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Biochemical and gene expression responses of two Iranian grape cultivars to foliar application of methyl jasmonate under boron toxicity conditions

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ABSTRACT

Boron (B) toxicity is a serious problem for the growth and development of plants in arid and semiarid areas. Jasmonates enhance the capacity of plants to grow under stressful environmental conditions. The objective of this experiment was to study the effect of foliar application of methyl jasmonate MeJA (at 0 and 100 µM) on biochemical and gene expression responses of two Iranian grape cultivars ('Bidaneh Ghermez' and 'Rashe') under B toxicity conditions (0, 10 and 20 mg B /kg soil). With increase in B levels of the soil, the leaf B content, malondialdehyde (MDA), hydrogen peroxide, total phenolics, total flavonoids, total protein and proline content, as well as total antioxidant activity and the activity of antioxidant enzymes (except for the ascorbate peroxidase) were significantly ($P \leq 0.01$) increased. Under B toxicity conditions, foliar application of MeJA at 100 µM enhanced the activity of catalase, superoxide dismutase and phenylalanine ammonia lyase enzymes, and decreased MDA and proline content in the leaves of both cultivars. In addition, MeJA significantly ($P \leq 0.01$) influenced the accumulation of B in grape leaves. B accumulation in the leaves of Rashe cultivar was lower than that of 'Bidaneh Ghermez'. The expression of *VvPAL* and *VvBOR1* genes in all treatments were lower than those of controls. MeJA enhanced the expression of *VvPAL* under B toxicity conditions while the expression of *VvBOR1* gene was declined. The expression of *VvBOR1* in 'Rashe' was significantly ($P \leq 0.01$) lower than that of 'Bidaneh Ghermez' cultivar. According to the results, Rashe cultivar performed better as compared with Bidane Ghermez cultivar in response to increased soil B levels and MeJA treatment.

1. Introduction

Table grapes are among the most important horticultural crops produced in commercial scale in Iran. 'Rashe' ('Siahe Sardasht') is one of the main grape cultivars with a high antioxidant capacity grown in Kurdistan province. 'Bidaneh Germez' is another popular seedless and red-skinned grape cultivar cultivated in different regions in Iran (Ataei et al., 2017). Gapes are commonly produced in arid and semiarid regions of the country with a high possibility for different environmental stresses. Boron (B) is an essential micronutrient for the normal growth and development of higher plants, but B toxicity may have undesirable effects on different growth processes, such as metabolism, root cell division, photosynthesis, leaf chlorophyll content and some biochemical responses (Reid, 2013). As a global problem in arid and semiarid regions, B toxicity decreases the growth and development of plants including grapes (Yermiyahu and Ben-Gal, 2006). The response of plants to B toxicity are found to be modulated by genetic (Nable et al., 1990), other nutrient (Samet et al., 2015), phytohormones and environmental conditions (Aftab et al., 2011; Wang et al., 2011).

Jasmonates (JAs) are a group of oxidized lipids (oxylipins) derived from linolenic acid and are considered as ubiquitous phytohormones in plants (Cheong and Choi, 2003). The most well-known function of JAs in plants is the regulation of plant defense-related genes under stress conditions (Wasternack, 2017). Methyl jasmonate (MeJA) is a methyl ester of JA with a ubiquitous signal in plants acting as a resistance mediating agent in response to pests, pathogens and different abiotic stresses (Wolucka et al., 2005; Jin et al., 2009). The main and common responses of plant to exogenous MeJA is the production and accumulation of different antioxidants, anti-stress compounds and secondary metabolites (Sun et al., 2013; Asghari and Hasanlooe, 2015).

Although reactive oxygen species (ROS) and free radicals are produced normally during plant cellular metabolism, but stress conditions results in excessive generation of ROS and free radicals leading to the oxidative damage of different biomolecules such as lipids and proteins

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(Molassiotis et al., 2006; Jajic et al., 2015). Like other stresses, B toxicity have been shown to enhance the production of ROS in grape (Gunes et al., 2006) and apple rootstocks (Molassiotis et al., 2006).

Plants employ multiple antioxidant systems such as superoxide dismutase (SOD), catalase (CAT), perosidases, vitamin C and E and other enzymatic and no-enzymatic antioxidants for detoxifying the ROS and free radicals (Foyer and Noctor, 2000; Mishra et al., 2006). Phenolic compounds are also among the most important secondary metabolites acting as powerful antioxidants in plants (Hossain et al., 2009). Phenylalanine ammonia lyase (PAL) is the first and key enzyme in the production of phenolic compounds via the phenylpropanoid pathway, and it has been demonstrated that JAs enhance the expression of PAL genes under normal and stressful conditions in some plants (Cocetta et al., 2015; Brouki Milan et al., 2017; Neocleous et al., 2012; Zhang et al., 2015).

Identification of the genes involved in B tolerance is a prerequisite for the development of B-tolerant or B-accumulating cultivars (Rámila et al., 2016). Under B toxicity condition, plants use disparate types of B transporters to cope with the narrow optimum range of B concentration (Yoshinari and Takano, 2017). BOR1, a member of the BOR family of transporter proteins, exports borate out of cells toward the xylem and contributes to the translocation of B from roots to shoots (Takano et al., 2002, 2010).

VvBOR1, the grapevine ortholog of *AtBOR1*, encodes an efflux B transporter located in the plasma membrane and is differentially expressed. However, in the presence of B toxic levels, *BOR1* is degraded via endocytosis (Takano et al., 2005; Pérez-Castro et al., 2012), and overexpression of *BOR1* gene results in a reduced plant growth (Miwa et al., 2006).

Since the role of MeJA in modulating different stress related genes including B toxicity resistance ones is unknown, then this study was conducted to determine the effects of exogenous MeJA on gene expression and biochemical behavior of different grape seedlings under B toxicity conditions.

