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BRIEF COMMUNICATION

Changes in free polyamines and expression of polyamine metabolic genes under drought and high-temperature in *Citrus sinensis*

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Abstract

Polyamines play important roles in plant responses to various environmental stresses. In this study, changes in free polyamine content and transcription of genes participating in polyamine metabolism were investigated in sweet orange (*Citrus sinensis* Osbeck cv. Jincheng) seedlings under drought (DR), high-temperature (HT), and combined DR and HT (DRHT) stresses. Content of putrescine (Put) and spermidine (Spd) was the highest and that of spermine (Spm) was the lowest under the DR stress, whereas Put and Spd content was the lowest, and Spm content was the highest under the HT stress. Content of the three polyamines under the DRHT stress ranged between the values detected under the DR and HT at most time points. The fluctuation of Put content during the three stresses was irregular; significant amounts of Spd under the DRHT and Spm under all the three stresses were accumulated. Expression of genes encoding arginine decarboxylase, Spd synthase, and Spm synthase was significantly induced under the DR but not under the HT. The relief from all the stresses did not cause an expected decrease or increase in polyamine content and expression of related genes.

Additional key words: putrescine, spermidine, spermine, sweet orange.

Polyamines are low molecular mass aliphatic polycations ubiquitously distributed in all living organisms. In plants, the major polyamines are diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm). Biosynthesis and degradation of polyamines have been well characterized and defined (Martin-Tanguy *et al.* 2001, Kusano *et al.* 2008). Polyamine Put is synthesized from arginine and/or ornithine in a reaction catalyzed by arginine decarboxylase (ADC; EC 4.1.1.19) and ornithine decarboxylase (ODC; EC 4.1.1.17), respectively. Putrescine can be converted to Spd by Spd synthase (SPDS, EC 2.5.1.16), and Spd can be converted to Spm by Spm synthase (SPMS, EC 2.5.1.22). Biosynthesis of Spd and Spm requires an additional aminopropyl donated by decarboxylated S-adenosyl-L-methionine. It is catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) using *S*-adenosylmethionine as substrate. Polyamines are oxidatively degraded by two amine oxidases, diamine oxidase (DAO, EC 1.4.3.6) and polyamine oxidase (PAO, EC 1.5.3.11). Diamine oxidase catalyzes oxidation of Put producing pyrroline, hydrogen peroxide, and ammonia, whereas PAO mainly catalyzes oxidation of Spd and Spm to pyrroline and 1-(3-aminopropyl)-pyrroline, respectively, with concomitant production of hydrogen peroxide (Martin-Tanguy *et al.* 2001, Liu *et al.* 2007).

The roles of polyamines in response to environmental stresses, such as salinity, drought (DR), low temperature,

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Abbreviations: ADC - arginine decarboxylase; DAO - diamine oxidase; DR - drought, DRHT - drought + heat; HT - high temperature; ODC - ornithine decarboxylase; PAO - polyamine oxidase; Put - putrescine; qPCR - quantitative PCR; SAMDC - *S*-adenosylmethionine decarboxylase; Spd - spermidine; SPDS - Spd synthase; Spm - spermine, SPMS - Spm synthase.

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high temperature (HT), and osmotic stress (Gill and Tuteja 2010, Fu *et al.* 2011, 2014, Wang *et al.* 2011a,b) have been well documented in plants. For example, water-stress increases Put, Spd, and Spm in chickpea and soybean (Nayyar *et al.* 2005). Yang *et al.* (2007) reported a high content of free Spd and Spm and increased activities of SAMDC and SPDS in leaves of DR-resistant rice. Heat stress increases Spd and Spm content and DAO and PAO activities but decreases Put content in wheat (Goyal and Asthir 2010). Moreover, an exogenous application of polyamines or over-expression of polyamine biosynthetic genes improve tolerance to environmental stresses in various plants (Farooq *et al.* 2009, Wang *et al.* 2011a,b, Fu *et al.* 2014). Although the roles of polyamines have been demonstrated under different stress conditions, most of these studies focused on a single stress at one time. Thus, differences in polyamine responses to different types of stresses or a combination of stresses are still unclear. In many cases, stress leads to an accumulation of polyamines, but a decrease or negligible alteration of polyamines has also been demonstrated in numerous reports (Liu *et al.* 2006, 2007, 2008). These contradictory results are probably because the experiments were carried out under different conditions (Liu *et al.* 2006). Consequently, a consistent conclusion regarding the response of polyamines to various stress factors is lacking. Drought and heat are two major environmental stresses causing considerable losses in yield of many herbs and trees including citruses. To better understand the roles of polyamines in citrus under DR, HT, and combined DR and HT (DRHT) stresses, we examined changes in content of free polyamines and transcription of genes participating in polyamine metabolism under these conditions.

