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Effects of Salicylic Acid Pretreatment on the Seed Germination, Seedling Growth and Leaf Anatomy of Barley under Saline Conditions

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In this work, the effects of salicylic 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 salicylic acid pretreatment. On the other hand, it was determined that the mentioned plant growth regulator affected in different degrees on the various parameters of leaf anatomy of barley seedlings, and this difference was statistically important.

Keywords: barley, leaf anatomy, salicylic 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 (Dudley 1992; 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; Bilir 2011; Kaya 2012).

Salicylic acid (SA) is an endogenous growth regulator from group phenol compounds that influence many physiological processes such as seed germination (Singh et al. 2010),

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seedling growth (Sakhabutdinova et al. 2003), flowering (Khurana and Cleland 1992), membrane permeability (Barkosky and Einhelling 1993), ion intake to roots and stomatal conductivity (Raskin 1992), photosynthesis (Hayat et al. 2012), respiration (Kapulnik et al. 1992) and leaf senescence (Morris et al. 2000). SA is a conservative compound of some biological stresses and it is an important molecular signal for adjustment reaction of plant to environmental stresses (Krantev et al. 2006).

SA dose-dependently plays important roles in the seed germination and seedling growth under normal and saline conditions. While low concentrations of exogenous SA significantly increase the seed germination and seedling growth (Szepesi et al. 2005; Güneş et al. 2007), its high concentrations have the opposite effect on these parameters (Rajjou et al. 2006; Xie et al. 2007; Zahra et al. 2010). On the other hand, it has not been encountered any study concerning effects of SA 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 SA 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 salicylic 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. Salicylic acid (SA) concentration used in the experiments was 1 μM. SA and NaCl concentrations were determined in a preliminary investigation.

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 SA 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). Twenty-five 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 (Ungar 1974) long for germination to take place. At the end of the 7^{th} 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 NaCI 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. They were examined under a binocular light microscope (Olympus CX41) at 100 magnification. 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:

$$\text{Stomata index} = \frac{\text{Stomata numbers in unit area}}{\text{Stomata numbers in unit area+ epidermis cell numbers in unit area}} \times 100$$

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

Effects of SA on the seed germination and seedling growth

The findings related to the effects of SA on seed germination and various growth parameters of seedlings in distilled water and saline medium are presented in Table 1.

In distilled water medium, SA pretreatment partly increased the final germination percentage, coleoptile percentage and fresh weight according to the control (C) while it dramatically reduced the radicle length and number. In addition, it statistically showed the same effect as the C on the coleoptile length (Table 1).

Salt, in the parallelism of concentration increase, increased its inhibitive effect on all examined growth parameters. For example, while C seeds germinated in distilled water medium displayed 87% germination on the 7^{th} day, this value became 53%, 24%, 6% and 0%, respectively, in 0.325, 0.350, 0.375 and 0.40 M salinity. SA 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 SA demonstrated 15% germination in this high salt level. SA also continued its success on the seedling growth such as the seed germination. Especially at 0.375 and 0.40 M salinity, the mentioned growth regulator illustrated a prominent performance compared to the C (Table 1).

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

NaCl (M)	Pretreatment (μM)	Growth parameters					
		Germination percentage (%)	Coleoptile percentage (%)	Radicle length (mm)	Coleoptile length (mm)	Radicle number	Fresh weight (mg/seedling)
0.0	C	87±2.3^{h}*	85±2.0^{g}	102.4±0.3^{j}	104.5±3.6^{e}	9.2±0.3^{g}	347.5±5.0^{f}
	SA	91±3.8^{i}	91±3.8^{h}	98.6±0.5^{i}	103.8±1.7^{e}	4.9±0.0^{f}	355.0±5.7^{g}
0.325	C	53±3.8^{fg}	36±0.0^{f}	24.7±0.3^{g}	19.0±3.6^{cd}	3.3±0.0^{e}	112.5±5.0^{d}
	SA	55±6.0^{g}	34±2.3^{f}	26.5±0.5^{h}	21.2±1.7^{d}	3.1±0.2^{e}	126.0±0.5^{e}
0.35	C	24±0.0^{d}	11±2.0^{c}	16.1±0.2^{e}	16.7±1.8^{cd}	3.0±0.0^{de}	92.5±5.0^{b}
	SA	50±2.3^{f}	29±2.0^{e}	23.6±0.1^{f}	18.8±2^{cd}	2.7±0.3^{cd}	114.5±0.5^{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}
	SA	45±2.0^{e}	14±2.3^{d}	15.7±1.4^{d}	14.3±1.8^{bc}	2.6±0.4^{c}	100.5±1.0^{c}
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}
	SA	15±2.0^{c}	7±2.0b	14.1±0.3^{c}	10.6±7.1^{b}	2.2±0.1^{b}	112.7±2.6^{d}

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

Effects of SA on the leaf anatomy of the seedlings

The findings related with effects of SA on the some parameters of the leaf anatomy of barley seedlings grown in distilled water and saline medium are presented in Table 2a, b.