2. Material and methods

2.1. Plant materials, treatments and experimental design

A pot culture experiment was conducted to evaluate the effects of MeJA (at 0 and 100 μM) on biochemical responses and gene expression of two Iranian grape (*Vitis vinifera* L.) cultivars ('Rashe' and 'Bidaneh Germez') under different B concentrations (0, 10, and 20 mg/kg B in pot soil). One-year-old own-rooted seedlings of the two cultivars were planted in plastic pots with a diameter and height of 20 and 24 cm, respectively (one plant per pot). The pots were filled with air dried soil and sand of 2:1 ratio while the seedlings were dormant during the winter. Pots were transferred to the greenhouse with day/night temperatures of 38 °C/19 °C. During the experiment, standard care procedures were applied, and water of pot soil was kept at field capacity by irrigation with tap water. Each pot was individually weighed, and decreased amounts of water from field capacity were added to pots on a daily basis. After bud breaks, all vines were trimmed to a single shoot. Uniform vines were selected, and the seedlings with shoots of 25–30 cm in length (60 days after planting) were treated with different B and MeJA levels.

B was added to the pot soils in the form of boric acid (Applichem, Germany). Foliar spray of MeJA (Sigma, Aldrich) was carried out twice during the experiment. The first spray was performed at the same time with B treatments, and the second treatment was applied 48 h before leaf sampling for biochemical and molecular evaluations. Half of the vines were sprayed with 100 μM MeJA in deionized water with 0.1% (v/v) Tween-20. The remaining plants were sprayed with carrier solution in separate section of the greenhouse. Concentrations of MeJA were sprayed over the foliage to run off. After 24 h, all vines were positioned in the same greenhouse. Four weeks following the treatment, three young fully developed leaves were harvested per plot, immediately frozen in liquid N, and kept at −70 °C until biochemical and molecular evaluations.

2.2. Determination of B concentration in the leaves

B concentrations in the leaves were determined by azomethine-H method (Wolf, 1971). The samples were reduced to dry ash in a furnace at 550 °C for 6 h. After digestion, the absorbance of the samples was read by spectrophotometer at 420 nm. Different concentrations of boric acid were used to draw a standard curve. Leaf B content was determined in terms of milligrams per kilogram fresh weight.

2.3. Total phenolics (TP) content evaluation

TP content in the methanol extracts were measured using Folin–Ciocalteu reagent, as described by Singleton and Rossi (1965). Gallic acid (GAE) was used as a standard, and the results were expressed as mg gallic acid equivalents per g fresh weight.

2.4. Determining total flavonoids (TF) content

To measure TF content, 50 μL of methanolic extract was mixed with 150 μL sodium nitrite (5%) and kept for 5 min at room temperature. Then, 300 μl of 10% aluminum chloride was added and after 5–10 min, 1 mL of NaOH (1 M) was added, and the total volume of the material reached to 5 mL. TF content was spectrophotometrically measured at 360 nm and expressed as mg quercetin- equivalent per gram fresh weight (Chang et al., 2002).

2.5. Total protein content

Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 mL of 95% ethanol. To this solution 100 ml of 85% (w:v) phosphoric acid was added. The resulting solution was diluted to a final volume of one liter. Inside the cells, 2.5 mL of the prepared Coomassie blue solution was poured and then 100 μL of leaf extract from each sample was added to the cells. Ultimately, the absorbance of samples and standard solutions was read through spectrophotometer at 595 nm. Total protein content was obtained after drawing a standard curve and expressed as mg per 100 g of fresh weight (Bradford, 1976).

2.6. Proline content determination

Leaf samples (0.5 g) was used to prepare alcoholic extract according to Irigoyen et al.'s (1992) method. One mL of alcoholic extract was diluted with 10 mL distilled water and mixed with 5 mL acid-ninhydrin and 5 ml of glacial acetic acid in a test tube. The mixture was incubated in water bath for 45 min at 98–100 °C and allowed to cool at room temperature. The mixture was extracted with 10 mL benzene, and the absorbance was recorded at 515 nm, expressed as mg per gram of fresh weight (Paquin and lechasseur, 1979).

2.7. Evaluation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents

0.1 *g* chopped fresh leaves were ground with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation at 18,000 × g for 10 min, an aliquot of 0.5 mL of the supernatant was added to 5 mL of 0.5% (w:v) TBA in 20% (w/v) TCA. The samples were incubated at 95 °C for 30 min. Thereafter, the reaction was stopped using an ice bath. Centrifugation was accomplished at 10,000 × g for 5 min, and the absorbance of the supernatant was recorded at 532 nm with spectrophotometer. The values were adjusted for non-specific turbidity by subtracting the absorbance at 600 nm, and MDA content was expressed as micromole per gram fresh weight (Popham and Novachy, 1991).

H_2O_2 levels were determined according to the method of Velikova et al. (2000). Leaf tissues (0.5 *g*) were homogenized in ice bath with 3 ml 0.1% (w:v) TCA. The homogenate was centrifuged at 12,000 × g for 15 min, 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH = 7.0), and 1 mL 1 M KI. The absorbency of the supernatant was read at 390 nm and expressed as micromole per gram fresh weight.

2.8. Total antioxidant activity (AA)

The free radical-scavenging activity of the leaves was measured using the method described by Espin et al. (2000) with modifications. 0.5 *g* leaf tissue was mixed with 10 ml ethanol (80%) and centrifuged at 4000 × g for 10 min. 50 µL ethanol extract was mixed with 1000 µL DPPH 0.1 mM and kept for 30 min at room temperature. The absorbance of the reaction mixture was measured at 517 nm with spectrophotometer.

2.9. Antioxidant enzymes activity evaluation

To prepare 100 mL of extraction buffer, 0.607 *g* of Tris-base was dissolved with 0.05 g of polyvinylpyrrolidine in 90 mL of distilled water and then, with HCl, the pH of the solution was adjusted to 8, and then the solution was dissolved in a final volume of 100 mL. The buffer was used to extract protein and antioxidant enzymes. To extract the samples, first, 0.5 *g* samples stored in the refrigerator at −70 °C were thoroughly crushed with liquid nitrogen and completely homogenized after adding 2 mL of extraction buffer. The mixture was centrifuged for 15 min at 13,000 × g, and then the upper phase was used to read out the antioxidant activity of the enzymes.

