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EFFECTS OF HUMIC ACID PRETREATMENT ON SOME PHYSIOLOGICAL AND ANATOMICAL PARAMETERS OF BARLEY (*HORDEUM VULGARE* L.) EXPOSED TO SALT STRESS

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Abstract

The effects of humic acid (HA) pretreatment on the seed germination, seedling growth and leaf anatomy of barley under both normal and saline conditions were studied. HA application partly reduced the final germination percentage, coleoptile percentage, radicle lenght, radicle number and fresh weight of barley germinated under normal conditions while it showed statistically the same effect as the control on the coleoptile length. In parallel with concentration rise, salt inhibited the seed germination and seedling growth of barley. The inhibitive effect of salt on the seed germination and seedling growth was alleviated in varying degrees by HA pretreatment. Moreover, salinity of the medium caused changes in the leaf anatomy of seedlings. HA affected in different degrees the various parameters of leaf anatomy of barley seedlings grown in both normal and saline conditions, and this difference was statistically important.

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.* 2008).

Humic substances including humic, fulvic and hymatomelonic acids occur widely in mineral soils, peats and natural waters (Muscolo *et al.* 2007, Marino *et al.* 2008). One of the most important of humic substances is humic acid (HA), and it is a promising natural resource that can be used as an alternative to synthetic fertilizers to increase crop production. There are few studies about the effects of HA on the seed germination and seedling growth under normal and saline conditions. Some experimental studies have shown that exogenous application of HA stimulates the germination percentage and early seedling growth of barley, cowpea, wheat, bean, watermelon, geranium, cucumber and marigold seeds germinated in distilled water and saline medium (Hartwigsen and Evans 2000, El-Hefny 2010, Szczepanek and Wilczewski 2011, Silva-Matos *et al.* 2012). Unfortunately, it has not been encountered any study concerning effects of HA on the leaf anatomy of barley seedlings grown in both normal and saline conditions until now, especially on the parameters examined in this study.

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

Materials and Methods

Barley (*Hordeum vulgare* L. cv. Bülbül 89) seeds were used. Salt (NaCl) concentrations used were 0.0, 0.25, 0.275, 0.30, 0.325 and 0.35 M. Humic acid (HA) concentration used in the experiments was 28 mg/l.

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) or aqueous solution of HA for 24 hrs at room temperature. At the end of this pretreatment, the solutions were filtered immediately and the seeds were dried in vacuum. 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 seven days. It was assumed that the radicle should be 10 mm long for germination to take place. At the end of the seventh 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 four times.

The seedlings from the seeds germinated in the incubator at 20°C for seven 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. Anatomical sections were taken from the second leaves 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 ten times as three 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 DMRT (Duncan 1955).

Results and Discussion

Results showed that HA application partly reduced the final germination percentage, coleoptile percentage, radicle lenght, radicle number and fresh weight of barley germinated under normal conditions while it had no effect on the coleoptile length (Table 1). Szczepanek and Wilczewski (2011) reported that HA had no effect on the germination percentage of barley and wheat seeds in distilled water medium. This result were in agreement with our findings. Howewer, some researchers (Hartwigsen and Evans 2000, Chang *et al.* 2012, Silva-Matos *et al.* 2012) also observed that this pretreatment increased the radicle length, coleoptile length and fresh weight of the seedlings and this was not consistent with our findings. It can be said that HA can show different effects on seed germination and seedling growth depending on the plant species and the concentrations used.

Salt, in the paralellism of concentration increase, increased its inhibitive effect on all examinated growth parameters. For example, while control seeds germinated in distilled water medium displayed 77% germination on the seventh day, this value became 49, 40, 28, 19 and 4%, respectively in 0.25, 0.275, 0.30, 0.325 and 0.35 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. Our results showing the decrease in the fresh weight and water content of the seedlings in saline medium may be explained that by failure to receive sufficient water by roots due to high osmotic pressure of medium. The inhibitive effect of salt on the coleoptile length, radicle length and radicle number may result from reducing cell division, nucleic acid and protein synthesis (Mccue and Hanson 1990, Al-Karaki 2001).