In distilled water medium, SA pretreatment increased the stomata width, epidermis cell width and length in both surfaces in comparison with the C seedlings while it decreased the epidermis cell number and stomata number in both ones. Although the mentioned growth regulator reduced the stomata length in the upper surface, it had no effect on this parameter in the lower surface. This applying caused a partial decrease on the stomata index in the upper surface, but it markedly stimulated this parameter in the lower one. In addition, it showed a positive effect on the leaf thickness and distance between vascular bundles (Table 2a, b).

A 0.25 M salinity increased the stomata number and index in both surfaces in the seedlings non-pretreated with SA, in comparison with the leaves of C seedlings grown in distilled water medium, but it reduced the leaf thickness and distance between vascular bundles. This salt level stimulated the epidermis cell width and length in the upper surface while it decreased these parameters in the lower one. In addition, it increased the somata width and length in the upper surface of the leaf, but did not show a meaningful effect in the lower surface. Although the mentioned salinity leaded to a reduction on the epidermis cell number in the upper surface, it had no effect on this parameter in the lower one. On the other hand, SA pretreatment increased the epidermis cell width, stomata width and index 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 number and stomata length in both surfaces. This applying dramatically stimulated the leaf thickness and distance between vascular bundles. In addition, it reduced the stomata number in the upper surface, but it increased

Table 2a. Some parameters of leaf anatomy of barley seedlings grown for 20 days in various concentrations of NaCl at 25°C after SA pretreatment

NaCl (M)	Pretreatment (µM)	Epidermis cell number		Epidermis cell width (µm)		Epidermis cell length (µm)		Leaf thickness (µm)	Distace between vascular bundles (µm)
		Upper	Lower	Upper	Lower	Upper	Lower		
0.0	C	24.9±2.1^{de}*	20.9±2.5^{c}	4.9±1.1^{ab}	6.0±1.4^{ab}	8.0±0.6^{a}	9.4±1.3^{abc}	60.1±0.6^{b}	79.7±11.6^{bc}
	SA	16.1±3.0^{bc}	15.7±2.2^{ab}	7.6±1.7^{e}	7.0±2.1^{c}	10.4±1.7^{bc}	12.9±3.6^{d}	64.7±0.4^{c}	94.8±15.0^{d}
0.25	C	23.9±2.7^{d}	20.4±2.2^{c}	5.8±1.2^{bcd}	5.1±1.3^{a}	9.3±1.3^{ab}	8.4±2.0^{a}	57.2±3.4^{a}	68.2±18.1^{a}
	SA	15.1±3.8^{ab}	14.5±2.6^{a}	6.5±2.0^{cde}	6.9±1.7^{b}	9.2±2.0^{ab}	10.2±3.2^{abc}	65.1±2.0^{c}	89.4±9.8^{cd}
0.275	C	28.9±2.9^{f}	20.1±2.7^{c}	4.4±1.0^{a}	5.4±0.9^{a}	8.6±1.5^{a}	8.7±1.0^{ab}	67.0±2.5^{d}	68.5±13.6^{a}
	SA	14.0±2.5^{a}	17.2±3.8^{b}	7.1±2.3^{e}	7.0±1.7^{c}	11.3±2.9^{c}	11.0±4.6^{c}	61.2±0.4^{b}	72.2±16.3^{ab}
0.30	C	26.5±3.2^{e}	20.6±2.3^{c}	5.4±1.0^{abc}	5.5±0.9^{a}	9.3±1.9^{ab}	9.4±1.7^{abc}	67.1±2.4^{d}	69.0±18.8^{ab}
	SA	17.9±2.5^{c}	17.0±1.6^{b}	6.7±1.7^{de}	5.9±1.3^{ab}	10.7±2.7^{c}	10.5±2.8^{bc}	59.9±0.2^{b}	70.5±21.0^{ab}

