Appl Biol Chem (2018) 61(1):113–124
https://doi.org/10.1007/s13765-017-0329-1

Online ISSN 2468-0842
Print ISSN 2468-0834

ARTICLE

Potential objectives for gibberellic acid and paclobutrazol under salt stress in sweet sorghum (*Sorghum bicolor* [L.] Moench cv. Sofra)

Amir Hossein Forghani[1] · Abbas Almodares[1] · Ali Akbar Ehsanpour[1]

Received: 27 August 2017 / Accepted: 7 November 2017 / Published online: 31 January 2018
© The Korean Society for Applied Biological Chemistry 2018

Abstract The phytohormones are important in plant adaptation to abiotic and biotic stresses by facilitating a wide range of adaptive responses. Application of gibberellic acid (GA_3) and paclobutrazol (PBZ) as GA_3 inhibitors have been shown to affect salinity tolerance through modulating phytohormones. The aim of this study was to find out the potential objectives for GA_3 and PBZ as affected by salinity through altering the phytohormones and biochemical parameters in sweet sorghum. Following seed germination, seedlings were cultured in Hoagland nutrient solution containing NaCl supplemented with GA_3 and PBZ for 12 days. The results were analyzed by principal component analysis to identify the best target(s) for salinity, GA_3, and PBZ in sweet sorghum. Paclobutrazol associated with salt improved root/shoot length, carotenoid, and total chlorophyll by modulating cytokinin (CK)/GA_3, indole acetic acid (IAA)/GA_3, and total polyamines/GA_3 ratios. Gibberellic acid-treated plants not exposed to salinity treatments notably improved phytohormones content such as cytokinin, auxin, abscisic acid (ABA), and polyamines resulting in increased stem growth. Moreover, the main objectives of GA_3 were ABA, spermidine, and ABA/GA_3 ratio in response to salinity. Though GA_3 and PBZ have different roles against salt stress, ABA/GA_3 ratio was a similar target of GA_3 and PBZ. This work suggests that altered levels of GA_3 resulting from PBZ- and GA_3-treated plants cause different allocation patterns in sweet sorghum by regulation of CK/GA_3, IAA/GA_3, and total polyamines/GA_3 ratio. Also, accumulation chlorophyll pigments, carotenoids, and water soluble carbohydrates of sorghum plants under salinity regulated by total polyamines/GA_3 and ABA/GA_3 ratios positively correlated with PBZ application.

Keywords Auxin · Chlorophyll · Cytokinin · Hormone cross-talk · Polyamines

Introduction

Salinity is a major problem in agriculture and crop production. High concentration of toxic ions creates salty soil regions around the world. Sodium and chloride are main ions in most of the salty areas, and NaCl is the most damaging compound for plant growth and development. The negative effects of salinity on plant growth and leaf senescence are related to osmotic stress and ion toxicity [1–3].

Sweet sorghum (*Sorghum bicolor*) has usually been grown in areas with relatively low rainfall, high temperatures, and saline soils. This plant, as a moderately salt tolerant crop, has been adapted to water limitation and is more tolerant to salinity at germination stage than in the later stages of growth [4, 5]. Moreover, sorghum is an important food and bioenergy source as well as a model plant for studying the mechanisms of drought and salt tolerance in cereals [3, 6]. A number of methods have been developed for screening salt tolerance in plants because majority of physiological processes (e.g., germination, photosynthesis, biomass production, and chlorophyll content) are highly sensitive to salt stress [3]. Plants can adjust their growth and development in response to salt stress by signaling molecules. Phytohormones are active members of signal cascade involved in the induction of salt stress responses [7]. Several phytohormones such as abscisic acid

✉ Ali Akbar Ehsanpour
ehsanpou@sci.ui.ac.ir

[1] Department of Biology, University of Isfahan, Isfahan, Iran

(ABA), cytokinin (CK), auxin (Aux), polyamines, and gibberellic acid (GA_3) have been used to reduce the negative effects of salt stress on seed germination, plant growth, and fruit setting [8]. Recently, GA_3 has been considered as a growth regulator to ameliorate salt stress. This hormone has been used to increase wheat and rice growth under salinity condition [8]. The function of phytohormones is complicated due to their interactions with other plant growth regulators. In case of GA_3 action, no specific target of GA-induced genes has been identified which may propose interaction with other hormones plays a key role in response to stress [7]. How gibberellins treatment may induce salt tolerance in plants has not been clearly answered yet [9]. It has been shown that GA_3 and ABA play antagonistic roles in many plants' developmental processes such as germination, growth, and flowering. Moreover, the antagonistic effects of GA_3 and CK on some growth parameters have been previously reported [7]. In contrast, it has been indicated that GA_3 and auxin frequently act synergistically, especially in stem elongation [7]. Recently, it was reported that auxin is positively associated with GA_3 by promoting degradation of DELLA protein [7]. It has also been known that, salt stress reduces endogenous cytokinins and improves ABA and polyamines levels [7], especially spermine in sorghum [10]. Studies have shown that polyamines have positive effects in plants facing environmental stresses [10]. Although polyamines are essential for cell division and root initiation [10], little is known about signal transduction of polyamines and their interaction with GA_3. Triazoles such as paclobutrazol (PBZ) affect the isoprenoid pathway and alter the levels of certain plant hormones by inhibiting gibberellin synthesis, reducing ethylene evolution and increasing CK and ABA levels [11]. Triazole compounds also protect plants against various stresses, and they have been characterized as plant multi-protectants [12]. Application of PBZ has been reported to mitigate salt stress in some plant species by reducing the level of GA_3 via inhibition of monooxygenases which catalyze oxidative steps from *ent*-kaurene to *ent*-kaurenoic [13]. However, the actual mechanism of stress tolerance by PBZ remains unclear. It seems GA_3 and PBZ may increase salt tolerance by different mechanisms and they may have different physiological, biochemical, and hormonal objectives. To the best of our knowledge, no comparative study has been reported in plants using GA_3 and PBZ under salt stress. In the current study, we aim to understand: (1) How GA_3 and its inhibitor (PBZ) interact with other plant hormones under salt stress conditions; and (2) among carbohydrates, photosynthetic pigments, polyamines, and phytohormones, what are the best objectives for GA_3 and PBZ to ameliorate the negative effects of salt stress in sweet sorghum as a model plant.

