Journal of Applied Biological Sciences 9 (3): 09-17, 2015
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

Effects of 24-Epibrassinolide on Growth and Some Antioxidant Enzymes of Cotton (*Gossypium hirsutum* L.) Cultivars under NaCl Stress

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Received: August 11, 2015
Accepted: September 20, 2015

Abstract
The present study was conducted to investigate the anti-stress effects of 24-Epibrassinolide (EBL), an active brassinosteroid, in cotton cultivars against the NaCl stress. Nine cotton cultivars were tested for their germination responses to varying NaCl concentrations. According to germination test result, two tolerant (Nazilli 84-S and Carmen) and two sensitive (Sahin 2000 and Beyaz Altin 119) cultivars were selected for the experiments. Seeds of four cultivars were soaked in 3 µM EBL for 24 h and seedlings were irrigated with solution containing the various concentration of NaCl (0, 50, 100 and 150 mM). Germination, growth (fresh weight, dry weight, shoot and root length) and pigment content (chlorophyll a, chlorophyll b and carotenoid) reduced under salinity stress whereas the treatment with EBL alleviated the inhibitory effects of salt stress. Under high NaCl stress, superoxide dismutase (SOD), guaiacol peroxidase (GPX) and proline contents increased in 21-old-days seedlings; however EBL further increased the activity of antioxidant enzymes and proline content in cotton cultivars.
Keywords: 24-Epibrassinolide, cotton, NaCl, germination, growth, pigment content, enzyme activity

INTRODUCTION

Salinity is one of the important environmental problems that pose a severe threat to the growth and development of plant. According to the FAO Land and Nutrition Management Service (2008), over 6% of the world's land is affected by either salinity or sodicity which accounts for more than 800 million ha of land [1]. Having regard to this situation, it can be said that salinity is a major problem to crop production in many parts of the world, particularly in irrigated lands of arid and semiarid regions. Salt stress causes morphological, physiological, biochemical and molecular alterations in plants [2]. The general effect of salinity on plants is to reduce germination percentage of seeds, growth rate (decrease in leaf area, stem thickness, reduced shoot and root weight) and developmental characteristics such as root/shoot ratio and maturity rate. Most of abiotic stress, including salinity is an induced production of reactive oxygen species (ROS) which namely superoxide radical (O^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-). Salinity also adversely affects the photosynthesis, lipid metabolism, DNA, RNA, protein synthesis as well as mitosis [3-7].

Salt tolerance is a relative value based upon cultural conditions under which crop was grown. Although cotton is placed in the moderately salt-tolerant group of plant species with a salinity threshold level 7.7 dS m^{-1} [8], it is more sensitive to high salinity at germination and seedling stage [8, 9]. Germination of cotton seeds and emergence of seedlings decrease with the increasing of salt concentration and cottons' growth and seed yield severely reduce at high salinity levels (with a 50% reduction in yield at 17 dS m^{-1}) [9]. However, variations have observed among cultivars of cotton in response to salinity [7, 8, 10].

Many researchers investigate how to reduce the negative effect of salinity in plant growth and they have focused more on the mechanisms of salt tolerance in plants. Different approaches are being employed to maximize plant growth and development under high salt stress. In addition, many attempts have been made to overcome this disorder, including appropriate management and exogenous application of plant growth regulators [11].

Brassinosteroids (BRs) are new group of the phytohormones with significant growth promoting properties. BRs were first isolated and characterized from the pollen of rape plant (*Brassica napus* L.). Subsequently, they have been reported from 44 plants and descriptived of the six groups of phytohormones. It has been found that BRs at very low concentrations are effective in cell division, elongation and expansion, photo-morphogenesis, development of reproductive organs, leaf senescence, the increase in total biomass and yield as well as regulatory functions such as growth and development. In addition to this, BRs promote tolerance to a broad range of plant stressors, including high and low temperature stress, drought and heavy metals [12-15]. BRs have been shown to increase the degree of tolerance to salinity in rice [16], bean and barley [17], wheat [18, 19], pea [20], pepper [21] and eggplant [22]. Kumaro and Takatsuto (1999) remarked that the role of brassinosteroids in protecting plants against environmental stresses will be an important research theme and may contribute greatly to the usage of brassinosteroids in agricultural production [24].

In this study, we examined the effects of 24-Epibrassinolide (EBL) on early seedling growth, pigment content, proline level and the activity some antioxidant enzymes (superoxide dismutase and guaiacol peroxidase) in cotton cultivars subjected to different concerations of NaCl.

MATERIAL AND METHODS

Plant material

Seeds of nine cotton cultivars [Carmen, Flora, Celia, Nazilli 84-S, Nazilli 503, Sahin 2000, Beyaz Altin (BA) 125, Beyaz Altin 308 and Beyaz Altin 119] were obtained from cotton seed companies and Nazilli Cotton Research Institute.

