Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Selim, J Plant Pathol Microb 2015, 6:10
DOI: 10.4172/2157-7471.1000311

Research Article Open Access

Effectiveness of *Trichoderma* Biotic Applications in Regulating the Related Defense Genes Affecting Tomato Early Blight Disease

Mohamed E. Selim*

Agricultural Botany Department, Faculty of Agriculture, Menoufiya University, Egypt

Abstract

Early blight disease caused by *Alternaria solani* fungus is one of the most important diseases attacking tomato especially in humid regions with high temperatures (24-29°C). Controlling the early blight disease using fungicides has become unfavorable in the last years due to their environmental and human health concerns. Biotic and abiotic induction of host plants defense mechanisms could be applied as an alternative management strategy against the disease. Nowadays, different *Trichoderma* species could be used as one of promising bio-control agents affecting development and disease incidence of *Alternaria solani* on tomato plants. In present study, the direct and indirect effects of different *Trichoderma* isolates application on *Alternaria solani* infection as well as on the gene expression levels of some related genes to defense mechanisms in tomato plants was investigated. Results indicated that *Trichoderma* species reduced either the mycelial growth or the disease incidence of *Alternaria solani*. Treating tomato roots with *Trichoderma harzianum*-T10 isolate affected the relative expression levels of eight different genes within tomato leaves. Three genes of them i.e., Les.21895, Les.19403 and Les.1097, which involved in auxin, ethylene and lignin pathway respectively, were up regulated while the other three genes i.e., Les.20348, Les.3129 and Les.9833, which related to pyruvate kinase pathways, were down regulated. In addition, treating tomato plants with *Trichoderma harzianum* T10 regulated the expression level of some Pr-protein genes i.e., Pr-1 and Pr-5. These findings suggested that induction of systemic defense mechanisms using the mutualistic *Trichoderma* isolates such as T10 is candidate to be among the mechanisms that can play a crucial role in controlling tomato early blight disease caused by *Alternaria solani*.

Keywords: *Trichoderma* species; *Alternaria solani*; rt-PCR; Gene expression; Defense related genes in tomato; Pr-proteins

Introduction

Tomato (*Solanum lycopersicum* Mill.) is one of the most important vegetables, ranking first, based on production levels, accounting for 14% of the total fruit and vegetable production worldwide [1]. The global tomato production area covers approximately 4 million hectares of arable lands, annually yielding approximately 100 million tons with an estimated value of 5 to 6 billion US$ [2]. Noteworthy, Egypt is among the top 10 tomato producing countries worldwide covering an estimated 181.000 ha and yielding 6, 4 million tons in annual production [1,2].

The fungus *Alternaria solani* (Ellis & Martin) Sorauer, the causal agent of early blight disease, is a major pathogen of tomato [3] causing considerable yield losses all over the world, particularly in humid regions with fairly high temperatures (24-29°C). Epidemics can however also occur in semi-arid climates, like those of Egypt, where frequent and prolonged nocturnal damp periods occur [4]. Within the species, *Alternaria solani* is divided into races, which show significant variability in morphology. These races can also show variations in pathogenicity on various crops, like tomato and potato, and their cultivars [5]. When infecting, all above ground parts, including leaves, stems collars and fruits, can be attacked resulting in stem lesions on the adult plant, collar and fruit rot [6]. On leaves, *A. solani* causes circular concentric rings with yellow halo, which is known as early blight (EB). Earlier it was reported that EB infections in tomato, causes up to 79% of yield losses [7] whereas collar rot only is responsible for about 50% of these losses on tomato seedlings [8].

Control of early blight disease using chemical fungicides has become increasingly difficult due to the limited number of effective fungicides, some of which were recently withdrawn from the market due to the environmental and human health concerns [9]. Moreover, the use of fungicides during fruiting is discouraged, making the chemical control of *A. solani* virtually impossible. The search for alternative control methods is thus quite important and necessary. Although the use of resistant varieties which may be considered as a good alternative especially within integrated pest management strategies, unfortunately has also significant drawbacks due to the lack of suitable resistant tomato germplasm against *A. solani* [10,11]. Alternatively, no suitable biological control agents are available as well [12,13].

Research has characterized several mutualistic endophytic fungi with biological control activity against fungal pathogens or plant parasitic nematodes as well [14,15]. These mutualistic bioagents are considered eco-friendly and have no reported negative effects on non-targeted organisms, including humans, the useful microflora and host plants. In such tripartite interactions between the host plant, fungal pathogen and mutualistic endophyte agent, different mechanisms of action were reported and considered responsible for protecting the host plants from pathogens and parasites [16-18]. In some cases, the endophyte has the ability to trigger systemic resistance responses in different host plants. Different hormones such as auxin and ethylene were found to be related to complex defense signaling pathways against pathogens and parasites [19,20]. Furthermore, the

***Corresponding author:** Mohamed E Selim, Agricultural Botany Department, Faculty of Agriculture, Menoufiya University, Egypt, Tel: 2224347-2048; E-mail: m_elwy76@yahoo.com

Received October 08, 2015; **Accepted** October 27, 2015; **Published** November 02, 2015

Citation: Selim ME (2015) Effectiveness of *Trichoderma* Biotic Applications in Regulating the Related Defense Genes Affecting Tomato Early Blight Disease. Essential Oil. J Plant Pathol Microb 6: 311. doi:10.4172/2157-7471.1000311

studies of Hamberger and Hahlbrock [21] on *Arabidopsis thaliana* proved the direct or indirect association of CoA Ligase isoenzymes in the biosynthesis of lignin and cell wall-bound phenylpropanoid derivatives. Nicholson and Hammerschmitt [22]; Bhuiyan et al. [23] mentioned also that lignification process is involved in plant defenses against pathogen infection. Moreover, pyruvate kinase (PK) enzymes have been known to affect numerous physiological and biological processes in plants [24,25]. Different studies suggest that PK activity can be used as a remarkable physiological indicator of K^+, Mg^{2+}, and Ca^{2+} contents in plant tissues [26,27].

