Investigation in expression of key genes involved in sanguinarine biosynthetic pathway in *Papaver* genus under drought and salinity

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Abstract

The presence of morphine alkaloids, anti-microbial and anti-cancer metabolite, sanguinarine, in different species of *Papaver* genus has made it one of the most valuable plants in the pharmaceutical industry. With regard to the influence of drought and salinity on the increase of secondary metabolites, the present study attempted to investigate the effect of the mentioned stresses on the key genes expression involved in the sanguinarine biosynthetic pathway. Therefore, the expression of *BBE1*, *DIOX2* and *DBOX* genes involved in the biosynthesis of sanguinarine was investigated under drought (50% FC), salinity (100 mM NaCl) and non-stress conditions in four species of *P. somniferum*, *P. bractateum*, *P. armeniacum* and *P. argemone* in a factorial experiment based on a completely randomized design with three replications. Total RNA was extracted from leaves and roots of non-stressed and stressed plants, and then cDNA was synthesized and used for quantitative real-time PCR reactions. The results showed that, increases in the expression of *BBE1*, *DIOX2* and *DBOX* genes in each of four species under drought condition were more pronounced than salinity. In addition, the level of gene transcripts in roots was more than leaves. The expression of each of the three genes was higher in *P. somniferum* and *P. bractateum* compared to *P. armeniacum* and *P. argemone*; so that the highest transcription levels of *BBE1* (seven-fold), *DIOX2* (four-fold) and *DBOX* (six-fold) in *P. bractateum* were related to root under drought, leaf under salinity and root under drought stresses, respectively. *BBE1* expression in the studied species was more than *DIOX2* and *DBOX* genes, under the mentioned stresses. In fact, according to sanguinarine biosynthesis pathway, *BBE1* is a branching point gene and it seems that these species divert the biosynthesis pathway of benzylisoquinoline alkaloids towards the production of sanguinarine by increasing *BBEI* gene expression under stress conditions. In conclusion, our results support the idea that *P. somniferum* and *P. bractateum* could be used as suitable candidates for metabolite engineering and high yield extraction of sanguinarine.

Key words: *BBE1*, *DBOX*, *DIOX2*, Gene expression, Sanguinarine**.**

INTRODUCTION

Opium poppy is one of the plants used as drug ever since the beginning of humanity (Brownstein, 1993). This plant is the commercial source of the analgesics morphine, codeine and semi-synthetic analogs including oxycodone, hydrocodone, buprenorphine and naltrexone (Berenyi *et al*., 2009). Among other benzylisoquinoline alkaloids (BIAs) found in opium poppy, the antimicrobial agent sanguinarine, the muscle relaxant papaverine and the cough-suppressant and potential anticancer drug noscapine can be highlighted. Around 2500 different kinds of BIAs have been identified in various nitrogen-containing