In vitro germination test

Nine different cultivars of *Gossypium hirsutum* L. (2n = 4x = 56) were tested for their germination responses to varying salinity. In vitro germination test was conducted in petri dishes and the seeds of each cultivar were placed on moist filter paper (Whatman No. 2) [24]. Germination petries were placed in darkness where temperature ranged between 28 and 30 °C. The seeds irrigated daily with five different NaCl solutions (0, 50, 100, 150, 200 mM). Every 3 days, germinated seeds were counted and the percentages of germination were calculated. Only the plants that had completed cotyledon leaves on the surface of filter paper were considered. Germination experiments repeated four times.

Experimental setup and treatments

Among nine cultivars, Nazilli 84-S and Carmen were chosen for representing of a salt tolerant cultivar, while BA 119 and Sahin 2000 were as salt sensitive cultivars according to in vitro germination test results. These four cultivars were used in the subsequent experiments. The seeds of cultivars (Nazilli 84-S, Carmen, BA 119 and Sahin 2000) were soaked in 3 µM 24-Epibrassinolide (EBL) (Sigma-E 1641) or double distilled water as control for 24 hours (h) before sowing. After pre-treatment, seeds were sown in plastic pots which filled with steriled peat. The plastic pots were irrigated daily with water (0) or 50, 100, 150 mM NaCl solutions. Cultures were maintained at 28±2ºC under 12/12 h photoperiod of 2000 lux light intensity. The highest concentration of NaCl (200 mM) was not performed due to poor seedling growth. The plants were harvested at seedling stage (21 days after sowing) for analyzing growth parameters and biochemical assays. The layout of the experiment was a random-parcel-experimental design. There were five pots per treatment where each pot represented one experimental unit.

Growth parameters

At 21 day stage, a number of ten seedlings from each treatment were randomly selected and separated as shoots and roots. The shoots and roots were washed with distilled water and fresh weights recorded. Dry weight was noted after drying at 70°C for 36 hours. The shoot and root length of plants were measured by using a meter scale after removal from the pots.

Pigment content

Total chlorophyll (Chl) and carotenoids were extracted using the method described by Strain and Svec [25]. Frozen leaf tissue was extracted in 90% acetone and then the homogenate was centrifuged at 5000 rpm for 15 min. Absorbance of the supernatant was measured spectrophotometrically at 663, 645 and 450 nm. The pigment concentrations were expressed as mg g^{-1} fresh weight.

Free proline level

The free proline content in frozen leaves was determined using the procedure used by Bates et al. [26]. The results were calculated using a standard curve prepared with pure proline and was expressed in µmol g^{-1} fresh weight.

Assay of antioxidant enzymes

Frozen leaves (0.5 g) were homogenized in liquid nitrogen with 3 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2% (w/v) polyvenylpyrolidone (PVPP) and 1 mM disodium ethylenediaminetatraacetic acid (Na-EDTA). After centrifuging at 13000 *g* for 20 min at 4ºC, the supernatant was used for the determination of the protein contents and enzymes activities. Total soluble protein estimation was determined following the method of Bradford [27]. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured following the procedure as described by Beauchamp and Fridovich [28]. The test tubes were placed in the light for 20 min and the decrease in the absorbance was read at 560 nm. One unit of SOD was defined as the amount of enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%. Guaiacol peroxidase (GPX) (1.11.1.7) activity was measured by an increase in 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) according to the method described by Lee and Liu [29].

Statistical analyses

All parameters were analyzed by one-way analysis of variance (F-test). The least square difference (LSD) and Duncan's Multiple Range test were used for post hoc analyses. All data were evaluated by using the SAS version 11.0 software (SAS Institute Inc, USA).

RESULTS AND DISCUSSION

In vitro germination

The effect of different concentrations of NaCl treatments on germination of cultivars is shown in Table 1. The germination percentages of nine cultivars were ranged between 100% and 92.5% (Table 1) under unstressed conditions. Germination of seeds also decreased with the increasing concentration of NaCl in all cotton cultivars. While germination percentage differences were small at low salinity level (50 mM NaCl) among cultivars, became large at higher salinity levels (100 and 150 mM NaCl) when compared to control (without NaCl). The lowest germinations were observed in BA 119 and Sahin 2000 cultivars at 200 mM NaCl concentration, 22.5% and 27.5%, respectively (Table 1). In 200 mM NaCl concentration, the highest germination percentages were determined in Nazilli 84-S (72.5%), Carmen (70.0%) and Nazilli 503 (67.5%) cultivars (Table 1). The germination almost 35-40% decreased at 200 mM NaCl treatment in BA 125 and BA 308 cultivars when compared to their respective controls. The 200 mM NaCl treatment resulted in more than 45% reduction in germination percentage in the Flora and Celia cultivars (Table 1). The findings are in accordance with some researchers who reported that salt has a negative effect on germination of cotton seeds and this negative effect shows differences among cultivars [8, 30-33]. Germination of seeds in saline conditions is useful and simple parameter due to several parameters.

