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ORIGINAL ARTICLE

Physiological and molecular response of annual *Medicago* species to juglone

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Abstract The current study was conducted to evaluate the physiological and molecular response of self-regenerating annual *Medicago* species (*M. polymorpha* and *M. lupulina*) to juglone exposure. A randomized complete block design was performed in which two treatment groups consisted of a control and juglone (10^{-4} M) allotted to main plots and genotypes assigned to subplots. A significant increase in the concentration of GSH and the GSH/GSSG ratio was observed in both annual *Medicago* species in response to juglone exposure. However, such response was greater in *M. lupulina* than *M. polymorpha*. The activity of all antioxidant enzymes (CAT, APX, GST, and GPOX) was significantly increased by juglone. In response to juglone, the expression of *WRKY* was significantly decreased. The transcription of *CBF4*, *Zpt2-2*, *CAT*, and *GST* genes was highly induced by juglone in annual *Medicago* species. Higher expression of *CBF4*, *Zpt2-2*, *CAT* and *GST* genes in *M. lupulina* which showed more juglone-tolerance can be associated with higher tolerance against allelochemical stress. It can be concluded that *M. polymorpha* is juglone-sensitive because it started to show chlorosis of leaves after a week subjecting to juglone. Though *M. lupulina* seems to be more juglone-tolerant at least in short term, long-term exposure of juglone should also be examined to find out its suitability to grow in walnut orchards. Considering the presence of cross-talk between different stresses, we can propose that *M. lupulina* is more tolerant to other abiotic stresses compared to *M. polymorpha*.

Keywords Allelopathy · Antioxidant enzyme · Gene expression · Oxidative stress · Transcription factor

Introduction

Juglone, 5-hydroxy-1,4-naphthalenedione ($C_8H_6O(OH)_2$), is a naturally occurring compound in plants of Juglandaceae family, particularly the black walnut (*Juglans nigra*) (Sytykiewicz 2011). Juglone has toxic and growth-retarding effects on other plants and because of that, a large space between walnut trees remains uncultivable. In fact, juglone is a highly bioactive substance that serves as an oxidant stimulant for the production of reactive oxygen species (ROS) in juglone-exposed plants (Sytykiewicz 2011). A complicated network of interrelationships is evolved to overcome cellular oxidative damages in stressed organs (Gill and Tuteja 2010). It is interesting to know that there are some plants, which are tolerant to juglone. However, little information exists about gene expression in response to juglone stress. Chi et al. (2011) analyzed transcriptome responses in rice seedlings exposed to juglone stress. Using microarray analysis, they found changes in transcript levels of genes underlying cell growth, cell wall formation, chemical detoxification, and abiotic stress response in plants that were exposed to juglone (Chi et al. 2011). Sytykiewicz (2011) focused on the analysis of glutathione-*S*-transferase (*GST*) gene

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expression patterns in maize seedlings exposed to juglone. This study showed that juglone treatment significantly enhanced the transcriptional activity of *GST* in maize seedlings when compared to control plants.

Considering an area of 100 m^2 per walnut tree, there is a plenty of room in walnut orchards that can be used by self-regenerating plants with minimal farming operations that are tolerant to juglone. Annual *Medicago* species belonging to the Fabaceae family are suitable plants. Annual *Medicago* species may have unique beneficial genes that *Medicago sative* lack (Tivoli et al. 2006). This provides a potential for plant breeders to take an advantage of such beneficial potential for improving the resistance of plants to different stresses. The present study aims at studying the tolerance to juglone of self-regenerating annual *Medicago*, and identifying responsible genes in annual *Medicago* that confer resistance to the plant, which can be exploited in genetic improvement programs. We focused on the expression profile of transcription factors (TFs) because TFs interact with *cis*-elements in the promoter regions of genes that are involved in stress response (Agarwal and Jha 2010; Rafiei et al. 2015). Considering the presence of crosstalk between different abiotic stresses, we evaluated the expression of TFs which respond to salinity stress in *Medicago* genome. In this regards, the effects of juglone treatmeant on expression profiles of *CBF4*, *MYB*, *WRKY*, and *Zpt2-2* genes in two annual *Medicago* species (*M. polymorpha*, and *M. lupulina*) were studied. Also, we measured the expression pattern of *GST* and catalase (*CAT*) as crucial antioxidant genes. Some physiological traits including glutathione concentrations and changes in enzymatic antioxidants in leaves of annual *Medicago* species were determined. We tried to establish relationships between gene expression profile and the level of tolerance to juglone stress.

