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Article

Gene expression changes in response to combination stresses in *Phaseolus vulgaris* L. (Fabaceae)

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ABSTRACT
Plants are continuously exposed to a broad range of environmental stresses. Common bean (*Phaseolus vulgaris* L.) is found to be seventh major world food crop widely attacked by several pests including a generalist herbivore, *Tetranychus urticae* Koch. We investigated the transcriptome signature of some genes (two Pathogen Related Protein (*PR3* and *PR4*), phenylalanine ammonia-lyase (*PAL*), Lipoxygenase (*LOX)*, phenylalanine ammonia-lyase *(PAL)* and (E)-β-ocimene synthase (*OS*)) in four common bean accessions which have been previously recognized to be resistant to *Tetranychus urticae* (two varieties with the highest degree of resistance, i.e. Naz and Ks41128, and the two varieties with the lowest degree of resistance, Akthar and G11867) in response to single stress (only *T. urticae* infestation) versa dual stress combinations (moderate drought stress and *T. urticae* infestation). Drought pretreatment significantly modified the transcriptome signature of some *Phaseolus vulgaris* varieties infested with *T. urticae*. The obtained results addressed for the impact of combined stresses at the transcriptome level, with some gene expression (*PAL* in Naz, Akhtar, *LOX* in Naz and Akhtar, *PR4* in Akhtar and Ks41128, *PR3* in Ks41128 and *OS* in Ks41128 and G11867) increasing after multiple stresses but not after single stress. Drought stress could alter the gene expression pattern in genotypes regardless of their resistibility/susceptibility to the spotted spider mite. Collectively, the results highlight the complex nature of multiple stress responses and that common bean varieties responses to multiple stresses are complicated as well as unpredictable.

KEY WORDS: Common bean; drought stress; resistance; *Tetranychus urticae*; variety.

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INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) serves as one of the world's most important grains so that it provides protein and other valuable nutrients and can be a good alternative to animal protein (Majnon Hoseini 2007). Common bean is found to be seventh major world food crop widely attacked by several pests including *Tetranychus urticae* Koch (Acari: Tetranychidae) among many others. This is one of many polyphagous pests of agricultural crops in the world which feed on 960 hosts belonging to 100 plant families (Khanjani and Haddad-Iraninejad 2006). *Tetranychus urticae* is a pest inhabiting economically important crops and if not controlled, it can destruct 10–15% of some crops. The importance of *T. urticae* is attributed to its huge damage, a wide host range, high rate of reproduction and the ability to develop resistance to pesticides. It is characterized by high reproduction rate and is the first greenhouse pest which shows resistance to pesticides. Altogether such traits have led *T. urticae* to be a dangerous pest which degrades quantity and quality of

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agricultural crops (Raworth 1986). Although there are no exact statistics about the damage of this pest in Iran but available reports show that it is most prevalent pest in common bean cultivation in areas like Fars, Chaharmahal and Bakhtiari, Lorestan, Zanjan, and Markazi provinces and possibly in other parts of the country (Tahmasebi *et al*. 2014). Plants inhabit the environments where they are often encountered with enormous stressors. Plant responses to different stresses are very complicated including transcription, cellular and physiological changes (Rizhsky *et al*. 2004).

Plant responses to drought involve adaptations in morphology, physiology and biochemistry (Chaves *et al*. 2003), which may affect plant resistance to insect herbivores in a complex way (Kanaga *et al*. 2009; Massad and Dyer 2010). It is important to include this aspect of plant resistance when assessing effects of abiotic stress on plant-herbivore interactions. The effects of drought on the fitness of herbivorous insects can be direct, resulting from water limited-sensitive insect traits, or indirect, resulting from the effects of drought stressors on host plants (Huberty and Denno 2004). However, the effects are often inconsistent, in part because water availability affects insects differently depending on the feeding guild (Bezemer and Jones 1998; Koricheva *et al.* 1998). Because herbivorous insects display physiological changes after feeding on resistant plants (Enayati *et al.* 2005; Li *et al.* 2007), insects should also respond to changes in the level of resistance of a host plant affected by drought stress. However, little is known about how herbivorous insects respond to both stressors. Foregoing interactions between biotic and abiotic stresses is controlled by different signal pathways that can induce each other or have an inhibitory effect on each other. Specificity in plant responses to stress is controlled by a variety of molecular mechanisms that work together in a complex regulatory network (Atkinson and Urwin 2012). Studies have shown that biotic and abiotic stresses such as drought and pests affect plant growth and development. Plant responses to them are associated with changes in gene complex network (Narusaka *et al*. 2004) and so expression pattern of genes on adaption or withstand to the stress is essential. Given the limited data on the role of drought stress in changing patterns of expression of some genes involved in immune genotypes responses to *T. urticae* and to investigate gene expression changes in both irrigated and drought states, the present research evaluates changes in the expression pattern of genes involved in the direct and indirect defence of resistant and susceptible common bean genotypes infected with *T. urticae* under drought stress.

