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The expression of resistance genes in tomato induced by abiotic and biotic factors against *Fusarium oxysporum* f. sp. *Lycopersici*

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Abstract : The vascular wilt pathogen, *Fusarium oxysporum* f. sp. lycopersici (Fol) is one of the most devastating pathogens for tomato crop. The aim of this study was to evaluate the expression of resistance genes in tomato plants treated by chemical and bio-agents factors before inoculation with Fol inoculum.
Methods :. The tomato plants revealing Fusarium crown and root rot symptoms, was collected and brought into laboratory, washed separately using tap water. Leaves and roots samples were macerated using liquid nitrogen, 250mg were taken for DNA extraction and for further Quantitative Real-Time PCR experiments.
Results : The genes *LECHI3*, *LECHI9*, and *LEGLUCA* were highly significant expressed in roots rather than in leaves after the treatment of plants with Salicylic acid +Fol, $CaCl_2$, $CaCl_2$+Fol. However the gene *LEGLUCB* was showed increasing in expression rate in leaves rather than in roots after the treatments with *Trichoderma harzianum*+Fol, and $CaCl_2$. Tomato plants grown in infected soil (A) supplemented with sterilized extract of tomato debrisM0revealed no expression in the genes in both roots and leaves.
Conclusion : It appears that the defense genes of tomato plants against Fol are stimulated by chemical inducers and bio-control agents and that will be helpful to identify pathogenicity mechanism involved in the tomato wilt disease development. Our findings could lead to the improvement in disease control strategies.
Keywords : Gene expression, Tomato diseases, *Fusarium oxysporum*, qPCR.

Introduction

Growth and productivity of tomato plants (*Lycopersicon esculentum* Mill.) are facing different challenges due to the outbreak of pest and diseases[1] (Highlands, 2015). Wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol), is highly destructive in both greenhouse and field causes economically loses for this crop[2,3](Girhepuje and Shinde, 2011; Bawa, 2016). Controlling of tomato wilt disease give significant defies because of the ability of this fungus to remain dormant in the soil in the form of spores[4] (Zeller *et al.*,2003).

Management of this disease through the application of fungicides may cause several environmental complications and could be toxic to non-target organisms as well[5] (Ramaiah and Garampalli, 2015).

Unfortunately, these chemical fungicides are tend to persist for a long time in the environment and numerous fungi have developed resistance to them[6] (Bajwa*et al.*, 2003). Use of natural products for the controlling of fungal diseases like *Trichoderma*or Salicylic acid could plays a significant influence in decreasing the prevalence of fungal disease[7,8](Metraux, 2001; Daw*et al.*, 2008).

It is quit necessary todevelop eco-friendly management like using plant debris, Trichoderma fungus, chemical compounds e. g salicylic acid, calcium chloride. Since these elements may have minimal environmental deterioration and limited risk to consumers, in contrast to synthetic pesticides, and give possible alternatives means to control Fol, by induce resistance in targeted plant[9] (Wilson *et al.*, 2015).

On other hand, Fol isolates caused wilt disease in the fields of tomato, middle of Iraq were identified using molecular approaches[10](Merjan and Al-Janabi, 2015). Also characterization and molecular identification of polygalacturonase enzyme from *F. oxysporum* were investigated[11] (Mohsen *et al.*, 2016). But the identification of the specific tomato genes related to defense responses remain unclear*TC116429*, *TC12440*,*TC118045*[12](Inra,2008). This work aimed to profiling the expression of root and leaves genes of tomato plants in health and Foliofected plants using biotic and abiotic factors as a resistance inducers.

Materials and Methods

Experimental Organism

The fungus *Trichoderma harzianum* isolate was obtained from the Laboratory of Advanced Mycology, Department of Biology, College of Science, University of Babylon, Iraq.

Isolation and Identification of Fusarium crown and root rot

The tomato plants revealing Fusarium crown and root rot symptoms, was collected and brought into laboratory, washed separately using tap water. The infected parts were cut into small pieces (1cm each), then surface sterilized (0.5% NaOCl for 3 min) and rinsed 3 time with sterile water to remove a traces of sodium hypochlorite solution. These parts were cultured on petri-plates containing fresh sterilized PDA (Potato Dextrose Agar)amended with 0.05g/L chloramphenicol. Petri-dishes incubated at 25± 1°C for 7 days. *Fusarium oxysporum* f. sp. *Lycopersici* isolates were identified morphologically[13] (Rahjoo*et al.*, 2008), and maintained on slant PDA medium and preserved at 4˚C until use.High pathogenic isolate of this pathogen was selected and used in this study.

Preparation of fungal inoculum

Millet seeds (*Panicum miliaceum* L.) were brought from local market, washed well and boiled in distilled water for 1 h, then completed to 1L with distilled watered packed separately in individual 500 ml conical flasks. Flasks were closed with cotton plugs and autoclaved at 15 psi for 1hr at 121°C. After cooling, and under laminar air flow cabinet, three discs (5 mm) from new colony of FOL and from*T. harzianum* cultures were inoculated into each flask, separately. Conical flasks were incubated at 25°C ± 1 for 3 weeks. To obtain uniform fungal growth on millet seeds and to break the mycelial mat, the flasks were shaken vigorously every 3 days. After 21 days the inoculum of Foland for *T. harzianum* in conical flasks were kept in refrigerator at 4°Cuntil use[14] (Dewan, 1989).

