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Research Article

The Effect of *Trichoderma harzianum* and nitrogen fertilizer on the gene expression of the cytokinin in tomato leaves

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Abstract
This work aimed to study the effect of two fertilizers agents of *Trichoderma harzianum* and nitrogen fertilizer on the gene expression related to cytokinin in tomato leaves. It was conducted in the College of Agriculture, Wasit University, Iraq. Treatments include the control group (T1) without any treatment, 100kg N(nitrogen fertilizer)/h^{-1} (T2), 160 kg $N.h^{-1}$ (T3), 20kg $N.h^{-1}$ (T4), 0kg N/h^{-1} with 100g of *T. harzianum* (T5), 100kg $N.h^{-1}$ with 100g of *T. harzianum* (T6), 160kg $N.h^{-1}$ with 100g of *T. harzianum* (T7) and, 200kg $N.h^{-1}$ with 100g of *T. harzianum* (T8). An experiment was carried out by planting tomato seeds in pots under growth chamber conditions to assess the expression of genes associated with cytokinin up-regulation and stimulation. The plants were treated with the above fertilizer agents after 12 and 27 days of seedlings, and sampled 72 hours later. The results showed that all the treatments up-regulator the expression of *SlRRA1* and *SlHK4* genes in tomato leaves and enhance cytokinin activity and T6 was superior in promoting the expression. The expression of *SIRRA1* and *SIHK4* genes in cytokinin expression of tomato leaves were affected when treated with two fertilizers agents of *T. harzianum* and nitrogen fertilizer after 15 and 30 dpi.

Keywords: Tomato, Fertilizer, SlRRA1, SlHK4, Gene expression.

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Introduction

Cytokinins are hormonal phytochemical organic compounds that encourage cell division and growth and delay aging in leaves. They are produced at the roots' tops, transmitted upward through the xylem, and promote axillary bud formation and cell differentiation (Kieber & Schaller 2018). The first discovery of cytokinin and its function in plants was through histidine kinase (HK), which contains five genes viz. *PtHK2, PtHK3a, PtHK3b, PtCRE1a*, and *PtCRE1b*. *Arabidopsis thaliana* has three genes, including *CRE1/WOL/AHK4, AHK2*, and *AHK3* that activate association with hormone by activating the phosphorylation process that stimulates the activity of cytokinin and increases its physiological functions in the plant (Shi et al. 2011; Lomin et al. 2018). Plants employ an expanded version of the basic cytokinin pathways (He et al. 2016; Arkhipov et al. 2019). Cytokinins bind to HK receptors, leading to autophosphorylation. The HpT proteins serve as mediators to receive the phosphoryl group and transfer it to BRRs containing DNA-binding receptors such as MYB at the extended C-terminus. Through this, cytokinin signaling is maintained by a system composed of histidine aspartate phosphorylase, which leads to the altered expression of cytokinin-regulating genes (Burr et al. 2020). Three genes in *A. thaliana* that act as cytokinin receptors are *AHK2, AHK3,* and *AHK4*. They stimulate cytokinin activity through phosphorylation by kinase protein and HpT phosphorylate. When cytokinin is not bound, it acts as a phosphatase (Argyros et al. 2008; Ishida et al. 2008).

Trichoderma harzianum plays an important role in

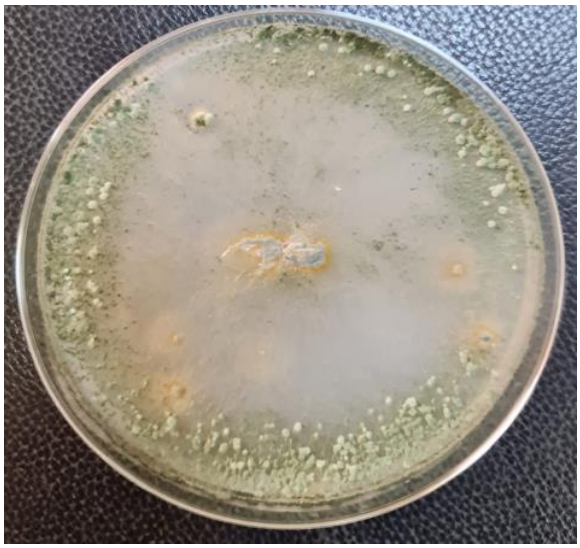

Fig.1. *Trichoderma harzianum* isolates on the potato dextrose agar after 7 days of incubation.

the protection and growth of plants by producing some secondary metabolites, polysacharids, enzymes, and hormones. etc, and other mechanisms that act as an internal stimulus inside hosts, playing a critical role in activating the genes and changing the state from the turn off to turn on (Alkooranee et al. 2017). This study aimed to determine the gene expression of *SlRRA1* and *SlHK4* in cytokinin expression of tomato leaves treated with two fertilizers agents of *T. harzianum* and nitrogen fertilizer at 15 and 30 days of seedlings.