Table 1. The germination percentages of nine cotton cultivars subjected to different NaCl concentrations (±SE, based on four replicates)

Cotton cultivars	Germination (%)				
	NaCl (mM)				
	0	50	100	150	200
Nazilli 84-S	100 ± 0.00	97.5 ± 1.80	95.0 ± 2.88	80.0 ± 8.63	72.5 ± 5.17
Carmen	100 ± 0.00	100 ± 0.00	87.5 ± 2.07	80.0 ± 5.00	70.0 ± 1.67
Nazilli 503	97.5 ± 2.16	95.0 ± 2.94	92.5 ± 4.33	95.0 ± 3.64	67.5 ± 3.71
BA 125	100 ± 0.00	97.5 ± 2.04	87.5 ± 3.71	70.0 ± 6.94	65.0 ± 4.76
BA 308	95.0 ± 3.53	95.0 ± 4.56	77.5 ± 9.34	65.0 ± 0.44	57.5 ± 5.46
Flora	97.5 ± 2.60	95.0 ± 4.12	82.5 ± 2.58	57.5 ± 4.44	45.0 ± 7.61
Celia	97.5 ± 1.50	95.0 ± 2.15	82.5 ± 5.45	62.5 ± 2.33	37.5 ± 2.56
Sahin 2000	100 ± 0.00	85.0 ± 4.30	62.5 ± 4.12	37.5 ± 3.34	27.5 ± 2.12
BA 119	92.5 ± 3.25	90.0 ± 3.35	65.0 ± 5.12	42.5 ± 4.50	22.5 ± 3.40

For example, salinity tolerance at germination stage has been shown to be a heritable trait which could be used as an efficient criterion for the selection of salt tolerant populations. However, seeds and young seedlings are usually resisted by much higher salinities than growing plants because germination generally occurs in surface of soils which accumulate soluble salts as a result of evaporation and capillary rise [32].

Salt tolerant (Nazilli 84-S and Carmen) and salt sensitive (BA 119 and Sahin 2000) cultivars were chosen by taking to consideration the results in vitro germination among nine cultivars. Figure 1 shows that the percentage germinations of salt tolerant and sensitive cultivars at different days after start of the experiment. Differences in germination percentage between the 50 and 100 mM NaCl treatments were relatively low in Nazilli 84-S and Carmen cultivars at all incubation periods (Figure 1). On the other hand, relatively lower germination percentages were observed at 150 and 200 mM NaCl treatments for incubation periods between 3 and 12 days in Sahin 2000 and BA 119 (Figure 1). Salt stress inhibits seed germination by limiting water absorption by the seeds [34], thereby arresting radicle emergence. Furtermore, salt stress affects the mobilisation of stored reserves [35] and the stuctural synthesis of protein in germinating embryos [36].

Growth parameters

In the present study, the effect of EBL on the growth parameters (fresh weight, dry weight, shoot and root length) was examined in salt tolerant (Nazilli 84-S and Carmen) and salt sensitive (Sahin 2000 and Beyaz Altin 119) cultivars of cotton subjected to different concentrations of NaCl. The most well-known symptom of high salt stress is the plant growth reduction. Salt stress can influence growth of plant in several ways [37]. Firstly, salt decreased the water uptake capacity of plants and causes the rapid decline in the growth rate. Secondly, salt accumulates in the leaves, causing salt toxicity and finally affect the biomass and yield in the plants. In addition to, salinity leading nutritional disorders which change the availability, absorption and transport of nutrients within the plant. Osmotic stress, nutrient deficiency and ion toxicity are reasons based on the detrimental effects of salt stress on plant growth and development [37].

The presence of NaCl significantly reduced the shoot and root length of all cultivars ($P \leq 0.01$) (Table 2). Shoot and root lengths were more negatively affected by treatment with 150 mM NaCl than the other applied NaCl treatments in comparison to control and the length of the root were inhibited more drastically compared to that of shoots under salinity conditions. However, EBL treatment alleviated the inhibitory effect of NaCl on the growth of seedlings in both tolerant and sensitive cultivars of cotton and it has been found statistically significant ($P \leq 0.01$). Moreover, it has been observed that EBL generally has more effective to increase shoot and root length at highest concentration of NaCl (150 mM) (Table 2).

Salt treatments impaired the fresh and dry weight of seedlings in all tested cultivars in a statistically significant way ($P \leq 0.01$) (Table 3). The lowest fresh weights were recorded in BA 119 (0.98 ± 0.12) and Sahin 2000 (1.00 ± 0.10) cultivars at 150 mM NaCl treatment. Similar rate decreases also were observed in fresh and dry weights in salt tolerant and salt sensitive cultivars with increasing concentration of NaCl when compared to control seedlings. Several previous studies have reported that a decrease in fresh and dry weight in different plant species under high NaCl concentrations [16, 31]. While salinity causes significantly decrease in fresh and dry weight, pre-treatment of EBL to seeds increased biomass significantly at 21-day-old seedlings ($P \leq 0.01$). Furthermore, treatment with EBL exhibited an approximately 22-30% and 25-33% increase in fresh and dry weight, respectively, as compared to 150 mM NaCl treatment alone in all cultivars (Table 3). Anuradha and Ram Rao [32] also observed similar positive effects of EBL and HBL in rice under salt stress. BRs promote plant essential processes as a result of the additive effect of re-established water uptake ability and induced activity of ATPase pump in vacuoles or acid growth promotion [38]. Turgor driven cellular expansion presents the integrated picture as healthy growth of plant in terms of fresh and dry weight and length of shoot and root in *Arabidopsis thaliana* [39] and *Pisum sativum* [40]. Similarly, improvements in growth of the root and/or that of the shoot as a result of BR treatment have been observed in various plants [41, 42].