2.9.1. Superoxide dismutase (SOD) activity

SOD activity was assayed by the nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich, 1971). This measurement is based on the ability of the superoxide dismutase enzyme to stop the photochemical regeneration of NBT by superoxide radicals in the presence of riboflavin and light. In this method, 50 µL of extract with one mL superoxide dismutase solution, which contains 50 µM of potassium phosphate buffer (pH = 7.8), 75 µM NBT, 13 µM L-methionine, 0.1 M EDTA and 2 µM riboflavin, was mixed. The absorbance was measured at 560 nm. The activity of SOD in the samples was expressed as U g^{-1}FW.

2.9.2. Catalase (CAT) activity

CAT activity was determined according to the method of Aebi (1984). 50 µL of extract was mixed with 1 mL of catalase measurement solution containing 50 mmol L^{-1} potassium phosphate buffer (pH = 7) and 15 mmol L^{-1} hydrogen peroxide (H_2O_2). Then, the absorbance was read by spectrophotometer at 240 nm for one minute. An enzymatic unit of catalase decomposes a micromole hydrogen peroxide in one minute.

2.9.3. Ascorbic peroxidase (APX) activity

APX activity was determined by following the decrease of ascorbate by change in absorbance at 290 nm (Nakano and Asada, 1981). 50 µL of extraction was mixed with 2 mL of potassium phosphate buffer (0.05 mM with pH = 7), and 20 µL of Hydrogen peroxide 5 µM in an ice bath and immediately was added to 50 µL of enzyme extracts. Finally, by adding 10 µL of ascorbic acid 50 µM, the absorption change curve was read at 290 nm for 1 min with spectrophotometer. An enzyme unit of ascorbic peroxidase decomposed a micromolar ascorbic acid in one minute.

2.9.4. Phenylalanine ammonia lyase (PAL) enzyme activity

PAL activity was assayed according to the method of D'Cunha et al. (1996). One mL of potassium phosphate buffer (50 mM, pH = 7), 0.5 mL of phenylalanine 10 mM, 0.4 mL of distilled water, and 0.1 mL of enzymatic extract mixed for one hour at 37 °C. The reaction was stopped by adding 0.5 mL of chloride acid 6 M and was measured at 290 nm. The level of PAL activity in the samples was expressed as milligram per gram fresh weight based on the cinnamic acid standard.

2.10. VvPAL and VvBOR1 gene expression analysis

2.10.1. RNA extraction and cDNA synthesis

Total RNA was extracted from the leaves (0.2 mg) using CTAB method. The quality and quantity of the RNA and complementary DNAs (cDNAs) were determined using a NanoDrop (Thermo 20030, USA). cDNAs were synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions. Negative control reactions including reverse transcriptase minus (RT-) negative control and no template control (NTC) were performed during cDNA synthesis to recognize genomic DNA and reagent contamination of RNA samples. To test the right working of the cDNA synthesis kit, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control RNA (1.3 kb), supplied with the kit, was also converted to cDNA, and PCR was carried out with specific primers. A fragment of 496 bp was observed in 1.5% agarose gel. All control reactions were carried out in line with the instructions provided in the kit.

2.10.2. Primer design and real time PCR reaction

Coding sequences of *VvPAL*, *VvBOR1*, *GAPDH* and *PEP* genes were obtained from the National Center for Biotechnology Information (NCBI). Specific primers were designed using Fast PCR 4.0 and Gene runner 3.05 softwares. Primers were blasted against nucleotide database in NCBI to ensure their specificity (Table 1).

Annealing temperature of the genes was optimized using PCR with 20 µL final volume containing 3 µL diluted cDNA (500 ng/µL), 2 µL 10 × PCR buffer (10 mM Tris−HCL, 50 mM KCl, pH = 8.3), 0.7 µL $MgCl_2$ (50 µM), 0.5 µL dNTP (10 µM), 0.25 µL Taq DNA polymerase (5 u/µL), 1 µL from each primer (10 pmol L^{-}) and 11.55 µL twice distilled water. PCR conditions were as follows: an initial denaturation step of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 40 s at 72 °C, followed by a final extension step of 5 min at 72 °C. PCR reactions were run in an Eppendorf thermal cycler. Amplified products were separated on 1.8% agarose gel and stained with ethidium bromide, and gel documentation system (Gel Logic 212 PRO, Carestream, USA) used

Table 1
Primer sequences, annealing temperature (Ta) and amplicon length of *VvPAL*, *VvBOR1*, *GAPDH* and *PEP* genes in grape.

Amplicon length (bp)	Ta (°C)	Primer sequence	Accession number	Gene
125	58/2	*ATCTCTCTGGAAGCCGAAAC* F: 5'- R: 5'- AGCACTTTCGACATGGTTGG	X75967.1	*VvPAL*
117	60	F: 5'- ACGCTTGAATGAGTTGAGG GCTTGAAGACGAACCATGAGG R: 5'-	XM_010653992.2	*VvBOR1*
125	57	TTCCGTGTTCCTACTGTTG F: 5'- R: 5'- CCTCTGACTCCTCCTTGAT	CN938023	*GAPDH*
128	58	F: 5' - CCTCCTCCTCCAGATTGC R: 5'- GGCTTGCTTGATTCCATTATC	NC_012018.3	*PEP*

for imaging.

Real-time PCRs were performed in a volume of 12.5 µL in Rotor-Gene Q (QIAGE, USA) using Maxima SYBER Green/Flourescein qPCR Master Mix (Fermentase, Germany) according to the manufacturer's recommendations. Temperature requirements were as follow: 10 min at 95 °C as initial denaturing temperature and 40 cycles of 95 °C for 30 s, 30 s at 57–60 °C (Table 1), 40 s at 72 °C. Three biological replicates for each sample were used for real-time PCRs. To verify the specificity of the amplicons, agarose gel (1.8%) and melting curve analysis (45–95 °C) were used. Out of the two reference genes (*PEP* and *GAPDH*) tested, *GAPDH* was used as a reference gene for data normalization.

