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EFFECTS OF ASCORBIC ACID ON THE SEED GERMINATION, SEEDLING GROWTH AND LEAF ANATOMY OF BARLEY UNDER SALT STRESS

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ABSTRACT

In this work, the effects of ascorbic acid pretreatment on the seed germination, seedling growth (coleoptile percentage, radicle length, coleoptile length, radicle number and fresh weight) and leaf anatomy of barley under saline conditions were studied. In parallel with concentration rise, salt stress inhibited the germination and seedling growth of barley seeds. The inhibitive effect of salt on seed germination and seedling growth was alleviated in varying degrees, and dramatically, by ascorbic acid application. On the other hand, it was determined that ascorbic acid affected in different degrees on the various parameters of leaf anatomy of barley seedlings, and this difference was statistically important.

Keywords: barley, leaf anatomy, ascorbic acid, salt stress, seed germination, seedling growth.

INTRODUCTION

Salinity is one of the most important problems in the agriculture areas of the world. Nearly 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity (Zhu, 2001). The salt-affected soils contain excess salts which affect plants by decreasing the osmotic potential of the soil solution (osmotic stress), interfering with normal nutrient uptake, inducing ionic toxicity, and associating nutrient imbalances (An *et al.,* 2003). Processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by high salt concentration, ultimately causing diminished economic yield and also quality of production (Sairam and Tyagi, 2004). In addition, it is evident that there are big changes in leaf morphology and anatomy of the plants growing in saline soils (Çavuşoğlu *et al.,* 2007, 2008).

Ascorbic acid (AA), the main source of vitamin C for humans, is a small and water-soluble antioxidant molecule. It is also an essential compound for plants, with important roles as an antioxidant and as a modulator of plant development through hormone signalling (Pastori *et al.,* 2003). In addition, AA plays important roles in many physiological processes such as seed germination (Tavili *et al.,* 2009), seedling growth (Özdener and Kutbay, 2008), flowering (Barth *et al.,* 2006), membrane permeability (Mukherjee and Choudhuri, 1985), ion intake to roots (Gonzalez-Reyes *et al.,* 1994), photosynthesis (Foyer and Lelandais, 1993), respiration (Bartoli *et al.,* 2006), senescence (Kim *et al.,* 2008), protein and nucleic acid contents (Garg and Kapoor, 1972) and activities of enzymes as peroxidase, superoxide dismutase (Ejaz *et al.,* 2012).

On the other hand, there are few studies about the effects of AA on the seed germination and seedling growth under normal and saline conditions. Some experimental studies have shown that exogenous application of AA stimulates the germination percentage and early seedling growth of wheat (Afzal *et al.,* 2006), bean (Shaddad *et al.,* 1990; Azooz and Al-Fredan, 2009), pea (Burguieres *et al.,* 2007), tomato (Barh *et al.,* 2008) and sorghum (Arafa *et al.,* 2009) seeds germinated in distilled water and saline medium. Unfortunately, it has not been encountered any study concerning effects of AA on the leaf anatomy of barley seedlings grown in both normal and saline conditions until now, especially on the parameters examined in this study.

The purpose of this study is to observe the influences of AA in the reducing of the inhibitive effects of salt stress on the seed germination, seedling growth and leaf anatomy of barley.

MATERIALS AND METHODS

The seeds, salt and ascorbic acid concentrations

In this study, barley (*Hordeum vulgare* cv. Bülbül 89) seeds were used. The seeds were surface sterilized with 1% sodium hypochloride. Salt (NaCl) concentrations used were 0.0, 0.25, 0.275, 0.30, 0.325, 0.35, 0.375 and 0.40 M. Ascorbic acid (AA) concentration used in the experiments was 1 µM. AA and NaCl concentrations were determined in a preliminary investigation conducted by us.

Germination of the seeds

Germination experiments were carried out at a constant temperature (20°C), in the dark in an incubator. Barley seeds in adequate amount were pretreated in the beakers containing sufficient volume of distilled water (control, C) or aqueous solution of AA for 24 h at room temperature. At the end of this pretreatment, the solutions were filtered immediately and the seeds were dried in vacuum (Braun and Khan, 1976). 25 seeds from every application were arranged into Petri dishes (10 cm diameter) lined by 2 sheets of Whatman No. 1 filter paper moistened with 7 ml of the salt solution. After sowing, Petri dishes were placed into an incubator for germination for 7 days. It was assumed that the radicle should be 10 mm long for germination to take place (Ungar, 1974). At the end of the 7th day, after determination of the final germination percentages, the coleoptile emergence

percentages and radicle numbers were also recorded, and the coleoptile and radicle lengths of the seedlings were measured in mm, and in addition, the fresh weights in mg/seedling were determined. All experiments were repeated 4 times.