MATERIALS AND METHODS

Genotype planting

The plants used in this study were four common bean accessions which have been previously recognized as resistant to *Tetranychus urticae* (Tahmasebi *et al*. 2014). These genotypes included Naz, Akhtar, KS41128 and G11867, which were resistant, susceptible, resistant and susceptible to *T. urticae*, respectively. All bean plants were grown in plastic pots (diameter 5 cm; depth 10 cm) in a climate controlled greenhouse under a 16:8 h L:D regime with temperatures of 25 °C (day) and 20 °C (night).

Drought stress regime

The bean plants were maintained well-watered in the greenhouse as described in the previous section until they had developed three expanded leaves (in about 2–3 weeks). Then plants were randomly divided in two groups: one group for the control and one group for the moderate drought stress treatment. Control plants were watered every 2–3 days to maintain the soil volumetric water content (θ) up to 70%. For moderate stress, watering was stopped for 7 days and thereafter plants were watered to maintain θ about 30%. The wilting point was avoided as it happens at θ = 16%. θ was determined gravimetrically by recording the pot weight of each plant pot.

Plant infection by T. urticae

A laboratory culture of two spotted spider mites was maintained on the susceptible *P. vulgaris* cv. Derakhshan under the same greenhouse conditions. All bean plants (both plants under normal irrigation and plants under water stress) were infested with spider mites, 50 adult females of *T. urticae* were transferred (Kant *et al.* 2004) to 5-week-old common bean plants using a fine paintbrush.

Quantification of gene expression via qRT-PCR

Fully expanded leaves of all the treatments were harvested at 0, 12, 24, 48 and 72 h after infestation. For each treatment, four biological replicates were used, each consisting of nine damaged leaves (three leaves from three plants). Leaf samples were immediately frozen by immersion in liquid nitrogen and stored at −80 °C for further RNA extraction. Total RNA was extracted and purified as indicated by the protocol RNA plant Kit (bioZOL™-P, Plant RNA Reagent). Measurement of RNA quality was done using a spectrophotometer and synthesis of cDNA was performed using oligodt 23-mer (Sina clone Company, Karaj, Iran) and Revert Aid MMULV reverse transcriptase (Sina clone Company, Karaj, Iran). Transcript levels of two Pathogen Related Protein (*PR3* and *PR4*), phenylalanine ammonia-lyase (*PAL*) and Lipoxygenase (*LOX)* that are all expressed in common bean after infection with *T. urticae* (Arimura *et al.* 2000), phenylalanine ammonia-lyase *(PAL)* that expressed in common bean either after *T. urtic*ae infection (Arimura *et al.* 2000) and drought stress (Khakdan *et al.* 2018) and (E)-β-ocimene synthase (*OS*) gene, which encodes the enzyme synthesis one of the most important volatile compounds ((E) -β-Ocimene) that attract predators and parasites feeding on *T. urticae* (Arimura *et al.* 2004) were quantified. As reference genes, metalloproteinase protease *Cons7* was used (Libault *et al.* 2008). The amplification products were also resolved onto agarose gel to verify amplicon size. The primer pairs used and the size of the expected amplicons are illustrated in Table 1. Primers, designed with the aid of the Primer3 (http://frodo.wi.mit.edu/primer3) were chosen to amplify a fragment between 140 and 160 bp (Thibivilliers *et al.* 2009).