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

Table 2b. Some parameters of leaf anatomy of barley seedlings grown for 20 days in various concentrations of NaCl at 25°C after SA pretreatment

NaCl (M)	Pretreatment (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^{bc}*	3.5±0.5^{bc}	6.4±0.5^{ab}	6.5±0.5^{a}	15.2±1.1^{cd}	16.8±1.0^{c}	14.5±0.7^{ab}	14.5±1.0^{a}
	SA	2.6±0.5^{a}	3.1±0.9^{ab}	9.0±1.0^{e}	8.4±1.4^{b}	17.1±2.9^{e}	16.6±2.7^{c}	13.9±1.0^{a}	16.4±0.4^{b}
0.25	C	4.4±1.1^{c}	4.1±1.0^{cd}	6.6±0.5^{bc}	6.1±0.7^{a}	16.0±0.7^{d}	16.5±1.5^{c}	15.6±0.6^{c}	16.3±0.7^{b}
	SA	3.6±1.3^{b}	4.2±1.4^{d}	7.9±0.9^{d}	8.5±1.7^{b}	13.1±2.0^{a}	13.9±2.6^{b}	25.4±1.2^{g}	22.6±1.2^{e}
0.275	C	5.2±0.7^{d}	4.4±1.0^{d}	5.9±0.7^{a}	5.8±0.6^{a}	14.9±1.3^{cd}	14.9±1.5^{b}	15.3±0.9^{c}	17.9±1.1^{c}
	SA	4.3±1.4^{bc}	4.7±1.1^{d}	7.2±1.2^{cd}	8.1±0.9^{b}	13.1±1.9^{a}	12.6±2.7^{a}	23.4±0.4^{f}	21.4±0.9^{d}
0.30	C	5.2±0.9^{d}	4.4±0.9^{d}	6.0±0.6^{ab}	5.9±0.7^{a}	14.5±1.0^{bc}	14.7±1.0^{b}	16.5±0.9^{d}	17.2±1.5^{bc}
	SA	4.1±1.4^{bc}	2.8±1.0^{a}	7.5±1.6^{d}	7.7±1.7^{b}	13.4±2.2^{ab}	15.1±1.6^{b}	18.8±1.3^{e}	14.3±0.6^{a}

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

this parameter in the lower one. Although the mentioned growth regulator caused an increase on the epidermis cell length in the lower surface, it statistically exhibited the same value as the C in the upper surface (Table 2a, b).

A 0.275 M salinity partly increased the stomata number and index in both surfaces of the leaves of seedlings non-pretreated with SA, in comparison with the leaves of C seedlings grown in distilled water medium, but it partly decreased the epidermis cell width in both the upper and lower surface. Although this salt level leaded to reductions on the epidermis cell length and stomata length in the lower surface, it had no effect on these parameters in the upper one. The mentioned salinity stimulated the leaf thickness while it decreased the distance between vascular bundles. In the upper surface, 0.275 M salinity caused an increase on the epidermis cell number and a decrease on the stomata width, but it did not show a meaningful effect on these parameters in the lower one. Alternatively, SA pretreatment reduced the epidermis cell number, stomata length and leaf thickness in comparison with the C seedlings grown in 0.275 M salinity while it increased the epidermis cell width and length, distance between vascular bundles and stomata width and index. Moreover, it exhibited an inhibitive effect on the stomata number in the upper surface, but had no effect on this parameter in the lower one (Table 2a, b).

A 0.30 M salinity increased the stomata number and index in both surfaces in the seedlings non-pretreated with SA, in comparison with the leaves of C seedlings grown in distilled water medium, but it decreased the stomata length in both ones. This salt level stimulated the epidermis cell number and length in the upper surface while it statistically showed the same values as the C in the lower surface. Although the mentioned salinity increased the leaf thickness, it reduced the distance between vascular bundles. On the epidermis cell width, it leaded to an increase in the upper surface and a decrease in the lower one. Moreover, it did not exhibit a meaningful effect on the stomata width in both the upper and lower surface. On the other hand, SA pretreatment increased the stomata width, epidermis cell width and length in both surfaces in comparison with the C seedlings grown in 0.30 M salinity while it reduced the epidermis cell number and stomata number in both ones. Although this applying caused an increase on the stomata index in the upper surface, it showed an inhibitive effect on this parameter in the lower one. The mentioned growth regulator decreased the leaf thickness, but it demonstrated the same effect as the C on the distance between vascular bundles. In addition, SA pretreatment reduced the stomata length in the upper surface while it had no effect on this parameter in the lower surface (Table 2a, b).

