The expression profile of *D4H* and *DAT* genes in *Catharanthus roseus* in response to drought, salinity and salicylic acid

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ABSTRACT

Catharanthus roseus L. is an important medicinal plant producing several terpenoid indole alkaloids (TIAs) such as vincristine and vinblastine secondary metabolites with anticancer activity. The TIAs biosynthetic pathways are affected by biotic and abiotic stresses. In this study the effect of drought (7 days), salinity (150 mM NaCl), foliar spray of salicylic acid (10^{-5} M) and salicylic acid in combination with drought and salinity were investigated separately on the expression of desacetoxyvindoline- 4-hydroxylase *D4H* and deacetylvindoline 4-O-acetyl transferase *DAT*, two late vincristine and vinblastine biosynthetic pathway genes. RNA samples were extracted from leaves and cDNAs were synthesized from all samples and used for analysis in Real time PCR. Data analysis based on Ct curves showed that the expression of these genes increased by the application of abiotic stresses. The maximum increases in the mRNA levels of *D4H* and *DAT* genes (537% and 440%, respectively) were noted in plants exposed to drought stress in comparison to the control condition. Also, the results showed that the effects of salinity, drought + salicylic acid and salinity + salicylic acid stresses were positive on the increase of *D4H* and *DAT* genes expression in comparison to the control plants. Salicylic acid treatment caused a slightly higher gene expression than in control and other treatments. Therefore, it can be concluded that the application of abiotic stresses has a significant role in increasing the expression of genes involved in the biosynthetic pathway of vincristine and vinblastine alkaloids.

Keywords**:** *C. roseus*, *D4H*, *DAT*, Drought, Salicylic acid, Salinity.

INTRODUCTION

Catharanthus roseuse prevalently known as periwinkle is a tropical perennial plant of Apocyanaceae family (Samad *et al*., 2008). It contains a large number of TIAs, among which over 130 substances have been isolated and recognized (Heijden *et al*., 2004). It manufactures several commercially valuable secondary metabolites including the anti-cancerous vinblastine, vincristine and anti-hypertensive alkaloids ajmalicine and serpentine (Verma *et al*., 2012). Catharanthine, tabersonine, lochnericine and horhammericine are other indole alkaloids discovered in *C. roseus* seedlings (Tikhomiroff and Jolicoeur, 2002).

During recent years, the biosynthetic pathway of TIAs has been largely explained, a number of the en-

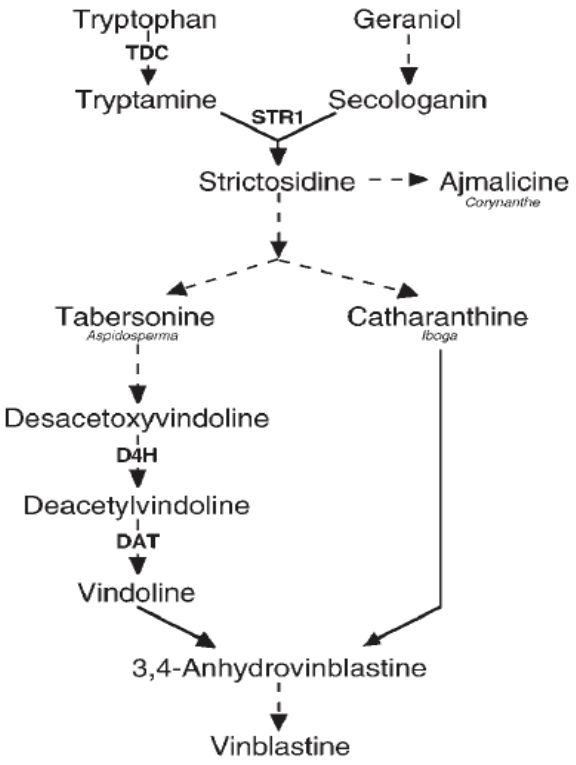

Figure 1. Terpenoid indole alkaloids (TIAs) biosynthetic pathway in *C. roseus* (St-Pierre *et al*., 1999).