The seeds of sweet orange (*Citrus sinensis* Osbeck) cv. Jincheng were sowed in a wet sand and germinated in a growth chamber at a temperature of 28 °C in the dark for 15 d. The seedlings were then grown at a temperature of 25 °C, a relative humidity of 60 %, a 16-h photoperiod, and an irradiance of 50 µmol m^{-2} s^{-1} for the next 15 d. Thereafter, the small seedlings of a similar size were transferred to a hydroponic solution composed of 4 mM $Ca(NO_3)_2$, 6 mM KNO_3, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 46 µM H_3BO_3, 6 µM $MnCl_2$, 0.7 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 1 µM H_2MoO_4, and 50 µM Fe-EDTA. After 3 months in the hydroponic culture, the seedlings of a similar size were selected and divided into 9 groups (15 seedlings per group) for different treatments. The first three groups of the seedlings were dehydrated *in vitro* at 25 °C for DR treatment. The second set of three groups of the seedlings were placed at 45 °C for HT treatment. The last set of three groups of the seedlings were dehydrated *in vitro* at 45 °C for DRHT treatment. These treatments were conducted simultaneously in two growth chambers. After 3 h of the stress treatments, all nine groups of the seedlings were allowed to recover under normal conditions for 3 h. Leaves were collected at 0, 0.5, 1, 2, and 3 h after the start of each stress treatment and at 1.5 and 3 h after the start of the recovery period (initiation of the stress treatments was considered as time point 0). The collected leaves were immediately frozen in liquid nitrogen and then stored at -80 °C until use.

Free polyamines were extracted and measured following the protocol described by Shi *et al.* (2010). Briefly, about 0.1 g of sample powder was extracted in 1 cm^3 of 5 % (m/m) perchloric acid on ice for 30 min. After centrifugation, the supernatant was derived with 0.4 cm^3 of dansyl chloride (10 mg cm^{-3} in acetone), meanwhile 0.01 cm^3 of 100 µM 1,6-hexanediamine was added as an internal standard. The dansylated polyamines were measured using a high performance liquid chromato-graphy (*Waters*, USA).

Transcription of polyamine biosynthetic and catabolic genes including *ADC*, *ODC*, *SAMDC*, *SPDS*, *SPMS*, *PAO*, and *DAO* were analyzed by real time quantitative PCR (qPCR). Isolation of RNA, cDNA synthesis, and PCR amplification were performed as described previously (Fu *et al.* 2014). The sequences of genes in citrus were retrieved by *BLAST* search against the *Citrus sinensis* genome (Xu *et al.* 2013) using relevant *Arabidopsis* and tobacco genes as query sequences (Table 1). Specific primers of these genes were designed using the *Primer-BLAST* program (http:// www.ncbi.nlm. nih.gov/tools/primer-blast/) available at *NCBI* (Table 1).

Table 1. Polyamine biosynthetic and catabolic genes and actin gene information in the sweet orange (*Citrus sinensis* Osbeck) genome, and primer pairs used for real-time quantitative PCR.

