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Plant-Derived 20-Hydroxyecdysone Alleviates Salt Stress in Cotton (*Gossypium hirsutum* L.) Seedlings

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ABSTRACT

The action of plant-derived 20-hydroxyecdysone (20E) on germination and seedling growth of three cotton varieties under various concentrations of NaCl and $MgSO_4$ were studied. Seed dormancy enforced by salinity (100 mM NaCl) was substantially alleviated and germination was significantly promoted by 20E from 31 to 39% in var. 'Namangan-77' and from 8 to 21% in 'AH-balut-2'. In contrast, seed germination of 'C-6524' variety was less affected by 20E. The 20E significantly stimulated seedling root and shoot growth of 'Namangan-77' and 'AH-balut-2' varieties at 10^{-4} M NaCl or $MgSO_4$. Our results showed that the application of 10^{-4} M 20E improves cotton seed germination and protects seedlings from saline stress, although this effect is dependent upon cultivar.

Keywords: germination, *Gossypium hirsutum*, phytoecdysteroids, salinity, seedling growth

INTRODUCTION

Phytoecdysteroids are widely distributed molecules in the plant kingdom (Dinan 2001) and have been isolated from fungi and red alga (Fukuzawa *et al*. 1986; Kovganko 1999). They are thought to function as phytohormones and regulate physiological processes in plants (Machackova *et al*. 1995; Golovatskaya 2004). Phytoecdysteroids may also protect plants against insect attack by acting as feeding deterrents (Lafont *et al*. 1991; Tanaka *et al*. 1994).

The effect of ecdysteroids on growth, cell size and biochemical properties in cyanobacterium *Nostoc* 6720 (Maršálek *et al*. 1992) and in *Chlorella vulgaris* (Bajguz and Dinan 2004) were also reported. Bajguz and Godlewska-Zylkiewicz (2004) studied the protective role of 20-hydroxyecdysone (20E) against lead stress in *C. vulgaris* cultures. In those experiments, 20-hydroxyecdysone (10^{-10}–10^{-8} M) increased the growth and chemical composition of *C. vulgaris* cells. In other studies, phytoecdysteroids showed allelochemical effects on other plants or microbes. Ecdysteroids isolated from *Chenopodium album* have been tested for their phytotoxicity on the seeds of *Lactuca sativa* L. 20E inhibited the germination of lettuce by 15% at 10^{-4} M (DellaGreca *et al*. 2005). Also, Bakrim *et al*. (2007) reported that 20E (10^{-4} M) fulfilled some bioactive actions during germination and seedling growth in tomato.

Ecdysteroids show antimicrobial activity against microbes inducing inflammatory and purulent processes (Ahmad *et al*. 1996; Shirshova *et al*. 2006; Sautour *et al*. 2008), although these compounds did not have antifungal activity against three *Candida* species (*C. albicans*, *C. glabrata* and *C. tropicalis*).

To date there have been no studies on the alleviation effect of plant-derived 20E on seed germination and plant growth as affected by salt stress. Since drought and salt stress responses in plants are often mediated by phytohormones, it is important to study plant-derived molecules which function as plant hormones in saline conditions which could facilitate plant growth in such harsh environments. Therefore our study aimed to determine if the application of 20E could alleviate the effects of salinity on the germination and seedling growth of cotton under saline conditions.

MATERIALS AND METHODS

Isolation of 20-hydroxyecdysone (20E) from aerial parts of *Silene viridiflora*

Isolation of 20E was carried out using extraction, TLC and column chromatographic methods. Air-dried ground aerial parts (2 kg) of *Silene viridiflora* L. grown in the Botanical Garden of the Institute of the Chemistry of Plant Substances (Tashkent, Uzbekistan) were extracted with CH_3OH (10L × 6 times). The extract was condensed to 0.5 L and diluted with an equal amount of water. The resulting precipitate was filtered off. The CH_3OH was evaporated using a rotary vacuum evaporator at 40°C. The aqueous solution was extracted with $CHCl_3$ (2 L) and butanol (0.5 L). Solvents were evaporated in vacuum to afford the butanol (110 g) fraction, which was chromatographed over a silica gel column (L 100-160, Chemapol, Praha, Czech Republic). Elution with chloroform: methanol (9:1 v/v) isolated 7 g of 20E (0.35% yield on a air-dried mass of aerial parts of the plant basis), $C_{27}H_{44}O_7$, mp 241-242°C (acetone), $[\alpha]_D^{20}$ +58.9 ± 2° (*ca*. 0.3; CH_3OH) (Mamadalieva *et al*. 2003).