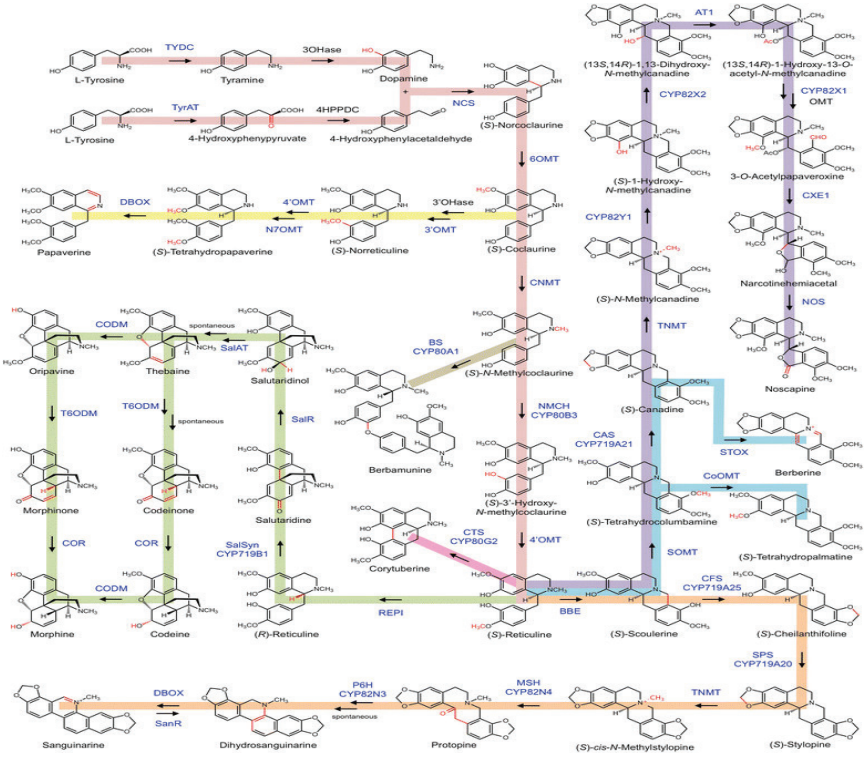

Figure 1. Biosynthesis pathway of benzylisoquinoline alkaloids sanguinarine, noscapine and morphine in opium poppy (Hagel *et al.,* 2015). (BBE: Berberine bridge enzyme, DBOX: Dihydrobenzophenanthridine oxidase, SOMT: Scoulerine -O methyltransferase).

metabolites. This BIAs can also be found particularly in the families of *Papaveraceae*, *Ranunculaceae*, *Berberidaceae* and *Menispermaceae* (Liscombe *et al*., 2005).

Benzophenanthridines as a subcategory of isoquinoline alkaloids (Figure 1) is produced via S-reticuline and S-scoulerine (Kutchan and Zenk, 1993). S-reticuline produces morphine (Weiczorek *et al*., 1986), and S-scoulerine acts as the precursor to sanguinarine biosynthesis (Cline and Coscia, 1988). S-scoulerine is produced from S-reticuline by acts of berberine bridge (*BBE1*) enzyme (Frenzel and Zenk., 1990). Generally, Benzophenanthridines are defensive compounds whose production is induced by biotic and abiotic stresses (Villegas *et al*., 2000). Scoulerine-O-demethylase (*DIOX2*) is another key gene in sanguinarine biosynthesis process, which converts S-scoulerine to 6-O-demethylation as another precursor in sanguinarine biosynthesis. In fact, 6-O-demethylation is the result of the first enzyme biosynthesis after S-scoulerine, which diverts the alkaloid synthesis process to the toxic sanguinarine production. *DIOX1* catalyzed the 6-O-demethylation of the betaine and oripavine, yielding codeinone and morphinone, respectively. Conversely, *DIOX3* catalyzes the 3-O-demethylation of codeine and betaine, yielding morphine and oripavine, respectively. *DIOX2* does not accept the betaine, oripavine or codeine as substrates (Hagel and Facchini, 2010). Dihydrobenzo phenanthridine (*DBOX*) is an important gene in the sanguinarine biosynthesis pathway, which has the responsibility of setting threshold sanguinarine cellular toxicity. Since the dihydrobenzo phenanthridine enzyme is responsible for the conversion of dyhidro sanguinarine to sanguinarine; it acts by increasing sanguinarine in the cell. Therefore, its expression is reduced in cells to keep the cells from sanguinarine poisoning (Cho *et al*., 2008).

Real-time PCR is one of the most accurate methods for determining changes in gene expression. The changes in gene expression is compared with an internal control known as the standard or reference gene, and then the normalized CT value, is measured with regards to a control sample (Pfaffl, 2001). Among the genes which can be utilized as an internal control in the process of gene expression, are *18S rRNA, ubiquitin (UBQ), actin (ACT), β-Tubulin (TU)* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH*). Housekeeping

Table 1. Accession number and characteristics of seeds of *Papaver* species used in this study.

Species	Country	Province	Family	Accession no.	Date of collection
P. somniferum	IRAN	Tehran	Papaveraceae	IBRC P1010901	6. 28. 2015
P. bracteatum	IRAN	Tehran	Papaveraceae	IBRC P10071107	7. 14. 2011
P. argemone	IRAN	Tehran	Papaveraceae	IBRC P1006478	5. 20. 2010
P. armeniacum	IRAN	Tehran	Papaveraceae	IBRC P1006433	9. 24. 2011

genes are expressed in all cells with the same copy number and their expression does not change under stress conditions (Jain *et al*., 2006). The *ubiquitin* (*UBQ*) gene which binds to the protein and causes its degradation was used as the reference gene, due to its high expression (Welchman *et al*., 2005).