The quality of each primer was determined before qRT-PCR analysis. All qRT-PCR experiments were performed in duplicate (technical replicates) and average values were used in the analyses. The thermal cycling program started with a step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of a 15 s step at 95 °C followed by 1 min at Ta indicated in Table 1. A normalization factor was calculated by geometrically averaging the threshold cycle (Ct) values of the constitutively expressed genes. Then the transcript level for each tested gene was calculated relative to the normalization factor using the $2^{-\Delta CT}$ method, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ (Livak and Schmittgen 2001).

Table 1. Gene-specific primer sequences and amplicon details.

Primers	Sequence 5' to 3'	Tm (°C)	Amplicon Length (bp)	Gene	Accession Number
Cons7-F **Cons7-R**	GGCATTAAGGCAGCTCACTCT ATGAATGACGGTTCCCATGTA	60	140	Metalloproteinase protease (*Cons7*)	AW310136
PR-3-F **PR-3-R**	ACTGAGAGGTGACAAGGTCAGAG TCGGATTCTTCAAGAGATACTGTG	60	150	Pathogen Related Protein3 (*PR3*)	M13968
LOX-F **LOX-R**	ATCTGTATCCCAAGATGTGTTGC TTGTAGGCAATGTAACTCCACCT	60	140	Lipoxygenase (*LOX*)	X63521
OS-F **OS-R**	ATCGAGGGAAGATTATTCTACGG TAAGCTAGCCTTTCTTGTCAACG	60	150	(E)-β-ocimene synthase (*OS*)	EU194553.1
PAL-F **PAL-R**	CTTAAGCTCATGTCTTCCACGTT TGTGGTAAGAGTCCTCTTGGAAA	60	160	Phenylalanine ammonia-lyase (*PAL*)	M11939
PR-4-F **PR-4-R**	CTTGCTTGCACCATCTATCTCTT CACTGACTTCGGTAGAGTTGGTT	60	140	Pathogen Related Protein4 (*PR4*)	X57187

RESULTS AND DISSCUSSION

Gene expression levels were quantified by Real-time PCR experiments with gene-specific primers for common bean genotypes infested with *T. urticae* alone and either stressed by moderate drought and the herbivorous mite (Figs. 1–4). Under moderate drought stress, expression ratio of *PR3* gene 24 hours after infection (peak regulation of target genes) in Akhtar (susceptible to spider mite) and KS41128 (resistance to spider mite) had increased in both genotypes compared to normal conditions (Figs. 2, 3). The up-regulation of these genes in KS41128 and Akhtar were 4.5 and 1.5 respectively. Lee *et al.* (2008) while assessing PR proteins response to drought stress in white clover showed that increased activity of this protein under drought stress than normal is closely associated with a decrease in leaf water potential. It can be noted that drought stress increases direct defense encoded by *PR3* gene in KS41128 genotype. Given the importance of this gene in direct defense, the information in this research can be promising to further studies in order to understand the complex responses of plants against two simultaneous stresses. *PR3* gene expression under drought stress has decreased compared to normal condition in genotypes Naz (resistance to spider mite) 2.5 times and G11867 (susceptible to spider mite) 2 times (Figs. 1, 4). Fountain *et al.* (2010) while assessing effects of drought stress on the transcription of three genes *PR10*, *GLX-I* and *TI-14* expression in different lines of corn found that drought stress during corn vegetative period affected the expression of genes in various genotypes differently. According to Rasmussen *et al.* (2013) simultaneous effects of stresses differed from those in individuals. According to them, exposing plants to multiple stresses may require the opposing responses or antagonists or agonists and unrelated response to the stress individually. Sharma *et al.* (2007) stated that when the effects of a biotic stress along with a pathogen or pest are tested, both positive and negative interactions depending on the timing, nature and severity of stress are observed.