zymes involved have been identified and some of the key genes have been cloned (Zhou *et al*., 2009). TIAs biosynthetic pathway in *C. roseus* is complex with various steps and it is under a severe molecular regulation (Liu *et al*., 2011). Vinblastine and vincristine, two dimeric TIA, are synthesized in the leaves of *C. roseus* by the compression of two monomeric TIA, catharanthine and vindoline (St- Pierre and De Luca, 1995; Schroder *et al*., 1999). The biosynthesis of vindoline results from a six enzymatic reaction intervened transmutation of tabersonine (Guirimand *et al*., 2011). The two early and two late TIAs biosynthetic pathway genes, tryptophan decarboxylase (TDC), strictosidine synthase (STR), desacetoxyvindoline- 4-hydroxylase (*D4H*) and deacetylvindoline 4-O-acetyl transferase (*DAT*) have been isolated, partly characterized and over-expressed (Figure 1) (Liu *et al*., 2011). TDC converts tryptophan to tryptamine and it is a key enzyme in the biosynthetic pathway because of its position on the interface of primary and secondary metabolism (Ouwerkerk *et al*., 1999). STR catalyzes the stereospecific condensation of tryptamine and secologanin to form the most significant central intermediate, strictosidine. The other two genes, *D4H* and *DAT*, catalyze the two end steps of synthesis of the leaf monomeric alkaloid in *C. roseus*, vindoline (Dutta *et al*., 2005). *C. roseus* has become a model species for the study of secondary metabolism in plants (Mu *et al*., 2012). Vinblastine and vincristine production cost from *C. roseus* is high, because they are present in very small amounts in leaves (around 0.0005% plant dry weight). Also their extraction is carried out in the presence of many other compounds with very similar chemical and physical properties. Nevertheless, this plant remains the only commercial source for these secondary metabolites (Loyola-Vargas *et al*., 2007; Liscombe *et al*., 2010).

Secondary metabolites are fundamentally produced by genetic processing; but, since the accumulation of secondary metabolites in plant is a common response of plants to biotic and abiotic stresses, concentrations of various secondary plant products are forcefully contingent on the growing situations, particularly stress conditions (Rezaeieh *et al*., 2012). The TIA pathways are not only regulated tissue-specifically and developmentally, but also are affected by exterior biotic and abiotic factors and several environmental conditions have been shown to influence the production of monoterpenoid indole alkaloids (MIAs) in *C. roseus* (Magnotta *et al*., 2007; Wei, 2010). Jing and Zaho (2010), Osman *et al*. (2007) and Idrees *et al*. (2011) found that the vincristine and vinblastine accumulation in the leaves of *C. roseus* increased significantly under drought, salinity and salicylic acid stresses. Using abiotic stresses such as jasmonate in *C. roseus* seedling, Vazquez-Flota and De Luca (1998) found that jasmonate induces the expression of some TIA biosynthetic genes, such as the genes encoding TDC and D4H.

Many researches on plant gene regulation and metabolic engineering have been based on gene expression profiling, which needs quantification of specific mRNA sequences (Schmittgen and Zakrajsek, 2000). One of the best methods utilized for the accurate quantification of gene expression is quantitative Real-time PCR, because of its high sensitivity (Guo and Ki, 2012; Bustin, 2000). In this study, the expression levels of two genes *D4H* and *DAT*, the two late TIAs biosynthetic pathway genes, were examined by quantitative Real time PCR under abiotic stresses: drought, salinity and salicylic acid.

Tabel 1. Primer pairs sequences used in the study of *D4H* and *DAT* genes expression in *C. roseus* under salinity, salicylic acid and their combination using Real- time PCR.