In this study, the potential of various isolates of *Trichoderma* species as promising biocontrol agent against *A. solani,* the causal pathogen of early blight disease, was evaluated under *in vitro* and *in vivo* conditions.

Moreover, in order to study the influence of *Trichoderma* application on eliciting the systemic defenses pathways in tomato, the relative expression levels of related defense genes which involved in the auxin biosynthesis, ethylene signaling, lignin biosynthesis, pyruvate kinase biosynthesis and Pr-proteins production were determined using qRT-PCR technique.

Materials and Methods

Endophyte and pathogen cultures

Pure culture of 5 bio-agents i.e., *Trichoderma hamatum (Th), T. viride (Tv), T. harzianum (Tz22)*, *Trichoderma* species (T32) and *Trichoderma koningii* (Tkg) were obtained from Institute of Crop Science and Resource Conservation (INRES), Bonn University, Lab of Prof. Dr. Richard Sikora. In addition, *Trichoderma harzianum*-T10 isolate and the pathogenic isolate of *Alternaria solani* were obtained from Plant Pathology Department, Faculty of Agriculture, Menoufiya University, Egypt. Pure cultures of all fungi were maintained in stored micro-bank tubes at -80°C. Conidial spores of tested *Trichoderma* isolates and *Alternaria solani* were obtained from pure cultures grown on PDA plates, supplemented with 150 mg l^{-1} of chloramphenicol at 25°C in the dark for two weeks. Five ml of ml sterile distilled water were added to each plate then the spores were scraped far away the mycelial mat using sterilized glass rod. The spore suspension was filtered through three layers of fine sterile cheesecloth. The prepared spore suspensions of tested *Trichoderma* isolates were adjusted at 1×10^7 spores/mL and at 2×10^3 spores/ml for the pathogen [28].

Plant growth

Tomato (*Solanum lycopersicum*) cv. Hellfrucht/Frühstamm that consider as susceptible cultivar to infection with *A. solani*, was used in all experiments of this study. Seeds were surface-sterilized by submersing them in a 75% ethanol solution for 1 min and 1.5% sodium hypochloride (NaOCl) solution for 3 min, respectively. The seeds were then thoroughly rinsed three times with sterile water. The seeds were then sown in plastic trays containing sandy clay soil (1:1, v/v), which was previously autoclaved at 121°C for 1 h. The plants were grown in a growth chamber at 25 ± 3°C with 16 h diurnal light, 60 to 70% humidity and fertilized weekly with 5 ml of 2 g/l N:P:K: (14:10:14; Aglukon, Düsseldorf, Germany). After 2 weeks, when reaching 10-15 cm in height, the seedlings were transplanted into separate pots filled with 300g of a mixture of sand and sandy loam soil in a ratio of 2:1 (v/v), and transferred to a greenhouse, maintained at 27 ± 5°C and 16 h diurnal light.

In vitro antagonism assay

The bio-control activities of tested *Trichoderma* isolates against the tested pathogenic isolate of *A. solani* was studied *in vitro* by the dual culture technique on plate [29]. Five millimeters diameter plugs of both two weeks old pathogen and biocontrol agent cultures were taken with a sterile cork borer. These plugs were placed equidistantly (60 mm) apart on PDA, amended with 150 mg l^{-1} of chloramphenicol, in a 90 mm Petri-dish. As a control, a plug with the pathogen was placed on PDA, amended with 150 mg l^{-1} of chloramphenicol, alone. Each treatment was replicated three times. The Petri-dishes were incubated at 23 ± 2°C. The radial growth of both tested bio-agents and the pathogen were recorded after 10 days and the relative mycelial growth inhibition was calculated according to Vincent [30].

In vivo antagonism assay

The antagonistic capabilities of tested *Trichoderma* isolates against *A. solani* were studied *in vivo*. In this respect, 3 ml of spore suspension of each individual *Trichoderma* species was inoculated into soil at transplanting stage of tomato seedlings to pots (10 cm diameter contains 300 g soil). One week after the inoculation of the *Trichoderma* isolates, spores of *A. solani* was sprayed over the true leaves of tomato plants [28]. Each treatment was replicated three times. The non-treated plants with *Trichoderma* spore suspension were served as control. Potential influence of the tested *Trichoderma* spp. on early blight disease severity was also compared with a Propamocarp HCl (72,2%) fungicide that is generally recommended (purchased from Syngenta company, Egypt). Therefore, three plants were sprayed with 2.5 ml/L of this fungicide at the same time with *Trichoderma* application. The early blight severity on each leaf of the sprayed plants was recorded three weeks after inoculation using disease index described by Vakalounakis [31] based on a scale of 0 to 5, where 0=no visible lesions on leaf; 1=up to 10% leaf area affected; 2=11%– 25%; 3=26%–50%; 4=51%–75%; and 5=more than 75% leaf area affected or leaf abscised. Percentage of disease severity was calculated according to the following formula [32]:

$$\text{Disease severity}(\%) = \frac{\text{sum of all ratings}}{\text{no. of leaves sampled - maximum disease scale}} \text{-} 100$$

Gene expression analysis by semi-quantitative real-time qRT-PCR

The effect of *Trichoderma harzianum*-T10 isolate on relative gene expression of eight selected genes coding for IAA6 (Les.21895), ER69 (Les.271), 4-coumarate--CoA ligase 2 (Les.1097), pyruvate dehydrogenase kinase (Les.20348) and two pyruvate kinases (Les.3129 and Les.9833) in addition to two pathogen related genes (PR-1 and PR-5) which were associated with either development signaling pathways or defense responses in tomato was assessed using quantitative PCR (Table 1).