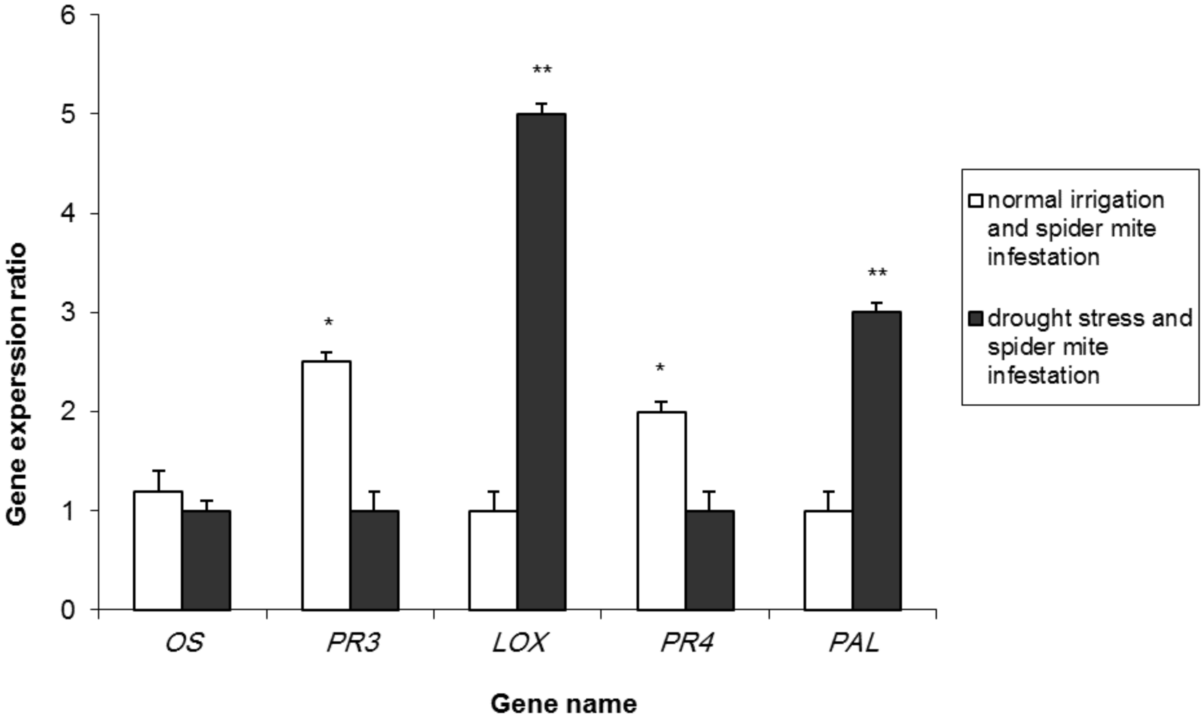

Figure 1. Relative gene expression of some genes (*OS, PR3*, *LOX*, *PR4* and *PAL*) by real-time PCR in *Tetranychus urticae* infected and control plants under drought stress in genotype Naz. Y-axis shows gene expression ratio. Asterisks indicate that the $2^{\Delta Ct}$ values were significantly different ($P < 0.01$; Student's t-test).