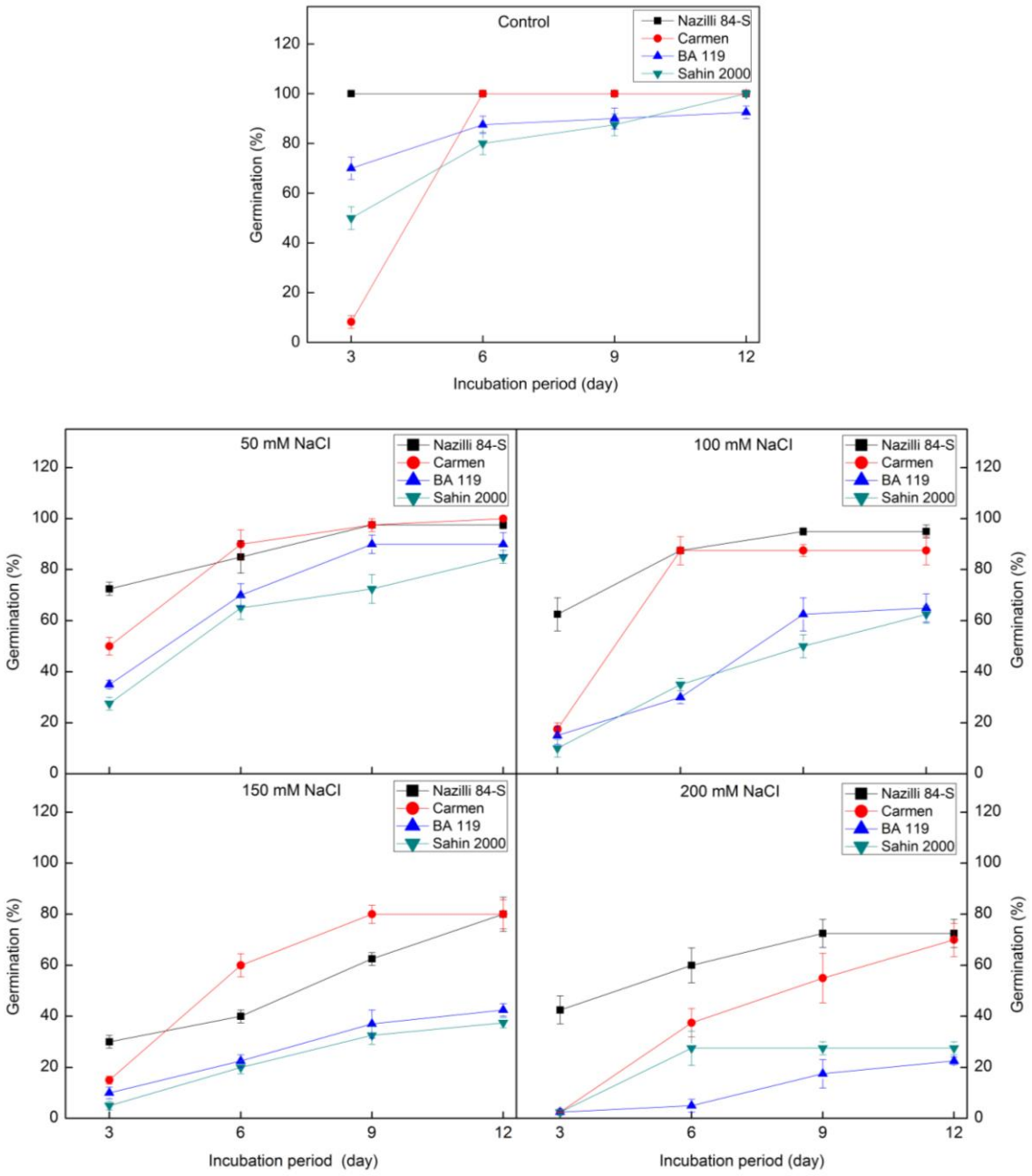

Figure 1. Percantage germination of Nazilli 84-S, Carmen, BA 119 and Sahin 2000 cultivar seeds when incubated in petri dishes moistened with sterile distilled water (control) or with different concentrations of NaCl solution (50,100, 150 and 200 mM), at different days after start of the experiment. Error bars represent standard errors (based on four replicates)

