leaf sampling, the amount of H_2O_2 produced in the cell is reduced. Based on the comparison of the mean concentration of H_2O_2 of foliar samples taken in different time points, the numerically highest concentration of H_2O_2 (122.12 µM) was found with the control seedlings only primed with *Bt*. However, there was no significant difference among H_2O_2 concentration in *Bt*-controls, *Fol*-controls, and *Fol*-treated *Bt*-primed seedlings 3 hat (Fig. 2). The least concentration of H_2O_2 (47.14 µM) was detected in *Fol*-treated *Bt*-primed seedlings 72 hat and was individually grouped in a unique group.

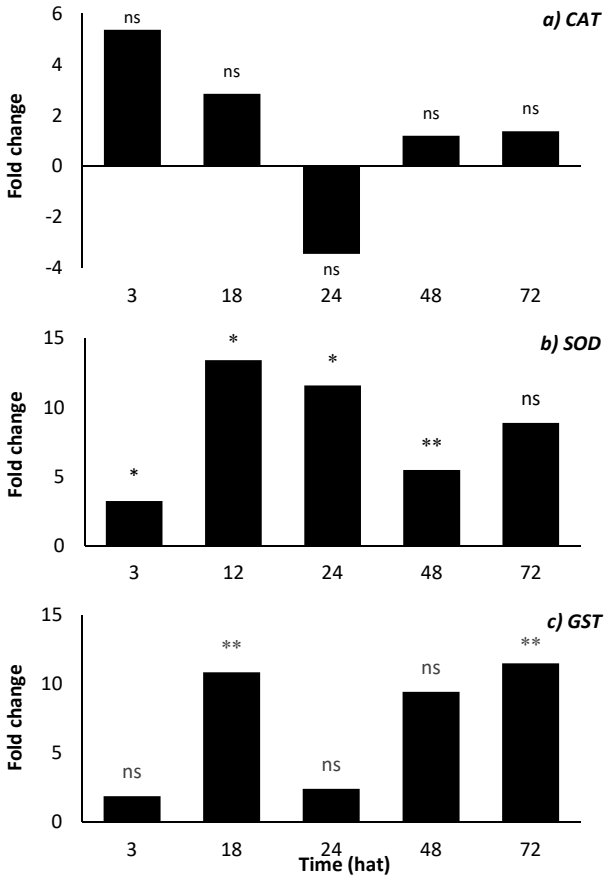

Figure 1. Relative *CAT*, *SOD*, and *GST* genes expression in tomato (*Solanum lycopersicum* L. cv. C. H.) seedlings primed with *Bacillus thuringiensis*, 3, 18, 24, 48 and 72 hours after treatment with *Fusarium oxysporum* f. sp. *lycopersici* in comparison with non-primed seedlings only challenged by the pathogenic fungus. ns, *, ** are not significant, P<0.05 and P<0.01, respectively.

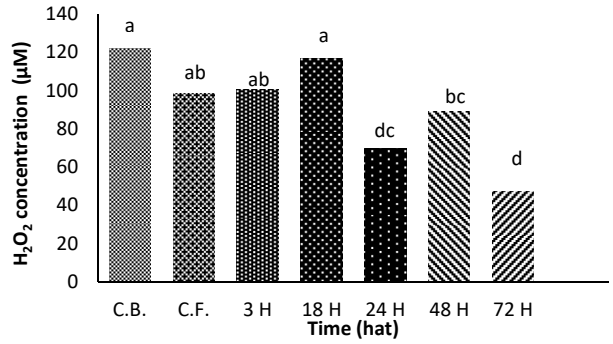

Figure 2. The concentration of hydrogen peroxide (H_2O_2) in fresh foliar tissue of tomato seedlings primed with *Bacillus thuringiensis* (*Bt*) applied as a biological control agent against fusarium wilt measured 3, 18, 24, 48 and 72 h after treatment (hat) with the pathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) as compared to controls treated with either *Bt* (shown as C.B.) or *Fol* (shown as C.F.). The time-points marked with at least a common letter are not of statistically significant differences in H_2O_2 concentration (alpha = 0.05).

DISCUSSION

Under normal conditions, the level of ROS generation is in balance to cell anti-oxidative capacity [10]. The increased rate of ROS is known as a permanent response to abiological and biological stresses, and a relevant signal for the activation of defensive genes [9]. Enzymatic antioxidants such as SOD and CAT prevent ROS free radicles from hurting cells. Thus SOD converts superoxide radicle to H_2O_2 and O_2, where the produced H_2O_2 is then detoxified by CAT [11].