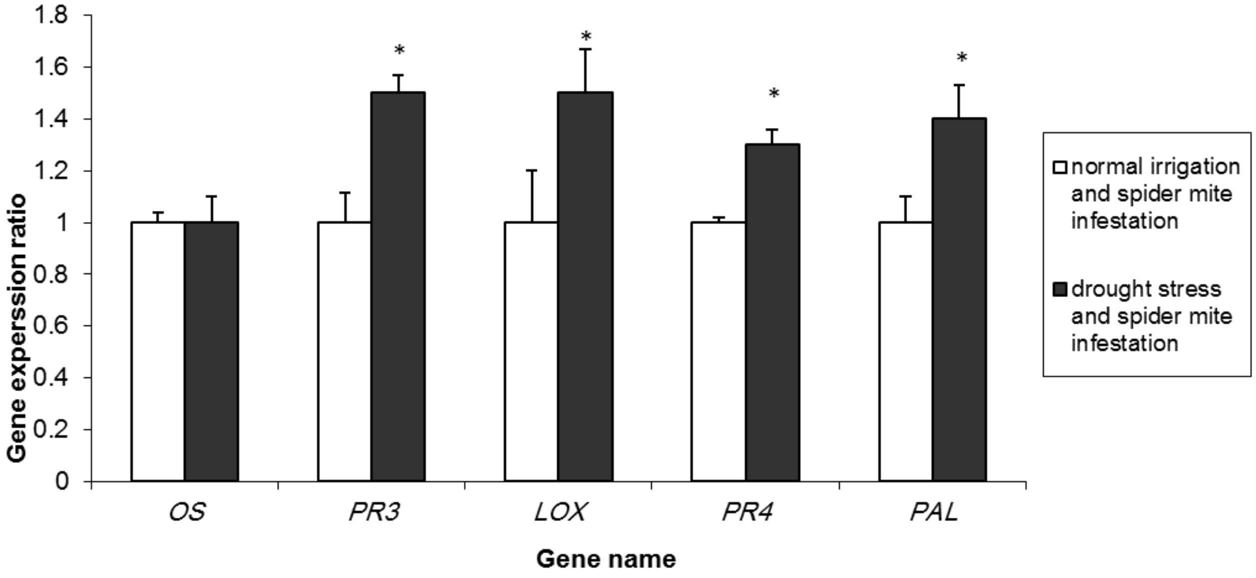

Figure 2. Relative gene expression of some genes (*OS, PR3*, *LOX*, *PR4* and *PAL*) by real-time PCR in *Tetranychus urticae* infected and control plants under drought stress in genotype Akhtar. Y-axis shows gene expression ratio. Asterisks indicate that the $2^{\Delta Ct}$ values were significantly different ($P < 0.01$; Student's t-test).

Under moderate drought stress, expression ratio of *PR4* gene, 24 hours after infection (peak regulation of target genes) in Akhtar (susceptible to spider mite) and KS41128 (resistance to spider mite) had increased in both genotypes compared to normal conditions (Figs 2, 3). Of the 17 groups of PR proteins, the PR-4 protein is one of the least extensively studied. The first PR-4 proteins to be described were from potato (Stanford *et al.* 1989), and were termed win-1 and win-2, from wound-inducible proteins. Focusing on PR-4 proteins, several studies showed the involvement of this family in plant defense responses regulated by signal molecules, such as salicylic acid (SA), abscissic acid (ABA), jasmonate (JA) and ethylene (ET).

Expression ratio of *LOX* genes 24 hours after infection in Naz and Akhtar under drought stress condition to normal condition increased. The up-regulation of this gene in Naz and Akhtar were respectively 5 and 1 times. Halitschke and Baldwin (2003) and Kessler *et al*. (2004) reported that *LOX* gene is a key enzyme in the octadecanoid pathway plays an important role in plant defense against herbivores. *LOX* gene is found to be key in direct defence and production of volatile compounds and indirect defence (Dicke *et al*. 2003). Common bean, like other plants takes various defence techniques to cope with pests. Such strategies are divided into two main groups: direct and indirect in direct defense, plants using physical and chemical barriers plant directly impose negative effect on the reproduction and biology of the pest's and in indirect defense, by attracting natural enemies reducing the pest population (Arimura *et al.* 2002). Sha *et al.* (2005) showed that in transgenic rice with Agrobacterium, *LOX* genes in response to drought stress, rice blast and bacterial blight of rice were expressed. In addition, Bruinsma *et al*. (2010) showed *LOX* suppression in plant defense both reduces directly and indirectly defenses. Ebel (1997) by measuring six volatile compounds in the leaves of apple trees, which are produced from *LOX* gene activity, showed that these compounds increased in response to drought stress and involved in the induction of leaf senescence. So he suggested that *LOX* gene is expressed under drought stress. Tahmasebi *et al.* (2014) reported that Naz genotypes under normal irrigation in direct defense (resistance to *T. urticae*) and indirect defence was superior to the three genotypes, in the present study drought stress in Naz genotype significantly increased *LOX* gene expression compared to normal conditions and increased expression in Naz has increased more than other genotypes of the gene. Given that the

LOX genes directly or indirectly involved in the defense, so Naz genotypes are promising candidates to study the role of *LOX* genes in common bean resistance to *T. urticae*. Also, *LOX* gene expression under the drought stress compared to the non-stress condition in genotypes KS41128 and G11867 decreased (Figs. 3, 4). It can be concluded that as *LOX* and *PR3* genes are involved in common bean defense against two spotted spider mite, their down regulation in G11867 genotype may lower plant resistance to *T. urticae*. Therefore drought stress may exacerbate the susceptibility of this genotype to *T. urticae*.