Materials and methods

Plant material and growth conditions

Seeds of *Medicago lupulina* and *Medicago polymorpha* were obtained from Iranian Forest, Range and Watershed Organization (http://www.frw.org.ir). Seeds were scarified in concentrated sulfuric acid and planted in the trays containing a mixture of cocopeat:perlite in 70:30 ratio, and grown in a greenhouse. Hoagland solution used to irrigate the plantlets and gradually concentrated from 25% in the first 2 days, and reached 100 % in a week. Two-week-old plants were subjected to an allelochemical (juglone) and a control treatment. Juglone (5-hydroxy-1,4-naphthoquinone, m.w. 174.15) was obtained from Sigma-Aldrich Ltd. and dissolved in Hoagland solution to provide the concentration of 10^{-4} M. This is the concentration of juglone which is naturally found in walnut (Teriz 2008). We had four replicates in our experiment. One week after exposure to juglone, *M. polymorpha* started to wilt and changing the color to yellow. At this time, we collected leave and shoot samples from all plants to measure physiological criteria.

Physiological traits

Total, oxidized, and reduced glutathione

The concentration of total glutathione content (TGSH) in plants was measured using the recycling enzymatic assay (May and Leaver 1993). In brief, 100 mg of fresh plant shoot was weighed and smashed in liquid nitrogen. The ground tissue was then extracted in 0.5 mL of 0.1 M Na phosphate buffer and 5 mM EDTA (pH 7.6). Following centrifuging at 9000*g* for 10 min, an aliquot of the supernatant (0.1 mL) was taken in a tube containing 1 mL reaction mixture (6 mM dithiobis (2-nitrobenzoic acid), 3 mM NADPH, and 2 U of glutathione reductase from *Saccharomyces cerevisiae*). All reagents were obtained from Sigma-Aldrich Ltd. Glutathione-dependent reduction of dithiobis was spectrophotometrically measured at 412 nm. TGSH was calculated using the equation of the linear regression obtained from a standard GSH curve. Oxidized glutathione (GSSG) was measured in the same extracts following derivatization of reduced GSH. A volume of 100 µL plant extract underwent derivitization in 0.5 mL of 0.5 M K phosphate buffer, pH 7.6, in the presence of 4 mL of 2-vinyl pyridine (Sigma-Aldrich) for 1 h at room temperature. Upon conjugation of GSH with 2-vinyl pyridine, the product was extracted in 1 volume of diethylether and the GSSG was measured by means of a spectrophotometer as described for total glutathione. Reduced glutathione (GSH) was deduced as the difference between TGSH and GSSG.

Assays of antioxidant enzyme activities

An amount of 100 mg fresh shoot tissue was homogenized in 1.5 ml of 50 mM sodium phosphate buffer containing 0.1 mM EDTA (pH 7.8) on ice. The homogenates were centrifuged at 12,000*g* for 20 min at 4 °C and the supernatants were used for enzymatic assays.

The activity of APX was measured according to Nakano and Asada (1981) with minor modifications. The reaction mixture consisted of 50 mM PBS (phosphate buffer solution), 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 ml of enzyme extract. Decrease in the absorbance at 290 nm was recorded as oxidation of ascorbic acid. APX activity (one unit of APX oxidizes ascorbic acid at a rate of

1 μmol min^{-1} at 25 °C) was calculated considering an extinction coefficient of 2.6 mM^{-1} cm^{-1} for ascorbic acid.

GPOX activity in shoot extracts was determined by the method described by Lin and Kao (1999). The assay mixture contained 9 mM guaiacol and 19 mM H_2O_2 in 50 mM phosphate buffer at 25 °C and the absorbance was measured at 470 nm. The extinction coefficient of 26.6 mM^{-1} cm^{-1} was considered to convert the absorbance values to specific activity. A unit of GPOX enzyme activity was defined as production of 1 μM tetraguaiacol in 1 min.

Glutathione-*S*-transferase activity was measured according to Carmagnol et al. (1981). Decrease in the absorbance at 340 nm was considered as glutathione (GSH) oxidation. The reaction mixture (2 ml) contained 100 mM PBS (phosphate buffer, pH 6.5), 75 mM GSH, and 30 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol. The reaction was started by adding 200 ml enzyme extract. Decrease in the absorbance was kinetically monitored at 340 nm for 5 min (EC of G-SDNB conjugate: 9.6 μM^{-1} cm^{-1}).

Catalase (CAT) activity was determined according to Aebi (1983). The sample was added to a reaction solution containing 10 mM H_2O_2 in phosphate buffer solution. Decrease in the absorbance at 240 nm was recorded kinetically. CAT activity was calculated considering an extinction coefficient of 0.039 mM^{-1} cm^{-1}. One unit of CAT gives a H_2O_2 decomposition rate of 1 μmol min^{-1} at 25 °C.