Gene	Primer sequence (5'–3')	Accession number	Amplicon length (bp)	GC (%)
D4H F	TTGGGACAAGCAAGCACTCA	DQ778071.1	118	55
D4H R	GCTCCAGGAATGAAGGGGAC			60
DAT F	TTCCCTCCGGAAGCCATAGA	DQ778074.1	125	55
DAT R	GCTGATTTCCCTGCTACCGT			55
18S*rRNA* F	GCAACAAACCCCGACTTCTG	HF565052.1	148	55
18S*rRNA* R	TGCGATCCGTCGAGTTATCA			50

MATERIALS AND METHODS

Plant growth and experimental design

The seeds of *C. roseus* L., cultivar G. Don were obtained from the Department of Medicinal Plants, Arak, Iran. Seeds surface were sterilized with a 0.2% w/v $HgCl_2$ solution for 5 min with frequent shaking and thoroughly washed three times with deionized water. Then they were sown in plastic pots filled with soil mixture containing moist vermiculite, farmyard manure and perlite at 2:2:1 ratio. This research was carried out based on a completely randomized experimental design (CRD) with three replicates in the glasshouse at 30 ± 2/20 ± 2°C in day/night, respectively, in Imam Khomeini International University, Iran.

Treatments and stress conditions

The pots were irrigated regularly up to 90 days after sowing with an interval of two days. Treatments were applied when the seedlings were at the stage of 10 – 12 leaves. The treatments were as follows: T_1, pots were irrigated with water in two-day intervals as a control. T_2, salinity stress was applied at 150 mM NaCl concentration (NaCl concentration was increased gradually, 50 mM every two days until the desired concentration (150 mM NaCl) was achieved), T_3, drought stress treatment was applied as seven-day interval drought, T_4, salicylic acid was sprayed, using a foliar spray, in concentration of 10^{-5} M three times with 10-day intervals for a period of one month, T_5, a combination of salinity stress (150 mM NaCl) + 10^{-5} M salicylic acid and T_6 a combination of drought stress + 10^{-5} M salicylic acid.

The relative water content (RWC) was determined for each treatment. Leaves Fresh weights (FW) were immediately recorded and then leaves were soaked for 16 hours in distilled water (in darkness) and weighed to determine fully turgid weight (TW). Dry weight (DW) was obtained after drying the leaf samples for 48 h at 70°C. RWC was calculated using the following formula (Khodadadi, 2013): RWC = [(FW – DW) / (TW – DW)] × 100.

RNA extraction and cDNA synthesis

Fresh leaves were harvested randomly, immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Total RNA was extracted from leaf tissues, using RNX-Plus Solution kit (Cinnagen. Co, Iran) according to the manufacture's protocol. Finally, RNA was dissolved in 50 µl diethyl pyrocarbonate (DEPC) treated distilled water. The extracted RNAs were kept at -20°C for further analyses. The RNA samples were treated with RNase-Free DNase I (Fermentas) according to the manufacturer's instructions to eliminate remaining genomic DNA. The concentration of RNA and its purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific Nanodrop 2000, USA). Also, RNA quality was evaluated by 1% agarose gel electrophoresis. The first-strand cDNA was synthesized from 1 µg of total RNA with Oligo $d(T)_{18}$ primer in a final reaction volume of 20 µl using Reverse Transcription Kit (Product No: RTPL12, Vivantis, Malysia) according to the manufacturer's instructions. The synthesized cDNA was diluted 50-fold for using as the template for real-time PCR. The cDNAs were stored immediately at -20°C.

Design of PCR primers

The Real-time PCR primers for genes *DAT*, *D4H* and 18S*rRNA* (as the housekeeping gene) were designed (with equal annealing temperatures 59-60°C) using SoftBerry (www.softberry.com) and primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) software. Oligo5 software was used to confirm the sequence specificity of the designed primer pairs (Table 1). The sequence of these three genes is available at

Table 2. Analysis of variance for RWC *C. roseus* under salinity, drought, salicylic acid and their combinations.

