International Journal of Environmental Research and Technology
E-ISSN:2667-4041 2(3): 37-43, 2019
Proceeding Book of 3th IPSAT Congress

Short-term Effects of Exogenous Application of Ascorbic Acid on Barley (*Hordeum vulgare* L.) Seedlings under Salinity Stress

Cüneyt Uçarlı[a]*

[a]Department of Molecular Biology and Genetics, Istanbul University, Vezneciler, 34134, Istanbul, Turkey

Corresponding author: ucarlicu@istanbul.edu.tr
This paper was presented at 3th IPSAT Congress, Afyon, Turkey, 18-20 December 2019

ABSTRACT

Ascorbic acid (AA) is one of the most important and abundant water-soluble antioxidants in plants, and plays important role in adaptation of plants to stress conditions including salt stress. Salt stress is first perceived by the plant roots and inhibits plant growth in the short-term by inducing osmotic stress caused by decreased water availability. The study focused on determination of alleviating effects of exogenous application of ascorbic acid (AA) on barley seedlings exposed to salt stress for short term. In hydroponic conditions, three-leaf-stage barley cv. Martı treated with 160 mM NaCl with or without 0.5 mM AA for 0, 2, and 26 h. The exogenous AA has been found to increase the accumulation of osmolytes by 10 and 25% in leaf and root, respectively, at 26 h after treatment compared to salinity conditions. Besides, AA decreased the enhanced levels of electrolyte leakage due to salt stress of root and leaf tissues from 88.6 to 78.2% and 37.5 to 32.9%, respectively. Furthermore, addition of 0.5 mM AA into root medium led to rapidly differentiation in expression of salt-stress responsive genes including *HvDRF*2, *HvWRKY12*, *HvBAS1*, *HvDHN3*, and *HvNHX1* in root and leaf of barley seedlings under salt stress.

Keywords: Ascorbic Acid, Salinity Stress, Barley, Osmolality, Ion leakage, HvDRF2, HvBAS1

INTRODUCTION

Plants are exposed to various adverse environmental conditions such as salinity, drought, cold, hot, excess water, and heavy metals during their life span. Salinity is one of the most deleterious environmental stress to limit plant growth and crop productivity in arid and semi-arid regions (Mahajan & Tuteja, 2005). Salt stress primarily causes osmotic stress and ionic toxicity resulting in oxidative stress through an accumulation of reactive oxygen species (ROS) in plants (Qureshi et al., 2013). Salinity stress adversely affects the physiological and metabolic processes in plants, including biosynthesis of proteins, enzymatic activities, cellular homeostasis, photosynthetic activity, and phytohormone regulation (Gupta & Huang, 2015). The effect of salt stress on the plant and the degree of injury varies depending on the plant species, plant development stages, salt concentration and the duration of exposure to salt stress (Ngara & Ndimba, 2014). Plants have developed various strategies to cope with the detrimental effects of salinity such as ion homeostasis, ion transport, biosynthesis of osmoprotectants, activation of antioxidant enzymes, and synthesis of antioxidant compounds (Parida & Das, 2005). A large number of genes are involved in the response to salt stress, which are generally grouped into two major categories; functional genes (e.g., *NHX1*, *LOX1*, *DHN3*, and *MT2*, and regulatory genes such as *DRF2* and *WRKY12*.

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop among grains in quantity produced after maize, rice and wheat in the world (Uçarlı & Gürel, 2019). Barley is characterized by wide adaptability and high salinity tolerance. As a result, it is used as a model plant in attempts to understand

salinity tolerance in cereals (Wu et al., 2013). Salinity tolerance may vary depending on the stage of plant growth. It has been reported that barley is more sensitive to salt stress during germination and young seedling phases than older plants (Al-Karaki, 2001).

Ascorbic acid (AA) is one of the most effective, water-soluble, non-enzymatic antioxidants found in plants that has an important function in preventing or reducing cell damage. AA is considered to be a very powerful ROS scavenger due to being an electron donor in many enzymatic and non-enzymatic reactions (Foyer & Noctor, 2011). It protects membranes by reacting directly with $O_2^{\bullet -}$ and H_2O_2 and producing α-tocopherol from tocoproxyl radicals (Gill & Tuteja, 2010). It has been reported that exogenous application of AA mitigated the deleterious effects of salt stress in plants (Agami, 2014; Wang et al., 2019). The present study was focused on whether exogenous application of ascorbic acid (0.5 mM) able to alleviate the adverse effects of salinity (160 mM NaCl) on barley seedlings in short time as 2 and 26 h in hydroponic conditions. After exogenous GA3 treatment, physiological changes on barley seedlings exposed to salts stress was determined by osmolyte accumulation and ion leakage. Besides, differential gene expression profiles of salt-responsive genes were determined by qPCR.