Identifying the pathway of secondary metabolites biosynthesis and how to set it up are the most important steps in the metabolite engineering. Recently, isoquinoline alkaloids biosynthetic pathway was identified, hence opening the way for metabolites engineering. For instance, the enzyme stimulation by elicitors is one of the employed methods for metabolite engineering (Sato *et al.,* 2001). Elicitors are compounds with either biotic or abiotic origins, which cause the biosynthesis and accumulation of secondary metabolites through the induction of defense responses (Namdeo, 2007). In this context, Choudhary *et al.* (2005) reported that *P5CS* (*D-pyrroline-5-carboxylate*) gene expression increased in drought condition (Choudhary *et al.,* 2005). Furthermore, an increase in the expression of *guanylate kinase* (signal transduction), *lytB* (antibiotic/drug resistance), *selenium-binding protein* (heavy metal stress), *polyprotein* (reverse transcriptase), and AC-like transposable element genes were also reported in response to drought and salinity condition in sunflower (Liu and Baird, 2003). Also another study revealed that the expression of *DREB2A* and *DREB2B* genes in *Arabidopsis thaliana* increased under salinity (Nakashima *et al*., 2000).

Since plants in *Papaver* genus contain compounds with medical properties and play a crucial role in the treatment of many diseases, more research is required for increasing their secondary metabolites. With regard to the effect of drought and salinity conditions on the increase of secondary metabolites and considering the arid and semi-arid coverage of many regions of the world, the present study attempted to investigate the effect of salinity and drought stresses on the key genes expression involved in the sanguinarine biosynthesis pathway.

MATERIALS AND METHODS

This research was conducted in the genomics laboratory of Imam Khomeini International University. Four species of *Papaver* genus were used to evaluate the expression of *BBE1*, *DIOX2* and *DBOX* genes in two different tissues (root and leaf) under drought, salinity and non-stress conditions. For this purpose, the seeds were provided from the Iranian Biological Resource Center (IBRC) (Table 1). To increase seed viability, they were treated with gibberellic acid solution with a concentration of 100 ppm for 24 hours and then sown. The seeds were sown in pots (20×20) in greenhouse conditions with factorial experiment based on a completely randomized design with three replications. The pots were irrigated normally up to 60 days after planting, with interval of 3 days. The treatments of drought (50% field capacity) and salinity (100 mM Nacl) stresses were performed on the four species of *Papaver* genus (*P. somniferum*, *P. bractateum*, *P. armeniacum* and *P. argemone*) at twelve-leaf stage for 14 days. Upon the appearance of signs of stress, leaf and root samples were taken and samples were quickly stored at –80 °C for RNA extraction.

Relative water content for each sampled treatment was calculated in three replications. In order to measure the relative water content (RWC), samples of fresh leaf tissues were weighed from the three youngest leaves (FW) and then leaves were soaked in distilled water for 16-18 hours, and turgid weight (TW) was measured after drying with tissue paper. Finally, to measure dry weight (DW), samples were placed in the oven for 48 hours at 70 °C. The samples relative water content was calculated using RWC=(FW-DW/TW-DW)×100 equation (Turner, 1981).

Design of qRT-PCR primers

The primers for *BBE1*, *DIOX2*, *DBOX* and *ubiquitin* genes were designed and then synthesized by Bioneer Company (Table 2). Primers were designed by the Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html) and Primer3 (http://bioinfo .ut.ee/primer 3-0.4.0/). Specificity of amplification of the

Table 2. List of primer sequences used in qRT-PCR reaction for *BBE1*, *DIOX2* and *DBOX* genes expression under drought and salinity in four species of *Papaver* genus.

Primer sequence (5'→3')	Amplified fragment length	GenBank accession number	Gene name
F TGTGAGAAACTGAAGAACACACAAT **R** AAGGACTCAGACCACTGAAAGACG	90	GQ500140.1	*DIOX2*
F GAGAACTGAGATAGCACCAGAAC **R** GTCTGATTACATCGCCCTACAA	107	AF025430.1	*BBE1*
F CACCCAACTCAACACAAACAC **R** CATGACCACCACTTCGTACTT	123	JX390714.1	*DBOX (ADOX5)*
F CTCCAGGGATTCGAACGGAG **R** TGT CCCTGTGAAATCAAGAA	120	kf614970.1	*Ubiquitin*

aforementioned genes were examined and confirmed by Primer BLAST software of the NCBI database (http://www.ncbi.nlm.nih.gov/). Furthermore, their performance was also evaluated by PCR reaction.