2.11. Statistical analysis

The study was conducted as a factorial experiment (Two grape cultivars, three B levels and two MeJA levels) based on randomized complete block design with three replications (each replication consisting of one table grape seedling). The ΔΔCT method was used for quantitative real-time PCR data analysis (Pfaffi, 2001). Normality test for the data and residuals was carried out by MINITAB 16. Software. The genes expression levels, for all the treatments, were compared with the control plants.

3. Results

3.1. B concentrations in grape leaves

In both cultivars with increase in soil B level, the B concentration of the leaves were significantly ($P \leq 0.01$) increased. In all treatments, B concentrations in the leaves of 'Bidaneh Ghermez' was higher than those of Rashe cultivar. Exogenous MeJA significantly enhanced the accumulation of B in the leaves of both cultivars (Fig. 1).

3.2. Biochemical responses of the cultivars to B and MeJA treatments

With increase in B level, the TP content of leaves was significantly enhanced. The highest TP content was observed in Rashe cultivar treated with 20 mg/kg B and 100 µM MeJA. Foliar application of MeJA increased TP and TF content in the leaves of 'Rashe' cultivar under boron toxicity conditions. In the applied B levels, no regular trend was observed for 'Bidaneh Ghermez' in view of TP and TF content (Table 2).

Both treatments significantly influenced total protein, proline, MDA and H_2O_2 contents ($P \leq 0.01$). With increase in B level of the soil the contents of protein, proline, MDA and H_2O_2 in the leaves were significantly increased. Foliar application of MeJA significantly increased total protein and H_2O_2 content, and decreased the proline and MDA content in the leaves of both cultivars, indicating the activation of resistance mechanisms and retention of cell integrity in response to MeJA treatment. In all treatments, the highest MDA content was recorded in 'Bidaneh Ghermez' while the highest concentration of H_2O_2 was observed in Rashe cultivar (Tables 2 and 3). Indicating that Rashe is tolerant than 'Bidaneh Ghermez' against B toxicity.

A significant difference ($P \leq 0.01$) in antioxidant activity (based on DPPH assay) was found between treatments (Table 3). The antioxidant activity was increased with B and MeJA treatments in both cultivars. The antioxidant activity of 'Rashe' leaves was 64.67% in control seedlings treated with only MeJA, while it was significantly increased up to 97.19% when the plants treated with 20 mg/kg B and 100 µM MeJA. In Bidaneh Ghermez cultivar, antioxidant activity was increased from 59.17% in control plants with no MeJA treatment up to 94.75% in plants subjected to 20 mg/kg B and treated with 100 µM MeJA (Table 3).

3.3. Antioxidant enzyme activity

With increase in B concentration of the pot soil, the activity of SOD and CAT was increased and MeJA substantially enhanced the activity of these enzymes in both cultivars under normal and B toxicity conditions. The highest CAT activity was recorded in plants of Rashe cultivar subjected to severe B toxicity (20 mg/kg) and treated with MeJA (Figs. 2 and 3). Showing that the more tolerant cultivar produces more antioxidant enzymes under B toxicity and in response to MeJA treatment. In contrast to SOD and CAT activities, B treatment significantly reduced the activity of APX in both cultivars. Foliar application of MeJA increased the APX activity in control plants but not B toxicity conditions (Fig. 4).

The activity of PAL in 'Rashe' cultivar was significantly higher than that of 'Bidaneh Ghermez' in response to increased B levels and MeJA treatment. With increase in B concentration, MeJA enhanced PAL activity in 'Bidaneh Ghermez' but the highest PAl activity in Rashe cultivar was recorded in plants treated with MeJA under moderate B levels (Fig. 5).

3.4. Expression of VvPAL and VvBOR1genes

The expression of *VvPAL* and *VvBOR1* genes was significantly ($P \leq 0.01$) different in two cultivars. The expression of *VvPAL* and *VvBOR1*genes in all treatments were lower than those in controls. Following an increase in B level from 10 mg/kg to 20 mg/kg, the expression level of *VvPAL* and *VvBOR1* genes was significantly increased in both cultivars, meanwhile *VvBOR1* expression in Bidaneh Ghermez cultivar was higher than 'Rashe' under B stress conditions. This may explain to some what the reason for the susceptibility of 'Bidaneh Ghermez' to B toxicity. Foliar application of MeJA enhanced the

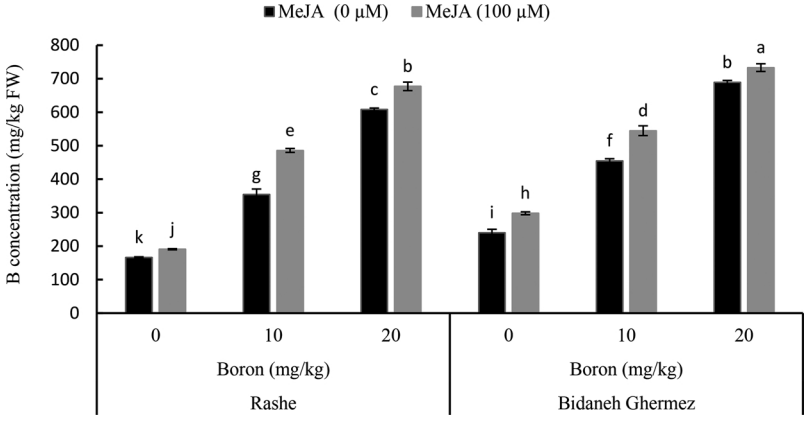

Fig. 1. Effect of B and foliar application of MeJA on the leaf B concentration in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on Duncan's multiple range test at $P < 0.01$, and the vertical bar indicated to ± SE (n = 3).

Table 2
Effect of increasing levels of B and MeJA on some biochemical traits in two grape cultivars.