RNA extraction, cDNA synthesis and RT-PCR

Samples of leaves for transcriptome analysis were harvested 24 h after juglone treatment. Each sample had three biological replicates. Total RNA was extracted by RNA isolation kit (Dena Zist Asia, Iran). DNase treatment and cDNA synthesis were carried out by Fermentase Kit (Hanover, MD) and the SUPERSCRIPT II first-strand synthesis system (Vivantis), respectively. Prior to use in RT-PCR, cDNAs were diluted to 1/4. Subsequently, an aliquot of diluted cDNAs (4 μL) was used as a template in 10 μL PCRs. Each biological replicate had three technical replications in RT-PCR.

A review of literature indicated that *MYB* and *CBF* gene families are contributed to tolerance to different abiotic stresses. The genome of *M. truncatula*—an annual *Medicago* species—is publicly available. Li et al. (2011) has already indicated that *CBF4* and *MYB112* play key role in tolerance to abiotic stresses in *Medicago* species. Also, Merchan et al. (2007) showed the TFIIIA-like TF, *Zpt2-2* is involved in recovery responses to salinity and cold stress in *M. truncatula*. Variety of studies reported the importance of WRKY TFs in biotic and abiotic stresses. Regarding the presence of cross-talk between different stresses, we examined the expression pattern of *CBF4*, *MYB*, *WRKY* and *Zpt2-2* under juglone treatment in annual *Medicago* species.

We also determined the expression pattern of *CAT* and *GST* among annual *Medicago* species under control and juglone treatment. The sequences of *CAT* (XM_003623068.1) and *GST* (XM_003623148.1) genes were obtained based on the analysis of microarray data, GSE14029 (available in NCBI). These microarray data conducted by Li et al. (2011) included data from transcriptome analysis of *M. truncatula* cv. A17 in salt stress condition. *MtActin* is considered as internal control gene in RT-PCR. The primer's information used in our study is summarized in Table 1.

Statistical analysis

Physiological data were subjected to randomized complete block design (RCBD) using SAS software (Version 9). We used $2^{-\Delta Ct}$ method to represent real-time PCR data as individual data points. In this formula,

$$\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{internal control}}$$

(Schmittgen and Livak 2008). The mean comparisons exceeded twofold change and *P* value less than 0.05 were expressed as significant result. A *t* test analysis was performed to determine significant differences for comparisons of the physiological traits and gene expression profile within each genotype.

Results

Physiological traits

Juglone significantly increased TGSH and reduced GSH contents in both annual *Medicago* species with

Table 1 Sequences of used primers for *CBF4*, *MYB112*, *WRKY53*, *Zpt2-2*, *GST*, *CAT* and housekeeping gene "Actine"

(5'→3')	Primer
CTTGACCTCCACTCTCGCTG	MYB112-F
GCATCCAAACGTGACGCAAT	MYB112-R
GGATTCCGGGAAGTGGGTTT	CBF4-F
CGCCGCCTTTTGAATATCCC	CBF4-R
AGACATCCTTGGAGCCATGC	WRKY53-F
GGACCAACCACATTGGAAGC	WRKY53-R
GGCAACGGACTTTCTACCTC	Zpt2-2-F
CTCCTCCCATCAGCCACCGTG	Zpt-2-2-R
TAAACCAGTGTTTAAGTGCT	GST-F
CTCCACCAAAGAAAGTCT	GST-R
GAGGCAAAAGTGCTTGGTCC	CAT-F
GTGTCAGGTATCGGGGCTTC	CAT-R
CCCACTGGATGTCTGTAGGT	Actine-F
AGAATTAAGTAGCAGCGCAAA	Actine-R

Table 2 The different responses of annual *Medicago* genotypes to juglone treatment

Genotypes	*M. polymorpha*		*M. lupulina*	
Treatment	Control	Juglone	Control	Juglone
TGSH Shoot (μmol g^{-1} FW)	80.07 (±2.06)	119.11 (±1.58)**	64.29 (±2.12)	115.56 (±1.39)**
GSSG Shoot (μmol g^{-1} FW)	19.8 (±3.98)	16.67 (±0.79)**	14.9 (±1.96)	22.57 (±1.7)**
GSH Shoot (μmol g^{-1} FW)	60.3 (±2.14)	102.44 (±0.93)**	49.4 (±2.51)	92.99 (±1.68)**
APX (μmol min^{-1})	0.048 (±0.003)	0.1285 (±0.001)**	0.0107 (±0.01)	0.0214 (±0.012)**
GPOX (μmol min^{-1})	1.912 (±0.13)	4.84 (±0.08)**	2.17 (±0.026)	2.54 (±0.052)*
GST (μmol min^{-1})	0.152 (±0.003)	0.491 (±0.034)**	0.508 (±0.05)	2.037 (±0.0008)
CAT (μmol min^{-1})	34.645 (±0.22)	284.77 (±3.021)**	41.5 (±0.22)	167.51 (±1.59)**