Gene	Gene ID	CDS length [bp]	Chromosome location	Primers (5′-3′) forward	reverse
ADC	Cs8g07560.1	2130	8	TCTTGCAGAGTGATGGTCCG	ACAAGAGGATGGCACCACAA
ODC	orange1.1t01512.1	1146	unknown	TCAGTTGATCTCCAACCTCCC	TATGAGACACTGGCTTACACG
SAMDC	Cs7g12410.1	1095	7	AGGTTCAGCTGGTGCAATGA	AAGCCAACACCCTCTCAACC
SPDS	Cs7g08430.2	1038	7	AACTGCCGCCAGATCTTCAA	TGCTGCTGTGTGGATCTCTG
SPMS	Cs9g18030.1	1110	9	GCATTAAGGCCTGGAGGTGT	GCAGCTGAGTGAATCTCCGA
DAO	Cs9g06760.1	1983	9	TTGCGGACAGTCAGAGTCAA	GGTGACCCACGCATTGTAGT
PAO	Cs7g02060.1	1491	7	TAGCGGCGGATTCGTACAAG	GCATACCCTCTCTCATCGGC
Actin	Cs1g05000.1	1134	1	CATCCCTCAGCACCTTCC	CCAACCTTAGCACTTCTCC

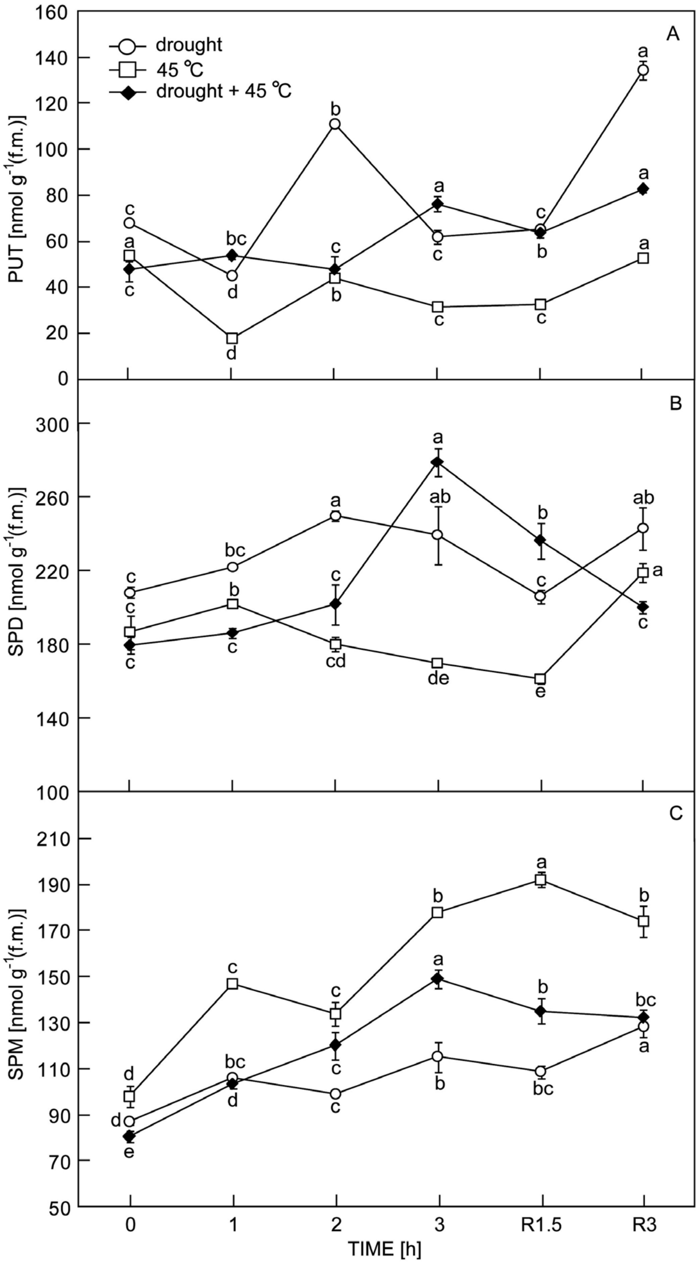

Fig. 1. Changes in putrescine (PUT; *A*), spermidine (SPD; *B*), and spermine (SPM; *C*) content under drought (DR), high temperature (HT, 45 °C), and combined DR and HT (DRHT) stresses. The samples were taken at 0, 1, 2, and 3 h of the stresses and 1.5 h (R1.5) and 3 h (R3) of recovery. Means ± SEs, $n = 4$. Different letters indicate significant differences among different time-points for each treatment at $P < 0.05$.

Data were analyzed by one-way analysis of variance (*ANOVA*) with the *SPSS* statistical software (*v. 17.0*, *SPSS Inc.*, Chicago, USA), and statistical differences were compared by Fisher's least significant difference (LSD) tests at $\alpha = 0.05$.