Seed germination and seedling growth

The seeds of three cotton (*Gossypium hirsutum*) varieties 'Namangan-77', 'C-6524' (salt-tolerant genotype) and 'AH-balut-2' (salt-sensitive genotype) were obtained from the Department of Plant Production, Tashkent State University of Agriculture, Uzbekistan and used for this study. Seeds were sorted to eliminate broken, small and infected seeds. Seeds of cotton were surface-sterilized for 5 min with concentrated sulphuric acid followed by 70% ethanol for 3 min and rinsed 5 times with sterile, distilled water. The sterility of seeds was tested on LC agar after incubation for three days. No fertility tests were conducted.

Seeds were germinated in covered, sterilized Petri dishes containing a single sheet of Whatman No. 3 filter paper moistened with either distilled water (control), or 60 and 100 mM of either NaCl or $MgSO_4$ solutions which are relevant salt compositions of

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cotton-cultivated areas in Uzbekistan and neighbouring countries. A concentration of NaCl greater than 100 mM showed a greater inhibitory effect on seed germination during preliminary tests.

Petri dishes were sealed with parafilm (Parafilm 'M,' American Can Co.) to prevent evaporation of water, thus minimizing changes in concentration of solutions. Thirty healthy and uniform seeds were sown in each Petri dish with three replications.

The effect of 20E on seed germination, and seedling growth were determined at 10^{-4} M and 10^{-5} M under non-saline and saline conditions. In our previous studies we observed that higher concentration of 20E inhibited the growth and development of wheat, soybean and cotton (unpublished results). Seeds were incubated in a growth chamber at 28°C and were considered germinated when the radical emerged. Germination rate of seeds was recorded until obtaining maximum of seed germination of control (only H_2O (distilled water) without 20E). Germination rate was calculated as a percent after 5 days. For determination of growth of cotton seedling, ten seedlings from control and treated samples were harvested randomly and then length of root and shoot which were more than 0.2 mm measured.

Analysis of variance was performed using the Excel program package version 11 for Windows 98 (Microsoft Corp.), Least significant differences (LSD) were applied to compare means at $P < 0.05$.

RESULTS AND DISCUSSION

The experimental data on seed germination are presented in **Tables 1** and **2**. The germination rate of cotton seeds were similar for 5 and 10 days and for all experiments the seeds were germinated for 5 days. The control (no salt) showed clear differences among the varieties regards seed germination from 81% in 'C-6524' to 87% in 'AH-balut-2' and to 94% in 'Namangan- 77'. Increased concentration of NaCl caused a decrease in germination in all cultivars. At the highest NaCl concentration (100 mM), the reduction in germination ranged from 31% in 'Namangan-77' to 19% in 'C-6524' and to 8% in 'AH-balut-2' (**Table 1**). Differences in varietal behaviour may affect adaptability to saline environments and such variation has been reported for other species such as amaranth (*Amaranthus* sp.) (Omami 2005), sugar beet (*Beta vulgaris* L.) (Ghoulam and Fares 2001), bean (*Phaseolus vulgaris* L.) (Çiftçi *et al.* 2011), turnip (*Brassica rapa* L.) and radish (*Raphanus sativus* L.) (Noreen and Ashraf 2008). Siddiqi *et al.* (2007) and Kaya *et al.* (2003) also observed considerable magnitude of variation for salt tolerance in a set of 10 available lines of safflower (*Carthamus tinctorius* L.) at the germination and seedling stages.

With increasing $MgSO_4$ concentrations, the seed germination in all varieties decreased. Exposure to 100 mM $MgSO_4$ depressed germination more than NaCl with germination reduction ranging from 18 to 20% in all varieties (**Table 2**). Jamil *et al.* (2006) reported that higher salinity levels resulted in a delay in germination of cabbage (*Brassica oleracea*), sugar beet and pack-choi (*Brassica rapa*) seeds.

Increasing salinity levels (60 and 100 mM) also decreased seedling growth, whereas the greatest reduction of root and shoot growth of wheat seedlings occurred with 100 mM NaCl and $MgSO_4$ conditions. Decrease in the length of roots was more pronounced than shoots. Considering roots 4.1 cm long and shoots 6.2 cm tall of 'Namangan-77' (control, no salt), 100% of analysed data shows that salt stress (60 mM NaCl) reduced the length of roots and shoots by as much as 44% and the reduction was higher (up to 84%) at 100 mM NaCl (**Table 3**). The highest reduction occurred when seeds were stressed by $MgSO_4$, whereas root length was reduced by 90% and shoot length by 84% at 100 mM (**Table 4**). The rate of decline in root length was most marked in 'AH-balut-2' with 77% at 60 mM $MgSO_4$. A high concentration of $MgSO_4$ totally inhibited seed germination of 'AH-balut-2' (**Table 4**).