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNX-Plus kit (CinnaGen, Iran) and its concentration was checked by Nano Drop spectrophotometer (Thermo, USA). Also, the quality of extracted RNA was evaluated by 1% agarose gel electrophoresis. Then extracted RNAs were treated with RNase-Free DNase I (Fermentas, Germany) according to the manufacturer's instruction to eliminate remaining genomic DNA. The first-strand cDNA was synthesized from 1 µg total RNA with oligo $(dT)_{18}$ primer in a final reaction volume of 20 µL using the Reverse Transcription Kit (Product No. RTPL12, Vivantis, Malaysia) according to the manufacturer's instruction. The synthesized cDNAs to be used as template in real-time PCR reaction were diluted 20-times and finally were stored at –20 °C.

Quantitative real-time PCR reactions and data analysis

Expression level of *BBE1*, *DIOX2* and *DBOX* genes was determined using real-time PCR detection system (Bio-Rad, USA). The PCR reaction mixture contained diluted cDNA (50 ng), SYBR Green qPCR Master Mix 1X (SYBR [Premix Ex TagII (Tli RNAase Plus), Bulk. Cod. RR820L]), gene specific primer pairs (0.4 µM) and distilled water in a final volume of 15 µl. The PCR program was set as follows: primary denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 10 sec, annealing step at 60 °C for 20 sec and extension at 72 °C for 25 sec. The specificity of the amplified fragments was investigated by melting curve analysis carried out from 60 °C to 95 °C at 60 cycles. Finally, the data were analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen , 2001).

RESULTS

Effect of drought and salinity stresses on relative water content

Data analysis revealed a significant difference ($P \leq 0.01$) among stress conditions in relative water content (RWC) after stress treatments. Mean comparison results showed that RWC significantly decreased under stresses, so that the highest (76.19%) and the lowest (52.82%) values for RWC were obtained for non-stress and drought conditions (Figure 2). Our finding on the reduction of RWC in plants under abiotic stresses was in agreement with the previous reports monitoring the level of RWC in plants under stress conditions (Altinkut *et al*., 2001; Vaezi *et al*., 2010).

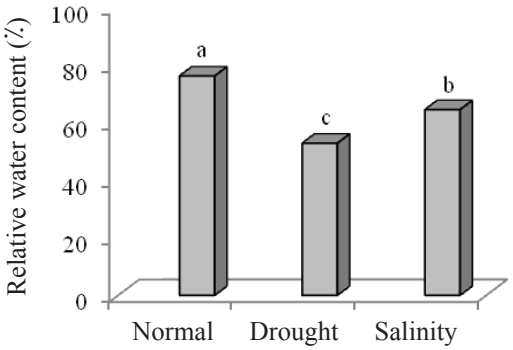

Figure 2. Mean comparison of RWC under drought, salinity and non-stress (normal) conditions in four *papaver* genus (Different letters above columns indicate significant difference with non-stress condition at P<0.01).

Expression profile of *BBE1*, *DIOX2* and *DBOX* in *P. argemone*, *P. armeniacum*, *P. bracteatum* and *P. somniferum*

In this research the expression of three genes in response to drought and salinity was investigated in four species of *Papaver* genus. Statistical analysis in

Table 3. Analysis of variance for *BBE1*, *DIOX2* and *DBOX* genes expression under drought and salinity stresses in *P. argemone*, *P. armeniacum*, *P. bracteatum*, and *P. somniferum*.