Source of variation	DF	Mean square of RWC
Treatment	5	448.76**
Experimental error	12	6.77
CV (%)		3.68

** Significant at 1% probability level.

National Center for Biotechnology Information Gene Bank sequence information.

Gene expression profiling

The expression profiling of *DAT*, *D4H* and 18S*rRNA* was measured with the Real-time PCR Detection System (Bio-Rad, USA). The PCR reaction mixture contained 3 µl of diluted cDNA, 7.5 µl SYBR Green qPCR Master Mix (SYBR [Premix Ex TagII (Tli RNAase Plus), Bulk. Cod. RR820L]), 0.3 µl of each gene-specific primer and 3.9 µl distilled water in a final volume of 15 µl. Thermal cycling conditions for the PCR were: first denaturation at 95°C for 2 min followed by 42 cycles of denaturation step at 95°C for 10 sec, annealing and extension steps at 60°C for 45 sec. Control PCR reactions with no templates were also performed for each primer pair. The specificity of the amplicons was checked by melting curve analysis performed from 60°C to 95°C at 60 cycles and by agarose gel electrophoresis. The data were further analyzed using $2^{-\Delta\Delta CT}$ method (Livak *et al*., 2001).

Statistical analysis

All data were subjected to one-way analysis of variance and Pearson correlation analysis using SPSS software (version 16.0) and the diagrams were drawn using the EXCEL software. Also, treatments mean were separated using the least significant difference (LSD) test.

RESULTS

Effect of abiotic stresses on leaf relative water content

The results showed that abiotic stresses had a significant effect on leaf RWC at $P < 0.01$ (Table 2). RWC decreased under salinity (53.473) to 39%, salinity + salicylic acid (63.680) to 36%, drought (65.977) to 24% and drought + salicylic acid (73.220) to 16% in comparison to control condition (87.053) plants, respectively. Decrease of RWC in plants treated with salicylic acid (80.987) was low and showed a non-significant reduction in comparison to control plants (Figure 2).

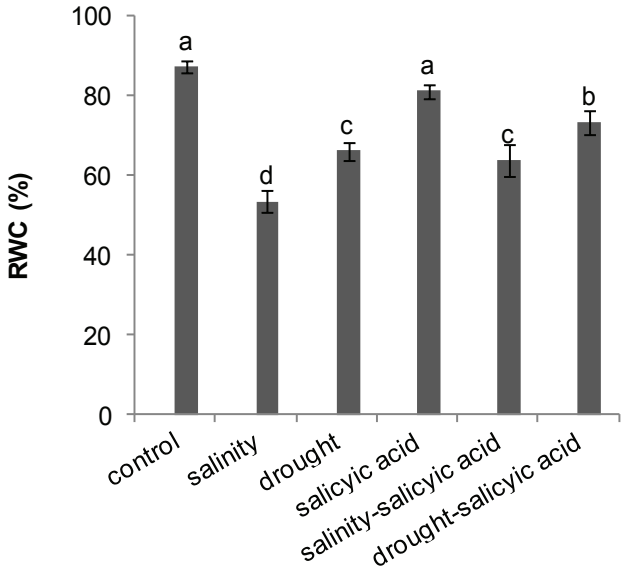

Figure 2. Mean comparisons of leaf RWC under abiotic stresses. Different letters above bars indicate significant differences with the control at the $P < 0.01$ level.

Effect of salinity and salicylic acid foliar spray on the expression of *D4H* and *DAT* genes

Melting curve and the amplification plot of *D4H* and *DAT* genes are shown in Figure 3. Melting curve analysis distinguishes PCR products on the basis of Tm and also it is a useful tool in product identification. Melting curve of *D4H* and *DAT* genes (Figure 3A and B) demonstrated that the correct target DNA had been amplified. The analysis of variance (Table 3) showed that at least one of treatments i.e. salinity, salicylic acid and salicylic acid in combination with salinity had a significant effect on the expression of *D4H* and *DAT* genes. By mean comparisons, the expression level of *D4H* was significantly higher in salinity (by 258%), whereas salicylic acid in combination with salinity (by 19%) and salicylic acid alone (22%) resulted a non-significant increase (Figure 4A). The expression level of *DAT* gene increased significantly under salinity (292%) and salicylic acid in combination with salinity (129%) compared to the control plants. Also a slight increase, but non-significant of *DAT* gene expression was observed due to the foliar spraying of salicylic acid in comparison to the control condition (Figure 5A).