Growth conditions of the seedlings from the seeds and anatomical observations

The seedlings from the seeds germinated in the incubator at 20°C for 7 days were transferred into the pots with perlite including NaCl solutions (0.0, 0.25, 0.275 and 0.30 M) prepared with Hoagland recipe and were grown in a growth chamber for 20 days. Growth conditions were: photoperiod 12-h, temperature 25±2°C, relative humidity 60±5%, light intensity 160 µmol/m^2/s PAR (white fluorescent lamps). Anatomical sections were taken from the second leave of 20-day-old seedlings by a microtome, in 6-7 µm thickness. Stomata and epidermis cells in a 1-mm^2 unit area were counted to determine the stomata index. These counts were made both in the lower and upper surfaces of each leaf 10 times as 3 replicates and the averages were calculated. After the determination of the number of stomata and epidermis cells in the leaf unit area, the stomata index was estimated according to Meidner and Mansfield's (1968) method.

Stomata width and length, epidermis cell width and length, leaf thickness and distance between vascular bundles were also determined in µm by using ocular micrometer. Statistical evaluation concerning all parameters was realized by using SPSS program according to Duncan's multiple range test.

RESULTS AND DISCUSSIONS

Effects of AA on the seed germination and seedling growth

Unless there are generally stress conditions, there is no need to add exogenously any plant growth regulator in germination process. But, there are few studies about the effects of AA on seed germination and seedling growth under normal conditions. Thus, we also wanted to test the effects of AA pretreatment on the germination and seedling growth in distilled water medium. Our results showed that AA application partly increased the final germination percentage, coleoptile percentage and radicle lenght of barley germinated under normal conditions, and dramatically reduced the coleoptile length, radicle number and fresh weight (Table-1). Burgeieres *et al.* (2007) and Barh *et al.* (2008) reported that AA stimulated the germination percentage, coleoptile percentage and radicle length of tomato and pea seeds in distilled water medium. These results were in agreement with our findings. Howewer, same researchers (Burgeieres *et al.,* 2007, Barh *et al.,* 2008) also observed that this pretreatment increased the coleoptile length and fresh weight of the seedlings and this was not consistent with our findings. It can be said that AA can show different effects on seed germination and seedling growth depending on the plant species and the concentrations used.

It was reported previously that saline conditions negatively affect growth and development events in general, even in halophytes. Howewer, the effect mechanism of salinity has not been completely clarified so far (Ghoulam and Fores, 2001). It is well known that salinity prevents seed germination (Demir *et al.,* 2003) and seedling growth (Ashraf *et al.,* 2002). Salt, in the paralellism of concentration increase, increased its inhibitive effect on all examinated growth parameters. For example, while C seeds germinated in distilled water medium displayed 87% germination on the 7th day, this value became 53%, 24%, 6% and 0%, respectively in 0.325, 0.350, 0.375 and 0.40 M salinity (Table-1). Salt stress can perform its preventive effect in many ways. It may interfere with seed germination by changing the water status of the seed so that water uptake is inhibited (Kabar and Baltepe, 1990). On the other hand, AA pretreatment markedly alleviated the inhibitive effect of salt stress on the seed germination. For instance, the C seeds showed no germination in 0.40 M salinity while the ones pretreated with AA demonstrated 26% germination in this high salt level. AA also continued its success on the seedling growth such as the seed germination. Especially at 0.375 and 0.40 M salinity, it illustrated a prominent performance compared to the C (Table-1). Many researchers have determined that AA application increases seed germination and seedling growth under saline conditions (Özdener and Kutbay, 2008, Azooz and Al-Fredan, 2009, Tavili *et al.,* 2009). The results obtained in this work are consistent with the above-mentioned research findings. It is possible that AA may be successful in alleviating the inhibitive effect of salt on germination and seedling growth by increasing nucleic acid and protein synthesis (Garg and Kapoor, 1972) by stimulating mitotic activity of embryo (Maiti and Sengupta, 1979), by providing stabilization of cell membranes (Rodriguez-Aguilera *et al.,* 1995) or by raising antioxidant enzyme activities (Ejaz *et al.,* 2012).