Material and Methods

The experiments were conducted at the Microbiology lab., Field crop Department, College of Agriculture, Wasit University. Tomato seeds were planted and grown in the growing chamber. Tomato seed (Majd cultivar) has a limited-growing hybrid produced by Seminis Company. The potassium sulfate and triple superphosphate fertilizers were used before planting, and urea fertilizer was applied after the seedlings and a second time after a month of planting.

The study was conducted with the isolate of *T. harzianum* collected in 2020 from the healthy tomato plants' rhizospheres. *Trichoderma harzianum* isolate was grown on potato dextrose agar (PDA) media (200g sliced potatoes, 20g dextrose, and 20g agar powder in 1000mL water). After seven days of incubation at 35±2°C, it showed the mycelium and conidia of fungus (Fig. 1). It is grown on potato dextrose broth (PDB) and incubated for two weeks at a temperature of 35°C for later use in experiments.

The characters of mycelia colour patterns varied noticeably between the *Trichoderma* isolates, from colourless to yellow and white; their shapes consisted of concentric rings, and hyphae were septated, smooth-walled, and hyaline. The conidia were green to yellow-green or dark green colour. Their shapes were rough and subglobose. Conidia production was more intense in the centre but declined toward the margins (Dubey et al. 2007).

Trichoderma harzianum fungus was added at a concentration of 100ml per pot to the root zone of the plants according to the treatments. The treatments were applied by adding the biological factor and nitrogen fertilizer 12 and 27 days after planting. The experiment includes a group without any fertilizer as control (T1), 100kg N(nitrogen fertilizer).h-1 (T2), 160kg N.h-1 (T3), 200kg N/h-1 (T4), 0kg N.h-1 with 100ml of *T. harzianum* (T5), 100kg N.h-1 with 100ml of *T. harzianum* (T6), 160kg N.h-1 with 100ml of *T. harzianum* (T7), and 200kg N.h-1 with 100ml of *T. harzianum* (T8). Samples of the leaves were taken after 72 hours post-inoculation (hpi). The collected samples have been preserved in Trizol to extract total RNA to measure gene expression.

Table 1. List the primers used for RT. PCR in the current study.

Gene symbol	Name	Reference	Primers
SlRRA1	Response Regulator	Shi et al. 2013	5'- TGTTGGTGATGTTGGCGAAC-3' 5'- TCGTCCCCTAAAGCATTCTC-3'
SlHK4	Histidine Kinase	Shi et al. 2013	5'- ACTGGAGAATACTCATCTCT-3' 5'- GAGTACTTTTGCTGAGTACA-3'
IPT1 *Reference gene*	Isopentenyl transferase genes	Wang et al. 2020	5'- CAGTCGAACCCTGTCAGCAAA-3' 5'- GGACCCGAGATTCGCCATTAT-3'

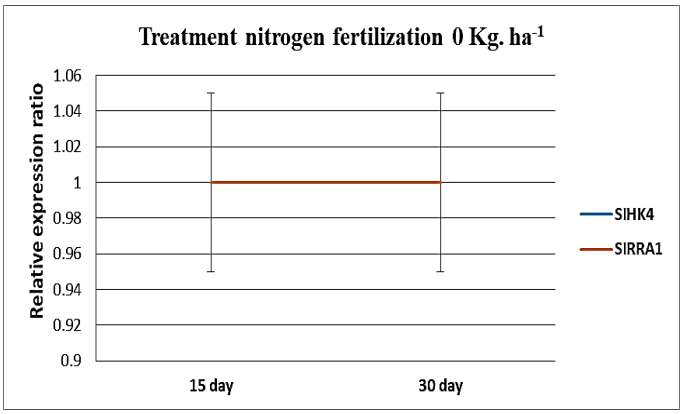

Fig.2. Expression levels of cytokinin-related genes of tomato plants non-treated.