Changes in Put content showed a similar tendency upon the DR and HT treatments. During the recovery period, Put content increased significantly at 3 h after the DR (Fig. 1*A*). Content of Spd firstly increased and then decreased after 3 h of both the DR treatment and the HT treatment. During the recovery period, Spd content continued decreasing in the first 1.5 h, followed by a significant increase at 3 h. Under the DRHT stress, Spd continuously increased until 3 h and sharply decreased during the recovery (Fig. 1*B*). Content of Spm gradually increased during 3 h under all the three stresses except for a small decrease at 2 h of the DR and HT treatments. During the recovery period, content of Spm decreased significantly only in the plants exposed to the DRHT treatment (Fig. 1*C*). Comparing the DR, HT, and DRHT conditions, content of Put and Spd was the highest under the DR stress, whereas Spm content was the highest under the HT stress. Content of the three polyamines under the DRHT ranged between the values observed in the DR or HT individually at most time points.

These results reveal various changes in Put, Spd, and Spm content among the three different treatments. They corroborate those of previous studies that found a significant increase of Spd and Spm levels in wheat (Liu *et al.* 2004), chickpea and soybean (Nayyar *et al.* 2005), and cacao (Bae *et al.* 2008) under DR stress, and a significant increase of Spm in *Arabidopsis* (Sagor *et al.* 2013), tobacco (Cvikrová *et al.* 2012), wheat (Goyal and Asthir 2010), and alfalfa suspension-cultured cells (Königshofer and Lechner 2002) under HT stress. The results presented herein also show that the content of Put,