Values represent means of $n = 4$ replicates

* Comparisons within each genotype are significantly different at $P \leq 0.05$

** Comparisons within each genotype are significantly different at $P \leq 0.01$

concomitant reductions in GSSG. Such increase, however, was much higher in *M. lupulina* than *M. polymorpha* (Table 2). Conversely, oxidized GSH was decreased by juglone in both species. *M. polymorpha* had significantly higher concentrations of GSH, GSSG, and TGSH than M. *lupulina* (Table 2).

In general, juglone exposure resulted in enhanced activities of antioxidant enzymes. The results showed juglone treatment enhanced APX activity in both *Medicago* species. Such an increase was 2 and 2.5 times in *M. lupulina* and *M. polymorpha,* respectively (Table 2).

GPOX activity was increased in juglone treatment compared to the control. Though *M. lupulina* experiences insensible increase, *M. polymorpha* had a 2.5 times increase in the activity of GPOX (Table 2).

We observed a fourfold and a threefold increase in GST activity, respectively, in *M. polymorpha* and *M. lupulina* following juglone exposure (Table 2).

Juglone exposure enhanced CAT activity ($P < 0.01$) in annual *Medicago* species. *M. lupulina* and *M. polymorpha* had eightfold and fourfold increase when subjected to juglone, respectively (Table 2).

Gene expression profile

The expression of *MYB112* did not show any significant change in leaves of annual *Medicago* genotypes under juglone treatment (Fig. 1).

The plants subjected to juglone stress had greater overexpression of *CBF4* than the plants on the control group. *M. lupulina* and *M. polymorpha* showed eightfold and sixfold increase, respectively, in overexpression of *CBF4*. *M. lupulina* was distinguished from *M. polymorpha* by higher expression of *CBF4* in the control and juglone exposure (Fig. 2).

Juglone caused an overexpression of *Zpt2-2* in the leaf of *M. lupulina* and *M. polymorpha.* The overexpression of

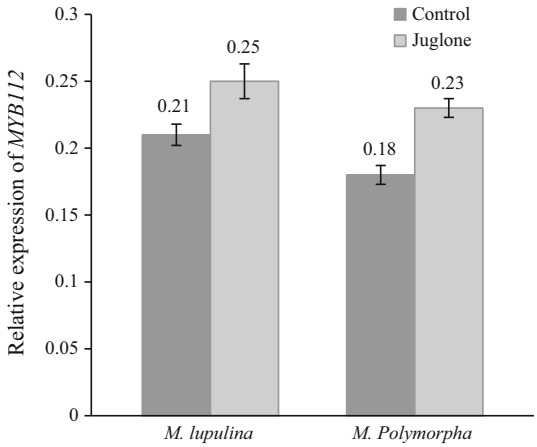

Fig. 1 Real-time RT-PCR analysis of *MYB*112 differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *MYB* calculated using $2^{-\Delta Ct}$ method

Zpt2-2 was similar across *Medicago* species (approximately threefold). Furthermore, *Zpt2-2* showed greater expression in *M. lupulina* than *M. polymopha* plants (Fig. 3).

Conversely, the expression of *WRKY53* declined in the leaf of *Medicago* species as a result of juglone stress with more remarkable change in *M. polymorpha* compare to *M. lupulina* (Fig. 4).

Juglone treatment intensified the overexpression of *GST* gene leaves of annual *Medicago* species (Fig. 5). Exposure to juglone increased the overexpression of *GST* in *M.polymorpha,* which was greater than *M. lupulina* (8.7-fold vs. 5.9-fold). Nevertheless, the expression of *GST* gene in *M. lupulina* was greater than *M. polymorpha* in the control and juglone stress (Fig. 5).

The expression of *CAT* gene showed an increase in the juglone treatment. Furthermore, *CAT* showed a greater expression in *M. lupulina* than *M. polymorpha* in juglone condition (Fig. 6).