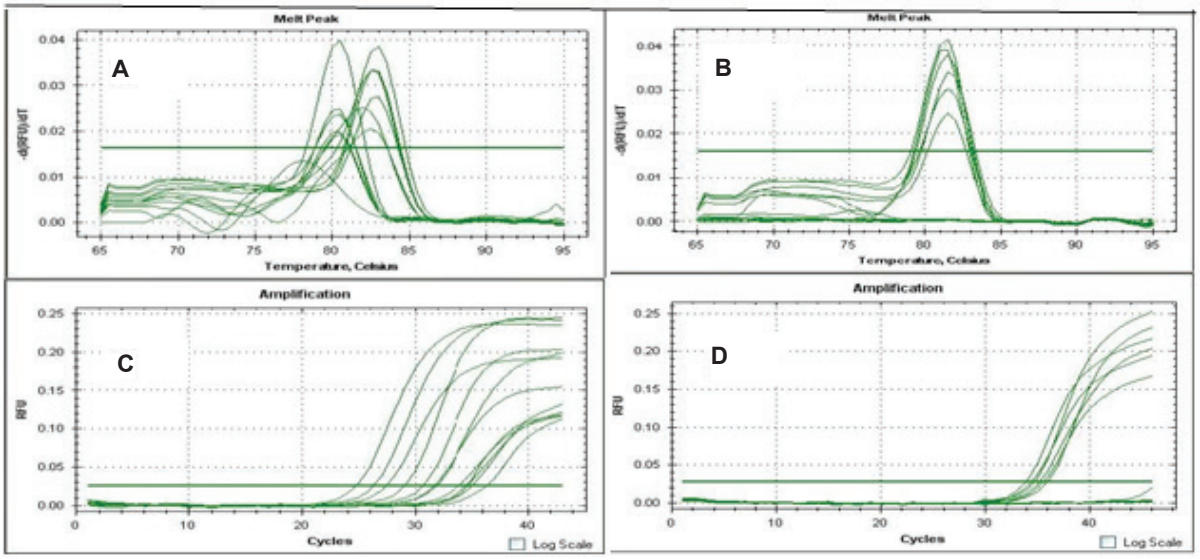

Figure 3. The melting curve of (a) *D4H* (b) *DAT* and amplification plot of (c) *D4H* (d) *DAT* genes under abiotic stresses using SYBER Green in the Real-time PCR.