Treatments	Traits			
	TP (mg GAE /g FW)	TF (mg QE/g FW)	Protein (mg/100 FW)	Proline (mg/g FW)
B1Cl1 M1	12.69 ± 0.035 ^{g}	1.57 ± 0.115 ^{c}	10.19 ± 0.115 ^{h}	4.81 ± 0.039 ^{h}
B1Cl2M1	12.22 ± 0.024 ^{g}	0.58 ± 0.090 ^{g}	6.42 ± 0.303 ^{i}	2.55 ± 0.039 ^{j}
B1Cl1 M2	17.20 ± 0.024 ^{f}	0.99 ± 0.125 ^{d}	12.57 ± 0.303 ^{g}	4.90 ± 0.067 ^{h}
B1Cl2M2	10.28 ± 0.035 ^{h}	0.64 ± 0.090 ^{f}	10.85 ± 0.403 ^{h}	3.84 ± 0.051 ^{i}
B2Cl1 M1	18.34 ± 0.180 ^{e}	1.57 ± 0.169 ^{c}	13.43 ± 0.066 ^{f}	6.87 ± 0.039 ^{e}
B2Cl2M1	8.60 ± 0.018 ^{j}	0.91 ± 0.107 ^{e}	11.84 ± 0.175 ^{g}	6.22 ± 0.039 ^{f}
B2Cl1 M2	21.8 ± 0.498 ^{c}	1.67 ± 0.021 ^{b}	14.62 ± 0.175 ^{de}	6.09 ± 0.025 ^{f}
B2Cl2M2	9.65 ± 0.061 ^{i}	0.42 ± 0.062 ^{i}	13.82 ± 0.066 ^{ef}	5.11 ± 0.039 ^{g}
B3Cl1 M1	30.61 ± 0.035 ^{b}	1.63 ± 0.149 ^{b}	18.58 ± 0.175 ^{c}	13.98 ± 0.039 ^{a}
B3Cl2M1	19.68 ± 0.035 ^{d}	0.52 ± 0.074 ^{h}	15.21 ± 0.066 ^{d}	9.86 ± 0.064 ^{c}
B3Cl1 M2	31.23 ± 0.018 ^{a}	2.7 ± 0.071 ^{a}	28.04 ± 0.229 ^{a}	11.81 ± 0.039 ^{b}
B3Cl2M2	18.82 ± 0.221 ^{e}	0.58 ± 0.090 ^{g}	19.51 ± 0.115 ^{b}	7.34 ± 0.025 ^{d}

Values in the same column with different letters are significantly different based on the Duncan's multiple range test at $P < 0.01$. Each value represents mean and ± SE (n = 3). B: Boron (B1 = 0 mg/kg, B2 = 10 mg/kg and B3 = 20 mg/kg), C: Cultivars (C1 = Rashe and C2 = Bidaneh Ghermez) and M: Methyl Jasmonate (M1 = 0 μM and M2 = 100 μM).

Table 3
Effect of increasing levels of B and MeJA on some biochemical traits in two grape cultivars.

Treatments	Traits		
	MDA (μmol/g FW)	H_2O_2 (μmol/g FW)	DPPH (%)
B1Cl1 M1	0.24 ± 0.009 ^{j}	0.24 ± 0.003 ^{g}	64.67 ± 1.280 ^{h}
B1Cl2M1	0.31 ± 0.004 ^{i}	0.15 ± 0.002 ^{j}	59.17 ± 1.095 ^{i}
B1Cl1 M2	0.07 ± 0.004 ^{k}	0.27 ± 0.006 ^{f}	82.02 ± 1.135 ^{f}
B1Cl2M2	0.09 ± 0.004 ^{k}	0.18 ± 0.002 ^{i}	64.92 ± 0.833 ^{h}
B2Cl1 M1	0.91 ± 0.006 ^{f}	0.31 ± 0.006 ^{e}	85.02 ± 1.265 ^{e}
B2Cl2M1	1.30 ± 0.010 ^{e}	0.19 ± 0.003 ^{h}	77.21 ± 0.631 ^{g}
B2Cl1 M2	0.71 ± 0.008 ^{h}	0.35 ± 0.007 ^{d}	92.94 ± 0.740 ^{c}
B2Cl2M2	0.83 ± 0.007 ^{g}	0.23 ± 0.005 ^{g}	88.26 ± 1.453 ^{d}
B3Cl1 M1	2.02 ± 0.006 ^{b}	0.51 ± 0.008 ^{b}	94.13 ± 1.147 ^{b}
B3Cl2M1	2.26 ± 0.008 ^{a}	0.32 ± 0.007 ^{e}	91.94 ± 1.365 ^{c}
B3Cl1 M2	1.35 ± 0.010 ^{d}	0.73 ± 0.007 ^{a}	97.19 ± 1.095 ^{a}
B3Cl2M2	1.75 ± 0.009 ^{c}	0.42 0.006 ^{c}	94.75 ± 1.075 ^{b}

Values in the same column with different letters are significantly different based on the Duncan's multiple range test at p < 0.01. Each value represents mean and ± SE (n = 3). B: Boron (B1 = 0 mg/kg, B2 = 10 mg/kg and B3 = 20 mg/kg), C: Cultivars (C1 = Rashe and C2 = Bidaneh Ghermez) and M: Methyl Jasmonate (M1 = 0 μM and M2 = 100 μM).

expression level of *VvPAL* and decreased *VvBOR1* expression under boron toxicity conditions in both cultivars (Figs. 6 and 7).

4. Discussion

As a new finding, we recorded the differences between the two studied cultivars in response to B toxicity and MeJA treatment. The resistant cultivar, Rashe, showed higher activation of resistance mechanisms including total antioxidant activity, lower B accumulation in the leaves and MDA production, higher PAL and antioxidant enzymes activity, phenolics, flavonoids, proline and proteins contents under B toxicity conditions. MeJA significantly enhanced the resistance mechanisms in both of the studied cultivars but the Rashe cultivar showed more increase in most of resistance mechanisms in response to MeJA treatment under B toxicity conditions. The activity of *VvBOR1* gene was higher in 'Bidaneh Ghermez' cultivar under B toxicity conditions.