Effects of AA on the leaf anatomy of the seedlings

AA pretreatment greatly affected the leaf anatomical structure of *Hordeum vulgare* seedlings grown under normal conditions. In distilled water medium, AA pretreatment increased the stomata length and index in both surfaces in comparison with the C seedlings while it decreased the epidermis cell length in both ones. Although AA application reduced the epidermis cell number in the upper surface, it had no effect on this parameter in the lower surface. This pretreatment caused a partial decrease on the stomata number in the upper surface, but it stimulated this parameter in the lower one. The mentioned application increased the epidermis cell width and stomata width in the upper surface while it statistically exhibited the same values as the C in the lower surface. In addition, it leaded to reduces on the leaf thickness and distance between vascular bundles (Table-2a, b). Unfortunately, it has not been encountered any study concerning effects of AA on the anatomy parameters examined in this work until now.

Table-1. Various growth parameters of the seedlings from barley seeds germinated in saline conditions for 7 days.

		Growth parameters					
NaCl (M)	**Treatment (µM)**	**Germination percentage (%)**	**Coleoptile percentage (%)**	**Radicle length (mm)**	**Coleoptile length (mm)**	**Radicle number**	**Fresh weight (mg/seedling)**
0.0	**C**	*87±2.3^{g}	85±2.0^{f}	102.4±0.3^{g}	104.5±3.6^{f}	9.2±0.3^{g}	347.5±5.0^{f}
	AA	90±2.3^{h}	89±2.0^{g}	114.4±0.3^{h}	96.0±4.1^{e}	5.1±0.2^{f}	280.0±5.7^{e}
0.325	**C**	53±3.8^{f}	36±0.0^{e}	24.7±0.3^{f}	19.0±3.6^{cd}	3.3±0.0^{de}	112.5±5.0^{c}
	AA	53±2.0^{f}	34±2.3^{de}	23.9±0.5^{e}	19.9±2.8^{d}	3.4±0.4^{de}	127.5±5.0^{d}
0.35	**C**	24±0.0^{c}	11±2.0^{b}	16.1±0.2^{c}	16.7±1.8^{cd}	3.0±0.0^{cd}	92.5±5.0^{b}
	AA	48±0.0^{e}	30±2.3^{c}	20.2±0.3^{d}	15.8±1.3^{bc}	3.4±0.2^{de}	127.5±5.0^{d}
0.375	**C**	6±2.3^{b}	0±0.0^{a}	10.0±0.1^{b}	0.0±0.0^{a}	2.0±0.1^{b}	87.5±5.0^{b}
	AA	36±0.0^{d}	32±0.0^{cd}	23.5±0.4^{e}	18.7±1.7^{cd}	3.6±0.2^{e}	125.0±5.7^{d}
0.40	**C**	0±0.0^{a}	0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	AA	26±2.3^{c}	12±0.0^{b}	16.2±0.3^{c}	12.9±0.6^{b}	2.7±0.6^{c}	87.5±5.0^{b}

*The difference between values with the same letter in each column is not significant at the level 0.05 (±SD)