Sources of variation	df	Mean of Squares (MS)											
		P. argemone			*P. armeniacum*			*P. bracteatum*			*P. somniferum*		
		BBE1	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*
Stress	2	4.26**	0.94**	0.99**	2.79**	0.73**	3.04**	14.03**	10.29**	13.36**	3.54**	0.04ns	1.57**
Organ	1	31.6**	3.35**	4.70**	14.56**	12.21**	4.45**	77.75**	0.20**	15.66**	13.33**	1.33**	0.34**
Stress × organ	2	4.26**	0.94**	2.29**	2.79**	0.73**	1.88**	14.03**	1.71**	18.91**	3.48**	0.70**	0.13**
Error	12	0.02	0.003	0.04	0.002	0.002	0.013	0.08	0.03	0.07	0.012	0.01	0.01
C.V.%		10.98	11.68	18.70	5.14	4.71	7.29	13.31	7.00	18.88	6.52	9.08	6.12

ns and **: non-significant and significant at P<0.01 level of probability, respectively.

P. argemone species showed significant differences at P<0.01 level in *BBE1*, *DIOX2* and *DBOX* genes expression in organs and stress×organ interaction in response to drought and salinity conditions (Table 3). Relative expression of genes in response to stress conditions in the four species is depicted in Table 4.

Table 4. Mean comparison of *BBE1*, *DIOX2* and *DBOX* genes relative expression in response to drought and salinity stresses in four species of *Papaver* genus.

Condition	*P. armeniacum*			*P. argemone*			*P. bracteatum*			*P. somniferum*		
	BBE1	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*	*BBE1*	*DIOX2*	*DBOX*
Non-stress	0.52c	0.52b	0.98b	0.50b	0.50c	1.02c	0.51c	1.00c	1.00b	1.00c	1.01b	1.03c
Drought	2.20a	0.001c	1.46a	1.69a	1.20a	2.38a	3.57a	2.51b	3.11a	1.54b	1.54ab	2.05a
Salinity	1.26b	0.78a	0.65c	0.51b	0.78b	1.33b	2.20b	3.61a	0.22c	2.52a	2.52a	1.63b

Based on stress×organ interaction mean comparison results in *P. argmone*, transcription level of *BBE1* increased in root in all conditions. However, the uttermost rate (fourfold) of *BBE1* expression was observed in drought stress in root tissues. While, no expression of *BBE1* was detected in leaves (Figure 3A). The data showed that *DIOX2* expression decreased in a similar manner to *BBE1* gene (Figure 3B). Based on results, *DBOX* gene expressed in both root and leaf tissues; however, the level of transcription in leaf tissues was lower in drought and salinity compared to the non-stress condition. In drought condition, root