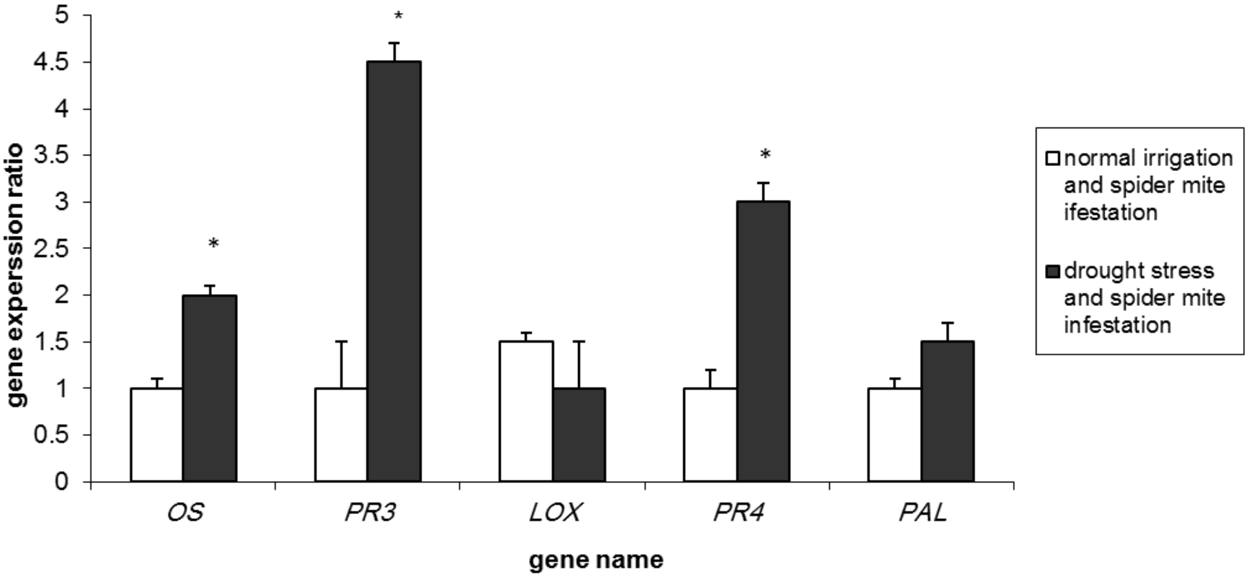

Figure 3. Relative gene expression of some genes (*OS, PR3*, *LOX*, *PR4* and *PAL*) by real-time PCR in *Tetranychus urticae* infected and control plants under drought stress in genotype KS41128. Y-axis shows gene expression ratio. Asterisks indicate that the $2^{\Delta Ct}$ values were significantly different ($P < 0.01$; Student's t-test).

Expression ratio of *PAL* genes 24 hours after infection in Naz, Akhtar, KS41128 and G11867 under drought stress condition to normal condition increased. (Figs. 1–4). Phenylalanine ammonia lyase (*PAL*) and its conversion of l-phenylalanine to trans-cinnamate and ammonia is the first committed step in the phenylpropanoid pathway, redirecting large amounts of fixed carbon from primary to secondary metabolism. It is crucial during the stage of the environmental and development stimulants (Khakdan *et al.* 2018). Phenylpropanoid compounds not only fulfill various essential functions during plant development, but also they act as important protectants against various biotic and abiotic environmental stresses. The biosynthesis of *PAL* and accumulation of phenylpropanoid structures have been reported up on pathogenic attack including viruses, tissue wounding, UV irradiation, low temperatures, low levels of nitrogen, phosphate and iron (Dixon and Paiva 1995; Ritter and Schulz 2004; Gholizadeh 2011). An increase in the activity of PAL enzyme has been recently reported in the cases of winter triticale and a drought resistant maize genotype (Hura *et al*. 2007; 2008).