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

NaCl (M)	Treatment (mg/l)	Growth parameters					
		Germination (%)	Coleoptile (%)	Radicle length (mm)	Coleoptile length (mm)	Radicle number	Fresh weight (mg/seedling)
0.0	C	*77±3.8^{k}	77±1.8^{k}	95.3±1.1^{h}	124.6±6.4^{g}	4.6±0.2^{h}	347.5±2.2^{i}
	HA	75±2.7^{j}	75±0±8^{j}	83.1±1.5^{g}	120.3±4.5^{g}	4.2±0.1^{gh}	322.5±3.0^{h}
0.25	C	49±3.8^{f}	49±1.2^{f}	41.1±3.3^{f}	35.4± 2.2^{f}	4.0±0.1^{fgh}	172.5±4.8^{g}
	HA	73±2.0^{i}	73±1.8^{i}	37.1±1.0^{ef}	29.2±1.8^{e}	3.8±0.1^{fg}	157.5±2.5^{f}
0.275	C	40±3.2^{e}	40±1.5^{e}	29.7±1.3^{cde}	24.7±1.3^{cde}	3.1±0.4^{cde}	142.5±1.5^{d}
	HA	60±0.2^{h}	60±0.2^{h}	32.1±2.1^{def}	26.7±1.2^{de}	3.7±0.3^{efg}	155.3±4.2^{ef}
0.30	C	28±0.5^{c}	28±0.3^{c}	26.9±2.4^{bcde}	22.6±2.3^{bcd}	3.1±0.3^{cde}	140.0±1.2^{d}
	HA	56±3.2^{g}	56±3.2^{g}	29.2±2.7^{cde}	24.2±2.3^{cde}	3.4±0.1^{def}	153.2±2.3^{e}
0.325	C	19±2.2^{b}	19±2.5^{b}	22.5±4.9^{abcd}	19.4±1.8^{abc}	3.0±0.6^{cd}	132.5±2.5^{bc}
	HA	33±1.3^{d}	33±2.7^{d}	20.7±1.4^{abc}	18.5±2.7^{abc}	2.6±0.2^{bc}	135.0±1.0^{c}
0.35	C	4±0.0^{a}	4±0.2^{a}	15.5±1.3^{a}	15.5±6.0^{a}	2.0±0.8^{a}	129.2±1.8^{b}
	HA	19±1.7^{b}	19±2.8^{b}	16.2±1.3^{ab}	16.8±1.1^{ab}	2.2±0.4^{ab}	122.5±1.5^{a}

*Values with the same letter in each vertical column is not significant at 0.05 level. C- control, HA- humic acid.

On the other hand, HA pretreatment markedly alleviated the inhibitive effect of salt stress on the seed germination. The seeds pretreated with HA demonstrated 73, 60, 56, 33 and 19% germination in the mentioned salt levels. HA also affected the seedling growth such as the seed germination. Specially at 0.275 and 0.30 M salinity, it illustrated a prominent performance compared to the control on the coleoptil percentage, radicle lenght, coleoptile length, radicle number and fresh weight of barley seedlings (Table 1). Some researchers have stated that HA application increase seed germination and seedling growth under saline conditions (El-Hefny 2010, Gülser *et al.* 2010). The results obtained in this work are consistent with the above-mentioned research findings. It is possible that HA may be successful in alleviating the inhibitive effect of salt on germination and seedling growth by increasing nucleic acid and protein synthesis, by stimulating mitotic activity of embryo, by providing stabilization of cell membranes or by raising antioxidant enzyme activities (Travisan *et al.* 2010, Garcia *et al.* 2012).