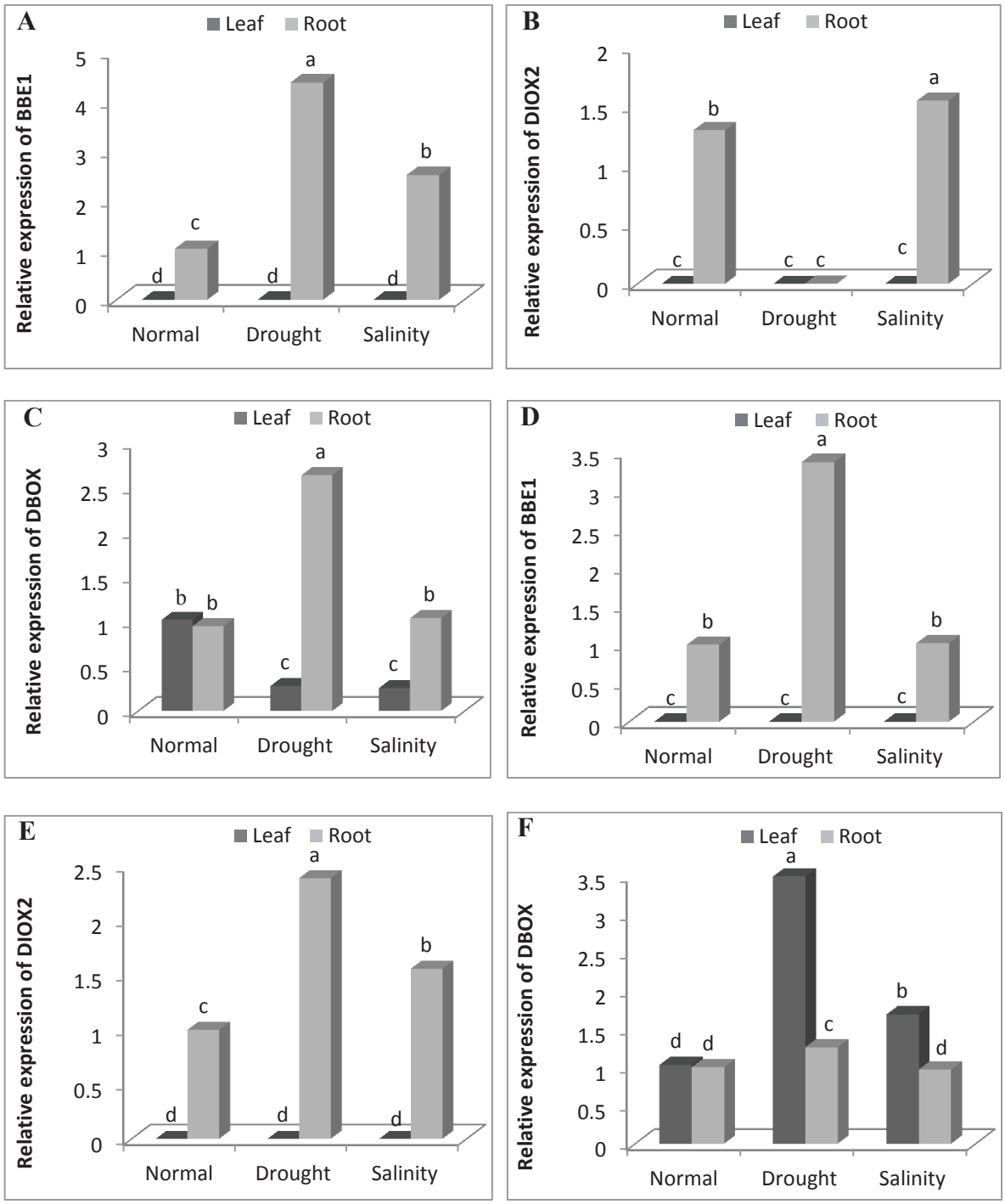

Figure 3. Relative expression of: **A:** *BBE1*; **B:** *DIOX2* and **C:** *DBOX* in *P. argemone*; **D:** *BBE1*; **E:** *DIOX2* and **F:** *DBOX* in *P. armeniacum* under drought, salinity and non-stress (normal) conditions in root and leaf tissues (Different letters above columns indicate significant differences at P≤0.01 level).

tissues had the uttermost level of *DBOX* transcription, while its expression was not significantly different between salinity and non-stress condition (Figure 3C).

Investigation of gene expression in *P. armeniacum* indicated that stresses, organs and stress×organ interaction had significant effects on each of the three studied genes (Table 3). In contrary to leaf tissues, transcription rate of *BBE1* in roots increased significantly at different conditions. The maximum rate of *BBE1* transcription (more than three-fold) was observed in drought stress in root tissues (Figure 3D). Similar to *BBE1*, no expression was observed for *DIOX2* gene in leaves, but the expression level of *DIOX2* increased in roots at different stress conditions, so that, the quantity of *DIOX2* transcription was significantly greater in drought stress (class A) than salinity (class B) and it was more than non-stress (class C) in salinity condition (Figure 3E). *DBOX* gene was expressed in both leaf and root tissues. Mean comparison revealed that the highest (more than three-fold) rate of transcription was related to leaf tissues in drought stress (Figure 3F).

Evaluation of expression level for *BBE1*, *DIOX2*, and *DBOX* genes among the four species revealed that the maximum transcription rate was related to *P. bracteatum*. Moreover, the expression level of *BBE1* in the root tissues showed a seven and four-fold increases in response to drought and salinity stresses, respectively (Figure 4A), while no *BBE1* expression was observed in leaf tissues in *P. bracteatum*. Comparing expression rate of *DIOX2* gene in both organs of *P. bracteatum* in three stress conditions showed that expression of *DIOX2* in leaf was significantly higher than root under drought stress, and vice versa; its expression in root was higher than leaf under salinity (Figure 4B). The highest level of *DBOX* transcripts (more than six-fold) was observed in root in drought stress. In contrary, transcription level of *DBOX* under salinity not only did not increase in root, but also decreased (however, not significantly) compared to non-stress condition (Figure 4C).