MATERIALS AND METHODS

Plant material, growth conditions and ascorbic acid treatment

After seeds of barley (*Hordeum vulgare* L.) cv. Martı (salt tolerant) were germinated in petri dishes for 7 days, seedlings were transplanted into plastic pots containing perlite. Seedlings were grown 3 weeks until 3-leaf stage in the pots irrigated with half-strength Hoagland solution under controlled conditions (16 h light/8 h dark photoperiod at 25 °C, relative humidity of 75% and light intensity of 20.000 lux). Three-leaf-stage seedlings were transferred into hydroponic systems containing half-strength Hoagland solution. Seedlings were incubated with or without 160 mM NaCl and 0.5 mM ascorbic acid (Sigma, A4403) for 0 (control), 2 and 26 h.

Ion leakage and osmolality assays

Ion leakage and osmolality assays were conducted as described by Tufan et al. (2017).

Total RNA isolation and quantitative real time polymerase chain reaction (qPCR)

Root and leaf samples (100 mg) were frozen in liquid nitrogen and then powdered with mortar and pestle. Total RNA was isolated with the TRIzol® (Invitrogen, 15596-026) according to manufacturer's instructions. The first strand cDNA was synthesized from 4 mg of total RNA using SuperScript™ First-Strand Synthesis System (Invitrogen, 11904-018) with oligo(dT)12-18 primers according to the manufacturer's instructions. qPCR amplification and analysis were conducted as described by Uçarlı and Gürel (2019). Primers shown in Table 1 were used in qPCR.

Statistical analysis

Statistical analyses were conducted using the one-way analysis of variance (ANOVA) with least significance difference (LSD) test function at $P<0.05$ in SPSS21(IBM) statistical software. Three biological and two technical replicates for each assay were used.

Table 1. List of primer sequences used for gene expression analysis by qPCR.

Gene	Accession No	Primer Sequences	Amplicon Size (bp)
HvACTIN	AY145451.1	5' CGTGTTGGATTCTGGTGATG 3' 5' AGCCACATATGCGAGCTTCT 3'	208
HvDRF2	AF521302	5' TGAGACGATCAAGCAATGGA 3' 5' CGAATTTCAGCAACCCACTT 3'	195
HvWRKY12	DQ840411.1	5' CTACCGGTGCACACATCAAG 3'	157

		5’ GACCTGCATCTGGGTGAGTA 3’	
HvNHX1	AY461511.1	5’ CCCGCTTTCATTCTTATCCA 3’ 5’ GAACGACAGTGATGGTGCTG 3’	200
HvLOX1	U83904.1	5’ AGCAGTGAAAGCGAGGAGAG 3’ 5’ AAGTCGTTGAGGTCCAGCAC 3’	142
HvDHN3	AF043089.1	5’ GTGATCAGCAGCAGACCGG 3’ 5’ CATGATGCCCTTCTTCTCGC 3’	176
HvMT2	BM816564	5’ TCAGTCGAATCAACACATGGA 3’ 5’ CACGAGGACGGAACTAAAGC 3’	266
HvASR1	AK252452.1	5' GAGAAGCACCACAAGCACAA 3' 5’ CTCCTCCTCGATCTTGTGCT 3’	132
HvBAS1	Z34917.1	5’ CGTCACCAAATCGATCTCAAA 3’ 5’ TCCACACTACGGCCAATACC 3’	141

RESULTS AND DISCUSSION

Physiological effects of exogenous ascorbic acid on barley seedlings under salt stress