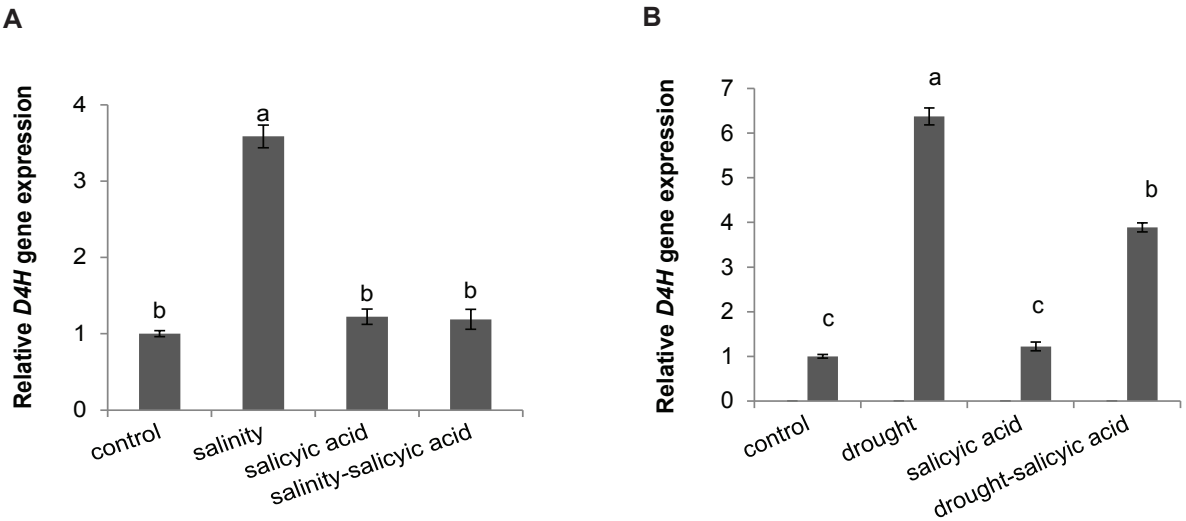

Figure 4. Effects of (A) salinity, salicylic acid and their combination, (B) drought stress, salicylic acid and their combination on *D4H* gene expression in *C. roseus*. Different letters above bars indicate significant differences with the control at the P < 0.01 level.

Effects of drought and salicylic acid foliar spray on the expression of *D4H* and *DAT* genes

The results of ANOVA indicated that drought stress, salicylic acid and salicylic acid in combination with drought stress increased significantly ($p < 0.01$) the expression levels of *D4H* and *DAT* genes (Table 4). *D4H* gene under drought stress had the highest expression level (by 537%) and salicylic acid in combination with drought stress (by 289%) was in the second order. However, salicylic acid upon increasing to 22% showed a non-significant enhancement in comparison to the control plants (Figure 4B). *DAT* gene expression increased significantly in drought stress (by 440%) and salicylic acid in combination with drought stress (by