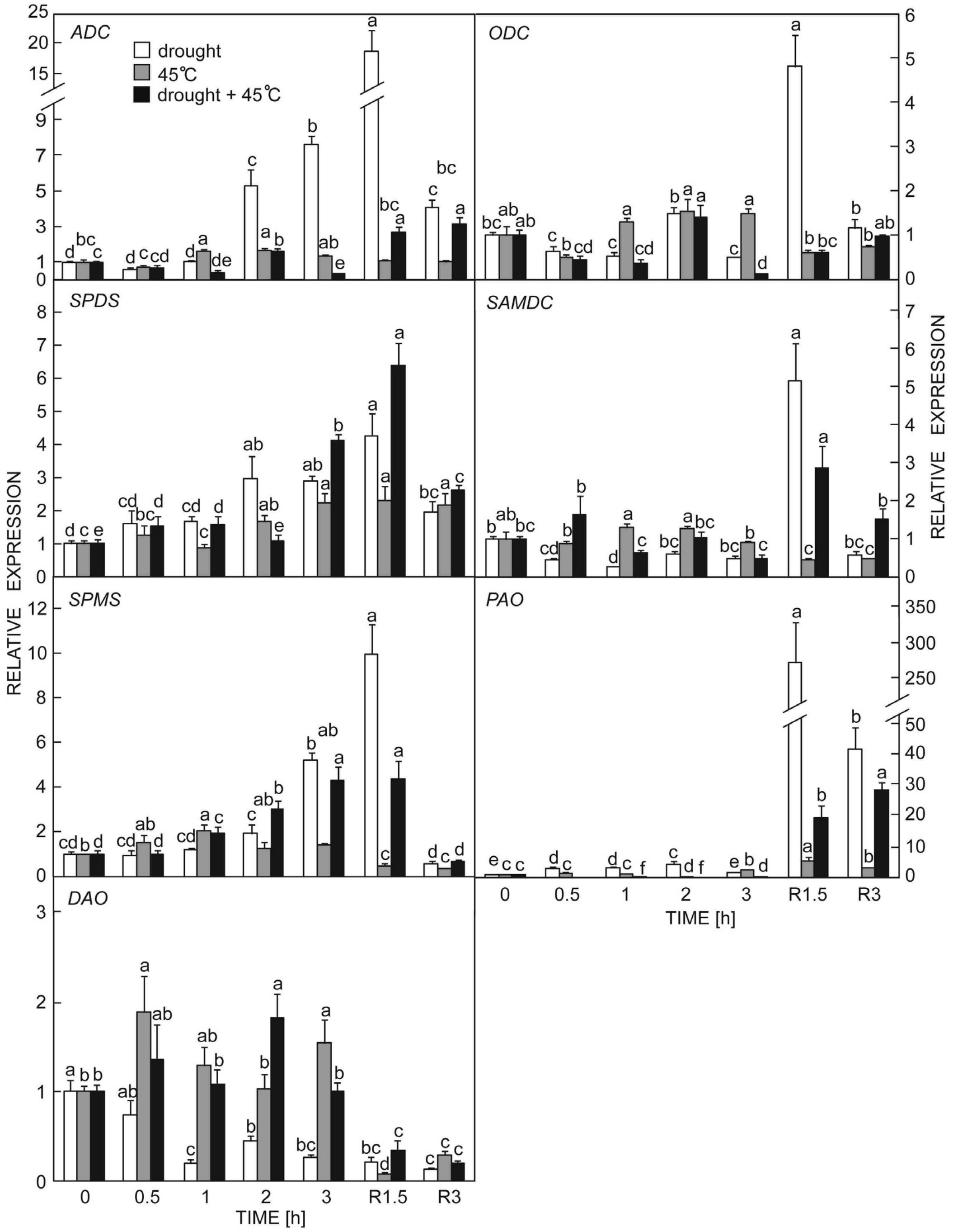

Fig. 2. Transcriptions of *ADC*, *ODC*, *SPDS*, *SAMDC*, *SPMS*, *PAO*, and *DAO* genes under drought (DR), high temperature (45 °C; HT), and combined DR and HT (DRHT) stresses at 0, 0.5, 1, 2, and 3 h of stress treatments and 1.5 h (R1.5) and 3 h (R3) of recovery. Means ± SEs, $n = 4$. Different letters indicate significant differences among different time points at $P < 0.05$.

Spd, and Spm in plants exposed to the DRHT stress conditions were neither higher nor a simple superposition of the values measured under the DR and HT treatments. This suggests that the plants, although suffering the double stress, did not accumulate more polyamines. Such a response to DRHT stress conditions is probably caused by the highly controlled homeostasis of polyamines in plants (Martin-Tanguy 2001, Kusano *et al.* 2008, Moschou *et al*. 2008b). Spermine was significantly induced in all the stress treatments indicating that Spm possibly plays a dominant role in mediating DR, HT, and DRHT stresses in plants. As tetra-amine, Spm contains a high positive charge that allows strong binding to negative charges, thereby stabilizing membranes more effectively compared to Spd and Put (Yang *et al.* 2007, Shi *et al.* 2010). Unexpectedly, termination of stress conditions did not cause an immediate decrease or increase of polyamines except of Spd and Spm after the DRHT treatment. Similar results were also reported in previous studies (Todorova *et al*. 2007, Cvikrová *et al.* 2012). We speculate that these results might be due to inappropriate tested time points or complex regulation processes such as biosynthesis, degradation, conjugation, and back-conversion that collectively control free polyamine content (Kusano *et al*. 2008, Moschou *et al.* 2008a,b). Expression of *ADC* was significantly up-regulated under the DR conditions, whereas there was no significant change under the HT or DRHT conditions (Fig. 2). A relatively high *ADC* expression persisted after the recovery from the DR and DRHT conditions for 1.5 and 3 h. Expressions of *ODC* and *SAMDC* were irregular and insignificantly up- or down-regulated except for a very high expression detected 1.5 h after the end of the DR (for *ODC*) and DR and DRHT (for *SAMDC*) treatments. The three stresses induced expressions of *SPDS* and *SPMS* at various degrees. In particular, the DR and DRHT treatments triggered expressions of these genes at late stages of the treatments, and high expressions were observed at 1.5 h of the recovery period. A significant increase in expression of *PAO* was observed only under the DR stress, and the expression further increased during the recovery period. In contrast, the expression of *DAO* was significantly down-regulated under the DR stress; the expression was very low during the recovery period after the three treatments.