Discussion

Unless there are generally stress conditions, there is no need to add exogenously any plant growth regulator in germination process. But, there are controversial reports on the effects of SA on seed germination and seedling growth under normal conditions. Thus, we also wanted to test the effects of this growth regulator on the germination and seedling growth in distilled water medium. Some researchers have argued that SA prevents seed germination and seedling growth in normal conditions (Larque-Saavedra 1978; Shettel and Balke

1983; Yang et al. 2002) and some also have declared that it increases these parameters (Gutierrez-Coronado et al. 1998; Jung et al. 2001; Singh et al. 2010). Our results showed that SA pretreatment stimulated the germination percentage, coleoptile percentage and fresh weight of the seedlings, and reduced the radicle length and number (Table 1). It can be said that SA can show different effects on 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. However, the effect mechanism of salinity has not been completely clarified so far (Al-Karaki 2001; Ghoulam and Fores 2001). It is well known that salinity prevents seed germination (Chartzoulakis and Loupanak 1997; Hosseini et al. 2002; Demir et al. 2003) and seedling growth (Dash and Panda 2001; El-Mashad and Kamel 2001; Ashraf et al. 2002). The seedling growth and germination of barley seeds, as expected, were inhibited under salinity conditions (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, SA pretreatment markedly removed the inhibitor effect of salt stress on the germination and seedling growth (Table 1). Many researchers have reported that SA increases seed germination and seedling growth under salinity conditions (Khodary 2004; El-Tayeb 2005; Dolatabadian et al. 2009). The results obtained in this work are consistent with the above-mentioned research findings. It is possible that this growth regulator may be successful in alleviating the inhibitive effect of salt on the germination and seedling growth by increasing nucleic acid and protein synthesis (Çanakçı 2008), by stimulating mitotic activity of embryo (Sakhabutdinova et al. 2003), by providing stabilization of cell membranes (Güneş et al. 2005) or by reducing endogenous amount of abscisic acid (ABA) in seeds (Wang et al. 2001).

After 7-day germination period of seedlings coming out of seeds treated or not treated with SA, those that were in distilled water were transferred into pots containing Hoagland solution, and also those that were in saline medium into pots containing NaCl concentrations prepared with Hoagland solution. After that, they were grown for 20 days, salinity of the medium added to dramatic changes in the anatomic properties of the seedlings' leaves. Salt stress mostly increased the epidermis cell number, width and length in the upper surface, the stomata number and index in both surfaces and the leaf thickness in the seedlings non-pretreated with SA, in comparison with the leaves of C seedlings grown in distilled water medium. In addition, the mentioned stress reduced the stomata length, epidermis cell number, width and length in the lower surface and the distance between vascular bundles (Table 2a, b).

On the other hand, it was reported previously that salt stress caused changes on epidermis cell number (Curtis and Lauchli 1987), width (Kılıç et al. 2007) and length (Çavuşoğlu et al. 2008), stomata number (Hwang and Chen 1995), width (Çavuşoğlu et al. 2007), length (Bilir 2011) and index (Bray and Reid 2002), leaf thickness (Hu and Schmidhalter 2001) and distance between vascular bundles (Kaya 2012). 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 the stomata number) properties (Strogonov 1964). On the other part, stomata can close as a response to salt stress due to the increase in Na^+ and Cl^- ions and the decrease in K^+ amount in the leaves of the plants, and so they can survive since transpiration and water loss decrease (Robinson et al. 1983). Moreover, stomata can close and water loss can decrease since ABA content in leaves having salt stress increases as well (Cramer and Quarrie 2002).

In this study, SA pretreatment generally increased the epidermis cell width and length, stomata width and index in both surfaces and distance between vascular bundles in comparison with the C seedlings grown in saline conditions. However, it decreased the stomata number and length in the upper surface, the epidermis cell number in both surfaces and the leaf thickness (Table 2a, b). SA provides adaptation to saline conditions by reducing the stomata number and length in all salt levels, 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 both lower and upper surface.

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 SA. 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 still rather poor. This work may serve to provide new conceptual tools for designing hypotheses of salt tolerance in plants.

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