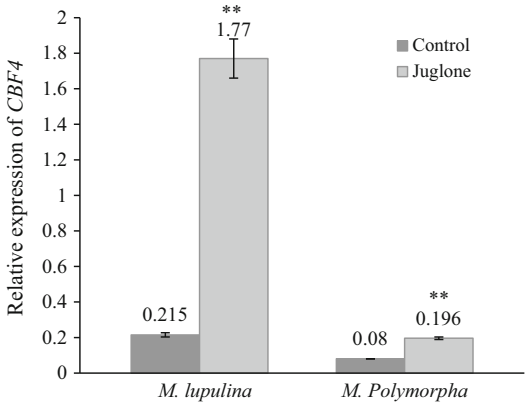

Fig. 2 Real-time RT-PCR analysis of *CBF4* differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *CBF4* calculated using $2^{-\Delta Ct}$ method

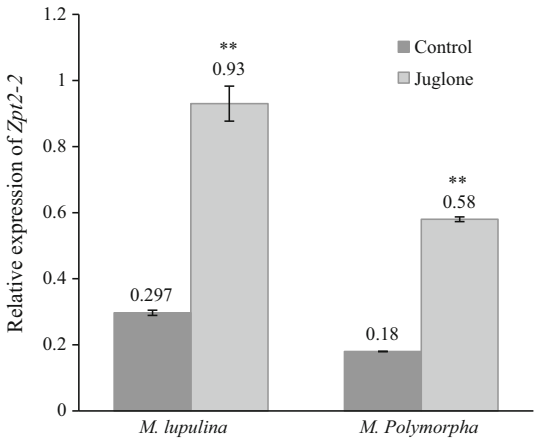

Fig. 3 Real-time RT-PCR analysis of *Zpt2-2* differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *Zpt2-2* calculated using $2^{-\Delta Ct}$ method

Discussion

Crucial role of GSH in plant antioxidant defense typifies this compound as a stress marker. GSH provides the most important intracellular defense against ROS (Mullineaux and Rausch 2005). It also plays a central role in detoxification of xenobiotics as well as the expression of stress-responsive genes (Ding et al. 2007; Golisz et al. 2008; Sytykiewicz 2011). Though the concentration of GSSG in both annual *Medicago* species significantly decreased, GSH concentration and consequently the ratio of GSH/GSSG were significantly increased in both annual *Medicago* species in response to juglone exposure. Although *M. ploymorpha* had higher concentration of GSH content, but the elevation of GSH as a result of juglone stress was more prominent in *M. lupulina.* Similar observation has been

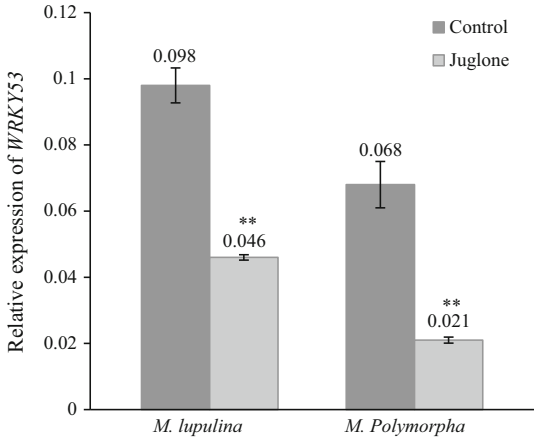

Fig. 4 Real-time RT-PCR analysis of *WRKY53* differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *WRKY53* calculated using $2^{-\Delta Ct}$ method

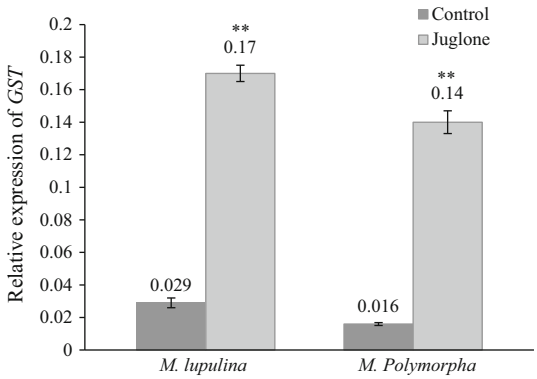

Fig. 5 Real-time RT-PCR analysis of GST differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *GST* calculated using $2^{-\Delta Ct}$ method

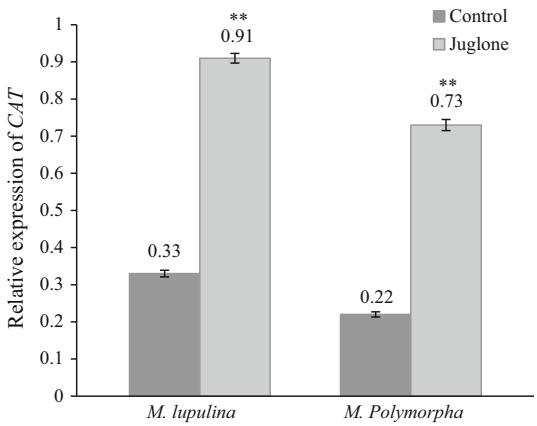

Fig. 6 Real-time RT-PCR analysis of *CAT* differentially expressed between annual *Medicago* species in response to juglone stress. Numbers on the *X axis* indicate individual expression of *CAT* calculated using $2^{-\Delta Ct}$ method