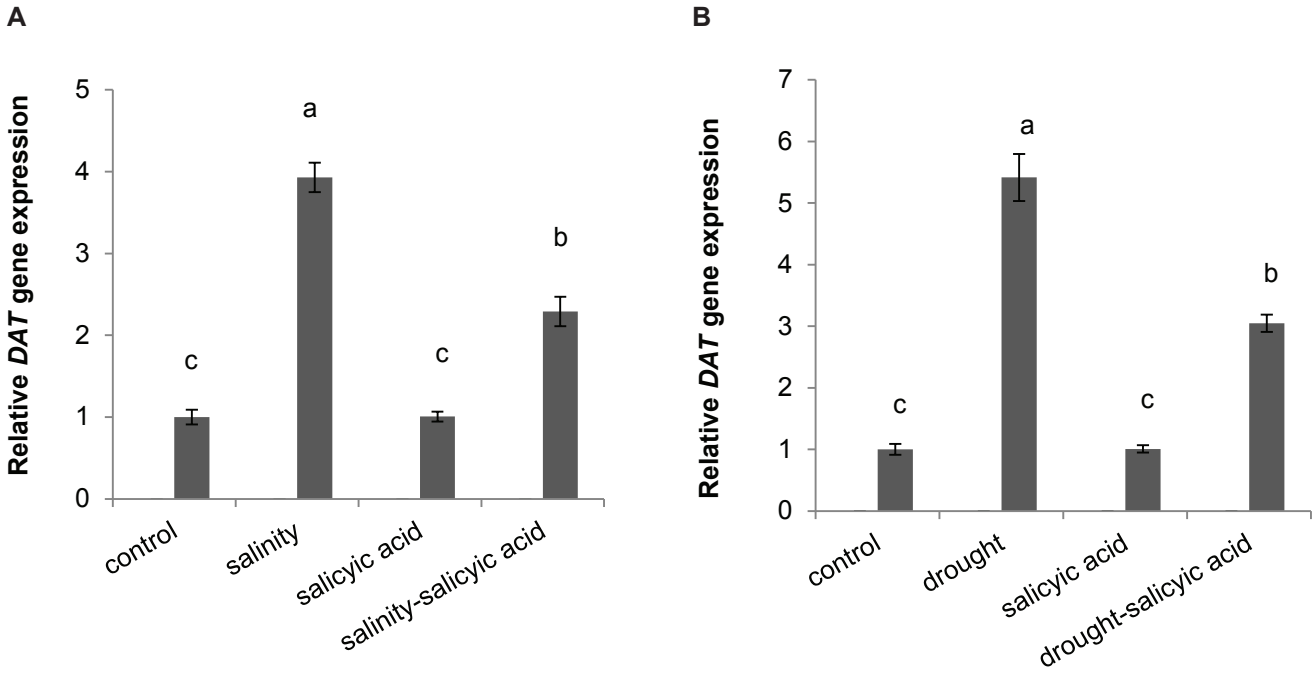

Figure 5. Effects of (A) salinity stress, salicylic acid and their combination, (B) drought stress, salicylic acid and their combination on *DAT* gene expression in *C. roseus*. Different letters above bars indicate significant differences with the control at the $P < 0.01$ level.

Table 3. Analysis of variance for *D4H* and *DAT* genes expression in *C. roseus* under salinity, salicylic acid and their combination.

Source of variation	DF	Mean square	
		D4H	*DAT*
Treatment	3	4.5215**	5.7821**
Experimental error	8	0.0128	0.0191
CV (%)		4.46	6.72

** Significant at 1% probability level.

Table 4. Analysis of variance for *D4H* and *DAT* genes expression in *C. roseus* under drought, salicylic acid and their combination.

Source of variation	DF	Mean square	
		D4H	*DAT*
Treatment	3	19.2862**	13.2169**
Experimental error	8	0.0146	0.0440
CV (%)		4.21	8.01

** Significant at 1% probability level.

204%) treatments in comparison to non-stress condition. Salicylic acid treatment showed a non-significant increase on *DAT* gene expression in comparison to the control plants (Figure 5B).

The comparison of *DAT* and *D4H* genes expression under abiotic stresses

The expression profile of *D4H* and *DAT* genes in leaves under different conditions are shown in Table 5. The configuration of increasing in mRNA levels of *DAT* gene was in drought, salinity, drought + salicylic acid, salinity + salicylic acid, salicylic acid and control (non-stress) treatments. However, mRNA levels of *D4H* gene were in order of drought, drought + salicylic acid, salinity, salinity + salicylic acid, salicylic acid and control (non-stress) treatments, respectively. The results showed that both *DAT* and *D4H* had the highest expression levels under drought stress. The effect of salinity and salinity + salicylic acid in increasing *DAT* gene expression was more than *D4H* expression. Furthermore, the expression levels of *D4H* were higher under drought + salicylic acid and salicylic acid stresses than in the *DAT* gene (Table 5).

Correlation analysis

Correlation analysis between the expression of *D4H*

Table 5. Mean compression of *D4H* and *DAT* genes relative expression levels under salinity, drought, salicylic acid and their combinations.

Treatment	*D4H*	*DAT*
Control	1.001 ± 0.04^{d}	1.002 ± 0.09^{e}
Salinity	3.585 ± 0.15^{c}	3.931 ± 0.18^{b}
Drought	6.376 ± 0.19^{a}	5.415 ± 0.38^{a}
Salicylic acid	1.223 ± 0.10^{d}	1.007 ± 0.06^{e}
Salinity + Salicylic acid	1.189 ± 0.13^{d}	2.291 ± 0.18^{d}
Drought + Salicylic acid	3.889 ± 0.10^{b}	3.050 ± 0.14^{c}

Means within a column with the same letter are not significantly different ($p < 0.01$).

and *DAT* genes under abiotic stresses showed highly positive (r=0.93) and significant ($p < 0.01$). Also the results indicated that there was a significant ($p < 0.01$) and negative correlation (r=-0.98) between the *DAT* gene expression in conditions of salicylic acid and salinity treatments. In addition, significant ($p < 0.01$) and positive correlations were observed for *D4H* gene expressions under salicylic acid and salinity, salinity and salinity + salicylic acid, salicylic acid + salinity and salicylic acid conditions.