During the transfer of the seedlings to the pots (as described in plant growth paragraph above), roots of three individual plants were dip-inoculated with spore suspension of *T. harzianum* -T10. Control plants were treated only with tap water. Four weeks after T10 inoculation, shoots plus leaves of inoculated and control plants were separately collected in 50 ml falcon tubes and frozen in liquid nitrogen. The leaves were ground into a fine powder using liquid nitrogen and a pre-cooled mortar and pestle. The ground plant tissue powder was kept frozen using liquid nitrogen, transferred into fresh 50 ml falcon tubes and stored at -80°C until further use. Total RNA was extracted from 100 mg tissue powder using the Macherey-Nagel total RNA extraction kit and further purified using the Machey-Nagel RNA clean up kit, both according to the manufacturer's recommendations. The RNA integrity was assessed by gel electrophoresis and the RNA concentration was measured photometrically at 260 nm using the Nanodrop 2000 C (Fisher Scientific, Germany). To assess RNA integrity, 6 µl of the total

Oligo- Name	Putative function according to NCBI	Sequence (5^{-} - 3^{-})	
		Forward- primer	Reverse- primer
Les.21895	auxin-regulated (IAA6)	TGGTCAGTGTGCCAGTGATAAGA	AGAACCATTGAGAAGATCCATCAAG
Les.271	Ethylene-responsive methionine synthse (ER69)	GGTATCGGCCCTGGTGTGTA	TTGTTAACTCTGACGGCAATCTCT
Les.1097	4-coumarate--CoA ligase 2	CGTTACACTCGTATTGTTTCGAAAAT	TTCGCCCCGTCGATTAAA
Les.20348	pyruvate dehydrogenase Kinas (PK)	CTCCTGATTGTGTGGGCTATATACA	ATTCGCGCAAGCAAATAGAAC
Les.3129	pyruvate kinase (PK)	CCTGTTTTGACTACAGACTCTTTCGA	CAAGCCCCTATATACCAAACTGTGT
Les.9833	pyruvate kinase (PK)	CAGTTCCCATGAGTCCTTTGG	ACTTCCTCCCCTGGTTAACACA
Les.20078	PR-1 (pathogenesis-related protein 1)	CACTCGTATCATGAGTCTTC	CCCTATATACCCTGGTTA
(PR-5)	PR-5 (pathogenesis-related protein 5)	CCCATGAGTCCTTGTGGGCTA	ACTCTGACGGCATATATACC

Table1: Genes selected for conducting qRT-PCR within inoculated and non-inoculated tomato plants with *Trichoderma harzianum* -T10.

RNA was mixed with 2 µl 6 x gel loading dye and loaded on a 1% agarose gel supplemented with 0.5 µg/ml ethidium bromide, (Figure 1). Of this total RNA sample, 1 µg was used to synthesis complementary DNA (cDNA) via the reverse transcription kit, according to the manufacturer's recommendations. The pipetting scheme and the PCR program is indicate in the Tables 2 and 3, respectively.

The cDNA was used for qPCR analysis by measuring the relative accumulation of the eight selected genes. For this, the cDNA was diluted ten times with DNA/RNA free water and 1 µl transferred into a well of a 96-well qPCR plate. For each well 19 µl master mix was added to 1 µl of the sample cDNA (the pipetting scheme is indicated in Table 4). For each sample, three biological replicates were prepared. The plate was covered with a qPCR foil, the reaction components were mixed and the plate was briefly centrifuged for 10 sec at 4000 × g. The thermal cycling parameters are indicated in Table 5. After qPCR, 6 µl of the amplification product was mixed with 2 µl 6 x gel loading dye and loaded on a 1% agarose gel, supplemented with 0.5 µg/ml ethidium bromide, for a gel electrophoresis at 60 mV to assess amplification. The accumulation of the genes of interest was normalized to actin transcript accumulation and the fold change in transcript accumulation relative to the control sample was calculated via the $2^{-\Delta\Delta Ct}$ – method described by Livak and Schmittgen [33].

Statistical analysis

The data were analyzed according to the standard analysis of variance procedure with SPSS 14 for Windows. Differences among treatments were tested using one way analysis of variance (ANOVA) followed by the Tukey test for mean comparison in case the F-value was significant [34].

Results

The antagonistic potentialities of the six tested *Trichoderma* isolates viz., *Trichoderma hamatum (Th), T. viride (Tv), T. harzianum (Tz22), T. harzianum* (isolate T10), *Trichoderma* species (T.32) and *Trichoderma koningii* (Tkg) were investigated under *in vitro* conditions onto PDA plates for determining their abilities in inhibiting the mycelial growth of *Alternaria solani*. The results indicate that all tested bio-agents significantly inhibited the mycelial growth of the pathogen compared with the control (Figure 2) where the inhibition % were ranged between 66% with Tz22 to 75% with T10. Conversely, the radial growth of tested *Trichoderma* isolates was not impaired by *Alternaria solani*. Thus, it could be concluded that T10 was the best performing tested isolate followed by Tkg with regard to inhibiting the mycelial growth of *Alternaria solani*.