Salinity causes hyperosmotic stress resulting in water deficit in plants. Plants basically counteract the negative effects of osmotic stress due to salinity by the synthesis and accumulation of osmolytes including ammonium compounds, sugars, and amino acids (Golldack et al., 2011). At physiological level, osmotic adjustment is an adaptive mechanism involved in salinity tolerance and permits the maintenance of turgor pressure under stress conditions (Singh et al., 2015). Salt stress (160 mM NaCl) significantly induced the osmolyte accumulation in leaves and roots of barley seedlings within hours. 160 mm NaCl was found to significantly increase osmolyte level from 442 to 987.5 mosmol kg^{-1} in leaves at 26 h after treatment (Table 2). The osmolality was measured lower in roots than in leaves after NaCl treatment (Table 2). The exogenous application of ascorbic acid (0.5 mM) resulted in enhanced osmolyte level in both root and leaf tissues of salt-stressed barley seedlings at 26 h after treatments. Similarly, Wang et al. (2019) have reported that 100 mM NaCl significantly increased proline, known as one of the most important osmolytes produced in plants in response to stress, content compared with the control in okra (*Abelmoschus esculentus* L.) seedlings. In comparison to treatment with 100 mM NaCl, spray of 0.1 mM ascorbic acid did not significantly change the proline content but increased the content of soluble proteins. In contrast, application of AA (0.5 mM) was reported to induce the proline content in rice calli and resulted in higher osmolyte level than 200 mM NaCl-treated calli (Alhasnawi et al., 2016). Chandrasekar et al. (2000) also reported that proline concentration was higher in drought-stress tolerant compared to susceptible cultivars of wheat. Increased osmolyte content by AA may be suggested protecting the barley seedlings under salt stress by affecting osmotic adjustment in addition to its antioxidant activity depending on concentration and plant genotype.

The ion leakage is a sensitive measure of loss-of-membrane integrity and shows the level of membrane damage as a result of oxidative damage. Salt stress (160 mM NaCl) was found to increase the ion leakage progressively depending on duration of exposure to salinity in roots and leaves of barley seedlings compared to control conditions. In 26 h, ion leakage increased from 6.5 to 37.5% in leaves and from 39.4 to 88.6% in roots (Table 2). On the other hand, addition of 0.5 mM AA into root medium alleviated the damage of salt on cells of leaf and root by decreasing ion leakage with 12.3 and 11.7%, respectively, compared to salt- stressed seedlings at 26 h of treatment (Table 2). Similarly, Agami (2014) has reported salinity stress (100 and 200 mM NaCl) increased ion leakage in barley cv. Giza 124 compared to the control while the 1 mM AA slightly decreased the ion leakage in leaves of barley under salt stress. In another study, application of 100 mM NaCl led to a sharp increase in ion leakage by 69.2% in *Citrus aurantium* L. seedlings, whereas ion leakage values were measured lower in response to salinity with the presence of 0.5 mM AA (Kostopoulou et al., 2015). Ascorbic acid (AA) could reduce the membrane

damage by decreasing salt-stress-induced ROS production in plants providing electron donors due to antioxidant properties.

Table 2. Osmolality and ion leakage in the leaves and roots of barley seedlings treated with dH_2O (C), 160 mM NaCl for 2 h (2h-NaCl), 160 mM NaCl and 0.5 mM AA for 2h (2h-NaCl+AA), 160 mM NaCl for 26 h (26h-NaCl), 160 mM NaCl and 0.5 mM AA for 26 h (26h-NaCl+AA).

Treatment	Osmolality (mosmolkg^{-1})		Ion Leakage (%)	
	Leaf	Root	Leaf	Root
Control	442.0±28.5 a	173.7±26.3 ab	6.5±0.9 a	39.4±3.0 a
2h-NaCl	486.7±20.3 a	240.0±32.2 b	15.3±1.4 a	52.7±5.2 ab
2h-NaCl+AA	483.3±37.1 a	143.3±23.3 a	14.6±0.6 a	55.80±2.4 b
26h-NaCl	897.5±20.9 b	186.7±29.6 ab	37.5±4.9 b	88.6±6.8 c
26h-NaCl+AA	983.3±54.9 b	233.3±6.7 b	32.9±1.3 b	78.2±2.8 c

The data presented are the means ± standard errors (SE) of three replicates. Values in vertical columns followed by different letters are significantly different at $P < 0.05$ level.

Differential gene expression profiles of salt-responsive genes in leaves and roots

qPCR experiments were conducted to investigate salt stress-related gene expression patterns in roots and leaves of 3-leaf-stage barley seedlings after 160 mM NaCl with or without 0.5 mM AA for 0. 2 and 26 h. qPCR data were visualized as graph (Fig. 1).