DISCUSSION

Plant growth and productivity are adversely affected by various abiotic and biotic stresses (Jaleel *et al*., 2008a). Secondary metabolites represent a chemical interface between plants and their surroundings (Vazquez-Flota *et al*., 2004). Therefore, the concentrations of various secondary plant products are strongly dependent on the growing conditions (Ramakrishna and Ravishankar, 2011). The production of indole alkaloids is strongly regulated by environmental conditions and abiotic stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH (Tikhomiroff and Jolicoeur, 2002; Jaleel *et al*., 2007b). Furthermore, the application of various exogenous chemicals can improve alkaloid production of *C. roseus* (Zhou *et al*., 2009) such as salicylic acid treatment (Xu *et al*., 2011).

Our results showed that RWC decreased significantly under abiotic stresses and exogenous chemicals in comparison to the control condition. A similar behavior was reported in *Solanum lycopersicum* L. (Tuna, 2014) and *Gossypium hirsutum* L. (Saleh, 2012) under salt stress and in *Triticum aestivum* (Amirjani and Mahdiyeh, 2013) and *Cicer arietinum* L. (Rahbarian *et al*., 2011) under drought stress. In this experiment, the reduction rate of RWC in salicylic acid treated plants was much lower than that of other treatments. This may have resulted from the fact that salicylic acid plays an important role in the response to abiotic stresses (Rowshan *et al*., 2010).

The results indicated that the application of salinity, drought, salicylic acid, salinity + salicylic acid and drought + salicylic acid treatments caused increases in *D4H* and *DAT* genes expression levels. Also, the results showed that drought stress, drought + salicylic acid and salinity stress had the highest effect on increasing the transcription and expression levels of *D4H* and *DAT* genes. Jing-Yan and Zhao-Pu (2010) showed that the concentrations and yields of vindoline, catharanthine, vinblastine and vincristine increased under seawater stress. In addition, it is reported that salinity and drought stresses increased significantly the content of leaf and root alkaloids in *C. roseus* (Misra and Gupta, 2006; Jaleel *et al*., 2007c; Elfeky *et al*., 2007; Jaleel *et al*., 2008a, b, c; Amirjani, 2013). Furthermore, Idrees *et al*. (2011a) found that foliar spray of salicylic acid not only overcame the adverse effect of stress but also improved the content of vincristine and vinblastine in drought stressed plants. Similar results have been reported that anticancer alkaloids production in *C. roseus* can be increased by salicylic acid, nickel toxicity (Idrees *et al*., 2013) and salinity stress (Idrees *et al*., 2011b). It is reported previously that the abiotic stresses can enhance the expression of TIAs biosynthetic pathway genes in the leaves of *C. roseus* (DH *et al*., 2011). Also, further correlation analysis between alkaloids content and the expression levels of TIAs biosynthetic pathway genes indicated that vincristine accumulation was significantly correlated with the expression of *D4H* and *DAT* genes. In the study of Methyl jasmonic acid (MeJA) effects on the expression changes of TIA biosynthetic pathway genes, it was reported that transcription levels of D4H, TDC and STR are all induced by MeJA, but with different induction patterns (Wei, 2010). Vazquez-Flota and De Luca (1998) indicated that light treatment caused increase levels of D4H transcripts in *C. roseus* seedlings. Our results showed that there was a positive correlation between D4H and DAT transcript levels.

Finally, the finding of this research suggests that drought, salinity and salicylic acid treatments practically can be used to increase the expression of TIAs biosynthetic pathway genes in the leaves of *C. roseus* seedlings.

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