Gene expression assay: Total RNA Extraction was done using MagPurix® Total RNA Extraction Kit (ZP02015). The first-strand cDNA synthesis by WizScript™ cDNA Synthesis Kit and quantitative real-time polymerase chain reaction (qRT-PCR) were used with primers as shown in Table 1. In addition, the IPT1 gene was used as a housekeeping gene.

Results and Discussion

The results showed the expression profile of cytokinin-related genes in tomato leaves treated with *T. harzianum* spp. and 0, 100, and 160Kg.ha^{-1} of nitrogen fertilization during different time periods (15- and 30-days post seedlings = dps) (Table 1). In the treatment of nitrogen fertilization 0Kg.ha^{-1}, the expression levels of *SIHK4* and *SIRRA1* had a peak at 15 and 30 dps in the tomato leaves (Fig. 2). In, the treated tomato with nitrogen fertilization of 100Kg.ha^{-1} (Fig. 3), the expression levels increased, where expression of the *SIHK4* increased by 1.049 times at 15 to 11.392 times at 30 dps than the control one.

The expression of the *SIRRA1* gene increased by 3.024 times at 15 compared to control treatment but decreased by 2.394 times at 30 dps. The expression levels of *SIHK4* peaked at 15 and 30 dps in the tomato treated with nitrogen fertilization of 160Kg.ha^{-1} (Fig. 4) increased expression by 1.071 times at 15 dpi to 10.556 times at 30 dps, whereas *SIRRA1* was increased by 3.732 times at 15 dps and decreased by 0.716 times at 30 dps. The expression level of the *SIHK4* increased in the plants treated with nitrogen fertilization of 200Kg.ha^{-1} (Fig. 5), which peaked at 15-30 dpi, increased by 1.013 times at 15 dpi to 7.260 times at 30 dpi; whereas *SIRRA1* expression increased by 2.219 times at 15 dpi but decreased by 1.464 times at 30 dpi. Nitrate-specific signaling is primarily involved in producing amino acids and nucleic acids. This pathway regulates the expression of a large number of genes. Cytokinin-mediated signaling is primarily associated with regulating nitrogen cycling and development (Sakakibara 2006). This mechanism is recognized by nitrogen-dependent cytokinin synthesis and the activation of His-Asp phosphorelay pathways

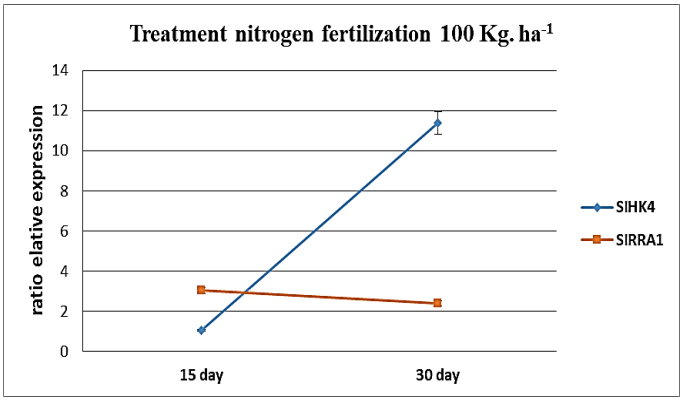

Fig.3. Expression levels of cytokinin-related genes of tomato plants treated with nitrogen fertilization 100Kg.ha^{-1}.

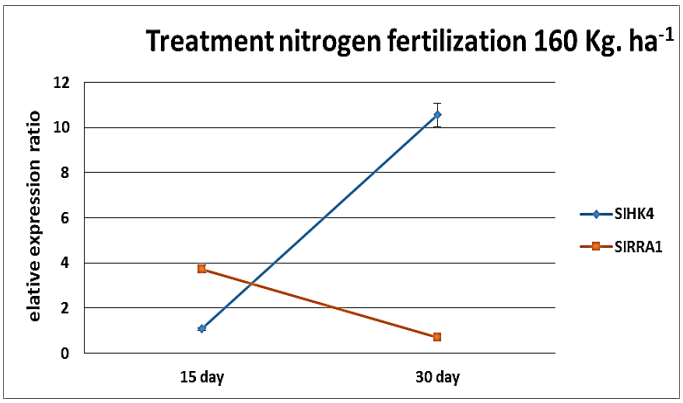

Fig.4. Expression levels of cytokinin-related genes of tomato plants treated with nitrogen fertilization 160Kg.ha^{-1}.