Expression ratio of *OS* gene 24 hours after infection in G11867 plants infected under the drought stress had significantly decreased and increased in KS41128 genotype compared to normal condition (Figs. 3, 4). Also, *OS* down regulation observed in Naz and Akhtar respectively 1.2 and 1.1 times (Figs. 1, 2) under drought stress compared to normal condition. Arimura *et al*. (2004) showed that this gene, by participating in the synthesis of (E) -β-Ocimene play an important role inducing defence responses in *Lotus japonicus* against *T. urticae*. In addition, Copolovici *et al.* (2014) while investigation of volatile compounds produced from *Monsoma pulveratum* (Retzius)

(Hymenoptera: Tenthredinidae: Allantinae) larvae feeding on tree foliage under drought stress showed that the volatile compounds (E) -β-Ocimene and DMNT in plants under drought stress were increased substantially than those under normal irrigation. Given that the *OS* is an important gene in the synthesis of (E) -β-Ocimene and is an important component in attracting predators and as Naz is resistant genotype and under irrigation in normal conditions successfully attract *T. urticae*, this result demonstrates that it is possible to drought stress reduces Naz indirect defence, although predator's attraction in drought condition should be further studied.

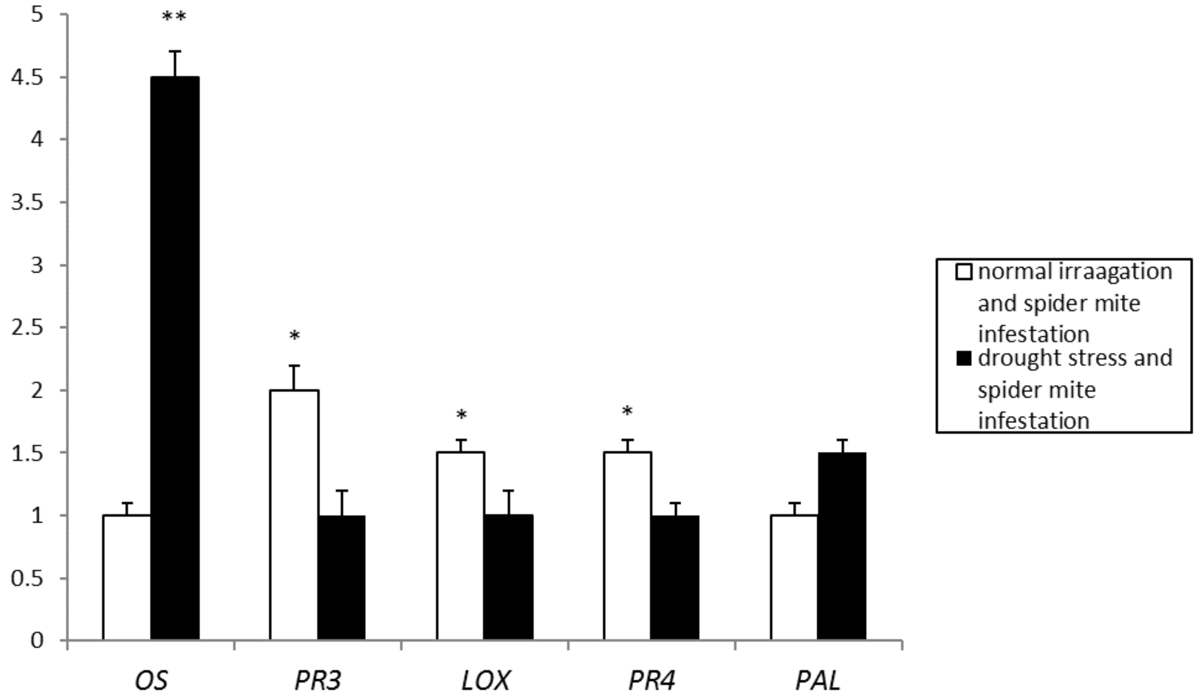

Figure 4. Relative gene expression of some genes (*OS*, *PR3, LOX*, *PR4* and *PAL*) by real-time PCR in *Tetranychus urticae* infected and control plants under drought stress in genotype G11867. Y-axis shows gene expression ratio. Asterisks indicate that the $2^{\Delta Ct}$ values were significantly different ($P < 0.01$; Student's t-test).