The transcription rate of *GST* and especially *SOD* rose in *Fol*-challenged *Bt*-primed tomato seedlings compared to that in the seedlings only treated with *Fol*, however, no statistically significant increase was detected with comparative *cat* gene transcription rate in *Fol*-challenged *Bt*-primed seedlings. SOD directly related to stress initiates the first defense line converting radicle $O_2^{\bullet-}$ to H_2O_2. SOD (EC 1.15.1.1) activity is found in plant cell chloroplasts, peroxisomes, cytosol, mitochondria as well as apoplast [26]. A *tau* (one of 13 structure-based classes of cyctosolic GST genes) GST gene, *LrGSTU5* is markedly inducible by signaling agents like salicylic acid and ethylene, and it is also induced after inoculation with *Fusarium oxysporum* [27]. Furthermore, constitutive expression of *LrGSTU5* gene from *Lilium regale* by tobacco led to up-regulation of defense-related genes encoding osmotin, PR-1b, chitinase, and SOD enzymes. Additionally, the heterologous expression of the gene resulted in significantly higher activity of three relevant antioxidant enzymes, GST, SOD, and ascorbate peroxidase (APX) in transgenic lines after inoculation with *F. oxysporum*, and led to increased resistance to *F. oxysporum* infection [27]. So, the overexpression of *GST* gene in *Fol*-challenged *Bt*-primed tomato seedlings can be regarded as a sign of enhanced resistance to *Fol* infection. Furthermore, considering the potential of the fungus in the production and secretion of mycotoxins such as fusaric acid, and fumonisin [28, 29], another important aspect of *GST* gene up-regulation is revealed. Considering the role of GSH metabolism in fungus-infected plants [13], the observed up-regulation of *GST* gene expression can lead to enhanced GSH metabolism and increased detoxification of *Fol* mycotoxins.

Based on its genome, *Fusarium oxysporum* is known as a necrotrophic phytopathogenic fungus [30]. Necrotrophic fungi destroy host plant tissues usually by toxins and feed on the remains of dead cells. The damage is accompanied by the generation of ROS that lead to further cell dysfunction and finally to cell death [31], and subsequent development of the necrotroph in host tissue. The role of plant *GST* genes in the resistance to necrotrophic fungi has already been discovered in various pathosystems [13]: *Alternaria brassicicola- Arabidopsis thaliana* [32], *Botrytis cinerea- A. thaliana* [33], *B. cinerea- Vitis vinifera* [34], *Elsinoe ampelina- Vitis flexuosa* [35], *Rhizoctonia solani- A. thaliana* [36], *Sclerotinia sclerotiorum- Brassica napus* [37, 38], *Verticillium dahliae- Gossypium arboretum* and *V. dahliae- A. thaliana* [39]. Various roles are attributed to GSTs: transportation of phytoalexins to extracellular medium even in the absence of pathogen infection or any elicitation of plant defenses [33] as well as the regulation of ROS content through reduction with GSH that also affects SA content [39]. Increased transcription of *SOD* and *GST* genes in *Fol*-challenged *Bt*-primed seedlings can lead to reduced deleterious activity of superoxide and H_2O_2 and consequent resistance to necrotrophic *Fol*. In agreement with our findings, Arabidopsis *esr1-1* (enhanced stress response 1) mutant with up-regulated activity of *GSTF8* promoter fragment fused to the luciferase reporter gene expressed enhanced resistance to the necrotrophic fungal pathogen, *F. oxysporum*. *ESR1* gene encodes a KII domain-containing RNA-binding protein. Transcriptome sequencing of *esr1-1* revealed altered expression of several genes involved in responses to biotic and abiotic stresses and hormone signaling pathways [36].

The results obtained from the molecular part of this study were also confirmed with the findings from its biochemical part. While the concentration of H_2O_2 was high in *Bt*-controls and *Fol*-controls, its concentration in the *Fol*-challenged *Bt*-primed seedlings decreased 18 hat (Fig. 2). The concentration of H_2O_2 increased 24 h after inoculation (2.6 folds) as the initial response of tomato plants to *Fol* [40], however, the subsequent decreased concentration of H_2O_2 in the present study can be explained by the detected significant increase in *GST*. The plant antioxidant system can be induced by interaction with beneficial and/or pathogenic microorganisms [41]. The reduced concentration of H_2O_2 in *Fol*-challenged *Bt*-primed tomato seedlings would lead to reduced cell dysfunction and cell death, and this could mean increased resistance of *Bt*-primed tomato seedlings to the necrotrophic fungus, *Fol*. GST (EC 2.5.1.18) is found in plant cell chloroplasts, cytosol, and mitochondria [26]. It conjugates GSH and electrophilic substrates, in its active sites, thus metabolizes toxic compounds and