HA pretreatment greatly affected the leaf anatomical structure of *Hordeum vulgare* seedlings grown under normal conditions (Figs 1a, b, 2 and 3). In distilled water medium, HA increased the epidermis cell number and cell width, and stomata width in both surfaces in comparison with the control seedlings while it decreased the stomata index in both. Although HA application reduced the epidermis cell lenght in the lower surface, it had no effect on this parameter in the upper surface. HA caused an increase on the stomata number and stomata length in the upper surface,

but it partly decreased these parameters in the lower one. The mentioned pretreatment increased the distance between vascular bundles while it reduced the leaf thickness (Table 2a, b).

Table 2a. Some parameters of leaf anatomy of HA pretreatment barley seedlings grown for 20 days in various concentrations of NaCl.

NaCl (M)	Treatment (mg/l)	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	*5.9±1.6^{a}	7.8±1.3^{a}	10.1±1.3^{abc}	9.8±1.2^{ab}	21.3±1.3^{cd}	21.4±1.8^{e}	99.3±1.3^{c}	180.8±2.7^{a}
	HA	18.5±1.5^{g}	18.1±1.5^{e}	16.1±1.7^{d}	19.1±1.3^{c}	21.8±1.9^{cd}	18.1±1.5^{bcd}	79.3±2.8^{a}	193.1±1.1^{c}
0.25	C	9.7±2.3^{b}	8.8±2.1^{a}	8.5±1.9^{a}	11.5±1.4^{b}	22.6±1.6^{cd}	16.9±1.4^{bc}	108.2±2.5^{e}	181.3±1.4^{a}
	HA	17.5±1.6^{fg}	15.2±1.6^{c}	9.2±1.8^{ab}	11.5±1.5^{b}	16.7±1.3^{ab}	16.8±1.4^{ab}	99.3±2.5^{c}	244.3±2.3^{f}
0.275	C	15.7±1.7^{de}	11.7±2.6^{b}	12.2±1.4^{c}	12.6±1.8^{b}	24.3±1.9^{d}	19.7±1.7^{cd}	121.3±1.7^{g}	210.3±3.4^{d}
	HA	16.7±2.2^{ef}	16.8±1.5^{d}	10.1±1.9^{abc}	7.6±1.2^{a}	13.9±1.6^{a}	13.2±2.3^{a}	85.8±3.6^{b}	180.5±1.3^{a}
0.30	C	14.9±1.6^{d}	16.2±2.1^{cd}	11.5±1.8^{bc}	12.1±1.3^{b}	19.8±1.8^{bc}	19.8±1.6^{cd}	161.6±1.2^{f}	238.6±2.9^{e}
	HA	13.5±1.1^{c}	12.4±1.1^{b}	8.6±1.6^{a}	8.2±1.3^{a}	17.3±1.6^{b}	18.9±1.9^{bcd}	103.5±1.7^{d}	185.4±2.6^{b}

* Values with the same letter in each vertical column is not significant at 0.05 level. C- control, HA- humic acid.

Table 2b. Some parameters of leaf anatomy of HA pretreatment barley seedlings grown for 20 days in various concentrations of NaCl.

NaCl (M)	Treatment (mg/l)	Stomata number		Stomata width (µm)		Stomata length (µm)		Stomata index	
		Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
0.0	C	*4.4±0.7^{bc}	5.7±1.2^{d}	19.1±1.3^{ab}	18.5±1.4^{bc}	40.8±1.6^{bc}	36.1±1.5^{b}	41.9	44.2
	HA	5.0±1.6^{c}	4.5±0.8^{c}	27.5±1.4^{e}	20.5±1.4^{cd}	46.5±2.5^{e}	32.6±1.4^{a}	22.9	19.5
0.25	C	4.1±1.1^{ab}	5.1±0.6^{c}	17.3±2.6^{a}	15.7±1.3^{a}	39.5±1.3^{b}	38.2±1.7^{bc}	31.9	35.7
	HA	3.9±1.2^{ab}	2.7±0.9^{a}	22.8±1.2^{d}	19.2±1.8^{cd}	42.6±2.7^{cd}	41.1±1.3^{de}	18.4	15.2
0.275	C	4.2±1.9^{b}	3.5±0.8^{b}	19.7±1.5^{bc}	16.3±1.2^{a}	35.3±1.2^{a}	40.3±1.8^{cde}	21.3	21.8
	HA	4.1±1.3^{ab}	3.2±1.1^{ab}	22.2±1.8^{d}	18.7±1.8^{cd}	42.5±2.3^{cd}	38.8±1.9^{cd}	17.6	17.0
0.30	C	3.2±1.3^{a}	3.4±0.7^{b}	21.3±1.8^{cd}	16.7±1.2^{ab}	45.5±2.4^{de}	42.3±1.7^{f}	19.3	18.6
	HA	3.6±0.8^{ab}	3.1±0.8^{ab}	20.1±1.6^{bc}	20.6±1.6^{d}	45.5±1.2^{de}	49.2±1.5^{g}	21.6	19.7