Phytohormones, as the active members of signal transduction pathways in the plants, are involved in the induction of plant stress responses (Pedranzani et al., 2003). Abiotic stresses including B toxicity negatively affect plant metabolism and development, and to deal with these stresses, exogenous application of plant hormones might have a great importance (Jaleel et al., 2009; Reid, 2013). Our results demonstrate that B toxicity significantly affects the biochemical compounds

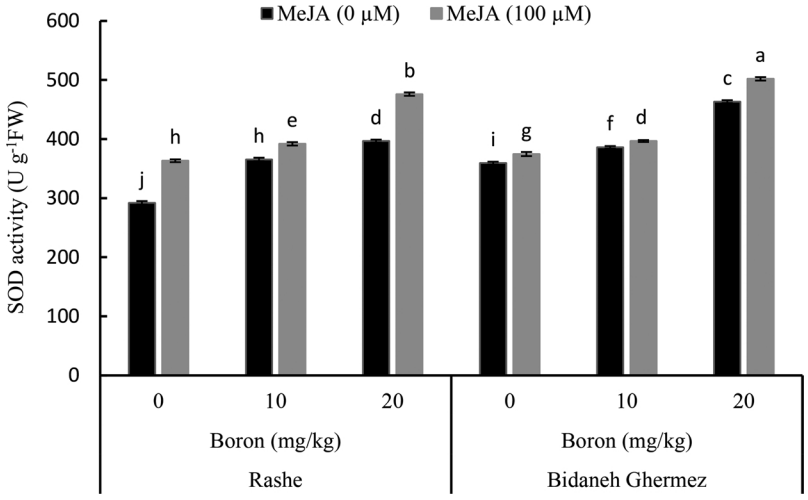

Fig. 2. Effect of B and foliar application of MeJA on the SOD activity in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at p < 0.01, and the vertical bar indicated to ± SE (n = 3).

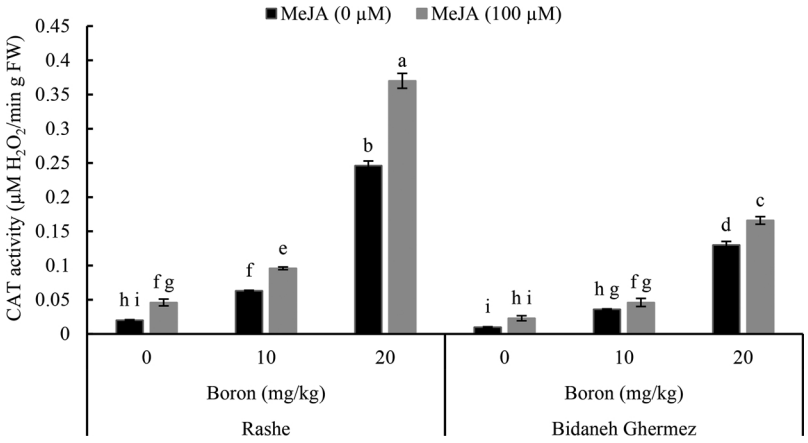

Fig. 3. Effect of B and foliar application of MeJA on the CAT activity in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at $p < 0.01$, and the vertical bar indicated to ± SE (n = 3).

and gene expression responses of grape seedlings, and the responses of two cultivars are different. By increase in the levels of soil B, the concentration of B in the leaves of the seedlings was significantly increased. It seems that the resistant genotypes reduce the absorption and intracellular B levels under B toxicity conditions (Rámila et al., 2016). Therefore, B accumulation in the leaves of resistant cultivars is lower than those in sensitive ones. Our results show that Rashe cultivar has a series of strong protection mechanisms for reducing B accumulation and negative effects. Piotrowska et al. (2009) reported that jasmonic acid in a concentration-dependent manner increased lead (Pb) accumulation in *Wolffia arrhiza* plants under lead toxicity conditions.

This study indicated that increase in B levels improved phenolics and flavonoid contents, especially in the case of Rashe cultivar, and MeJA significantly enhanced the production and accumulation of these compounds in both cultivars. Phenolic compounds play important roles in plant growth and development, particularly in defense mechanisms. Moreover, plant phenolic compounds are the first defense mechanisms of the plants in dealing with different stresses. Jasmonates as stress mediating phytohormones have been shown to enhance phenolic compounds production in different plants (Wolucka et al., 2005; Hura et al., 2008; Rahmati et al., 2014; Asghari and Hasanlooe, 2015).

In this study, total proline and total protein content was significantly increased in response to increased soil B levels. Proline is the most widely distributed compatible solute accumulating in plants during adverse environmental constraints and playing key roles in plant stress tolerance. Increase in proline level and enhanced H_2O_2 accumulation has been considered as a common response of plants under B toxicity conditions (Karabal et al., 2003). Proline is also known as the activator of the Krebs cycle reactions, thereby increasing the photosynthesis reactions rate and subsequent energy flow of plants (Gunes et al., 2006). Besides acting as an excellent osmolyte, proline acts as an inducer of metal chelators, antioxidative defense systems and a signaling molecule for resistance mechanisms activation (Hayat et al., 2012). Higher accumulation of proline and proteins in resistant genotypes of camelina plants under stress conditions have been reported by Ahmed et al. (2017). Foliar application of MeJA, under normal conditions, enhanced proline content but decreased the rate of increase in proline content in response to elevated B levels. Proline accumulates in response to ROSs, and MeJA enhances the antioxidant capacity under stress conditions leading to decreased ROSs and proline content (Sun et al., 2013; Rejeb et al., 2014). In addition, MeJA induces the production of different defense proteins under stress conditions leading to an increase in total protein levels (Cheong and Choi, 2003). Increase in total protein levels were reported in strawberry plants under salinity stress conditions (Neocleous et al., 2012).