transport them into vacuoles [42]. GST also plays roles in peroxide breakdown, hormone biosynthesis, stress signaling as well as glutathione peroxidase (GPX) activity accelerating [43]. In addition to CAT and GST, there are other enzymatic and non-enzymatic antioxidants involved in H_2O_2 detoxification [26]. APX as well as GPX can detoxify H_2O_2 via its conversion to water and molecular oxygen. AsA-GSH cycle or Asada-Halliwell cycle is known as the major antioxidant defense pathway involved in H_2O_2 detoxification. The system consists of non-enzymatic antioxidants [such as ascorbic acid (AsA), glutathione (GSH), and beta-carotene] as well as four relevant enzymes [ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)], where AsA-GSH cycle is of a key role in minimization of H_2O_2 and redox homeostasis [44]. Among non-enzymatic antioxidants, AsA and GSH are the most abundant soluble antioxidants in higher plants playing a vital role as electron donors and ROS scavengers directly via AsA-GSH cycle [45]. Therefore, it would be interesting to study these antioxidants in *Fol*-challenged *Bt*-primed tomato seedlings. In this research, the samples for real-time analyses were taken at 3, 18, 24, 48 and 72 hat. It is generally believed that plant resistance is resulted from fast and adequately intense response of the challenged plant. This time range is routine in the studies where qRT-PCR technique is applied, for instance [46-48].

B. thuringiensis can modify plant antioxidant system to improve tomato resistance against fusarium wilt caused by the necrotrophic soilborne fungus. Considering other abilities of the bacterium such as the production of durable endospores, and antagonism, it seems that potent strains of *B. thuringiensis* can be very profitable in the establishment of sustainable systems for the production of agricultural crops such as tomato here applied as a model.

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پاسخ آنتی-اُکسیداتیو گیاهان گوجه‌فرنگی پیش‌اَنگیخته با *Bacillus thuringiensis* به *Fusarium oxysporum* f. sp. *lycopersici*

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چکیده

در شرایط گرمایش جهانی، کاهش عملکرد گوجه‌فرنگی به دلیل آفات و بیماری‌های قارچی مانند پژمردگی فوزاریومی انتظار می‌رود. استفاده از مهارگرهای زیستی کارآمد در کنترل هر دو گروه آفات و بیماری‌ها از دیدگاه زیست‌محیطی و اقتصادی منطقی می‌باشد. در این پژوهش، تاثیر *Bacillus thuringiensis* در پیش‌انگیزی (Priming) پایستگی گوجه‌فرنگی در برابر پژمردگی فوزاریومی ناشی از قارچ *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) بررسی شده است. پیش‌انگیزی گیاهچه‌های گوجه‌فرنگی رقم فلات سی. اچ. با *Bt* در مرحله ۴ تا ۵ برگی انجام شد و نمونه‌های برگ ۳، ۱۸، ۲۴، ۴۸ و ۷۲ ساعت پس از تیمار با *Fol* ارزیابی شدند. میزان پراُکسیدهیدروژن (H_2O_2) و تغییرات رونویسی نسبی ژن‌های آنزیم‌های آنتی‌اُکسیدانی مانند سوپراُکسید دیسموتاز (SOD)، کاتالاز (CAT)، و گلوتاتیون S-ترانسفراز (GST) با روش qRT-PCR ارزیابی گردید. هیچ تغییر معنی‌داری در رونویسی نسبی ژن *CAT* مشاهده نشد. رونویسی نسبی ژن‌های *SOD*، و *GST* در گیاهان تیمار شده در مقایسه با گیاهان شاهد با گذشت زمان افزایش یافت. بیشترین میزان تغییر در رونویسی نسبی *SOD* در ۱۸ و ۲۴ ساعت پس از تیمار و در *GST* در ۱۸ و ۷۲ ساعت پس از تیمار مشاهده شد. افزایش رونویسی ژن‌های آنتی اکسیدانی با کاهش مقدار H_2O_2 در بافت گیاه پیش‌انگیخته با *Bt* همخوانی داشت. این نتایج با کارآمدی *Bt* در انگیزش پایستگی سیستمیک گوجه‌فرنگی در برابر قارچ *F. oxysporum* f. sp. *lycopersici* همخوانی دارند.

کلمات کلیدی: کنترل زیستی، کاتالاز، qRT-PCR، تنش، پژمردگی.