*Values with the same letter in each vertical column is not significant at 0.05 level. C - control, HA - humic acid.

Salinity of the medium caused changes in the leaf anatomic properties of seedlings (Figs 1a, b, 2 and 3). 0.25 M salinity decreased the epidermis cell width and stomata length in the upper surface; the epidermis cell length in the lower surface; the stomata number, stomata width and stomata index in both surfaces; and the leaf thickness in the seedlings non-pretreated with HA in comparison to control. This salt level stimulated the epidermis cell number in the upper surface;

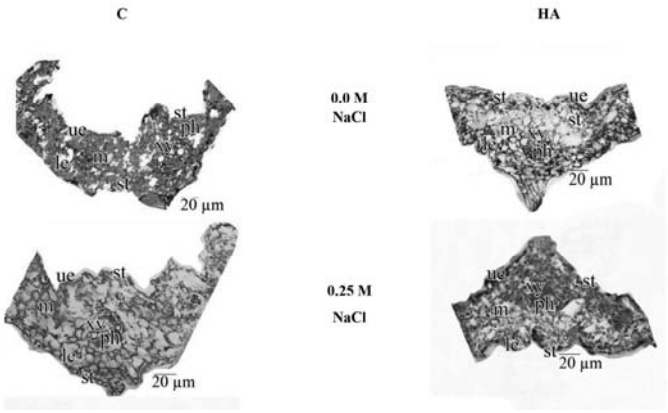

Fig. 1a. Leaf cross sections from distilled water and HA pretreated barley seedlings grown in various concentrations of NaCI at 25°C (le: lower epidermis, ue: upper epidermis, m: mesophyll, st: stomata, xy: xylem, ph: floem). C - control, HA - humic acid.

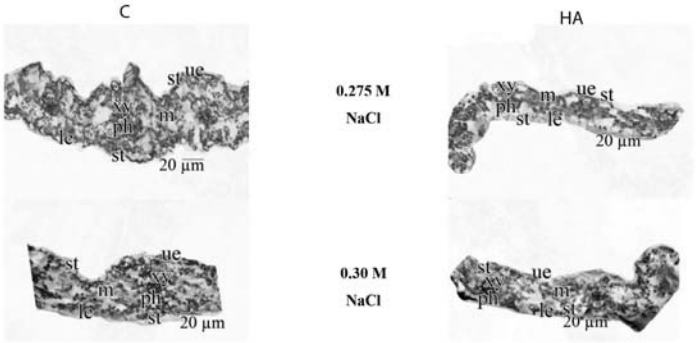

Fig. 1b. Leaf cross sections from distilled water and HA pretreated barley seedlings grown in various concentrations of NaCI at 25°C (le: lower epidermis, ue: upper epidermis, m: mesophyll, st: stomata, xy: xylem, ph: floem). C - control, HA - humic acid.

the epidermis cell width and stomata length in the lower surface. In salinity level of 0.275 M increased the epidermis cell length and stomata length in the upper surface; the stomata length in the lower surface; the epidermis cell number and epidermis cell width in both surfaces; and the leaf thickness and distance between vascular bundles. This salinity reduced the stomata length in the upper surface; the epidermis cell length and stomata length in the lower surface; the stomata number and stomata index in both surfaces. 0.30 M salinity increased the stomata width in the upper surface; the epidermis cell number, epidermis cell width and stomata length in both surfaces; and the leaf thickness and distance between vascular bundles, and decreased the stomata width in the lower surface; the epidermis cell length, stomata number and stomata index in both surfaces (Table 2a, b). On the other hand, it was reported previously that salt stress caused positive or negative effects on the leaf anatomical parameters of barley and radish seedlings (Çavuşoğlu *et al.* 2007, 2008).