Salinity of the medium caused changes in the leaf anatomic proporties of seedlings. 0.25 M salinity increased the stomata index in both surfaces in the seedlings non-pretreated with AA, in comparison with the leaves of C seedlings grown in distilled water medium, but it decreased the leaf thickness and distance between vascular bundles. This salt level stimulated the stomata length, epidermis cell width and length in the upper surface while it reduced these parameters in the lower one. In addition, the mentioned salinity decreased the epidermis cell number in the upper surface of the leaf, but it had no effect on this parameter in the lower surface. In the lower surface, 0.25 M salinity caused an increase on the stomata number and a reduce on the stomata width, but it did not show a meaningful effect on these parameters in the upper one. As for 0.275 M salinity, it considerably increased the stomata number and index in both surfaces, but it reduced the epidermis cell width, stomata width and length in both ones. Although this salt level leaded to increases on the epidermis cell number and length in the upper surface, it did not show a meaningful effect on these parameters in the lower surface. The mentioned salinity stimulated the leaf thickness while it decreased the distance between vascular bundles. 0.30 M salinity increased the stomata number and index in both surfaces, but it reduced the stomata width and length in both ones. This salt level stimulated the epidermis cell number and length in the upper surface while it statistically exhibited the same values as the C in the lower surface. Although the mentioned salinity increased the leaf thickness, it decreased the distance between vascular bundles. On the epidermis cell width, it leaded to an increase in the upper surface and a reduce in the lower one (Table-2a, b). On the other hand, it was reported previously that salt stress caused positive or negative effects on the epidermis cell number, epidermis cell width, epidermis cell length, stomata number, stomata width, stomata length, stomata index, leaf thickness and distance between vascular bundles (Çavuşoğlu *et al.*, 2008, 2013, 2014). These observations indicate that barley leaves acquire both succulent (for example, in the upper surface the increase in epidermis cell width) and xeromorphic (for example, in the lower surface the decrease in epidermis cell width and in the upper surface the increase in stomata number) properties (Strogonov, 1964).

In this study, AA pretreatment increased the epidermis cell number and stomata number in both the upper and lower surface in comparison with the C seedlings grown in 0.25 M salinity while it decreased the epidermis cell width, stomata length and index in both surfaces. AA application reduced the leaf thickness, but it stimulated distance between vascular bundles. In addition, it statistically demonstrated the same values as the C on the epidermis cell length in both surfaces. Although this pretreatment caused a decrease on the stomata width in the upper surface, it had no effect on this parameter in the lower surface. In 0.275 M salinity, AA pretreatment reduced the epidermis cell length, stomata index, leaf thickness and distance between vascular bundles. AA application illustrated a possitive effect on the stomata width and length in the lower surface, but it had no effect on these parameters in the upper one. This pretreatment

decreased the epidermis cell number in the upper surface while it increased this parameter in the lower surface. In addition, the mentioned application stimulated the epidermis cell width in the upper surface, but it inhibited this parameter in the lower one. Although AA caused a decrease on the stomata number in the upper surface, it statistically showed the same value as the C in the lower surface. As for 0.30 M salinity, AA pretreatment markedly increased the epidermis cell number in both surfaces while it decreased the epidermis cell width, stomata index, leaf thickness and distance between vascular bundles. Although AA application caused reduces on the epidermis cell length in the upper surface, it had no effect on this parameter in the lower one. In addition, it did not demonstrate a meaningful effect on the stomata width and length in both surfaces. Moreover, this pretreatment stimulated the stomata number in the lower surface, but it illustrated the same value as the C in the upper surface (Table-2a, b). AA pretreatment makes water and food transport easy by reducing the distance between vascular bundles in 0.275 and 0.30 M levels of NaCl. Moreover, the mentioned application provides adaptation to saline conditions by decreasing the stomata number in 0.275 M salinity, especially in the upper surface of the leaf, and so decrease transpiration and water loss. In addition, it can lead to the same aim by causing a reduction of leaf area as a result of decreasing the epidermis cell number of the upper surface in the same salt level.

Table-2a. Some parameters of leaf anatomy of barley seedlings grown for 20 days in various concentrations of NaCl at 25ºC after AA pretreatment