The influence of the six tested *Trichoderma* isolates i.e. *Th, Tv, Tz22,* T10, T.32 and Tkg on the incidence and the severity of early blight disease caused by *Alternaria solani* in tomato was determined under greenhouse conditions. As a reference for efficacy, treatment with the fungicide (Propamocarp Hcl) was included in the experiment. Comparing with control treatment (infected plants with *Alternaria solani* only), the results indicate that all tested bio-agents significantly reduced disease severity (Figure 3) which ranged from 50% in case of using *T. harzianum* Tz22 to 70% with *T. harzianum*-T10. The second best disease reduction was obtained with *Trichoderma koningii* (Tkg). On the other hand, *T. harzianum*-T10 came therefore close to the disease reduction % obtained with fungicide treatment, which averaged 77.5% (Figure 3).

The indirect reaction of tomato plants against *Alternaria solani*, the causal of early blight disease in response to treating the plants with *T. harzianum*-T10 as the best performing bio-agent was evaluated through determining the relative expression of eight different genes, six genes related to vital and essential bioprocess and two genes involved in defence mechanisms pathways, in tomato plants. The changes in these genes expression, Les.21895, Les.271, Les.1097, Les.20348, Les.3129 and Les.9833 in addition to two genes related to Pr-proteins i.e., pr-1 and pr-5, was analyzed through using the real-time PCR analysis. The obtained results (Figure 4) show that treating tomato plants with *T. harzianum*-T10 led to induction and accumulation of three genes i.e., Les.21895, Les.271 and Les.1097 which involved and related with synthesis of Indol acetic acid, Ethylene and coumarate Ligase pathways respectively, where the fold changes in gene expressions of these genes were increased 2.5, 7.1 and 3.7 folds respectively, comparing to control plants (un-treated with T10 isolate). Also, the relative expression levels of the other three genes i.e., Les.20348, Les.3129 and Les.9833 were down regulated up to two fold changes comparing to un-treated plants with T10. It is remarkable that all these three genes were found to be related to pyruvate kinase metabolisms. With regard to the two pathogenesis related proteins genes i.e., Pr-1 and Pr-5, results indicate that T10 isolate increased the relative expression of Pr-1 while it down regulated the expression of Pr-5. Moreover, the fold change was reached up to three times with Pr-1 and two times with Pr-5 (Figure 4).

Discussion

Using mutualistic bio-agents is a natural way to improve agricultural production and decreasing yield losses either directly, by mechanisms such as mycoparasitism or indirectly, by competing with pathogens for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defense mechanisms and antibiosis, [35].

The present investigation aimed to study the direct and indirect effects of mutualistic *Trichoderma* application as a promising bio-agent against *Alternaria solani*, the causal pathogen of early blight disease on tomato. In this respect, six different isolates belonging to genus *Trichoderma* i.e., *Trichoderma hamatum (Th), T. viride (Tv), T. harzianum (Tz22), T. harzianum (*isolate T10), *T.* species (T.32) and T.

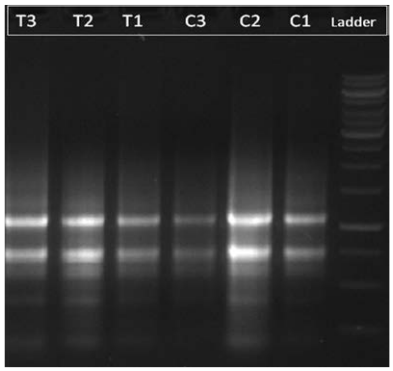

Figure 1: RNA isolated from leaves of three biological replicates (T1, T2 and T3) treated with *T. harzianum*-T10 isolate and three un-treated (control) replicates (C1, C2 and C3). Ladder is 1kbp ladder.

Components	Volume per reaction
10 x RT buffer	2 µ
dNTP mix (10 mM)	2 µl
Random Primers (10 mM)	2 µl
RNAase inhibitor (1 U/µl)	1 µl
Reverse transcriptase (5 U/µl)	1 µl
RNA (1 µg)	x µl
H_2Odest	0 - x µl
Total volume	**20 µl**

Table 2: Pipetting scheme containing reverse transcription reagents.

Step	Time	Temperature	Cycles
Anealling of Random primers	10 min	25°C	Hold
Reverse transcriptase activity	120 min	37°C	Hold
Reaction termination	5 min	85°C Hold	Hold

Table 3: Thermal cycling parameter for the cDNA synthesis/reverse transcription.

Components	Volume per reaction
Fast SYBR®- Green Master Mix	10 µl
Forward primer (10 mM)	0,5 µl
Reverse primer (10 mM)	0,5 µl
DNA (1:10 diluted)	1 µl
H_2Odest	8 µl
Total Volume	20 µl

Table 4: Pipetting scheme containing Real time PCR Fast SYBR®- Green Master Mix.