Salt stress progressively increased the expression of transcription factor *HvDRF2* in leaves of barley seedlings depending on exposure time compared to the control. Exogenous application of AA (0.5 mM) did not significantly alter the expression of *HvDRF2* in leaves of salt-stressed barley compared the salt stress conditions. On the other hand, in roots, *HvDRF*2 was promoted by 0.5 mM AA treatment compared to NaCl stress alone. The transcript level of *HvWRKY12* was found to be increased by salinity stress in 2 h then decreased as the level of controls in barley leaves in 24 h. Addition of AA into the root medium promoted the expression of *HvWRKY12* at 2 h after treatment compared with salt-stressed seedlings. The differential response of AA application under salt stress in leaves and roots may show a tissue-specific response of transcription factors *HvDRF2* and *HvWRKY12*.

Exogenous application of AA was found to increase the expression of *HvBAS1*, *HvMT2*, and *HvASR1* in leaves of barleys under salt stress compared to NaCl alone, whereas there was no significant increase in expression of other salt-stress-responsive genes including *HvNHX1*, *HvLOX1* and *HvDHN3*. Metallothioneins (MTs), cysteine-rich proteins, play role in detoxification of ROS (Yang et al. 2009). BAS1, also known as 2-cysteine peroxiredoxin, functions as a peroxide sensor and regulates ROS-related intracellular signaling (Dietz et al. 2006). Ullah et al. (2017) has reported that ascorbic acid is plays important role major role in plant stress signalling such as ROS scavenging and other enzymatic and non-enzymatic physiological processes in *Vigna angularis*. Similarly, qPCR analysis of this study shows that AA induced the expression of *HvBAS1* and *HvMT2*, which are functional in antioxidant activity in plants. In roots, transcripts of *HvNHX1* (coding Na^+/H^+ antiporter), and *HvDHN3* (a member of Dehydrins and functional in osmotic stress) were significantly enhanced at 26 h after 0.5 mM AA+160 mM NaCl treatment compared with 160 mM NaCl alone. Exogenous application of AA enhanced salinity tolerance in barley seedling via regulating the transport and compartmentalization of Na^+ by Na+/H+ antiporters and synthesis dehydrin proteins to adjust the osmotic potential in plant cells.

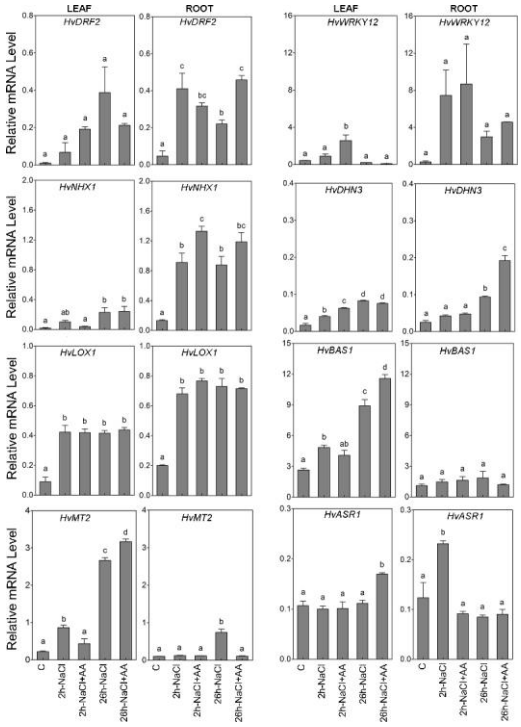

Fig. 1 Expression profiles of the salt-responsive genes in the leaves and roots of barley seedlings treated with dH_2O (C), 160 mM NaCl for 2 h (2h-NaCl), 160 mM NaCl and 0.5 mM AA for 2h (2h-NaCl+AA), 160 mM NaCl for 26 h (26h-NaCl), 160 mM NaCl and 0.5 mM AA for 26 h (26h-NaCl+AA).

CONCLUSION

In conclusion, exogenous application of ascorbic acid (AA) induced the physiological response of plant against salt stress by increasing osmolyte accumulation and decreasing the salt-elevated damage on cells of root and leaf within hours. Furthermore, ascorbic acid induced the expression of salt-stress responsive genes including *HvDRF2*, *HvWRKY12*, *HvBAS1*, *HvDHN3*, and *HvNHX1* in root and leaf of barley seedlings under salt stress within hours. Further studies including transcriptome analysis will be necessary to understand the roles of AA and the network with genes involved in the response of plant to salinity stress.