The generation of reactive oxygen species (ROS) is one of the earliest responses of plants to stress conditions (Jajic et al., 2015). ROS includes free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), as well as nonracial molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O2$), etc. (Sharma et al., 2012). All of these activated oxygen species are extremely reactive and cytotoxic in all organisms attacking different cell organelles as well as membranes. If not detoxified, they cause oxidative damage resulting in lipid peroxidation, protein denaturation and MDA production. MDA production rate is an

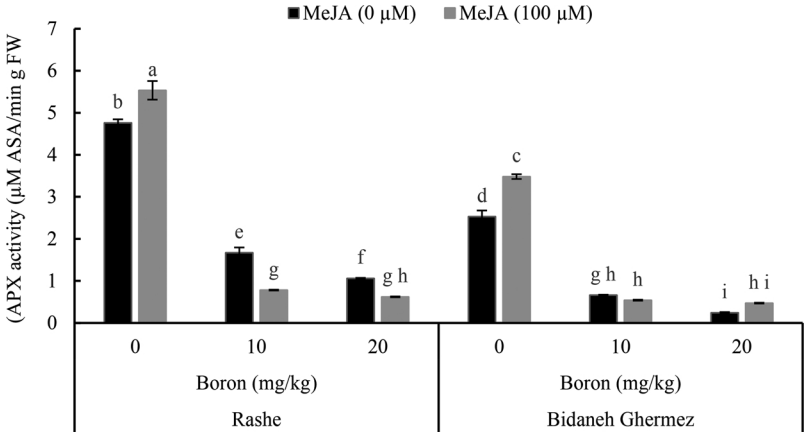

Fig. 4. Effect of B and foliar application of MeJA on the APX activity in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at $p < 0.01$, and the vertical bar indicated to ± SE (n = 3).

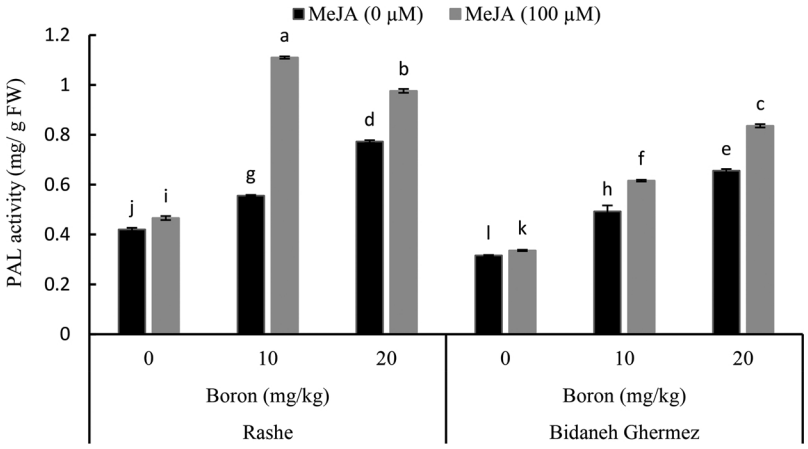

Fig. 5. Effect of B and foliar application of MeJA on the PAL activity in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at $p < 0.01$, and the vertical bar indicated to ± SE (n = 3).

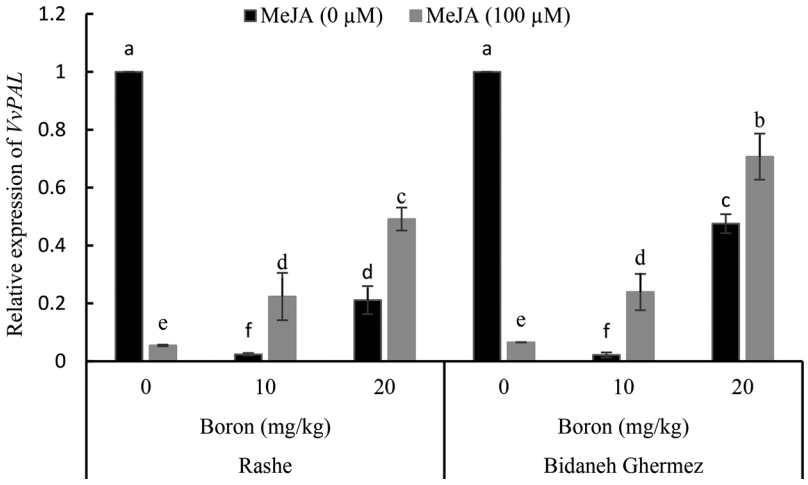

Fig. 6. Effect of B and foliar application of MeJA on the relative expression of *VvPAL* gene in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at $p < 0.01$, and the vertical bar indicated to ± SE (n = 3).

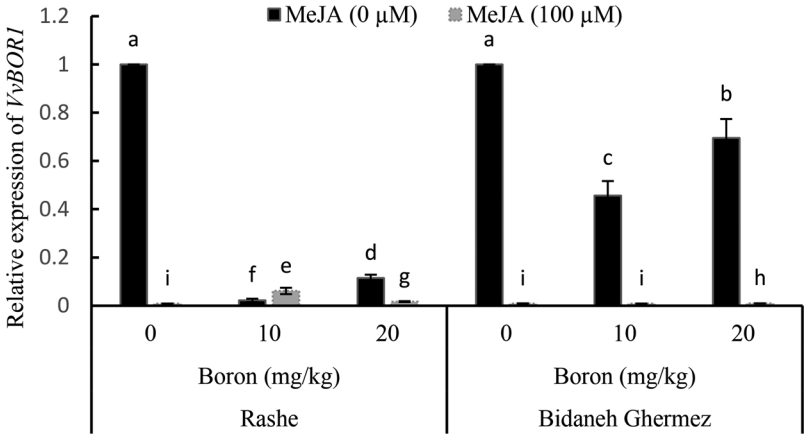

Fig. 7. Effect of B and foliar application of MeJA on the relative expression of *VvBOR1* gene in Rashe and Bidaneh Ghermez cultivars. Columns with different letters are significantly different based on the Duncan's multiple range test at $p < 0.01$, and the vertical bar indicated to ± SE (n = 3).

indicator of cell membrane damage extent due to stress conditions. Under stressful conditions, ROS and free radicals are produced to enhance the activation of different resistance mechanisms. Accordingly, the cells employ different antioxidant systems to detoxify the ROS and free radicals. If the cells are able to produce enough amounts of antioxidants, they will combat the oxidative stress (Foyer and Noctor, 2000; Mishra et al., 2006). In this study, the increased levels of different antioxidants and decreased rate of MDA production in response to MeJA treatment under B toxicity conditions indicate that MeJA is able to retain cell integrity by enhancing the antioxidant capacity of the cells. MeJA has been reported to reduce MDA accumulations in banana plants under water stress conditions (Mahmood et al., 2012).