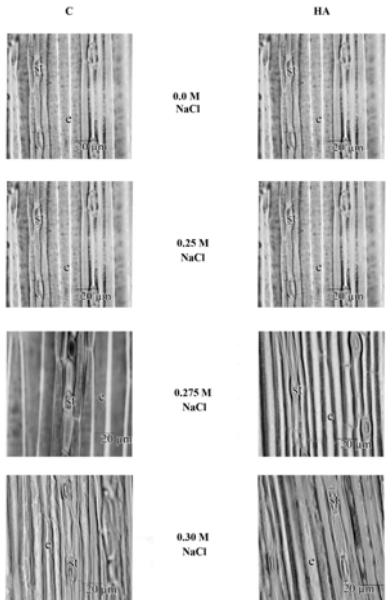

Fig. 2. Leaf lower surface sections from distilled water and HA pretreated barley seedlings grown in various concentrations of NaCI at 25°C (e: epidermis, st: stomata). C - control, HA - humic acid.

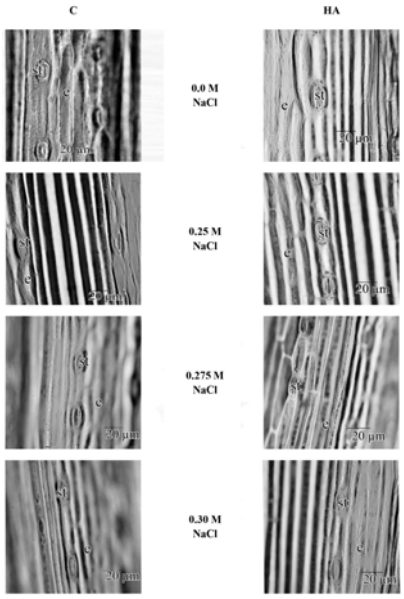

Fig. 3. Leaf upper surface sections from distilled water and HA pretreated barley seedlings grown in various concentrations of NaCI at 25°C (e: epidermis, st: stomata). C - control, HA - humic acid.

HA pretreatment increased the epidermis cell width in the upper surface; the epidermis cell number, stomata width and stomata length in both surfaces; and the distance between vascular

bundles in comparison with the control seedlings grown in 0.25 M salinity. This pretreatment reduced the stomata number in the lower surface; the epidermis cell length and stomata index in both surfaces; and the leaf thickness. In 0.275 M salinity, HA application stimulated the stomata length in the upper surface; the epidermis cell number and stomata width in both surfaces. This application decreased the stomata length in the lower surface; the epidermis cell width, the epidermis cell length, stomata number and stomata index in both surfaces; and the leaf thickness and distance between vascular bundles. As for 0.30 M salinity, HA increased the stomata number in the upper surface; the stomata width and stomata length in the lower surface; the stomata index in both surfaces. It reduced the stomata width in in the upper surface; the stomata number in the lower surface; the epidermis cell number, epidermis cell width and epidermis cell length in both surfaces; and the leaf thickness and distance between vascular bundles (Table 2a, b). HA pretreatment probably 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 and stomata index in 0.275 and 0.30 M salinity, especially in both surfaces of the leaves, and so decrease 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, epidermis cell width and epidermis cell length of both surfaces of the leaves in the same salt levels.

The adverse effects of salt stress on the seed germination, seedling growth and leaf anatomy of barley were significantly improved by exogenous application of HA. 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.

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