Preparation of tomato plant residues(TPR)

The residual extract of tomato plants were prepared according to the previous methods[15,16]of Weltzien (1992) and Znaidi(2002). Residues were collected from the fields of tomato (Al-majd and Saiahgh, Samawa Province) at the end of season during spring 2013.The residues were cut to small pieces (about 5cm each) and transferred to plastic container (10L size) without cover. Tap water was added at ratio 5:1 residue / water (V:V). The mixture was incubated for six days at 20±2 with continuous shaking for 10 min. The extracts were filtered through cheesecloth (250 µm). An appropriate volumes were taken from the extract and centrifuged at 800rpm for 15 min. Half of the extract was filtered through 0.2 µm and stored at 4°C until use.

Cultivation of tomato plants

Seeds of tomato *Solanum lycopersicum* var. Marira were obtained from the local market of Samawa City, Iraq, and highly susceptible to Fol was used in this study. Tomato seeds were surface sterilized by immersing them in in 0.2% sodium hypochlorite (NaOCl) for 3 min and washed several times with sterile distilled water. Then primed in the aerated solutions having~16.4 g/L NaCI, 30 g/ L $KN0_3$, for 24 h to enhance their germ inability, according to[17] the Farooq *et al.* (2005). The ratio of seeds weight to solution volume was 1:5 (g/mL). After priming for prescribed duration, seeds were washed with distilled water three times and planted in four propagative cages (20 × 30 cm), containing autoclaved sandy soil and peat moss (1: 1), 100 seeds per group, 100 seeds per each, containers were covered by a transparent plastic lids opened from the top. Incubated in growth chamber for one month at 25 ± 2 °C, irrigated with tap water where necessary. Tomato seedlings were used in the following experiments.

Inoculation

To achieve this experiment, field soils were prepared, wetted, double autoclaved and packed into plastic pots (20cm diameter). Uniform tomato plants one month old from previous experiment were selected and planted 24 h after the following treatments: (A) healthy control (no fungus) in sterilized soil; (B) healthy control (no fungus) in unsterilized soil;(AFol) infested control in sterilized soil; (BFol) infested control in unsterilized soil; (T) soil amended with *T. harzianum*; (T+Fol) infested soil amended with*T. harzianum*; (M0) soil amended with sterilized extract of tomato residues; (M0+Fol) infested soil amended with sterilized extract of tomato residues; (M1) soil amended with unsterilized extract of tomato residues, (M1+Fol) infested soil amended with unsterilized extract of tomato residues; (SA) soil planted with tomato plant sprayed separately with salicylic acid; (SA+Fol) infested soil planted with tomato plant sprayed separately with salicylic acid; ($CaCl_2$) soil planted with tomato plant sprayed separately with $CaCl_2$; ($CaCl_2$+Fol) infested soil planted with tomato plant sprayed separately with $CaCl_2$.

All treatments were carried out 24 h before transferring tomato plants to the pots, the inoculum of *T. harzianum* and/ or FOL was mixed thoroughly with the soil in ratio 5%, millet seeds: soil, V:V)., the salicylic acid and $CaCl_2$ were separately sprayed directly to the foliage of plants at concentration 2mM in sterilized distilled water. Sterilized and unsterilized extract of tomato debris were mixed with soilin similar way as done with fungi.

Each pot was planted with 4 plants and then transferred to the plastic house[18] (Schwarz *et al*., 2014). Each treatment consisted of four replicates, they were watered every 48h with equal amount of tap water. Plants were thinned to 1 plant per pot after one week post planting. Two months after the transfer of seedlings into pots and during the appearance of symptoms on tomato plant, samples were collected by taking individually 1gm from the youngest fully expended leaves and from the tips of the roots as well.

Extraction of DNA

Leaves and roots samples were macerated using liquid nitrogen, 250mg were taken for DNA extraction and for further Quantitative Real-Time PCRexperiments.

Total RNA extraction

The total RNA was determined using the method described by[19,20]Van Kan*et al*. (1995) and Sambrook*et al*. (1989).

Measure the concentration of RNA

The concentration of the extracted RNA was measured using Nanodropspectrophotometer. The concentration and purity measured by reading the degree of absorbance (260/280 nm)[19] (Van Kan*et al*., 1995).

The cDNA synthesis

The cDNA was synthesized from the extracted RNA using the method described by[21] He *et al*. (2002). The samples stored at -20 until used for Real-time PCR.

Quantitative Real-Time PCR (qPCR)

The rate of gene expression activity was measured using qPCR. The primers(Table 1) were designed according to[22]Aimé*et al*. (2008).

Table 1.primers types used in the study

Primer		Sequence	Amplicon Size bp	Reference
Actin	F	AGGCACACAGGTGTTATGGT	177	22
	R	AGCAACTCGAAGCTCATTGT		
LEGLUCA	F	GGTCTCAACCGCGACATATT	250	22
	R	CACAAGGGCATCGAAAAGAT		
LECHI9	F	GAAATTGCTGCTTTCCTTGC	235	22
	R	CTCCAATGGCTCTTCCACAT		
LEGLUCB	F	TCTTGCCCCATTTCAAGTTC	202	22
	R	TGCACGTGTATCCCTCAAAA		
LEPR1A	F	TCTTGTGAGGCCCAAAATTC	246	22
	R	ATAGTCTGGCCTCTCGGACA		
ECHI3	F	TGCAGGAACATTCACTGGAG	248	22
	R	TAACGTTGTGGCATGATGGT		