In general, the results of this study showed that among the various common bean genotypes studied under drought stress conditions compared to normal conditions are different in terms of direct and indirect defence-related gene expression. As results showed, drought stress reduced direct defense (*PR3* and *LOX* genes) in genotype G11867. This genotype is susceptible to *T. urticae* and drought stress can exacerbate genotype susceptibility to *T. urticae*. Also, it increased indirect defence (*OS* and *LOX* genes) in Akhtar genotype which can be used as a method of improving resistance to *T. urticae*. In this experiment, we found that there is no particular association in terms of gene expression in common bean genotypes in response to single stresses such as drought and *T. urticae* separately, with plant responses to stress at simultaneous two stresses. Gutbrodt *et al.* (2012) stated that drought causes some chemical changes in plants and lead to coping responses against herbivores in plants. These changes include reducing the concentration of secondary compounds in plants under stress without effects on its nitrogen content. In addition, the drought causes the opposite patterns in nutritional preferences between herbivores species. Here, effects of biotic and abiotic stresses are studied simultaneously so that according to Rasmussen *et al.* (2013) effects of stresses simultaneously is differed from those individually. In general integration of two drought stress and insects is much more complicated than when they are applied in a separate manner. According to Atkinson and Urwin (2012) interactions between biotic and abiotic stresses can be controlled by signaling pathways that can induce each other or impose inhibitory effects on each other. It is recommended that this experiment is performed in farm conditions with many genotypes. Farm condition is not controlled and many environmental factors can affect defence compounds including defence metabolites in plants. This implies the importance of further study on the role of moderate drought stress on increasing the plant's defense against some insects, especially *T. urticae*.

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تغییرات بیان ژن در *Phaseolus vulgaris* L. (Fabaceae) در پاسخ به ترکیب چند تنش

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چکیده

گیاهان به‌طور مداوم در معرض محدودهٔ گسترده‌ای از تنش‌های محیطی قرار دارند. لوبیا (*Phaseolus vulgaris* L.) هفتمین محصول غذایی اصلی جهان است که به طور گسترده‌ای توسط آفات مختلف از جمله کنهٔ گیاهخوار عمومی، *Tetranychus urticae* Koch، مورد حمله قرار می‌گیرد. در این پژوهش، الگوی بیان چند ژن را در چهار ژنوتیپ لوبیا که در مطالعه‌ای پیشین میزان مقاومت آنها به *T. urticae* ارزیابی شده بود (دو رقم با بیشترین درجهٔ مقاومت، ناز و Ks4128 و دو رقم با کم‌ترین درجهٔ مقاومت، اختر و G11867) را در پاسخ به تنشی منفرد (تنها آلودگی با *T. urticae*) در مقابل ترکیب دو تنش (تنش خشکی ملایم و آلودگی با *T. urticae*) ارزیابی شد. تنش خشکی الگوی بیان ژن‌ها را در برخی ژنوتیپ‌های *Phaseolus vulgaris* آلوده به *T. urticae* تغییر داد. نتایج این پژوهش در مورد ترکیب تنش‌ها نشان داد که بیان برخی ژن‌ها در ترکیب تنش‌ها تغییر می‌کند اما در تنش به تنهایی تغییر نمی‌کند. تنش خشکی می‌تواند الگوی بیان ژن را در ژنوتیپ‌ها بدون توجه به حساسیت یا مقاومت آنها به کنه تارتن دولکه‌ای تغییر دهد. در مجموع، نتایج بر طبیعت پیچیدهٔ پاسخ به چند تنش تاکید می‌کند و پاسخ رقم‌های لوبیا به چند تنش پیچیده است و قابل پیش‌بینی نیست.

واژگان کلیدی: لوبیای معمولی؛ تنش خشکی؛ مقاومت؛ *Tetranychus urticae* Koch؛ رقم.

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