In response to different stress conditions H_2O_2 is produced as a result of SOD activity which converts superoxide radicals to H_2O_2, leading to the activation of resistance mechanisms. The toxic H_2O_2 then should be eliminated by catalase (CAT) and other peroxidases (Mishra et al., 2006), thereby maintaining membranes of plant tissues and decreasing MDA production. B toxicity induces oxidative stress by increasing the level of H_2O_2 and lipid peroxidation (MDA) in the leaves (Gunes et al., 2006; Molassiotis et al., 2006; Wang et al., 2011). However, there are contradictory results for the effects of MeJA on H_2O_2 content in different plants under stress conditions (Gunes et al., 2006; Mahmood et al., 2012). In this study, MeJA enhanced the CAT activity leading to the scavenging of H_2O_2.

Higher levels of antioxidants measured in this study as DPPH activity was associated with an increase in B levels, and foliar application of MeJA improved total antioxidant activity in both cultivars. It has been demonstrated that exogenous MeJA enhances the antioxidant capacity of pomegranate fruits (Sayyari et al., 2011). An increase in leaf antioxidant capacity under salinity stress was attributed to the raised total phenolics content (Neocleous et al., 2012). According to our results, the effect of MeJA on increasing total antioxidant activity of grape cultivars under B toxicity condition is the result of the increase in total phenolic compounds and different antioxidant enzymes such as CAT and SOD. Rashe cultivars with a higher level of total phenolic and antioxidant activity exhibited a better tolerance against B stress.

PAL is the key enzyme in phenylpropanoid pathway producing different phenolic compounds with powerful anti-stress and antioxidant activities (Singh et al., 2010). The resistant cultivars show the higher PAL activity and phenolic compound production and MeJA has been demonstrated to enhance the activation of PAL enzyme in some plants (Hura et al., 2008; Nadernejad et al., 2012). In this study, Rashe cultivar showed higher *VvPAL* gene expression and enzyme activity. MeJA significantly enhanced the expression levels of *VvPAl* gene under B toxicity condition. In addition, MeJA enhanced the activity of PAL enzyme in both normal and B toxicity conditions resulting in an increase in different phenolics and flavonoid compounds.

Based on the results of this study, although there was an increase in the expression of *VvPAL* gene with an increase in B toxicity, in all treatments, this expression was found to be lower than control plants (plant received no MeJA under normal conditions). The down-regulation of *VvPAL* gene in plants treated with B and MeJA might be explained by the sampling time (Four weeks after treatment with B). It is possible that the expression of *VvPAL* gene is first up-regulated at the time of B stress and MeJA treatment, while with progress in stress conditions the byproduct of the expressed genes, phenolics, are accumulated leading to the down-regulation of the same genes. Similarly, Zhang et al. (2015) reported that most of the pathogenesis-related genes were up-regulated under drought stress, but the expression of these genes was down-regulated under a prolonged stress. A positive effect of MeJA on vv*PAL* gene expression have been reported in basil (Brouki Milan et al., 2017). Belhadj et al. (2006) also found that, in MeJA-treated grape leaves, the expression of *PAL* gene increased rapidly reaching the peak after 18 h and then slowly decreased until 72 h after the treatment. Similarly, Farooq et al. (2016) also reported that the exogenous application of MeJA further regulated the activities of PAL, PPO, and CAD as well as their relative mRNA levels.

VvBOR1 encodes an efflux B transporter located in the plasma membrane and is differentially expressed in response to different stimuli (Pérez-Castro et al., 2012). *VvBOR1* expression was down-regulated under B toxicity levels compared to control plants (especially in Rashe cultivar). It has been shown that in the presence of B toxic levels, *BOR1* is degraded via endocytosis (Takano et al., 2005; Pérez-Castro et al., 2012), and over-expression of *BOR1* gene culminates in a reduced plant growth (Miwa et al., 2006). However, the degradation of *BOR1* in the presence of excessive B levels has been demonstrated (Takano et al., 2005; Pérez-Castro et al., 2012). Exogenous MeJA extremely decreased *VvBOR1* expression in the leaves of both studied grape cultivars. However, the expression of *VvBOR1*gene in Rashe cultivar was significantly lower than Bidane Ghermez cultivar at toxic concentrations of B (10 and 20 mg/kg B). It has been reported that *BOR1* expression in Arabidopsis is regulated by translational suppression, which, together with the selective degradation of BOR1, causes a reduction in the accumulation of BOR1 under high B concentration (Aibara et al., 2018). Surgun et al. (2016) found that *BOR1* expression level was significantly down-regulated in the leaves of Arabidopsis plants subjected to B toxicity treatments as compared to the control. They also found that the application of brassinosteroids (BRs) declined *BOR1* expression in leaves and roots of Arabidopsis plants under B toxicity conditions (Surgun et al., 2016). However, the effect of these stimuli can be different depending on the applied concentrations (Farooq et al., 2016; Surgun et al., 2016).

5. Conclusion

The results of this study demonstrate that B toxicity affects biochemical and gene expression responses of Rashe and Bidaneh Ghermez grape cultivars and induces oxidative stress leading to the membrane damage and MDA production. Two cultivars exhibited different levels of antioxidants, phenolics and flavonoid compounds and PAL and *VvBOR1* genes expression levels under B toxicity conditions. MeJA significantly enhanced the resistance of both cultivars against B toxicity stress via enhancing PAL and different antioxidant enzymes activity, phenolic compounds, flavonoids, total antioxidant activity and *VvPAL* gene expression levels. Furthermore, MeJA decreased MDA production and *VvBOR1* gene expression leading to the more tolerance of the plants against stress conditions. By increasing the expression levels of *VvPAL* gene and PAL enzyme activity, MeJA increased total phenolics and flavonoids content leading to enhanced total antioxidant activity of the grape cultivars under B toxicity levels. We can conclude that Rashe cultivar is resistant than Bidane Ghermez cultivar against elevated B concentrations of the soil, and the resistance extent of both cultivars can be increased by MeJA foliar spray. However, future studies may evaluate the expressions of other key genes involved in phenylpropanoid pathway and other B toxicity resistance mechanisms as well as the morpho-physiological parameters to clarify diverse aspects of grape cultivars resistance against B toxicity conditions in response to MeJA treatments.

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