Differential expression of selected genes was verified by real time RT-PCR using the same RNA samples from cell cultures. The actin gene was used as a reference gene[22](INRA,2008). Total RNA was treated by DNase using RQ1 RNase-Free DNase. First strand cDNA was synthesized from 1mg of total RNA using ImProm- II- Reverse transcription system according to the manufacturer's instructions. Real-time PCR reactions were carried out with 25 ng of cDNA, 500 nM of each primers,10 mL of the SYBR green master mix and RNase free water in a final volume of 20 mL. In the negative control cDNA was replaced by RNase free water. The program used for real-time PCR was 15 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 95^0C, annealing for 30 s at 58°C and extension for 30 s at 72°C, at the end of which the fluorescence was measured. Two replicates of real-time PCR reactions were performed for each sample. Primer titration and dissociation experiments were performed to confirm no formation of primer–dimers or false amplicons which could interfere with the results. After the real-time PCR experiment, Ct number was extracted for both reference gene and target gene with auto baseline and manual threshold. Gene expression levels relative to the actin gene were calculated for each cDNA sample using the following equation.

Relative ratio gene=actin=(Egene_- Ct gene) / (Eactin -Ct actin)

Statistical Analysis

This experiment has been conducted in Randomized Complete Block Design (RCBD) in three replicates. Statistical analysis were carried out using analysis of variance (ANOVA), and the significant differences between means were tested as described by[23]Snedecor and Cochran, (1969). The means were compared by LSD at probability (0.01).

Results

Expression profiles of resistance genes

The expression profile of tomato resistance genes were investigated in roots and leaves using qPCR. The results (Figure 1) showed that the gene LECHI3 was highly expressed in the root samples, basically for the treatments SA+Fol (17.4), $CaCl_2$+Fol (14.3), SA (13), and $CaCl_2$ (10.8) compared with that in leave samples and for the same treatments above (9.6, 6.7,7.9, and 5.7 respectively) at a significant rate.

For the gene LECHI9, gene expression was significantly higher in the treatment SA+Fol for root samples (20%) compared to the LECHI3. The other treatments were also showed significant differences by the

*rate*of their *gene expression* (Figure 2). The expression of LEGLUCA gene was significantly higher in root treatment SA+Fol (17.8), SA (12.3), $CaCl_2$+Fol (11.3), T (10.1), T+Fol (10), and $CaCl_2$ (9.8) respectively compared with that in leave treatment samples (15.8, 9.1, 9.4, 6.3, 7.6, and 6.7 respectively) (Figure 3).

In contrast, LEGLUCB gene was highly expressed in leave samples rather than in roots for the treatments T+Fol (20.6), T (15.1), and $CaCl_2$ (5.7) (Figure 4). However there was no variations in the expression rate for the LIBERIA gene in root and leave samples except the treatments SA+Fol and $CaCl_2$+Fol were recorded high rate of gene expression (26 and 18.2 respectively) (Figure 5). The results also showed that the treatments M0+Fol and A+Fol doesn't induce the expression of the five genes studied.