NaCl (M)	Treatment (µM)	Epidermis cell number		Epidermis cell width (µm)		Epidermis cell length (µm)		Leaf thickness (µm)	Distance between vascular bundles (µm)
		Upper	Lower	Upper	Lower	Upper	Lower		
0.0	C	*24.9±2.1^{bc}	20.9±2.5^{a}	4.9±1.1^{ab}	6.0±1.4^{d}	8.0±0.6^{ab}	9.4±1.3^{c}	60.1±0.6^{d}	79.7±11.6^{d}
	AA	21.7±2.1^{a}	20.6±2.1^{a}	4.6±1.1^{ab}	3.9±0.7^{a}	7.6±1.6^{a}	12.9±3.6^{d}	39.8±4.0^{a}	72.4±8.0^{bcd}
0.25	C	23.9±2.7^{b}	20.4±2.2^{a}	5.8±1.2^{c}	5.1±1.3^{bc}	9.3±1.3^{c}	8.4±2.0^{bc}	57.2±3.4^{c}	68.2±18.1^{bc}
	AA	31.0±2.0^{g}	22.9±1.8^{b}	5.2±1.4^{bc}	4.4±1.4^{ab}	9.4±1.3^{c}	10.2±3.2^{abc}	52.2±3.5^{b}	76.5±9.5^{cd}
0.275	C	28.9±2.9^{ef}	20.1±2.7^{a}	4.4±1.0^{a}	5.4±0.9^{cd}	8.6±1.5^{bc}	8.7±1.0^{c}	67.0±2.5^{e}	68.5±13.6^{bc}
	AA	27.5±3.8^{de}	25.3±2.5^{c}	5.3±1.3^{bc}	4.7±1.0^{bc}	8.1±1.3^{ab}	7.5±1.1^{ab}	40.6±3.2^{a}	63.8±10.4^{b}
0.30	C	26.5±3.2^{cd}	20.6±2.0^{a}	5.4±1.0^{bc}	5.5±0.9^{cd}	9.3±1.9^{c}	9.4±1.7^{c}	67.1±2.4^{e}	69.0±18.8^{bc}
	AA	30.2±3.1^{fg}	30.8±4.1^{d}	5.0±1.1^{ab}	5.1±1.3^{bc}	8.8±1.2^{bc}	8.7±1.6^{c}	51.5±4.4^{b}	52.1±7.2^{a}

* The difference between values with the same letter in each column is not significant at the level 0.05 (±SD)

CONCLUSIONS

It is clear that adverse effects of salt stress on the seed germination, seedling growth and leaf anatomy of barley were significantly improved by exogenous application of AA. The mechanisms by which salinity inhibits growth are complex and controversial. Moreover, they may vary according to species and cultivar. A universal mechanism has not been established yet. Although the causes of salinity have been characterized, our understanding of the mechanisms by which salinity prevents plant growth is stil rather poor. This work may serve to provide new conceptual tools for designing hypotheses of salt tolerance in plants.

There is no literature yet on the effects of AA on the leaf anatomy of seedlings grown under saline conditions. There is need for more comprehensive and detailed researches for this subject to be made clear. We believe that present study will contribute to future studies.

Table-2b. Some parameters of leaf anatomy of barley seedlings grown for 20 days in various concentrations of NaCl at 25ºC after AA pretreatment

NaCl (M)	Treatment (µM)	Stomata number		Stomata width (µm)		Stomata length (µm)		Stomata index	
		Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
0.0	C	4.2±0.5^{ab}	3.5±0.5^{a}	6.4±0.5^{c}	6.5±0.5^{b}	15.2±1.1^{b}	16.8±1.0^{bc}	14.5	14.5
	AA	3.9±0.6^{a}	4.2±0.6^{b}	7.0±0.6^{d}	6.5±0.7^{b}	18.3±0.9^{d}	17.4±1.3^{c}	15.3	16.9
0.25	C	4.4±1.1^{ab}	4.1±1.0^{ab}	6.6±0.5^{c}	6.1±0.7^{ab}	16.0±0.7^{c}	16.5±1.5^{b}	15.6	16.3
	AA	5.7±0.6^{c}	4.4±0.5^{b}	5.5±0.5^{a}	6.2±0.7^{ab}	14.2±1.0^{a}	15.2±1.2^{a}	15.5	16.0
0.275	C	5.2±0.7^{c}	4.4±1.0^{b}	5.9±0.7^{ab}	5.8±0.6^{a}	14.9±1.3^{ab}	14.9±1.5^{a}	15.3	17.9
	AA	4.6±0.9^{b}	4.4±0.9^{b}	5.6±0.6^{ab}	6.3±0.6^{ab}	15.0±1.5^{ab}	16.6±1.4^{bc}	14.4	17.5
0.30	C	5.2±0.9^{c}	4.4±0.9^{b}	6.0±0.6^{b}	5.9±0.7^{a}	14.5±1.0^{ab}	14.7±1.0^{a}	16.5	17.2
	AA	5.2±0.7^{c}	6.0±1.4^{c}	6.0±0.6^{b}	6.0±0.7^{a}	14.6±1.1^{ab}	14.5±0.9^{a}	14.8	16.2

*The difference between values with the same letter in each column is not significant at the level 0.05 (±SD)

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