Munns and Termaat (1986) previously reported that osmotic effects of salts on seedlings are a result of lowering of the soil water potential due to increasing solute concentration in the root zone. Salt stress results in a decline in metabolic activity of plant cells, which is inevitably reflected in inhibited growth (Kurth 1986; Cicek and Cakirlar 2002) and all the major processes such as protein synthesis, and energy and lipid metabolism are affected (Parida and Das 2005). A significant reduction in photosynthetic rate (net CO_2 assimilation rate) was also observed in rice (Raza *et al.* 2006), cotton (Desingh and Kanagaraj 2007), sunflower (Noreen and Ashraf 2008; Siddiqi *et al.* 2009) and pea (Yildirim *et al.* 2008) under saline conditions.

Table 1 Effect of 20-hydroxyecdysone pretreatments on rate of germination (mean ±S.E.) of three cotton genotypes seeds in 0, 60 and 100 mM NaCl solutions.

Salinity treatments	20-Hydroxy-ecdysone	Cotton varieties		
		Namangan-77	C-6524	AH-balut-2
60 mM	0	52 ± 1.5	40 ± 1.0	24 ± 1.0
	10^{-4} M	55 ± 1.0	33 ± 2.6	48 ± 2.0*
	10^{-5} M	53 ± 2.3	45 ± 1.5*	33 ± 2.5*
100 mM	0	31 ± 1.0	19 ± 1.0	8 ± 1.0
	10^{-4} M	39 ± 2.3*	12 ± 1.2	21 ± 2.3*
	10^{-5} M	33 ± 2.3	20 ± 2.1	11 ± 2.3

* Significantly different from the control at $P < 0.05$ (LSD)

Table 2 Effect of 20-hydroxyecdysone pretreatments on rate of germination (mean ±S.E.) of three cotton genotype seeds in 0, 60 and 100 mM $MgSO_4$ solutions.

Salinity treatments	20-Hydroxy-ecdysone	Cotton varieties		
		Namangan-77	C-6524	AH-balut-2
Distilled	0	94 ± 1.5	81 ± 1.0	87 ± 1.5
H_2O	10^{-4} M	95 ± 2.3	93 ± 1.2*	81 ± 1.2
	10^{-5} M	89 ± 2.3	79 ± 3.1	86 ± 2.1
60 mM	0	44 ± 2.5	32 ± 1.0	17 ± 2.0
	10^{-4} M	59 ± 2.3*	24 ± 2.0	45 ± 2.3*
	10^{-5} M	49 ± 3.1*	37 ± 2.6*	23 ± 3.6
100 mM	0	18 ± 1.5	20 ± 1.1	20 ± 1.0
	10^{-4} M	24 ± 3.5*	11 ± 2.3	25 ± 2.3*
	10^{-5} M	19 ± 2.3	5 ± 2.1	0

* Significantly different from the control at $P < 0.05$ (LSD)

It is thought that the repressive effect of salinity on germination could be related to a decline in endogenous levels of hormones (Debez *et al.* 2001). Sakhabutdinova *et al.* (2003) also reported that salinity resulted in a progressive decline in the level of phytohormones in the root system of plants. In this condition, soaking seed with plant growth regulators and the application of additional natural phytohormones supplied sufficient hormones for normal plant development and growth in saline conditions (Kabar 1987; Afzal *et al.* 2005). Cavusoglu and Kabar (2007) observed that the application of gibberellic acid and kinetin (at 900 and 100 μM, respectively) could alleviate the effects of high temperature on the germination of barley seeds.

There are several reports (Gregorio *et al.* 1995; Naidu 2001) showing that exogenous application of plant growth regulators such as gibberellic acid, auxin and kinetin can effectively improve seed germination. Khan and Weber (1986) and Gul *et al.* (2000) also observed that plant growth-stimulating compounds such as gibberellic acid, zeatin and ethephon can alleviate the effect of salinity on germination and growth of *Ceratoides lanata*, *Salicarnia pacifica* and *Allenrolfea accidentalis* (Khan *et al.* 2004). The addition of abscisic acid (ABA) to the nutrient solution reduced the negative effect of NaCl on common bean (*Phaseolus vulgaris*) and improved the response of bean symbiosis under saline stress (Khadri *et al.* 2006).