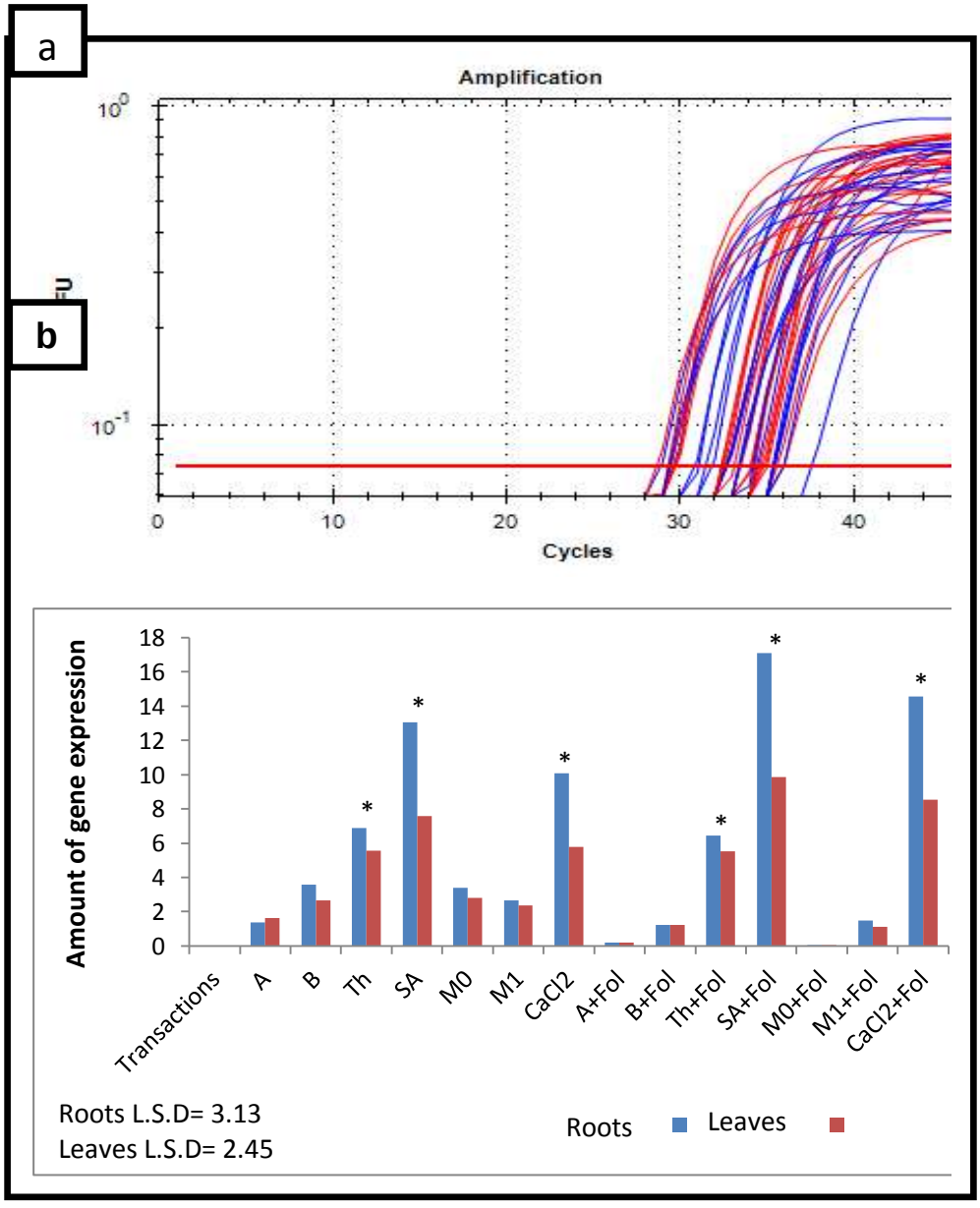

Figure 1: (a) The amount of expression in LECHI3 gene in tomato plant, blue samples roots, red samples leaves, (b): the amount of gene expression in LECHI3 resistance gene in the leaves and roots of tomato plants after the chemical and biological treatments.

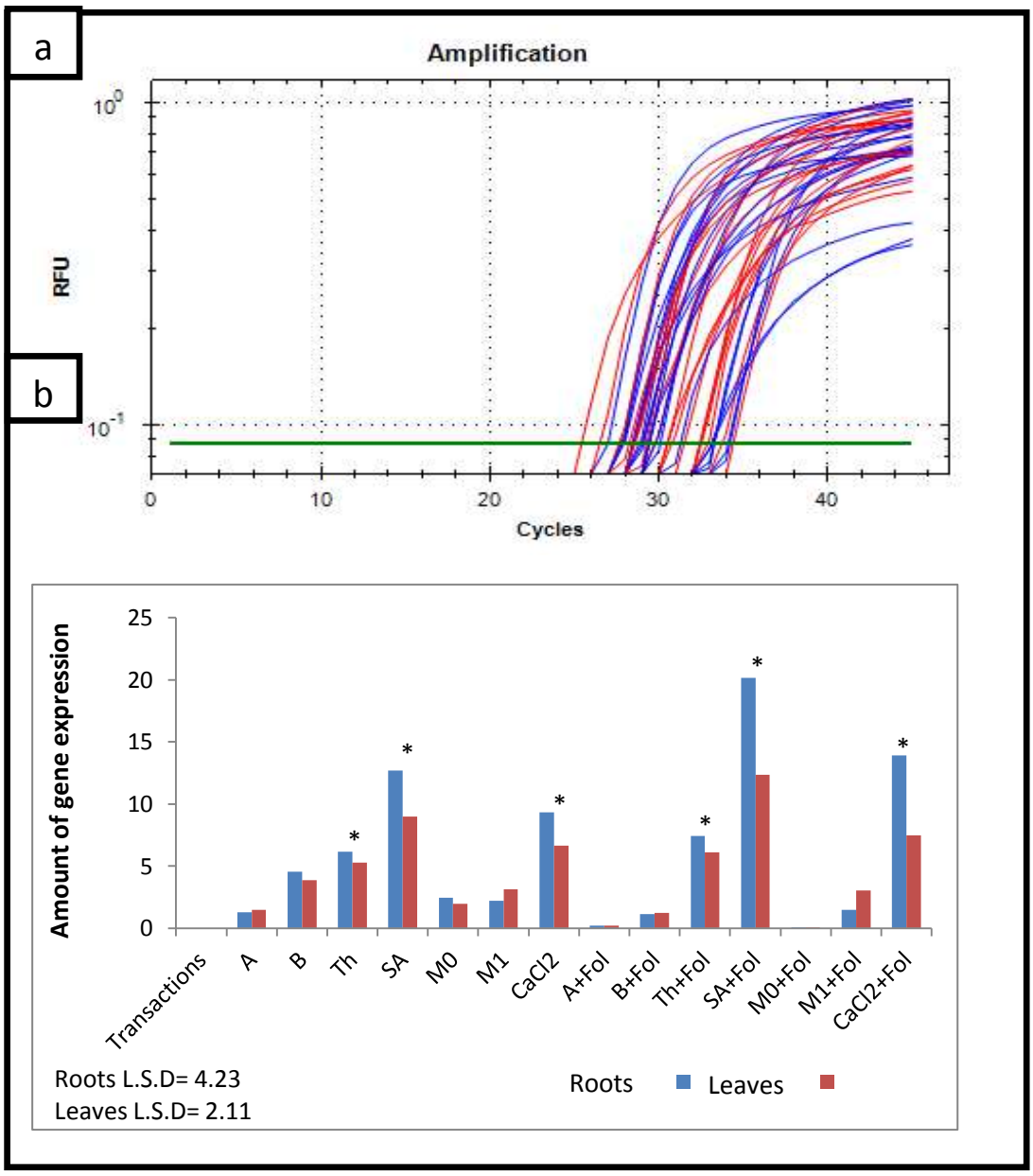

Figure 2: (a) The amount of expression in LECHI9 gene in tomato plant, blue samples roots, red samples leaves, b: the amount of gene expression in LECHI9 resistance gene in the leaves and roots of tomato plants after the chemical and biological treatments