REFERENCES

Agami, R. A. (2014). Applications of ascorbic acid or proline increase resistance to salt stress in barley seedlings. *Biologia Plantarum*, *58*(2), 341–347.

Alhasnawi, A. N., Che Radziah, C. M. Z., Kadhimi, A. A., Isahak, A., Mohamad, A., & Yusoff, W. M. W. (2016). Enhancement of antioxidant enzyme activities in rice callus by ascorbic acid under salinity stress. *Biologia Plantarum*, *60*(4), 783–787.

Al-Karaki, G. (2001). Germination, sodium, and potassium concentrations of barley seeds as influenced by salinity, *Journal of Plant Nutrition*, *24(3*), 511–522.

Chandrasekar, V., Sairam, R.K. & Srivastava, G.C. (2000). Physiological and biochemical responses of hexaploid and tetraploid wheat to drought stress. *Journal of Agronomy and Crop Science. 185,* 219–227.

Dietz, K. J., Jacob, S., Oelze, M. L., Laxa, M., Tognetti, V., De Miranda, S. M. N., … Finkemeier, I. (2006). The function of peroxiredoxins in plant organelle redox metabolism. *Journal of Experimental Botany*, *57*(8), 1697–1709.

Foyer, C.H. & Noctor G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology, 155,* 2–18.

Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry, 48*(12), 909–930.

Golldack, D., Lüking, I., & Yang, O. (2011). Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Reports*, *30*(8), 1383–1391.

Gupta, B., & Huang, B. (2014). Mechanism of salinity tolerance in plants: Physiological, biochemical, and molecular characterization. *International Journal of Genomics*, *2014*, 701596.

Kostopoulou, Z., Therios, I., Roumeliotis, E., Kanellis, A. K., & Molassiotis, A. (2015). Melatonin combined with ascorbic acid provides salt adaptation in Citrus aurantium L. seedlings. *Plant Physiology and Biochemistry*, *86*, 155–165.

Mahajan, S., & Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Archives of Biochemistry and Biophysics*, *444*(2), 139–158.

Ngara, R., & Ndimba, B. K. (2014). Understanding the complex nature of salinity and drought-stress response in cereals using proteomics technologies. *Proteomics*, *14*,(4–5), 611–621.

Parida, A. K., & Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety, 60*(3), 324–349.

Qureshi, M.I., Abdin, M.Z., Ahmad, J., & Iqbal, M., (2013). Effect of long-term salinity on cellular antioxidants, compatible solute and fatty acid profile of Sweet Annie (*Artemisia annua* L.). *Phytochemistry, 95,* 215e223.

Singh, M., Kumar, J., Singh, S., Pratap, S.V., Prasad, S.M (2015). Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. *Reviews in Environmental Science and Bio/Technology*, *14*, 407–426.

Tufan, F., Uçarlı, C., Tunalı, B., & Gürel, F. (2017). Analysis of early events in barley (Hordeum vulgare L.) roots in response to Fusarium culmorum infection. *European Journal of Plant Pathology*, *148*(2), 343–355.

Uçarlı, C., & Gürel, F. (2019). Differential physiological and molecular responses of three ‐ leaf stage barley (Hordeum vulgare L .) under salt stress within hours. *Plant Biotechnology Reports*, https://doi.org/10.1007/s11816-019-00586-5

Ullah, I., Waqas, M., Khan, M. A., Lee, I. J., & Kim, W. C. (2017). Exogenous ascorbic acid mitigates flood stress damages of Vigna angularis. *Applied Biological Chemistry*, *60*(6), 603–614.

Wang, Y. H., Zhang, G., Chen, Y., Gao, J., Sun, Y. R., Sun, M. F., & Chen, J. P. (2019). Exogenous application of gibberellic acid and ascorbic acid improved tolerance of okra seedlings to NaCl stress. *Acta Physiologiae Plantarum*, *41*(6), 1–10.

Wu, D., Shen, Q., Cai, S., Chen, Z.-H., Dai, F., & Zhang, G. (2013). Ionomic responses and correlations between elements and metabolites under salt stress in wild and cultivated barley. *Plant & Cell Physiology*, *54*(12*)*, 1976–1988.

Yang, Z., Wu, Y., Li, Y., Ling, H.Q. & Chu, C. (2009). OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Molecular Biology*, *70*, 219–229.