Taken together, the DR stress significantly induced expressions of *ADC*, *SPDS*, and *SPMS*, whereas the changes under the HT stress were not significant. Transcription of the tested genes under the DRHT conditions did not show a superposition of the values observed in the DR and HT treatments. Expressions of all tested genes except *DAO* were very high during the recovery period especially after the DR treatment. Our results corroborate other studies on the effect of DR stress; they reported a significant up-regulation of *ADC*, *SPDS*, and *SPMS* under DR (Bae *et al.* 2008, Shi *et al.* 2010, Alcázar *et al.* 2011, Wang *et al.* 2011b). Studies on gene expression under HT stress conditions are lacking. Comparison between the gene expression and polyamine content reveals a partial overlap in the two. For example, the higher transcription of the *ADC* and *SPDS* genes under the DR stress was in agreement with the higher Put and Spd content under the DR compared to HT stress. The high expressions of *ADC*, *SPDS*, *SPMS*, and *SAMDC* and the very low expression of *DAO* during the recovery period after all three stresses coincided with the relatively high content of polyamines, especially Put, during the recovery phase. However, our results also reveal a lack of a significant correlation between the gene expression and polyamine content. This is because polyamine content is regulated by complex processes including biosynthesis, degradation, conjugation, and back-conversion and therefore cannot be explained by expression of the studied genes only.

To the best of our knowledge, this is the first report on changes in polyamine content and transcription of polyamine metabolism genes under three different stress conditions in a perennial plant. Our findings provide not only a theoretical foundation for further study on the roles of polyamines in citrus response to DR and HT stresses but also a valuable information for relevant studies in other plants.

References

Alcázar, R., Bitrián, M., Bartels, D., Koncz, C., Altabella, T., Tiburcio, A.F.: Polyamine metabolic canalization in response to drought stress in *Arabidopsis* and the resurrection plant *Craterostigma plantagineum*. - Plant Signal Behav. **6**: 243-250, 2011.

Bae, H., Kim, S.H., Kim, M.S., Sicher, R.C., Lary, D., Strem, M.D., Natarajan, S., Bailey, B.A.: The drought response of *Theobroma cacao* (cacao) and the regulation of genes involved in polyamine biosynthesis by drought and other stresses. - Plant Physiol. Biochem. **46**: 174-188, 2008.

Cvikrová, M., Gemperlová, L., Dobrá, J., Martincová, O., Prásil, I.T., Gubis, J., Vanková, R.: Effect of heat stress on polyamine metabolism in proline-over-producing tobacco plants. - Plant Sci. **182**: 49-58, 2012.

Farooq, M., Wahid, A., Lee, D.J.: Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. - Acta Physiol. Plant. **31**: 937-945, 2009.

Fu, X.Z., Chen, C.W., Wang, Y., Liu, J.H., Moriguchi, T.: Ectopic expression of *MdSPDS1* in sweet orange (*Citrus sinensis* Osbeck) reduces canker susceptibility: involvement of H_2O_2 production and transcriptional alteration. - BMC Plant Biol. **11**: 55, 2011.

Fu, X.Z., Xing, F., Wang, N.Q., Peng, L.Z., Chun, C.P., Cao, L., Ling, L.L., Jiang, C.L.: Exogenous spermine pretreatment confers tolerance to combined high-temperature and drought stress in *vitro* in trifoliate orange seedlings *via* modulation of antioxidative capacity and expression of stress-related genes. - Biotechnol. Biotech. Eq. **28**: 192-198, 2014.

Gill, S.S., Tuteja, N.: Polyamines and abiotic stress tolerance in plants. - Plant Signal Behav. **5**: 26-33, 2010.

Goyal, M., Asthir, B.: Polyamine catabolism influences antioxidative defense mechanism in shoots and roots of five wheat genotypes under high temperature stress. - Plant Growth Regul. **60**: 13-25, 2010.

Königshofer, H., Lechner, S.: Are polyamines involved in the synthesis of heat-shock proteins in cell suspension cultures of tobacco and alfalfa in response to high-temperature stress? - Plant Physiol. Biochem. **40**: 51-59, 2002.

Kusano, T., Berberich, T., Tateda, C., Takahashi, Y.: Polyamines: essential factors for growth and survival. - Planta **228**: 367-381, 2008.

Liu, H.H., Dong, B.H., Zhang, Y.Y., Liu, Z.P., Liu, Y.L.: Relationship between osmotic stress and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings. - Plant Sci. **166**: 1261-1267, 2004.