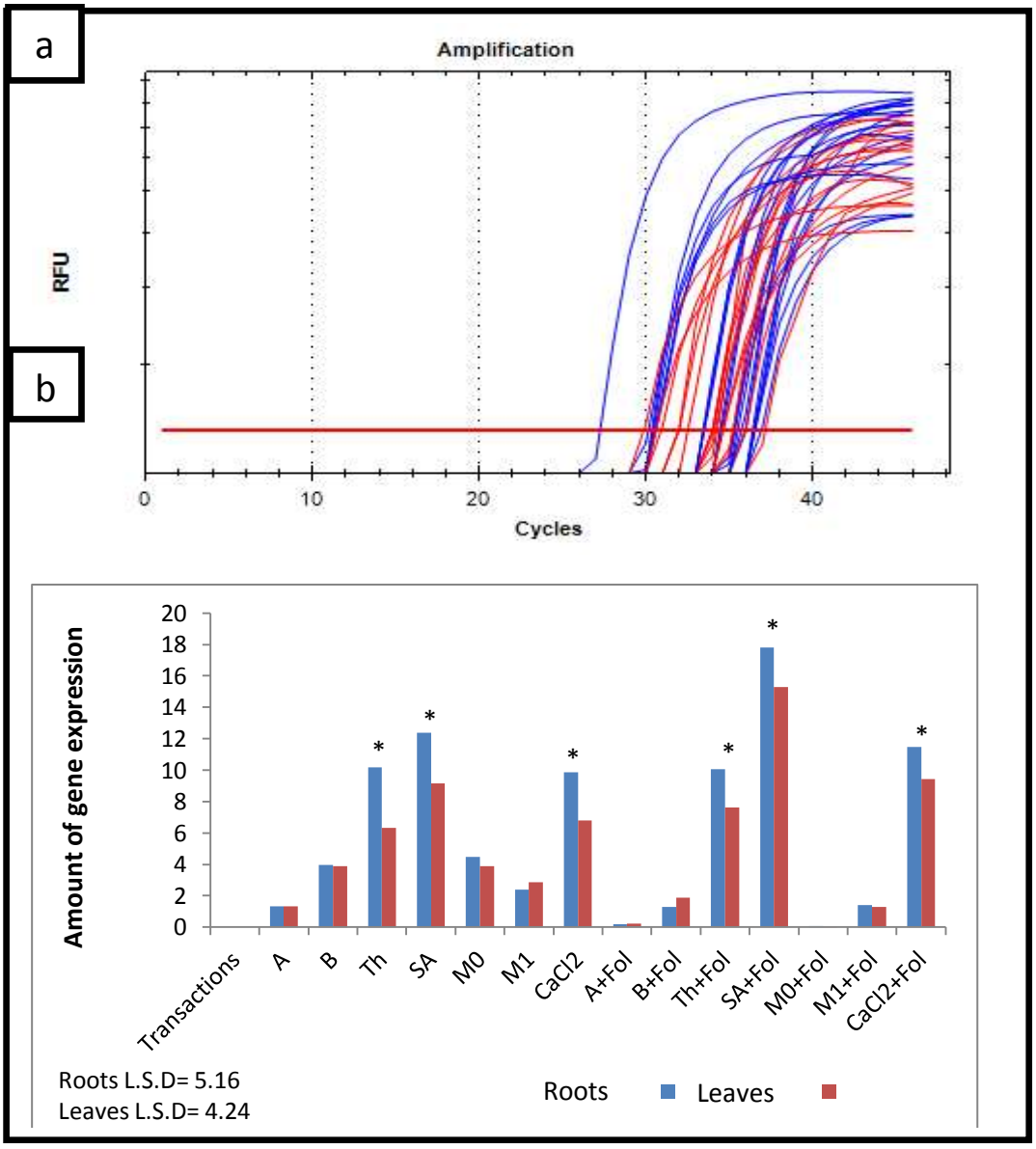

Figure 3: (a) The amount of expression in LEGLUCA gene in tomato plant, blue samples roots, red samples securities, b: the amount of expression in LEGLUCA resistance gene in the leaves and roots of tomato plants after chemical and biological treatments.