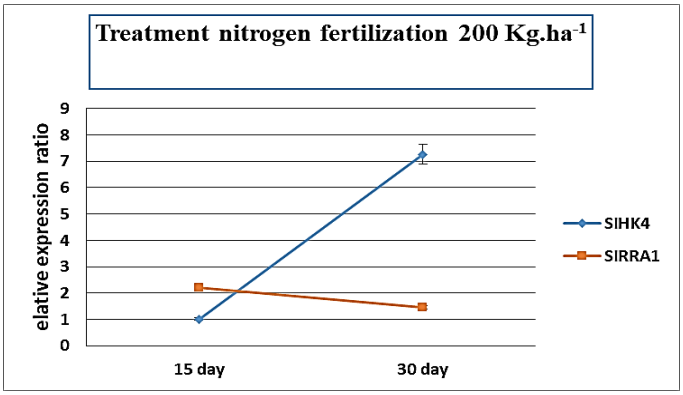

Fig.5. Expression levels of cytokinin-related genes of tomato plants treated with nitrogen fertilization 200Kg.ha^{-1}.

(Tesfay et al. 2019).

The results showed the genes were strongly affected by a combined inoculation of *T. harzianum* and nitrogen fertilization (Figs. 2, 6). The expression level of SIHK4 was up-regulated by 0.673 times peaked at 15 to 22.471 times at 30 dps in plants treated with *T. harzianum*, and the SIRRA1 was up-regulated by 0.607 times at 15 dpi to 2.738 at 30 dps when treated with *T. harzianum*. The expression level of the *SIHK4* increased in the plant treated with *T. harzianum* and nitrogen fertilization of 100Kg.ha^{-1}, which peaked at 1-15 dpi as 3.386 times to 27.665 times at 30 dpi. *SIRRA1* was up-regulated by 6.868 times at 15 dpi and 10.703 times at 30 dpi (Fig. 7). In the treatment of *T. harzianum* and nitrogen fertilization of 160Kg.ha^{-1} (Fig. 8), the expression levels increased, and the *SIHK4* was up-regulated by 1 time at 15 to 5.502 times at 30 dps, whereas the

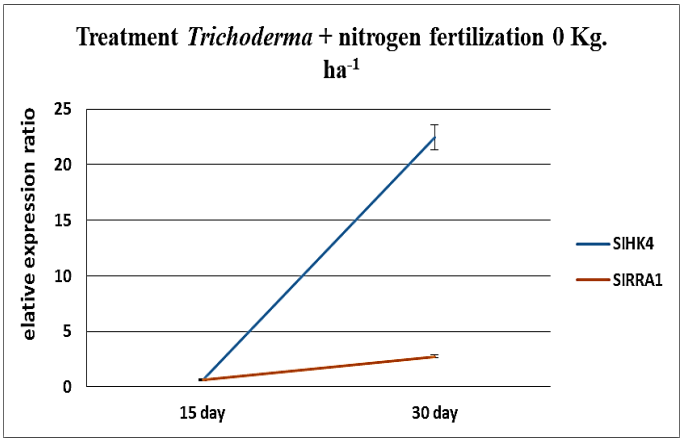

Fig.6. Expression levels of cytokinin-related genes of tomato plants treated with *Trichoderma* fungi + nitrogen fertilization 0Kg.ha^{-1}.