Pigment and proline content

High salt stress causes accumulation of Na^+ and Cl^- ions seriously inhibits the photosynthetic enzymes and affects electron transport chain and result in production of ROS. The salt stress disrupts the photosynthesis by changing the ultrastructure of the organelles, reduced the synthesis of pigments and enzymes, and inhibited the rubisco activity, stomatal conductance, CO_2 availability and photosynthetic enzyme activity [37]. In the present study, gradual decrease in pigment content was observed with the increase of NaCl. It was observed that chlorophyll a, b and carotenoid declined continuously with enhanced salinity level. At 150 mM NaCl chlorophyll a, b and carotenoid content was reduced about 49%, 34% and 49%, respectively, when compared to their respective control in salt tolerant cultivars (Table 4). Compared to the control, the above mentioned pigment contents were decreased by about 50%, 53% and 50%, respectively, in salt sensitive cultivars at the highest concentration of NaCl. İzci [43] also found that high NaCl concentrations decreased photosynthetic pigment contents (Chl a, b and carotenoid) of calli obtained from leaf explants of three cotton cultivars including Nazilli 84-S and Carmen cultivars. On the other hand, treatment with EBL led to significant enhancement of the pigment content of four cultivars in comparison with the seedlings exposed to NaCl alone. EBL treatment also caused relatively low increase in pigment content under unstressed conditions.

Table 2. Effect of EBL treatment on mean (±SE) shoot and root lenghts (cm) of 21-day-old of cotton seedlings subjected to different concentrations of NaCl (n=10; Ns: non-significant; *P ≤ 0.05; **P ≤ 0.01).

Cotton cultivars	NaCl (mM)	Shoot length (cm)		Root length (cm)	
		EBL (-)	EBL (+)	EBL (-)	EBL (+)
Nazilli 84-S	0	11.5 ± 0.41	11.4 ± 0.52	6.1 ± 0.44	6.0 ± 0.51
	50	10.5 ± 0.42	11.0 ± 0.20	5.8 ± 0.39	5.8 ± 0.17
	100	8.5 ± 0.84	10.3 ± 0.33	4.7 ± 0.77	5.5 ± 0.83
	150	7.7 ± 0.72	9.5 ± 0.70	3.9 ± 0.93	4.8 ± 0.11
Carmen	0	10.8 ± 0.73	11.3 ± 0.99	6.0 ± 0.84	6.2 ± 0.85
	50	9.8 ± 0.85	10.9 ± 1.33	5.4 ± 0.61	5.8 ± 0.68
	100	8.1 ± 0.55	9.8 ± 0.62	4.6 ± 0.38	5.3 ± 0.75
	150	7.5 ± 0.42	9.1 ± 0.41	3.8 ± 0.78	4.6 ± 0.57
BA 119	0	12.2 ± 0.92	13.5 ± 0.86	6.3 ± 0.18	6.2 ± 0.77
	50	10.3 ± 0.51	12.6 ± 0.81	5.7 ± 0.40	5.9 ± 0.30
	100	8.5 ± 0.38	9.8 ± 1.06	4.2 ± 0.32	5.0 ± 0.78
	150	7.3 ± 0.54	8.9 ± 0.63	3.5 ± 0.45	4.5 ± 0.45
Sahin 2000	0	12.6 ± 0.62	13.2 ± 0.64	6.5 ± 0.47	6.4 ± 0.20
	50	11.5 ± 0.50	12.6 ± 0.65	5.6 ± 0.70	5.8 ± 0.34
	100	9.5 ± 0.25	10.3 ± 0.62	4.3 ± 0.31	5.2 ± 0.29
	150	7.2 ± 0.43	8.8 ± 0.45	3.2 ± 0.43	4.5 ± 0.50

ANOVA (F test)	Shoot length	Root length
Cultivar	0.383**	Ns
Salt	0.383**	**0.463
EBL	0.271**	**0.327
Cultivar x Salt	0.765**	Ns
Cultivar x EBL	Ns	Ns
Salt x EBL	0.541*	Ns
Cultivar x Salt x EBL	Ns	Ns

Table 3. Effect of EBL treatment on mean (±SE) fresh and dry weights (g) of 21-day-old of cotton seedlings subjected to different concentrations of NaCl (n=10; Ns: non-significant; *P ≤ 0.05; **P ≤ 0.01).

Cotton cultivars	NaCl (mM)	Fresh weight (g plant^{-1})		Dry weight (g plant^{-1})	
		EBL (-)	EBL (+)	EBL (-)	EBL (+)
Nazilli 84-S	0	1.83 ± 0.25	1.83 ± 0.29	0.37 ±0.09	0.36 ± 0.07
	50	1.72 ± 0.17	1.79 ± 0.12	0.34 ± 0.03	0.35 ± 0.04
	100	1.40 ± 0.16	1.46 ± 0.09	0.26 ± 0.01	0.28 ± 0.06
	150	1.10 ± 0.08	1.41 ± 0.12	0.20 ± 0.03	0.26 ± 0.03
Carmen	0	1.74 ± 0.16	1.74 ±0.08	0.34 ± 0.02	0.36 ± 0.04
	50	1.69 ± 0.09	1.73 ± 0.08	0.33 ± 0.05	0.34 ± 0.03
	100	1.43 ± 0.14	1.48 ± 0.10	0.27 ± 0.05	0.28 ± 0.04
	150	1.11 ± 0.08	1.36 ± 0.08	0.20 ± 0.05	0.25 ± 0.02
BA 119	0	1.63 ± 0.13	1.64 ± 0.05	0.33 ± 0.03	0.33 ± 0.03
	50	1.63 ± 0.04	1.63 ± 0.08	0.32 ± 0.03	0.32 ± 0.03
	100	1.31 ± 0.06	1.40 ± 0.07	0.24 ± 0.08	0.27 ± 0.03
	150	0.98 ± 0.12	1.28 ± 0.11	0.18 ± 0.02	0.24 ± 0.04
Sahin 2000	0	1.74 ± 0.07	1.75 ± 0.14	0.36 ± 0.03	0.35 ± 0.03
	50	1.60 ± 0.05	1.59 ± 0.07	0.33 ± 0.06	0.31 ± 0.05
	100	1.32 ± 0.07	1.35 ± 0.08	0.24 ± 0.04	0.26 ± 0.03
	150	1.00 ± 0.10	1.27 ± 0.08	0.18 ± 0.04	0.23 ± 0.04