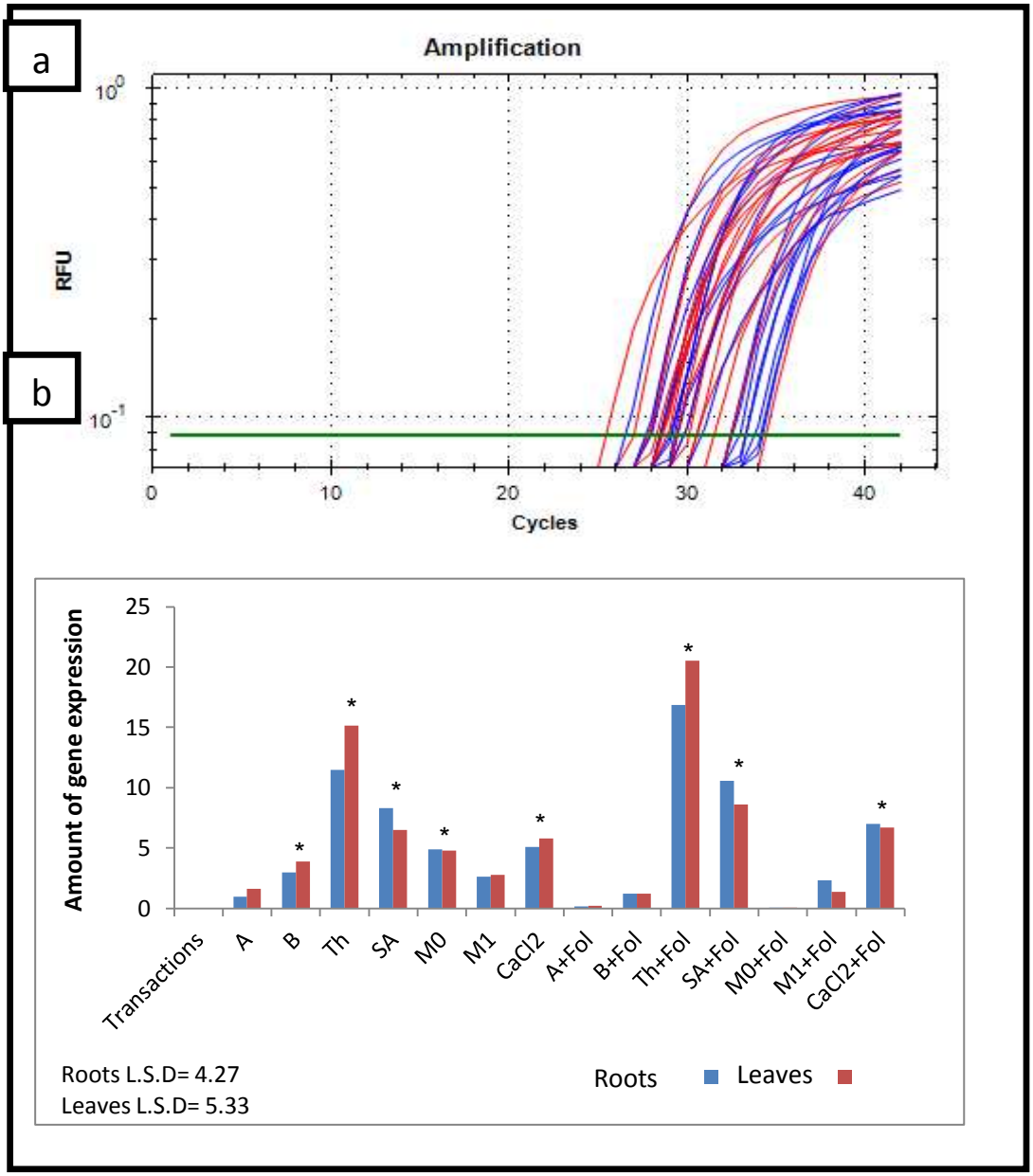

Figure 4: (a) The amount of expression in LEGLUCB gene in tomato plant, blue samples roots, red samples securities, b: the amount of expression in LEGLUCB resistance gene in the leaves and roots of tomato plants after the chemical and biological treatments.