Liu, J.H., Inoue, H., Moriguchi, T.: Salt stress-mediated changes in free polyamine titers and expression of genes responsible for polyamine biosynthesis of apple in *vitro* shoots. - Environ. exp. Bot. **62**: 28-35, 2008.

Liu, J.H., Kitashiba, H., Wang, J., Ban, Y., Moriguchi, T.: Polyamines and their ability to provide environmental stress tolerance to plants. - Plant Biotech. **24**: 117-126, 2007.

Liu, J.H., Nada, K., Honda, C., Kitashiba, H., Wen, X.P., Pang, X.M., Moriguchi, T.: Polyamine biosynthesis of apple callus under salt stress: involvement of ADC pathway in stress response. - J. exp. Bot. **57**: 2589-2599, 2006.

Martin-Tanguy, J.: Metabolism and function of polyamines in plants: recent development (new approaches). - Plant Growth Regul. **34**: 135-148, 2001.

Moschou, P.N., Delis, I.D., Paschalidis, K.A., Roubelakis-Angelakis, K.A.: Transgenic tobacco plants over-expressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. - Physiol. Plant. **133**: 140-156, 2008a.

Moschou, P.N., Paschalidis, K.A., Roubelakis-Angelakis, K.A.: Plant polyamine catabolism: the state of the art. - Plant Signal Behav. **3**: 1061-1066, 2008b.

Nayyar, H., Kaur, S., Singh, S., Kumar, S., Singh, K.J., Dhir, K.K.: Involvement of polyamines in the contrasting sensitivity of chickpea (*Cicer arietinum* L.) and soybean (*Glycine max* (L.) Merrill.) to water deficit stress. - Bot. Bull. Acad. sin. **46**: 333-338, 2005.

Sagor, G.H.M., Berberich, T., Takahashi, Y., Niitsu, M., Kusano, T.: The polyamine spermine protects *Arabidopsis* from heat stress-induced damage by increasing expression of heat shock-related genes. - Transgenic Res. **22**: 595-605, 2013.

Shi, J., Fu, X.Z., Peng, T., Huang, X.S., Fan, Q.J., Liu, J.H.: Spermine pretreatment confers dehydration tolerance of citrus *in vitro* plants *via* modulation of antioxidative capacity and stomatal response. - Tree Physiol. **30**: 914-922, 2010.

Todorova, D., Sergiev, I., Alexieva, V., Karanov, E., Smith, A., Hall, M.: Polyamine content in *Arabidopsis thaliana* (L.) Heynh during recovery after low and high temperature treatments. - Plant Growth Regul. **51**: 185-191, 2007.

Wang, B.Q., Zhang, Q.F., Liu, J.H., Li, G.H.: Overexpression of *PtADC* confers enhanced dehydration and drought tolerance in transgenic tobacco and tomato: effect on ROS elimination. - Biochem. biophys. Res. Commun. **413**: 10-16, 2011a.

Wang, J., Sun, P.P., Chen, C.L., Wang, Y., Fu, X.Z., Liu, J.H.: An arginine decarboxylase gene *PtADC* from *Poncirus trifoliata* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*. - J. exp. Bot. **62**: 2899-2914, 2011b.

Xu, Q., Chen, L.L., Ruan, X., Chen, D., Zhu, A., Chen, C., Bertrand, D., Jiao, W.B., Hao, B.H., Lyon, M.P., Chen, J., Gao, S., Xing, F., Lan, H., Chang, J.W., Ge, X., Lei, Y., Hu, Q., Miao, Y., Wang, L., Xiao, S., Biswas, M.K., Zeng, W., Guo, F., Cao, H., Yang, X., Xu, X.W., Cheng, Y.J., Xu, J., Liu, J.H., Luo, O.J., Tang, Z., Guo, W.W., Kuang, H., Zhang, H.Y., Roose, M.L., Nagarajan, N., Deng, X.X., Ruan, Y.: The draft genome of sweet orange (*Citrus sinensis*). - Nat. Genet. **45**: 59-66, 2013.

Yang, J., Zhang, J., Liu, K., Wang, Z., Liu, L.: Involvement of polyamines in the drought resistance of rice. - J. exp. Bot. **58**: 1545-1555, 2007.