ANOVA (F-test)	Fresh weight	Dry weight
Cultivar	**0.009	**0.05
Salt	**0.009	**0.05
EBL	**0.007	**0.04
Cultivar x Salt	**0.019	**0.10
Cultivar x EBL	**0.013	*0.07
Salt x EBL	**0.013	**0.07
Cultivar x Salt x EBL	**0.027	Ns

However, a maximum increase in chlorophyll a content (126%) was observed in Sahin 2000 cultivar treated with EBL and 150 mM NaCl together (2.65 ± 0.05) as compared to 150 mM NaCl alone (1.17 ± 0.02). Similar trends (about 63%) were observed in case of chlorophyll b in Sahin 2000 and BA 119 cultivar also. Furthermore, treatment with EBL increased the carotenoid content approximately 72% and 122% in salt toleant and salt sensitive cultivars, respectively, at 150 mM NaCl treatment. BRs induced transcription and/or translation of the enzymes involved in chlorophyll biosynthesis linked with a decrease in the level of catabolizing enzymes appears to be the plausible explanation to such an observation [44]. Similarly, exogenous application of BRs increased the pigment content in response to NaCl stress in bean [45], rice [46], soybean [47] and radish plants [48]. Moreover, an improvement in pigment level as a result of BR treatment in plant species can be one of the reasons for growth stimulation by BRs under salinity conditions [46].

One of the most common stress responses in plants is over producing of suitable organic solutes including proline [49].

Proline accumulates under stress conditions and shows defence against osmotic imbalance generally under salt stress conditions. Moreover, proline protects intracellular structures from free radicals and stabilizes enzymes of cells [50]. One of the results of the present study is that the proline contents in seedlings increased in response to different concentrations of NaCl. Compared with control, treatment with 100 mM NaCl caused increases in proline content in Nazilli 84-S and Carmen cultivars (26 and 21 %, respectively). However, the stress generated by 150 mM NaCl resulted in rises in proline content 64-63% in Sahin 2000 and BA 119 cultivars, respectively. On the other hand, seedlings germinated from EBL-treated seeds had greater proline content under salt stress in comparison with the seedlings treated with NaCl alone (Table 4). Treatment with EBL also showed 18%, 26% and 48% increases in proline content under different concentrations of NaCl (50, 100 and 150 mM, respectively) in Nazilli 84-S cultivar as compared to their respective controls.

In BA 119 and Sahin 2000 cultivars, pre-treatment of seeds with EBL increased proline content in 19 and 17% at 50 mM NaCl, 29 and 24% at 100 mM NaCl and 64 and 63% at 150 mM NaCl treatment as compared to their respective controls (Table 4). Sharma et al. [51] reported that the seedlings exposed to NaCl exhibited significant rise in proline content; however treatment with EBL further increased the proline content in rice. Moreover, it has been reported that BR treatments stimulate the expression level of proline biosynthetic genes as well as proline levels [52, 53].There are many reports that application of BRs increased proline level in various plants [54].

Antioxidant enzyme activities

ROS affect many cellular molecules and metabolites, and cause several harmful processes resulting in cellular degradation [55]. In this case, the plants increase the synthesis of the enzymes and antioxidant metabolites in order to endure and overcome the harmful effects of the ROS species such as superoxide radical, hydroxyl ions and hydrogen peroxide [4]. In the present study, salt stress led to the significantly changes in the activity of antioxidant enzymes. In all cultivars, salt stress significantly increased the activity of SOD enzyme which acts as first line of defense against ROS, dismutating O_2^- to H_2O_2.

Table 4. Effect of EBL treatment on mean (±SE) chlorophyll a, chlorophyll b, carotenoid (mg g^{-1} fresh weight) and proline (µmol g^{-1} fresh weight) of 21-day-old of cotton seedlings subjected to different concentrations of NaCl (n=3; Ns: non-significant; *P ≤ 0.05; **P ≤ 0.01).