Step	Time	Temperature	Cycles
Polymerase activation	20 sec	95°C	Hold
Denature	3 sec	95°	40
Anneal/Extend	30 sec	60°C	40
Melt curve	15 sec	95°C	Hold
Melt curve	1 h	60°C	Hold

Table 5: Thermal cycling parameter for the semi-quantitative RT-qPCR.

koningii (Tkg) were screened either *in vitro* or *in vivo* for this purpose. Also, the ability of *Trichoderma* to affect the expression levels of some related genes to defense mechanisms in tomato was evaluated using qRT-PCR technique.

The direct and indirect influence of all tested biocontrol isolates on *Alternaria solani* the causal of early blight disease was determined either under *in vitro* or in vivo conditions.

Moreover, the potential of the tested biocontrol *Trichoderma* isolates on incidence of early blight disease under *in vivo* conditions was compared with standard and recommended chemical fungicide, propamocarp Hcl, as well as with *Alternaria solani* infected tomato plants, which were treated with tap water only (control).

The obtained results from *in vitro* bioassay revealed that all tested isolates significantly reduced the mycelia growth of *A. solani* in dual cultures under laboratory conditions. The best results were recorded with *Trichoderma harzianum*-T10. Similar finding were reported by Rudresh et al. [36] who noticed that *T. harzianum* inhibited growth of *Rhizoctonia solani* and *Fusarium oxysporum* under *in vitro* conditions with 72.1% and 77.0%, respectively. The *in vitro* antagonistic activity of *Trichoderma* species against six fungal plant pathogens including *A. solani* was proved also by Bell et al. [37].

On the other hand, the *in vivo* bioassay of bio-agents illustrated that *Trichoderma harzianum*-T10 was the superior and the most promising isolate among the six tested bio-control isolates where it exhibited the maximum reduction % of disease severity comparing to control treatment. The realized reduction % of Disease severity as a result of treating tomato plants with *Trichoderma harzianum*-T10 came close to those obtained with fungicide treatment. These results are in agreement with those of Elad et al. [38]; Tu and Vartaja [39] who showed that *Trichoderma* species affected the growth and establishment of different plant pathogens including *Alternaria solani* on their hosts.

It is remarkable that mode of action in the tri-interaction between plant pathogen, *Trichoderma* and host plant still unclear. Several biocontrol agents are known to produce antimicrobial substances to achieve control over various plant ailments [40]. Furthermore, it is known that the genus *Trichoderma* comprises a great number of fungal strains exert biocontrol activity against fungal phyto-pathogens either directly or indirectly. These antagonistic properties are based on the activation of multiple mechanisms [35,40]. Recently, the ability of mutualistic bioagents to induce host plant systemic resistance pathways has been successfully tested. Results showed that some bio-agents including *Trichoderma* were capable to trigger host plant systematic resistance against hazardous plant parasites i.e. *Meloidogyne incognita* through altering the gene expression of some involved genes in different systemic resistance pathway [41].

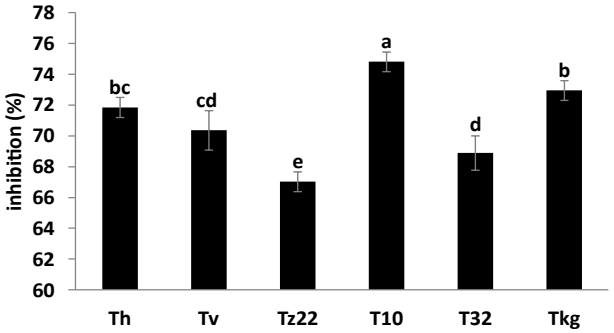

Figure 2: *In vitro* relative radial growth inhibition of *Alternaria solani* mycelial growth on PDA plates by the different *Trichoderma* isolates i.e., *T. hamatum* (Th), *T. viride* (Tv), *T. harzianum* (Tz22), *T. harzianum*-T10, *Trichoderma* sp. (T32) and *T. koningii* (Tkg). The relative growth inhibition was calculated by subtracting the radial growth of *Alternaria solani* in the direction of the biocontrol agent from the radial growth of *Alternaria solani* in the absence of a biocontrol agent (control) divided on radial growth of control , (n=3).

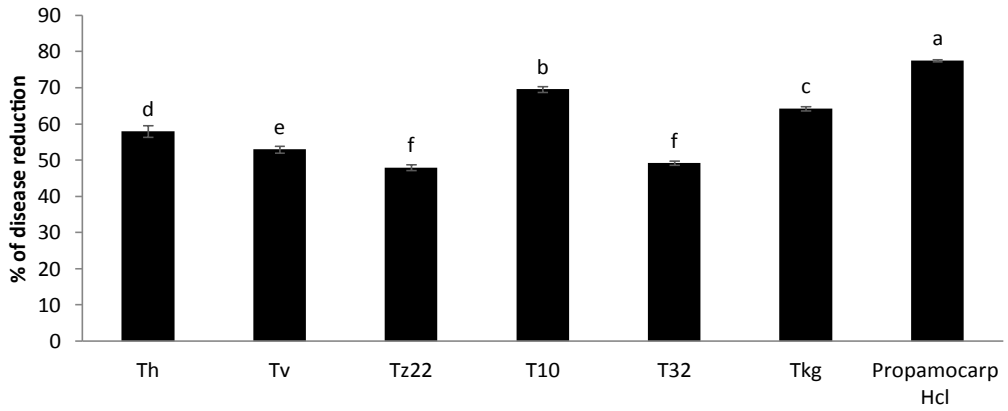

Figure 3: Reduction % of early blight disease recorded on tomato plants treated with the six individual isolates of *Trichoderma* species i.e., *T. hamatum* (Th), *T. viride* (Tv), *T. harzianum* (Tz22), *T. harzianum*- T10, *Trichoderma* sp. (T32) and *T. koningii* (Tkg) compared with treated plants with 2.5 ml/L of fungicide (Propamocarp Hcl).