Similarly, there was a significant difference among the stresses, organs and stress×organ interaction in *BBE1*, *DIOX2*, and *DBOX* expression for P. *somniferum* (Table 3). These findings indicated that the highest (four-times) and the lowest (0.4-times) transcription rates of *BBE1* occurred in salinity-root and drought-leaf interactions, respectively (Figure 4D). Maximum transcription level of *DIOX2* was observed in root in salinity stress (Figure 4E). Mean comparison of data showed that transcription rate of *DBOX* in different stresses and tissues were higher than those of *DIOX2* and *BBE* genes. In response to drought stress, the root of *P. somniferum* had the uttermost level of *DBOX* transcripts (Figure 4F). Generally, relative expression of genes in four species indicated that transcription rate of three genes increased in response to stress conditions, althogh expressions level under drought stress was more than that of salinity. In addition, the level of gene transcripts and expressions in roots was more than in leaves.

DISCUSSION

Sanguinarine biosynthesis in opium poppy is controlled by developmental and environmental factors (Facchini and Bird, 1998). This research was performed to investigate *BBE1*, *DIOX2* and *DBOX* expressions in response to drought and salinity stresses. Transcription level of *BBE1* in all four studied species, in roots was more than leaves, so that the peak of *BBE1* transcripts was observed in roots of *P. bracteatum* in drought stress. Similarly Rezaei *et al*. (2016) reported that the highest rate of *BBE1* transcription was observed in roots of *Papaver bracteatum* at the bud initiation stage. Also, in agreement with our results, Facchini *et al*. (1996) reported that the highest level of *BBE1* transcription was obtained in root tissues of *P. somniferum*. *BBE1* transcription in leaves of *P. somniferum* was observed and detected in our experiment. In agreement with these results, Facchini and park (2003) and Huang and Kutchan (2000) reported *BBE1* expression in leaves of *P. somniferum*. By considering the fact that the accumulation of sanguinarine exclusively occurs in roots (Facchini and De Luca, 1995), observation of the highest transcriptional level of *BBE1* in roots was expected. Actually, it is possible that sanguinarine intermediates are produced in aerial organs and then transported to the roots in order to produce sanguinarine (Facchini and Bird, 1998).

The research results of Facchini *et al.* (1996) revealed that, different fungal elicitors had various effects on *BBE1* gene expression, which is in agreement with our findings in relation to the expression of this gene under drought and salinity conditions. The expression of *BBE1* gene in four species in two stress conditions was more than those of *DIOX2* and *DBOX* genes. In fact, according to sanguinarine biosynthesis pathway (Figure 1), it seems that these species divert the biosynthetic pathway of benzylisoquinoline alkaloids towards the production of sanguinarine by increasing *BBEI* gene expression under stress conditions. The highest transcription of *DBOX* was detected in both