pinpointed under different environmental stresses (Esterbauer and Grill 1978; Polle and Rennenberg 1992). A comparative study in tomato confirmed that the cultivated tomato (salt sensitive) had no change in GSH concentration in response to salinity but its wild salt-tolerant relative revealed a significant increase in GSH (Mittova et al. 2003). Changes in the GSH/GSSG redox state have been reported to be toward more oxidized, more reduced, or not at all upon stress exposure depending on how successfully plants acclaim to the stress condition (Tausz et al. 2004). In an acclimation reaction, antioxidant concentrations are increased (Tausz et al. 2004). Accordingly, increase in GSH observed in our experiment indicates that plants were able to reach to a steady state. Higher increase in GSH in *M. lupulian* demonstrated that this species more effectively acclaimed to juglone stress compared to *M. polymorpha*, which strengthens the antioxidative defence systems.

Antioxidant enzyme activities (APX, GPOX, GST, and CAT) were significantly increased by juglone. Similar observations by different stressors have been reported in a variety of plants (Bais et al. 2003; Yu et al. 2003; Lara-Nunez et al. 2006; Ding et al. 2007). It seems that plant response to allelochemical compounds such as juglone is similar to the response to other abiotic stresses in which ROS levels increase inside plant cells (Ding et al. 2007; Golisz et al. 2008; Sytykiewicz 2011; Behdad and Maghamni 2013). Antioxidant enzymes have the potential to suppress cellular damage by ROS.

In our study, the induced activity of APX by juglone was higher in *M. polymorpha* which was juglone-sensitive species. APX is the most important peroxidase, which converts hydrogen peroxide to water. A similar increase in APX activity in response to stress was observed in *Hordeum vulgare* (Kim et al. 2005), *Plantago maritime* (Sekmen et al. 2007), and *Oryza sativa* (Turan and Tripathy 2013). These researchers found that the induction of APX activity was greater in the salt-sensitive genotypes than salt-tolerant counterparts.

In response to juglone, GPOX activity was increased in *M. polymorpha* but it had an insensible induction in *M. lupulina*. The activity of GPOX depends to a great extent on plant species and stress condition (Gill and Tuteja 2010). To elucidate the mechanism of interspecific interactions mediated by allelochemicals, Ding et al. (2007) compared the response of cucumber and figleaf gourd seedlings to cinnamic acid. They reported that the activity of GPOX was induced only in cucumber roots, but not in figleaf gourd. Ding et al. (2007) found interspecies difference in the recognition of allelochemicals, which induced oxidative stress along with root cell death in cucumber, an autotoxic plant, but not in figleaf gourd, a cucumber relative.

Exposure to juglone significantly induced GST activity in *M. polymorpha* and *M. lupulina*. Under stress condition, GST activity increased nearly by fourfold in the annual *Medicago* species. Plant GSTs conjugate electrophilic and highly hydrophobic toxins with glutathione to form non-toxic peptide derivatives (Dixon et al. 1998; Roxas et al. 2000). Cruz-Ortega et al. (2002) showed that under allelochemical stress, the GST activity was increased in tomato plant. The overexpression of GST genes was also reported in maize, rice and Arabidopsis under allelochemical treatment (Cruz-Ortega et al. 2002; Golisz et al. 2008; Chi et al. 2011).

CATs are able to directly dismutate H_2O_2 into H_2O and O_2 and are indispensable for ROS detoxification during stressed conditions (Garg and Manchanda 2009). In our study, CAT activity was increased in response to juglone stress in both *Medicago* species. Similarly, Singh et al. (2006) reported an increase in the CAT activity in *Cassia occidentalis*, *Amaranthus viridis*, *Triticum aestivum*, *Pisum sativum* and *Cicer arietinum* under alpha-Pinene allelochemical. According to Gill and Tuteja (2010), overexpression of ROS scavenging enzymes brings about abiotic stress tolerance in various crop plants owing to efficient ROS scavenging capacity. Pyramiding of ROS scavenging enzymes may also be used to obtain abiotic stress tolerance plants. In our study, the induction of CAT was much higher in *M. lupulina* which was the juglone-tolerance species. Therefore, *M. lupulina*, which is more potent to scavenge cellular ROS may be useful in selection for tolerance against harsh environmental conditions.