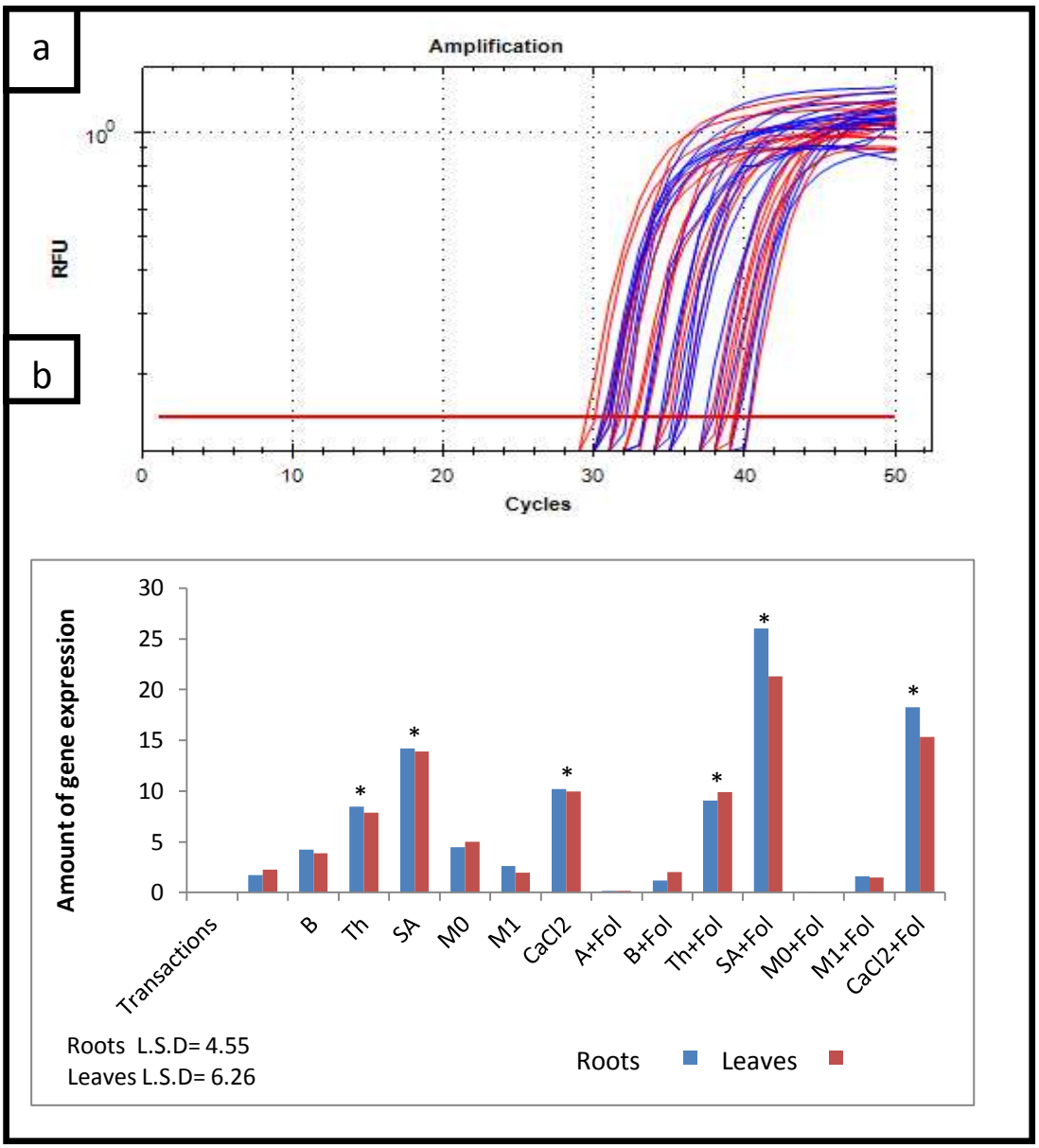

Figure 5: (a) The amount of expression in LIBERIA gene in tomato plant, blue samples roots, red samples securities, b: the amount of expression in LEPRIA resistance gene in the leaves and roots of tomato plants after the chemical and biological treatments.