In our study seed dormancy enforced by salinity (100 mM NaCl) was substantially alleviated and germination was significantly promoted by 20E from 31 to 39% in 'Namangan-77' and from 8 to 21% in 'AH-balut-2' (**Tables 1, 2**). In contrast, seed germination of 'C-6524' was less affected by 20E. The application of 20E also reversed the growth inhibiting effect of salt stress to a certain extent in both cotton shoots and roots (**Tables 3, 4**). 20E significantly stimulated root and shoot growth of 'Namangan-77' and

Table 3 Effect of 20-hydroxyecdysone pretreatments on seedling growth (mean ±S.E.) of three cotton genotype in 0, 60 and 100 mM NaCl solutions.

Salinity treatments	20-Hydroxy-ecdysone	Namangan-77		C-6524		AH-balut-2	
		Root	Shoot	Root	Shoot	Root	Shoot
Distilled H_2O	0	4.1 ± 0.7	6.2 ± 1.2	3.1 ± 0.8	5.0 ± 1.1	2.1 ± 0.8	4.0 ± 0.8
	10^{-4} M	4.6 ± 0.5	7.4 ± 0.7*	3.9 ± 0.9	5.5 ± 1.1	3.1 ± 0.7*	4.4 ± 0.9
	10^{-5} M	4.1 ± 0.7	5.5 ± 0.9	3.0 ± 0.8	5.1 ± 1.0	2.6 ± 0.7	4.0 ± 0.8
60 mM	0	2.3 ± 0.6	3.5 ± 0.7	1.4 ± 0.5	2.1 ± 0.7	0.9 ± 0.1	1.2 ± 0.4
	10^{-4} M	2.7 ± 0.5	3.7 ± 0.9	1.6 ± 0.5	2.7 ± 0.5	1.3 ± 0.5*	1.8 ± 0.4*
	10^{-5} M	2.2 ± 0.4	3.7 ± 0.8	1.5 ± 0.7	2.5 ± 0.5	0.9 ± 0.1	1.3 ± 0.4
100 mM	0	0.7 ± 0.2	1.0 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.7 ± 0.1
	10^{-4} M	0.7 ± 0.2	1.4 ± 0.3*	0.4 ± 0.2	0.8 ± 0.4	0.4 ± 0.1*	0.9 ± 0.1*
	10^{-5} M	0.6 ± 0.1	1.1 ± 0.1	0.3 ± 0.2	0.7 ± 0.4	0.3 ± 0.1	0.1 ± 0.1

* Significantly different from the control at $P < 0.05$ (LSD)

Table 4 Effect of 20-hydroxyecdysone pretreatments on seedling growth (mean ±S.E.) of three cotton genotype in 0, 60 and 100 mM $MgSO_4$ solutions.

Salinity treatments	20-Hydroxy-ecdysone	Namangan-77		C-6524		AH-balut-2	
		Root	Shoot	Root	Shoot	Root	Shoot
Distilled H_2O	0	4.1 ± 0.7	6.2 ± 1.2	3.1 ± 0.8	5.0 ± 1.1	2.1 ± 0.8	4.0 ± 0.8
	10^{-4} M	4.6 ± 0.5	7.4 ± 0.7*	3.9 ± 0.9	5.5 ± 1.1	3.1 ± 0.7*	4.4 ± 0.9
	10^{-5} M	4.1 ± 0.7	5.5 ± 0.9	3.0 ± 0.8	5.1 ± 1.0	2.6 ± 0.7	4.0 ± 0.8
60 mM	0	1.6 ± 0.1	2.4 ± 0.5	1.1 ± 0.4	1.3 ± 0.7	0.5 ± 0.1	0.9 ± 0.2
	10^{-4} M	1.7 ± 0.2	2.7 ± 0.4	1.0 ± 0.2	1.8 ± 0.5*	0.6 ± 0.1	1.1 ± 0.2*
	10^{-5} M	1.6 ± 0.2	3.4 ± 0.5	1.0 ± 0.2	1.7 ± 0.2*	0.6 ± 0.2	0.9 ± 0.1
100 mM	0	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0	0
	10^{-4} M	0.8 ± 0.3*	1.3 ± 0.3*	0.3 ± 0.2	0.7 ± 0.2	0.5 ± 0.1*	0.9 ± 0.1*
	10^{-5} M	0.6 ± 0.2	1.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0	0

* Significantly different from the control at $P < 0.05$ (LSD)

'AH-balut-2' at 10^{-4} M under saline conditions. To our knowledge, this is first report indicating the alleviation effect of 20E. In early studies, Dreier and Towers (1988) and Machackova *et al*. (1995) demonstrated a weak gibberellin-like activity of 20E in mung bean epicotyl assays. Schmelz *et al*. (2000) reported similar findings in which 20E showed phytohormone activity in spinach (*Spinacia oleracea*). Our results showed that the application of 10^{-4} M of 20E improves seed germination of cotton and protects seedlings from saline stress although this effect is dependent upon the cultivar.

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