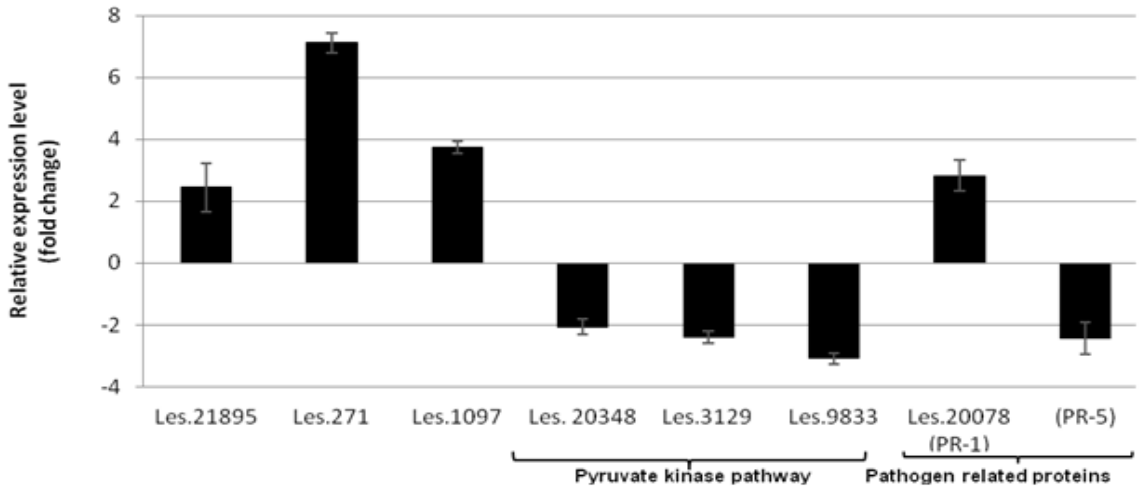

Figure 4: Relative expression levels of eight different related genes to essential bioprocess and defence mechanisms in tomato plants inoculated with bio-agent *Trichoderma harzianum*-T10. Bars indicating the means of fold changes in gene expression as generated by qRT-PCR. The target gene expression changes were normalized to the changes of the Actin gene as a reference gene. Data for three biological replicates.

In the present study, the influence of *Trichoderma* application on the expression levels of 8 different genes in tomato was investigated through quantitative real time PCR, qRT-PCR. The results showed that three genes, Les.21895, Les.271, and Les.1097, which related to auxin (IAA6), ethylene (ER69) and lignin (CL2) production pathways, respectively were up regulated. On the other hand, three genes i.e., Les.20348, Les.3129 and Les.9833 which coding for pyruvate kinase (PK) hormone were down regulated. Noteworthy that T10 affected also the expression levels of two pathogenesis related proteins including Pr-1 and Pr-5. Results demonstrated that *Trichoderma harzianum*-T10, induced the relative gene expression of Pr-1 while it decreased the relative expression of Pr-5 when compared with un-treated control plants. Davies [42] reported that the plant hormone auxin, typified by indole-3-acetic acid (IAA), regulates a variety of physiological processes, including apical dominance, tropic responses, lateral root formation, vascular differentiation, embryo patterning, and shoot elongation. The central role of the Aux/IAA gene family members in auxin signaling has been suggested by molecular genetics and biochemical studies of Woodward and Bartel [43]. Recently, studies of Overvoorde et al. [44] suggested that auxin hormone can regulate various aspects of plant growth and development. Their investigation showed also that the Aux/IAA proteins regulate auxin-mediated gene expression by interacting with members of the auxin response factor protein family. In the present study, the results showed that when tomato roots were treated with *Trichoderma* isolate (T10), there was an increase in the relative expression of auxin-regulated (IAA6) gene. This indicates that the tested *Trichoderma* isolate can trigger systemically the auxin signaling pathways, which involved in essential physiological and biological processes important for growth and health of tomato plants.

Furthermore, the results obtained from this present study illustrated that *Trichoderma* application increased the expression level of gene Les.271 which involved in ethylene signaling pathway. The change in gene expression was greatly high compared to control plants. Ethylene signaling pathway was found to play a distinct role in the response of host plant to pathogen infection as reported by Dowd et al. [45]. They detected differences between healthy and infected cotton plants with pathogenic *F. oxysporum f.sp.vasinfectum* in ethylene and auxin hormons concentrations. Moreover, they confirmed the role of

those two hormons in disease process by direct measurements of those hormone levels inside infected and non-infected plants. Nahar et al. [20] reported also that complex defense signaling pathways were found to be controlled by different hormones such as auxin and ethylene. Their results obtained from q-PCR revealed that Ethylene strongly activates jasmonic acid biosynthesis and signaling genes within roots of rice plants, which led to stimulate the systemic induced resistance (ISR) pathway against root knot nematodes. Based on these results, induction of ethylene pathway using *Trichoderma harzianum*-T10 is candidate to be one of the mechanisms that can be involved in management of early blight disease on tomato plants.