Cotton cultivars	NaCl (mM)	Chlorophyll a (mg g^{-1} fresh weight)		Chlorophyll b (mg g^{-1} fresh weight)		Carotenoid (mg g^{-1} fresh weight)		Proline (µmol g^{-1} fresh weight)	
		EBL (-)	EBL (+)	EBL (-)	EBL (+)	EBL (-)	EBL (+)	EBL (-)	EBL (+)
Nazilli 84-S	0	2.60 ± 0.09	2.68 ± 0.11	1.91 ± 0.03	1.97 ± 0.02	2.60 ± 0.09	2.68 ± 0.11	1.91 ± 0.03	1.97 ± 0.02
	50	2.46 ± 0.16	2.77 ± 0.09	1.74 ± 0.02	2.05 ± 0.02	2.46 ± 0.16	2.77 ± 0.09	1.74 ± 0.02	2.05 ± 0.02
	100	1.61 ± 0.09	2.29 ± 0.16	1.57 ± 0.05	1.92 ± 0.07	1.61 ± 0.09	2.29 ± 0.16	1.57 ± 0.05	1.98 ± 0.07
	150	1.26 ± 0.08	2.15 ± 0.11	1.24 ± 0.05	1.53 ± 0.06	1.26 ± 0.08	2.15 ± 0.11	1.24 ± 0.05	1.83 ± 0.06
Carmen	0	2.75 ± 0.07	2.79 ± 0.03	2.34 ± 0.01	2.45 ± 0.01	2.75 ± 0.07	2.79 ± 0.03	2.34 ± 0.01	2.45 ± 0.01
	50	2.73 ± 0.04	3.09 ± 0.09	2.07 ± 0.03	2.45 ± 0.04	2.73 ± 0.04	3.09 ± 0.09	2.07 ± 0.03	2.45 ± 0.04
	100	1.53 ± 0.07	2.15 ± 0.15	1.98 ± 0.03	2.40 ± 0.03	1.53 ± 0.07	2.15 ± 0.15	1.98 ± 0.03	2.40 ± 0.03
	150	1.49 ± 0.05	2.57 ± 0.05	1.55 ± 0.06	1.88 ± 0.09	1.49 ± 0.05	2.57 ± 0.05	1.45 ± 0.06	2.08 ± 0.09
BA 119	0	2.41 ± 0.04	2.49 ± 0.05	1.55 ± 0.11	1.62 ± 0.11	2.41 ± 0.04	2.49 ± 0.05	1.55 ± 0.11	1.62 ± 0.11
	50	2.11 ± 0.06	2.42 ± 0.16	1.14 ± 0.01	1.36 ± 0.10	2.11 ± 0.06	2.42 ± 0.16	1.14 ± 0.01	1.36 ± 0.10
	100	1.61 ± 0.09	2.49 ± 0.02	0.95 ± 0.05	1.25 ± 0.06	1.61 ± 0.09	2.49 ± 0.02	1.05 ± 0.05	1.35 ± 0.06
	150	1.22 ± 0.08	2.67 ± 0.07	0.70 ± 0.04	1.15 ± 0.06	1.22 ± 0.08	2.67 ± 0.07	0.70 ± 0.04	1.15 ± 0.06
Sahin 2000	0	2.36 ± 0.09	2.40 ± 0.07	1.80 ± 0.09	1.83 ± 0.09	2.36 ± 0.09	2.40 ± 0.07	1.80 ± 0.09	1.83 ± 0.09
	50	2.29 ± 0.11	2.65 ± 0.11	1.51 ± 0.08	1.76 ± 0.09	2.29 ± 0.11	2.65 ± 0.11	1.51 ± 0.08	1.76 ± 0.09
	100	1.23 ± 0.07	2.05 ± 0.07	1.34 ± 0.04	1.66 ± 0.07	1.23 ± 0.07	2.05 ± 0.07	1.34 ± 0.04	1.66 ± 0.07
	150	1.17 ± 0.02	2.65 ± 0.05	0.89 ± 0.03	1.45 ± 0.07	1.17 ± 0.02	2.65 ± 0.05	0.89 ± 0.03	1.45 ± 0.07

LSD	Chlorophyll a	Chlorophyll b	Carotenoid	Proline
Cultivar	**0.189	**0.408	**0.195	**0.035
Salt	**0.178	**0.372	Ns	**0.030
EBL	**0.134	**0.288	**0.138	**0.024
Cultivar x Salt	**0.378	Ns	**0.390	**0.064
Cultivar x EBL	**0.268	Ns	Ns	**0.049
Salt x EBL	Ns	Ns	Ns	**0.049
Cultivar x Salt x EBL	**0.535	**1.154	**0.551	**0.098

The activity of SOD was observed maximum at 150 mM NaCl treatment in BA 119 (30.42 ± 3.22) and Sahin 2000 (30.76 ± 2.01) cultivars (Table 5). The treatment with EBL further increased the activity of SOD enzyme in all cultivars ($P < 0.05$). However, a more increase was examined in the activity of SOD in salt sensitive cultivars (47-55%) than salt tolerant cultivars (23-33%) at the highest concentration of NaCl.