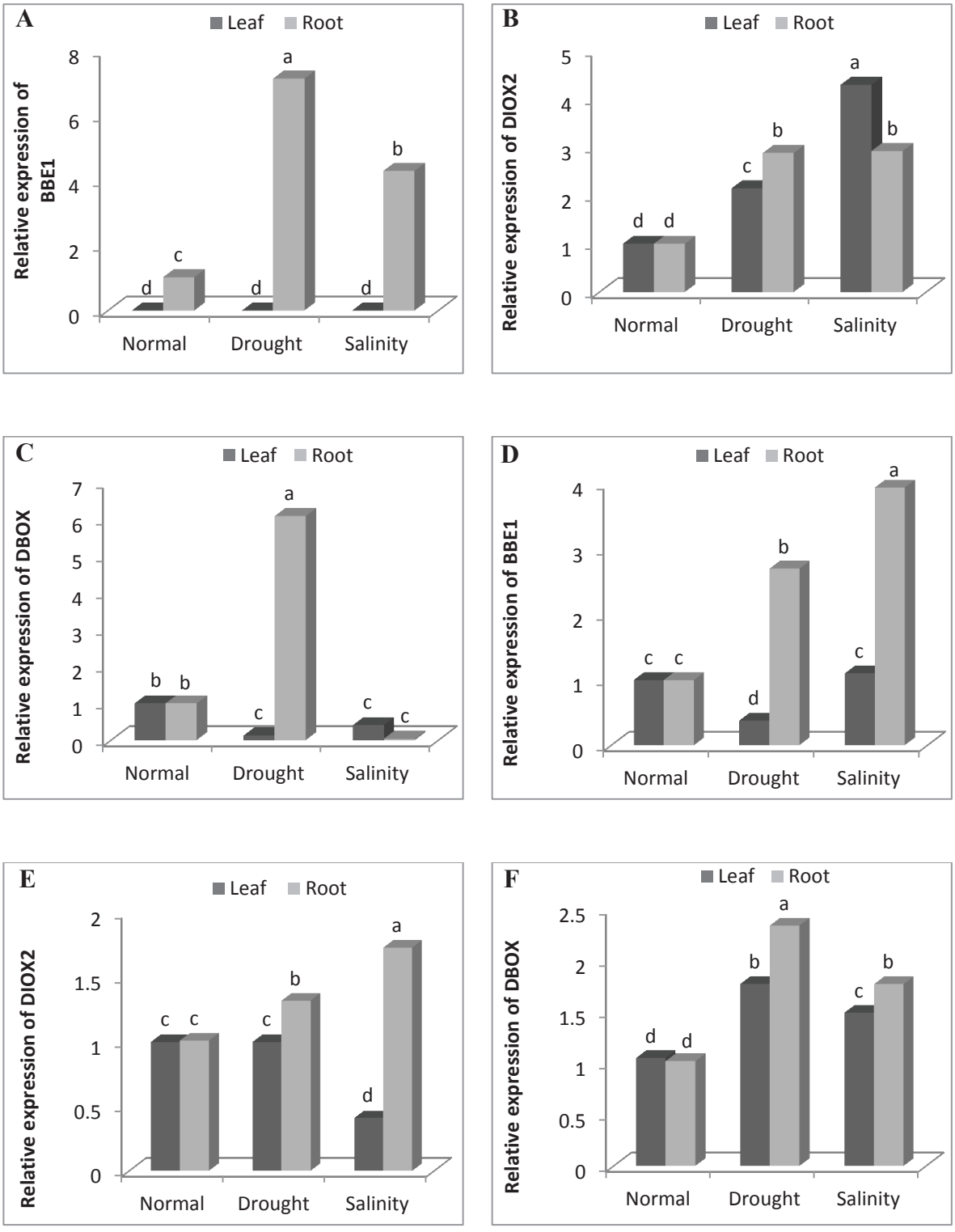

Figure 4. Relative expression of: **A:** *BBE1*; **B:** *DIOX2* and **C:** *DBOX* in *P. bracteatum*; **D:** *BBE1*; **E:** *DIOX2* and **F:** *DBOX* in *P. somniferum* under drought, salinity and non-stress conditions in root and leaf tissues (Different letters above columns indicate significant differences at P≤0.01 level).

leaves and roots of four studied species, with the highest level of transcription being in *P. bracteatum* and *P. somniferum*. Also, our findings showed that in all species (except for *P. armeniacum*), transcription of *DBOX* was higher in roots than in leaves. Similarly, Hagel *et al.* (2012) reported that among different organs, the peak of *DBOX* transcription was related to the roots. However, sanguinarine was not detected

in our research but, the higher level of *DBOX* transcriptions detected in roots, could support the idea that the final step of sanguinarine biosynthesis from intermediates of sanguinarine biosynthesis occurred in root tissues and for this reason, sanguinarine accumulated in roots. In this regard, Facchini and De Luca (1995) reported the accumulation of sanguinarine in the roots of opium poppy. Also, investigation on the effects of methyl jasmonate, salicylic acid and yeast extract on benzophenanthridine alkaloid accumulation showed that, by increasing sanguinarine in roots of *P. somniferum*, transcription level of *DBOX* gene reduced in cells to keep the cells from sanguinarine poisoning (Cho *et al*., 2008). Therefore, the high expression level of *DBOX* gene in *P. somniferum* compared with other species supports the idea that *P. somniferum* may have a high tolerance to sanguinarine toxicity. Based on our results, the uttermost transcription level of *DIOX2* was observed in *P. bracteatum*. Similar to our results, Khodayari *et al.* (2015) reported that transcription of *DIOX2* did not show profound changes compared to other genes (Khodayari *et al*., 2015). In addition, Hagel and Facchini found that *DIOX2* gene silencing had not considerable effects on the accumulation of alkaloids (Hagel and Facchini, 2010).

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