Moreover**,** the increase in expression level of gene Les.1097 which coding for 4-coumarate-CoA ligase 2 was observed on inoculated tomato plants with *Trichoderma harzianum*-T10 compared to control plants. According to studies of Hamberger and Hahlbrock [21] on *Arabidopsis thaliana*, two classes of CoA Ligase isoenzymes were identified. They reported that class I has been directly or indirectly associated with the biosynthesis of lignin and structurally related soluble or cell wall-bound phenylpropanoid derivatives, whereas class II isoenzymes have been associated with flavonoid biosynthesis. Furthermore, Nicholson and Hammerschmitt [22]; Bhuiyan et al. [23] mentioned that the lignifications, in partial related to CoA ligase isoforms activity, have the potential to act in several ways in plant defenses against pathogen infection. It can establish mechanical barriers to pathogen invasion, chemically modify cell walls to be more resistant to cell wall-degrading enzymes, increase the resistance of walls to the diffusion of toxins from the pathogen to the host and of nutrients from the host to the pathogen, produce toxic precursors and free radicals, and lignify and entrap the pathogen. According to these studies, it seems that the ability of isolate T10 to induce lignifications and/or secondary metabolites like phenylpropanoids in tomato plants can play a curtail role against *A. solani* on tomato.

In contrary, results revealed that inoculating tomato plants with *Trichoderma harzianum*-T10 reduced the relative expression level of three genes, Les.20348, Les.3129 and Les.9833. These three genes are involved in pyruvate kinase biosynthesis (PK). The enzyme pyruvate kinase (PK) had been shown to play a cretin role in various physiological processes in plants [24,25]. Different studies suggest that Pk activity can be used as a remarkable physiological indicator of K^+, Mg^{2+}, and Ca^{2+} contents in plant tissues [26,27]. Ruiz et al. [46] reported that increasing Ca^{2+} concentration in tobacco leaves resulted in decreasing the activity of pyruvate kinase. Moreover, they illustrated also that PK activities were inversely proportional to the leaf concentration of the free forms of K^+, Mg^{2+}.

In conclusion, *Trichoderma* species affected the growth and the establishment of *Alternaria solani*, the causal pathogen of tomato early blight disease, either *in vitro or in vivo*. Moreover, different mechanisms of action including stimulation of auxin, ethylene, lignin and pathogen related proteins can be involved in management of early blight disease on tomato plants using bio-control elicitors as *Trichoderma* species. On the other hand, *Trichoderma* application inhibited the expression level of three related genes to pyruvate kinase bio-synthesis pathway which may point to the role of this hormone in the interaction between host plants and bio–control elicitors with presence of *Alternaria solani* pathogen.

References

1. FAO (2003) Agricultural statistics.
2. Costa JM, Heuvelink E (2005) Introduction: The tomato crop and industry. In: Heuvelink, E. (Eds.). Tomatoes. CAB International, UK, pp. 1-19.
3. Peralta IE, Knapp S, Spooner DM (2005) New species of wild tomatoes (Solanum section Lycopersicon: Solanaceae) from Northern Peru. Syst Bot 30: 424-34.
4. Rotem J, Reichert I (1964) Dew - a principal moisture factor enabling early blight epidemics in a semiarid region of Israel. Plant Dis Rep 48: 211-15.
5. Kemmitt G (2002) Early blight of potato and tomato. The Plant Health Instructor.
6. Walker JC (1952) Diseases of Vegetable Crops. McGraw-Hill Book Co, New York, NY, 699 p.
7. Basu PK (1974) Measuring early blight, its progress and inluence on fruit losses in nine tomato cultivars. Canadian Pl Dis Survey 54: 45-51.
8. Sherf AF, MacNab AA (1986) Vegetable Diseases and their Control. John Wiley and Sons, New York, pp. 634-640.
9. Singh PC, Kumar R, Singh M, Rai A, Singh MC, et al. (2011) Identification of resistant sources against early blight disease of tomato. Indian J Hort 68: 516-521
10. Sikora RA, Schäfer K, Dababat AA (2007) Modes of action associated with microbially induced in planta suppression of plant-parasitic nematodes. Australasian Plant Pathology 36: 124-134.
11. Davies K, Spiegel Y (2011) Root Patho-Systems Nematology and Biological Control. In: Davies K. & Spiegel, Y. (Eds). Biological Control of Plant Parasitic Nematodes: Building Coherence between Microbial Ecology and Molecular Mechanisms, Chapter 12, Springer, The Netherlands.
12. Fageria MS (1997) Genetic analysis of resistance to *Alternaria* leaf spot in tomato. J Mycol Pl Pathol 27: 286-89.
13. Ganie SA, Ghani MY, Nissar Q, U-Rehman S (2013) Bioefficacy of plant extracts and biocontrol agents against *Alternaria solani*. African Journal of Microbiology Research 7: 4397-4402.
14. Alabouvette C, Lemanceau P, Steinberg C (1993) Recent advances in the biological control of Fusarium wilts. Pesticide Science 37: 365-373.
15. Olivain C, Alabouvette C (1997) Colonization of tomato root by a non-pathogenic strain of *Fusarium oxysporum*. New Phytologist 137: 481-494.
16. Alabouvette C, Edel V, Lemanceau P, Olivain C, Recorbet G, et al. (2001) Diversity and interactions among strains of *Fusarium oxysporum*: Application and biological control. In: M.J. Jeger and N.J. Spence (Eds.): Biotic interactions in plant-pathogen associations, pp 131-157. CAB International, London, England.
17. Fravel D, Olivain C, Alabouvette C (2003) *Fusarium oxysporum* and its biocontrol. New Phytol 157: 493-502.
18. Fuchs JG, Moënne-Loccoz Y, Défago G (1997) Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. Plant Dis 81: 492-496.
19. Abel S, Theologis A (1996) Early genes and auxin action. Plant Physiol 111: 9-17.
20. Nahar K, Kyndt T, De Vleesschauwer D, Höfte M, Gheysen G (2011) The jasmonate pathway is a key player in systemically induced defense against root knot nematodes in rice. Plant Physiology 157: 305-316.
21. Hamberger B, Hahlbrock K (2004) The 4-coumarate:CoA ligase gene family in *Arabidopsis thaliana* comprises one rare, sinapate-activating and three commonly occurring isoenzymes. Proc Natl Acad Sci USA 101: 2209-2214.
22. Nicholson RL, Hammerschmidt R (1992) Phenolic Compounds and Their Role in Disease Resistance. Annual Review of Phytopathology 30: 369-389.
23. Bhuiyan NH, Selvaraj G, Wei Y, King J (2009) Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. Journal of Experimental Botany 60: 509-521.
24. Lin M, Turpin DH, Plaxton WC (1989) Pyruvate kinase isozymes from the green alga, *Selenastrum minutum*. II. Kinetic and regulatory properties. Arch Biochem Biophys 269: 228-238.
25. Schulller KA, Plaxton WC, Turpin DH (1990) Regulation of phosphoenolpyruvate carboxylase from the green alga *Selenastrum minutum*: properties associated with the replenishment of tricarboxylic acid cycle intermediates during ammonium assimilation. Plant Physiol 93: 1303-1311.
26. Lavon R, Goldschmidt EE (1999) Enzymatic methods for detection of mineral element defeciencies in citrus leaves. a mini-review. J Plant Nutr 22: 139-150.