Though allelochemical toxicity can suppress plant growth, little information exists about the transcriptional changes involved in their response (Chi et al. 2011). Several studies have shown that allelochemical stress can trigger a wave of ROS, leads to a Ca^{2+} signaling cascade, induces genome-wide changes of gene expression, and ultimately results in the death in susceptible species (Bais et al. 2003). Figure 7 shows an overview for the complex regulation network of our studied genes in response to juglone stress across annual *Medicago* species. We used literature mining to extract the possible relations of our genes with morpho-physiological traits. As depicted in Fig. 7, the transcription level of *GST* and *CAT* was increased within 24 h in the leaves of *Medicago* species. Similarly, Sytykiewicz (2011) showed that the juglone treatment caused significant increase in the expression of *GST* in maize seedlings. It can be suggested that juglone treatment augments ROS production, which brings about up-regulation of genes that are involved in ROS. *M. lupulina* was distinguished from *M. polymorpha* by having a higher expression of *GST* and *CAT* in the control and/or juglone treatment. It seems that the higher expression of *CAT* and *GST* genes in *M. lupulina* directly related to higher activity of these enzymes in *M. lupulina* which is more tolerant to juglone.

Among genes, transcription factors are important messengers connecting a perceived stimulus to an induced response. Several reports demonstrated an overexpression of different TFs such as *AP2/ERF*, *HSF*, *NAC*, *C2H2*, *WRKY*, *MYB*, and *GRAS* in response to juglone treatment (Golisz et al. 2008; Chi et al. 2011).

The *MYB* family of proteins is large, functionally diverse and possesses recognition motifs which are involved in biotic and abiotic stress tolerance (Dubos et al. 2010). Although the over-expression of *MYB* was observed in rice (Golisz et al. 2008) and Arabidopsis (Chi et al. 2011) as a response to juglone stress, no changes were observed in expression of *MYB112* in annual *Medicago* species. Such discrepancy may account for the differences in plant species, developmental stages and stress level used in different studies.

In the present study, *CBF4* was significantly increased under juglone treatment in *M. polymorpha* and *M. lupulina*. The overexpression was higher in *M. lupulina* (eightfold) compared to that observed in *M. polymorpha* (sixfold). Likewise, individual expression of *CBF4 in M. lupulina* was greater than *M. polymorpha* under the control and juglone conditions. *CBF4*, one of the *AP2-EREBP* transcription factor family, plays important functions in plant growth and development, especially in hormonal regulation and response to biotic and abiotic stresses (Li et al. 2011). Researches showed that *CBF4* is overexpressed in response to abscisic acid (ABA), drought, salt, and cold stress (Li et al. 2011). Higher expression of *CBF4* in *M. truncatula* was highly correlated to lower electrolyte leakage (EL) and tolerance to salinity stress. Abundance of different stress-responsive *cis*-acting elements in the promoter of *CBF4* infers why this gene is induced by multiple stresses including juglone treatment (Li et al. 2011).

Juglone stress caused an overexpression of *Zpt2-2* in the leaf of *M. lupulina* and *M. polymorpha*. The expression of *Zpt2-2* was much higher in *M. lupulina* compared to *M. polymorpha*. In Arabidopis, the constitutive expression of *STZ*—one of the Arabidopsis *Zpt2*-related genes—augmented the expression of the oxidative stress-responsive genes *APX2*, *SOD1*, and *APX1* (Mittler et al. 2006). In our study, the induction of *GST* and *CAT* in response to juglone stress can be related to overexpression of *Zpt2-2* gene (Fig. 7). Literature mining showed that overexpression of *AZF1* and *AZF2* (the orthologs of *Zpt* genes in Arabidopsis) resulted in the reduced expression of genes involved in carbohydrate and lipid metabolism (Kodaira et al. 2011). Moreover, Guilfoyle and Hagen (2007) reported down-regulation of several auxin-responsive genes in the transgenic plants that overexpressed *AZF2*. Therefore, we speculate that growth suppression following down-regulation of these types of proteins is a critical response of plants to juglone stress (Fig. 7).

The expression of *WRKY* in leaves was down-regulated in response to juglone treatment. *WRKY* proteins are encoded by a large gene family in all higher plants and reported to play a critical role in plant defense processes (Rushton et al. 2010). Recently, Rushton et al. (2012) explored that *WRKY* TFs act downstream of ABA receptors and are key nodes involved in ABA signaling. They