Imposition of NaCl stress resulted in asignificant overall enhancement in the activity of GPX enzyme. The lowest activity of GPX enzyme was found in Carmen (1.13 ± 0.07), while highest GPX activity was determined in Sahin 2000 (1.74 ± 0.17) cultivar at 150 mM NaCl treatment (Table 5). However, the EBL treatment caused a significant increase in the activity of GPX enzyme in all cultivars ($P < 0.05$). Pre-treatment of seeds with EBL increased the activity of GPX in 46 and 43% at 50 mM NaCl, 58 and 51% at 100 mM NaCl and 70 and 79% at 150 mM NaCl treatment as compared to their respective controls in BA 119 and Sahin 2000 cultivars, respecvtively (Table 5). A maximum rise of 73% in GPX activity was observed in Carmen cultivar at binary combination of EBL and 150 mM NaCl (1.96 ± 0.24) as compared to seedlings treated with 150 mM NaCl alone (1.13 ± 0.07). A significant increase in GPX was also noticed in seeds treated with EBL in conjuction with NaCl in Nazilli 84-S cultivar ($P < 0.05$). Previous reports demonstrated that exogenous application of BRs modified antioxidant enzyme activity [56-58]. El-Khallal et al. [55] reported that BRs led to an up regulation of the genes controlling the synthesis of the antioxidant enzymes or an increased activation of constitutive enzymes pools in plants under stress conditions. Cao et al. [59] demonstrated on the basis of molecular, physiological and genetic approaches the elevation in antioxidant enzymes was the consequence of enhanced expression of *DET2* gene (encodes the steroid 5α-reductase responsible for an early step in the BR biosynthetic pathway), which increased the resistance to oxidative stress in *Arabidopsis*. Furthermore, Mazorra and Nunez [60] and Mazorra et al. [61] found that the effect of BRs on antioxidant activity was depent on concentration.

In conclusion, steroids are functional hormones in plants as in animals and brassinosteroids are a new group of phytohormones. Although high concentrations of NaCl cause stress and decreases in the germination, growth, and pigment contents in cotton cultivars, EBL treatments significantly favour growth, enhance the activities of antioxidants and proline content, and partly overcome the toxic effect of NaCl.

Table 5. Effect of EBL treatment on activity of SOD (Unit mg protein^{-1}) and GPX (ΔA470 min^{-1} mg protein^{-1}) enzymes of 21-day-old of cotton seedlings subjected to different concentrations of NaCl. The means (±SE) denoted by different letters present significant differences at $P < 0.05$ according to Duncan's Multiple Range test (n=3). Each cultivar was evaluated as a separate experiment in statistical analysis.

Cotton Cultivars	NaCl (mM)	SOD (Unit mg protein^{-1})		GPX (ΔA470 min^{-1} mg protein^{-1})	
		EBL (-)	EBL (+)	EBL (-)	EBL (+)
Nazilli 84-S	0	11.75 ± 1.65 f	15.79 ± 1.94 g	0.26 ± 0.05 f	0.35 ± 0.02 f
	50	22.77 ± 1.92 e	26.57 ± 1.81 d	0.87 ± 0.11 d	1.10 ± 0.17 de
	100	25.46 ± 1.32 d	34.40 ± 2.26 b	1.30 ± 0.11 cd	1.99 ± 0.17 b
	150	29.10 ± 1.26 c	38.76 ± 1.64 a	1.49 ± 0.05 c	2.46 ± 0.11 a
Carmen	0	12.23 ± 1.24 f	12.35 ± 1.75 f	0.41 ± 0.05 e	0.54 ± 0.17 cde
	50	17.55 ± 0.77 e	20.84 ± 1.34 d	0.68 ± 0.11 cde	0.98 ± 0.17 bcd
	100	20.10 ± 0.99 d	24.55 ± 2.24 c	0.96 ± 0.11 bcd	1.57 ± 0.09 ab
	150	29.96 ± 2.06 b	37.10 ± 3.69 a	1.13 ± 0.07 abc	1.96 ± 0.24 a
BA 119	0	12.25 ± 0.73 f	13.19 ± 1.79 f	0.26 ± 0.03 e	0.44 ± 0.05 de
	50	17.26 ± 1.15 e	23.75 ± 2.56 d	0.76 ± 0.11 d	1.11 ± 0.12 c
	100	25.55 ± 1.35 d	37.79 ± 3.62 b	1.22 ± 0.12 c	1.93 ± 0.17 b
	150	30.42 ± 3.22 c	44.94 ± 2.39 a	1.52 ± 0.23 bc	2.58 ± 0.45 a
Sahin 2000	0	15.34 ± 2.00 f	16.24 ± 1.86 f	0.28 ± 0.02 f	0.48 ± 0.05 f
	50	18.69 ± 2.21 ef	27.56 ± 2.03 d	0.51 ± 0.04 ef	0.73 ± 0.11 d
	100	26.52 ± 2.48 d	37.86 ± 2.50 b	1.37 ± 0.17 c	2.07 ± 0.15 b
	150	30.76 ± 2.01 c	47.81 ± 3.93 a	1.74 ± 0.17 bc	3.12 ± 0.24 a

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