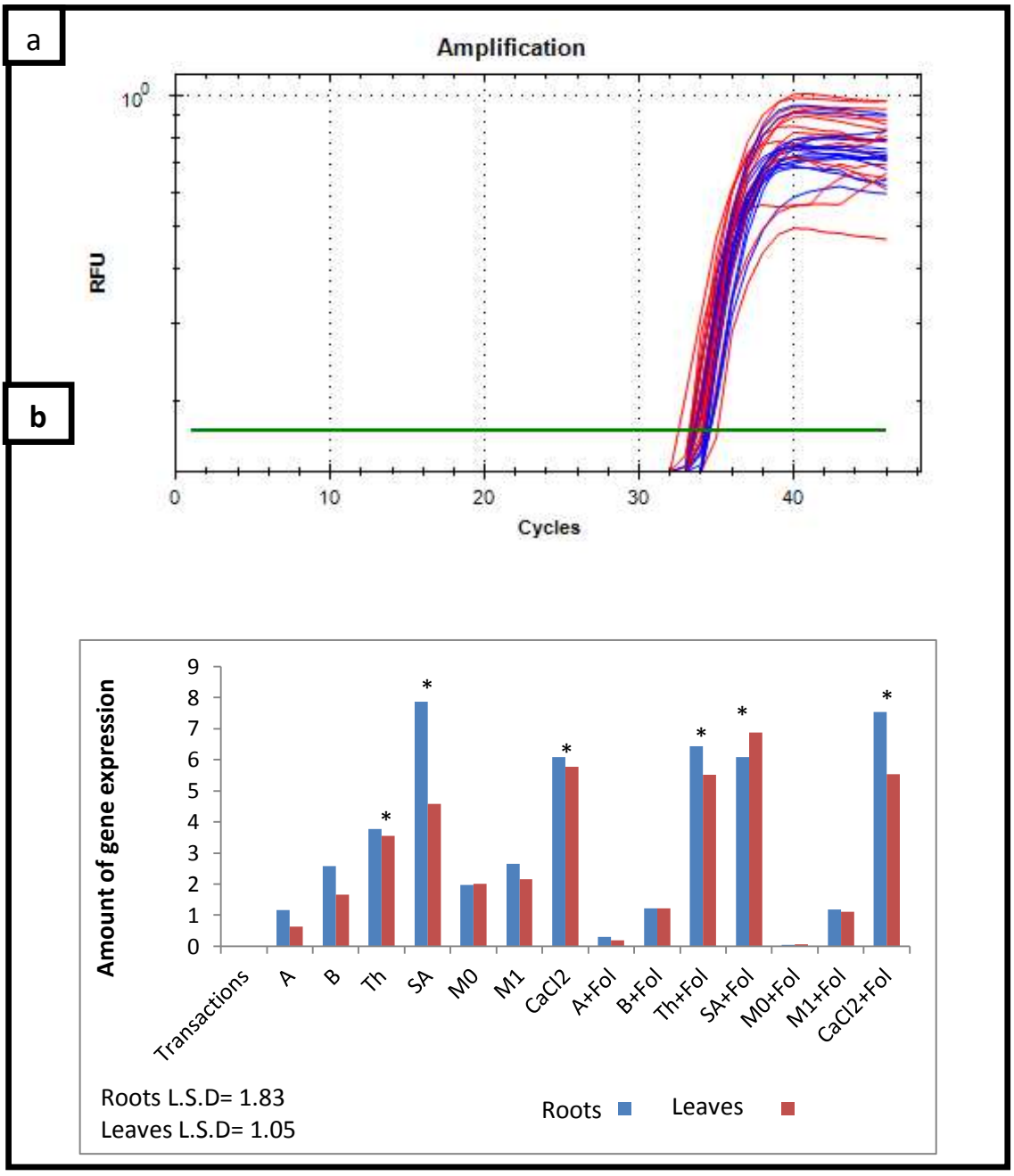

Figure 6: (a) The amount of expression in ACTIN controlgene in tomato plant, blue samples roots, red samples securities, b: the amount of expression in ACTIN resistance gene in the leaves and roots of tomato plants after the chemical and biological treatments.

Discussion

The role of Salicylic acid to stimulate systemic resistance to plant tomatoes in reducing the percentage of injuries and the severity of the injury, as well as participate in a vital industry for the compounds of defense such as Polyphenols and alkaloids of PR- protein[24] (Bai *et al.*,2012). The use of sterile and non-sterile extract showed a significant reduction in the concentration of the RNA. This may be attributed to the organic material contained in the extract which has worked to increase the growth and the activity of the fungus by increasing in the incidence and intensity compared with the control group[25] (Khan and Zhihui, 2010). The pathogen works to prevent the transmission of raw materials and water to plant, as well as the excretion of some toxins and acids

causes the death of the wood tissue cells, which have negatively impacted the decline in the purity and concentration of RNA of the plant and this is in line with the sentiments[26] (Van Peer and Schippers , 1992).

Gene expression

The results of current study showed that the treatment by salicylic acid with and without a pathogenic fungus (SA + Fol and SA) has outperformed significantly the rest of the transactions in both roots and leaves when compared with the control treatment. This action may be take place due to the resistance signal can be transmitted to the old parts of the plant by salicylic acid turned-Methyl Salicylate[27] (Aoki *et al.*, 2013). The salicylic acid act as inducer by stimulating the genes responsible for resistance to Turn-on. These genes manufacture certain enzymes involved in the vital industry for vehicles such as the defense and Alkaloids Polyphenols and PR- protein[24] (Bai *et al.*, 2012). Previous study done by[28] Van Loon et al, 2006 showed that the mechanism of action of SA in SAR is to stimulate the genes encoded for certain proteins associated with the disease, such as enzymes chitinase and 3,1-β-glucanases. The action of Salicylic acid was clearly explain in tobacco plant resistant to the virus CMV and hinders its movement from cell to cell by stimulating the resistance gene in the plant responsible for the topical response[29] (Wulff*et al*.,1998).

The treatment of plant with calcium chloride with and without a pathogenic fungus ($Cacl_2$ + Fol and $Cacl_2$) showed a highly rate of expression in all genes studied compared to the control group. The defense genes and signals mediated mechanism may sense the level of calcium in the root and leaves which led to stimulate the genes of defense as an increase in the level of calcium hampered the flow of nutrients to the nurse, and worked to increase membrane thickness and thus worked to limit the spread of the pathogen, as Ca activates many physiological functions of plants[30, 31](Dodd *et al.*, 2010; Batistic and Kudla, 2012).

Moreover, the increased calcium to a specified level within the plant leads to a change in the hormonal balance of growth hormones within the plant such as the ABA, which has fallen as a result of increasing the level of calcium to a certain extent, where it was noted that the decline in acid ABA cause increased resistance against pathogens can be explained by the incite close the stomata or by increasing the plant signals that you pay to stimulate the gene for the manufacture of a protein in response works to block the growth and development of the disease within the plant[32] (Bita and Gerats, 2013).

The results also proved that the impact of biological control using *T. harzianum*the resistance genes in the roots and leaves. This action of factors was due to its ability to secrete an enzymes such as SulailazAlklokanez B-1,3 glucanase, which works to break down Alklokan chains in the walls of a private threaded fungal wilt fungi[33] (Lichius and Lord, 2014). The Alklokan is the main component of multiple sugars and entering in the composition of the wall fungal cell and all the high-end fungus[34, 35](Sarkar *et al.*, 2009; Attaran*et al.*, 2014). Other enzymes is Chitinase and Glacanase, the most important compounds secreted by the fungus *T. harzianum* and these can control of pathogens by smashing Alkatin vehicles (Chitin) and Alklokan (1,3 glucanse) and polysaccharides necessary for the strength and stiffness walls of fungi[36] (El-Mohamedy*et al.*, 2011).

This may be because tomato plants in response to worker control biogenic of *T. harzianum* and the induction of resistance genes to the fungus that is able to increase the plant signals that is necessary to stimulate the resistance genes[37] (Karsa*et al.*, 2010). In a similar study, the induction of proteins associated with the disease in the potato plant defensive means against pathogens as it led to stimulate at least nine genes in the plant that has been treated with fungus control biogenic dubbed resistance genes systemic[38] (Mohsin*et al.*, 2010).

Conclusion:

In this study we have used biotic and abiotic factors against *F. oxysporum* infect tomatoes plant. These factors showed to play an important role in the induces of expression of defense genes in infected plant. The genes *LECHI3*, *LECHI9*, and *LEGLUCA* were highly expressed in roots rather than leaves.This could be eventually lead to improvement of Fusarium wilt disease resistance in tomatoes.

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