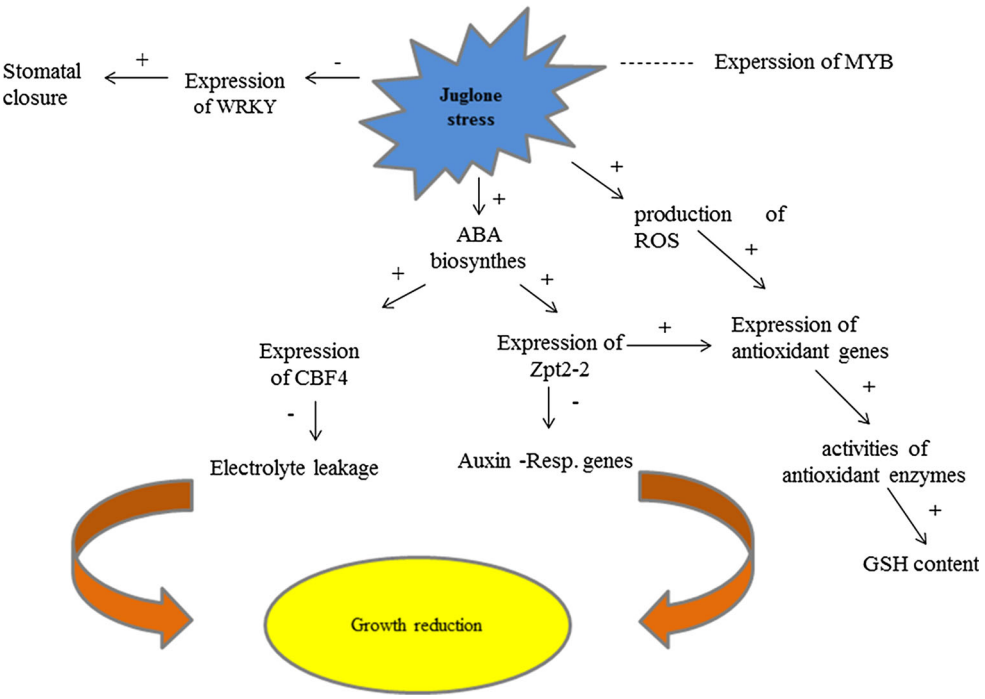

Fig. 7 Summary of the effect of juglone stress on anuual *Medicago* species based on the results of the present study and literature mining. Presence of "+" symbol means the positive effect, and "−" symbol means the negative effect

concluded that *WRKY* TFs are negative regulators of stomatal closure mediated through ABA and subsequent drought responses. It is worthy to know that juglone can inhibit photosynthesis, leaf stomatal conductance and respiration in different plants (Chi et al. 2011 and references therein). Down-regulation of *WRKY* in leaves of annual *Medicago* species in response to juglone suggests that juglone threatens photosynthetic system by stomatal closure enhancement.

Induction of *CBF4*, *Zpt2-2*, *GST*, and *CAT* genes as observed under juglone stress has also been observed by other abiotic stresses including salt, drought, and abscisic acid. This observation suggests crosstalk between these abiotic stresses. It seems that ABA is a key regulator in this pathway. ABA induces stress tolerance and causes growth inhibition in response to abiotic stresses (Kodaira et al. 2011). However, ABA was reported to have no effect in occurrence of allelopathic response at germination stage of *Lepidium sativum* exposed to myrigalone, an allelochemical of sweet gale but severely interfered with gibberellin metabolism (Oracz et al. 2012; Voegele et al. 2012).

Inconsistent expression of the genes across annual *Medicago* species speculates that genetic control networks governing transcription are highly complex and variable among genotypes (Karami et al. 2015). An augmented expression of *CBF4*, *Zpt2*-2, *GST*, and *CAT* in *M. lupulina* which showed more juglone-tolerance can be associated with higher tolerance against allelochemical stress. Similar results were reported by other researches who found that tolerant genotypes did not induce major changes at the transcriptional level in response to stress (Taji et al. 2004; Becher et al. 2004; Weber et al. 2004; de Lorenzo et al. 2007). The higher expression of stress-responsive genes in tolerant plants which was also observed in the present study is possibly an adaptive mechanism to cope with abiotic stress. Considering the presence of crosstalk between different abiotic stresses, we presumed that *M. lupulina* with higher expression in stress-respond genes is more tolerant to other abiotic stresses in comparison with *M. polymorpha*.

Conclusion

We concluded that *M. polymorpha* was more juglone-sensitive than *M. lupulina* as it manifested chlorosis of leaves within a week. Therefore, it may not be grown in walnut orchards. *M. lupulina* which showed more tolenace to juglone in short term, had higher expression of stress-respond genes. Long-term exposure of juglone should be experimented to find out if this *Medicago* specie can be a suitable candidate plant to grow in walnut orchards.

Author contribution statement Fariba Rafiei contributed to design of the experiment, data analysis, and preparation of the first draft of the paper; Zohre Torabi performed the experiments and collected data; Leila Shabani run enzyme assay, and the concentration of H_2O_2, and GSH; Abdol Razagh Danesh-Shahraki helped the team in running the experiment and applying juglone treatment.

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