27. Ruiz JM, Moreno DA, Romero L (1999) Pyruvate kinase activity as an indicator of the level of $K^{(+)}$, $Mg^{(2+)}$, and $Ca^{(2+)}$ in leaves and fruits of the cucumber: the role of potassium fertilization. J Agric Food Chem 47: 845-849.

28. Giri P, Taj G, Kumar A (2013) Comparison of artificial inoculation methods for pathogenesis studies of *Alternaria brassicae* (Berk.) Sacc. on *Brassica juncea* (L.) Czern. (Indian mustard). Afr J Biotechnol 12: 2422-2426.

29. Utkhede RS, Rahe JE (1983) Interaction of antagonist and pathogens in biological control of onion white rot. Phytopathology 73: 890-893.

30. Vincent JM (1947) Distortion of fungal hyphae in the presence of certain inhibitors. Nature 159: 850.

31. Vakalounakis DJ (1983) Evaluation of tomato cultivars for resistance to *Alternaria* blight. Ann Appl Biol 102: 138-139.

32. Pandey KK, Pandey PK, Kalloo G, Banerjee MK (2003) Resistance to early blight of tomato with respect to various parameters of disease epidemics. J Gen Pl Pathol 69: 364-71.

33. Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Elsevier methods 25: 402-408.

34. Plake BS, Kemmerer BE (1987) Statgraphics: Neil W. Polhemus and the Statistical Graphics Corporation. Rockville, MD: STSC. Computers in human behavior. 3: 289-298.

35. Benítez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7: 249-260.

36. Rudresh DL, Shivaprakash MK, Prasad RD (2005) Potential of *Trichoderma* spp. as bio control agents of pathogens involved in wilt complex of chickpea. J Biol Control 19: 157-166.

37. Bell DK, Wells HD, Markham CR (1982) In vitro antagonism of *trichoderma* species against six fungal plant pathogens. Phytopathology 72: 379-382.

38. Elad Y, Chet I, Katan J (1980) *Trichoderma* harzianum, a biocontrol agent effective against Sclerotium rolfsii and Rhizoctonia solani. Phytopathology 70: 119-121.

39. Tu JC, Vartaja V (1981) The effect of hypoparasite (*Gliocladium virens*) on *Rhizoctonia solani* on *Rhizoctonia* root rot of white beans. Canad J Bot 59: 22-27.

40. Yang Z, Yu Z, Lei L, Xia Z, Shao L, et al. (2012) Nematicidal effect of volatiles produced by *Trichoderma* sp. Journal of Asia-Pacific entomology 15: 647-650.

41. Selim ME, Mahdy ME, Sorial ME, Dababat AA, Sikora RA (2014) Biological and chemical – dependant systemic resistance and their significance for the control of root-knot nematodes. Nematology 16: 917-927.

42. Davies PJ (1995) Plant Hormones: Physiology, Biochemistry and Molecular Biology, 2nd ed. (Dordrecht, The Netherlands: Kluwer Academic Publishers).

43. Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot 95: 707-735.

44. Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, et al. (2005) Functional genomic analysis of the auxin/indole-3-acetic acid gene family members in *arabidopsis thaliana*. The Plant Cell 17: 3282-3300.

45. Dowd C, Wilson IW, McFadden H (2004) Gene expression profile changes in cotton root and hypocotyls tissues in response to infection with *Fusarium oxysporum* f.sp. vasinfectum. Molecular plant-microbe interactions 17: 654-667.

46. Ruiz JM, Lopez-Cantarero I, Romero L (2000) Relationship between calcium and pyruvate kinase. Biological Plantarum 43: 359-362.