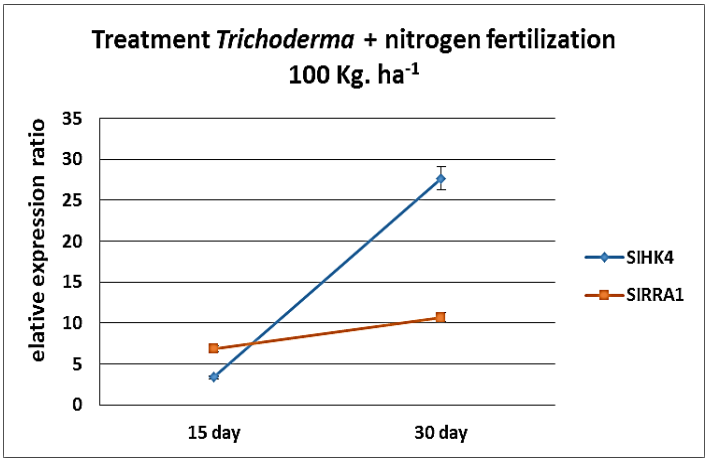

Fig.7. Expression levels of cytokinin-related genes of tomato plants treated with *Trichoderma* fungi + nitrogen fertilization 100Kg.ha^{-1}.

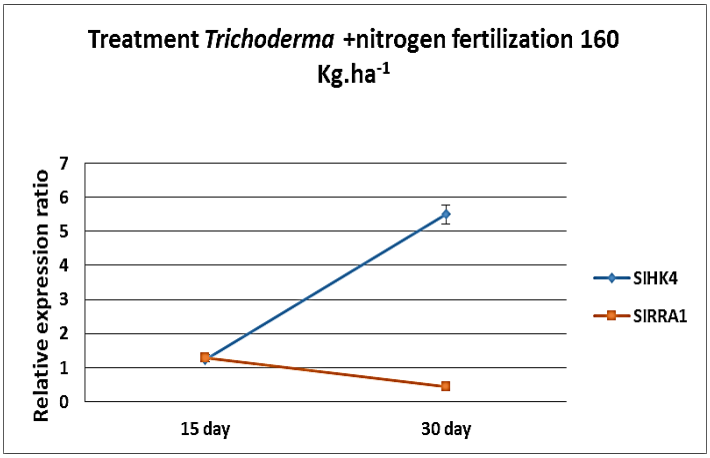

Fig.8. Expression levels of cytokinin-related genes of tomato plants treated with *Trichoderma* fungi + nitrogen fertilization 160Kg.ha^{-1}.

SIRRA1 was decreased by 1.301 times at 15 to 0.438 times 30 dps. The expression of *SIHK4* in tomatoes treated with *T. harzianum* and nitrogen fertilization of 200Kg.ha^{-1} was up-regulated by 18.802 times at 15 to 14.723 times at 30 dps; however, *SIRRA1* expression increased by 1.986 times at 15 dpi to 2.271 times at 30 dps (Fig. 9). According to Contreras-Cornejo et al. (2018) and Moya et al. (2020), some strains of *T. harzianum* can form intimate associations with plant roots, providing an endemic level of biological control or stimulating plant growth. The ability to produce soluble forms of

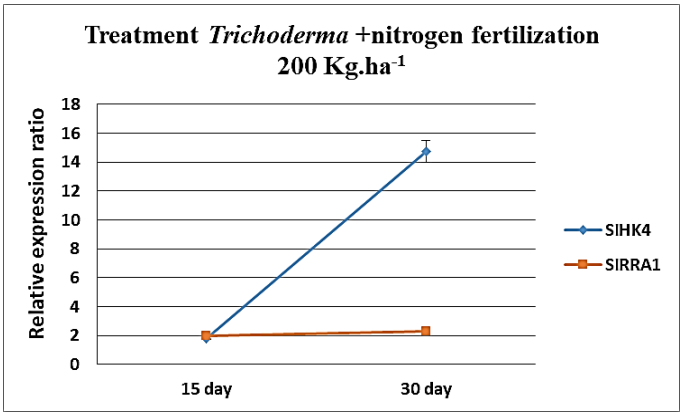

Fig.9. Expression levels of cytokinin-related genes of tomato plants treated with *Trichoderma* fungi + nitrogen fertilization 200Kg.ha^{-1}.

mineral nutrients and growth-promoting metabolites is one of the most important characteristics of these strains. These include regulation of hormonal balance, solubilization and mineralization, production of volatile organic compounds and microbial enzymes, and suppression of alleviation of abiotic stresses and plant pathogens (Zhang et al. 2018). In conclusion, the expression of *SlRRA1* and *SlHK4* genes in cytokinin expression of tomato leaves were affected when treated with two fertilizers agents of *T. harzianum* and nitrogen fertilizer after 15 and 30 dpi.

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