Materials and methods

Hydroponic culture

Fresh seeds of sweet sorghum (*Sorghum bicolor* [L.] Moench cv. Sofra) were supplied from the seed stock University of Isfahan, Iran. Seeds were surface sterilized for 1–2 min in 95% ethanol, followed by 15% sodium hypochlorite (v/v) for 20 min. Seeds were then washed three times using sterilized distilled water and transferred into the plastic pots containing sterile perlite and watered with Hoagland nutrient solution. Pots were then kept in the culture room at 25 ± 1 °C with 16/8 h photoperiod under 100 μmol m^{-2} s^{-1} light intensity derived from white fluorescent lamps. After germination, five seedlings were transferred to the new pots and each pot was moved inside a 200 mL container filled with Hoagland nutrient solution [14]. After 4 days, the nutrient solution of pots was replaced by fresh Hoagland medium containing 0, 100, 150, 200, and 250 mM NaCl, supplemented with either PBZ (17 μM) or GA_3 (17 μM). Pots with neither salt nor PBZ or GA_3 were used as control. After 12 days, treated plants were harvested and their biochemical and physiological parameters were measured as described below.

Growth parameters

A number of leaves, stem and root lengths as well as fresh weight (FW) of plants were measured after 12 days. To determine the dry weight, samples were dried in a 70 °C oven for 48 h. Shoot water content was measured by calculating the difference between the fresh and dry weights of the plants.

Photosynthetic pigments

For photosynthetic pigments' measurements, leaves (0.1 g FW) of plants were ground in 80% cold acetone and centrifuged at 5000 g for 10 min. The absorbance of the purified chlorophyll samples was measured at 470, 646, and 663 nm (Shimadzu UV-160, Kyoto, Japan). Chlorophyll and carotenoid contents were calculated according to Lichtenthaler and Wellburn [15].

Carbohydrate content

Water soluble carbohydrates (WSC) were determined based on the phenol–sulfuric acid method described by Dubois et al. [16]. To prepare carbohydrate extract, 10 mg of dry leaf was homogenized with 10 mL 80% ethanol. The extracts were centrifuged at 6000 rpm, and 0.5 mL of supernatant was mixed with 0.5 mL of 5% phenol and

2.5 mL 96% sulfuric acid. The samples were then vortexed gently for 30 min, and soluble carbohydrates were measured at 490 nm.

Measurement of phytohormones using HPLC

The level of indole acetic acid, cytokinin, and gibberellic acid was determined by method described below. Extraction: Approximately 2 g of tissue was ground in 30 mL cold 80% methanol and homogenized in darkness at 4 °C, then centrifuged at 5000 rpm at 4 °C for 15 min. The supernatant was filtered through 0.45 µm Whatman™ filter to remove suspended particles [17–19]. The filtered supernatant was treated sequentially with methanol–acetic acid (100:1, v/v), methanol–water–acetic acid (50:50:1, v/v/v), methanol–water–acetic acid (30:70:1, v/v/v), and finally with water before transferring 100 mL of filtrate to 10 C18 SPE columns (J.T. Baker, Phillipsburg, NJ, USA; 500 mg, 3 mL). The columns were washed with 10 mL water adjusted to pH 3 with acetic acid (acidified water). The phytohormones were eluted with 5 mL ethanol–water–acetic acid (80:20:1, v/v/v). The eluate was evaporated at room temperature under vacuum and finally, the evaporated eluate was dissolved in 1 mL methanol [17]. Prior to HPLC analysis, this reconstituted eluate was filtered using a 0.45 µm Whatman™ glass microfiber filter.

Evaluation by HPLC: Analysis of phytohormones was performed based on Ge et al. [20] and Ma et al. [17], using a HPLC system (Unicam, Crystal 200, Cambridgeshire, England) linked simultaneously to a photodiode array (PDA) system. Ten µL of extract was injected into a C18 reverse phase column (Zorbax SB-C18 100A°, 3.5 µm, 150 mm length, 2.1 mm diameter). The column thermostat was set at 25 °C. The initial HPLC column running conditions was initialized isocratically with methanol–formic acid buffer (10:90, v/v) for 5 min, then a linear gradient to methanol–formic acid buffer (30:70, v/v) in 5 min, which was later maintained isocratically for 10 min, before switching to a linear gradient toward a methanol–formic acid buffer (45:55, v/v) in 35 min and finally isocratically at methanol–formic acid buffer (45:55, v/v) for 15 min. Under the separation conditions, all compounds were successfully separated within 45 min. After each analysis, the column was washed with 95:5 methanol–formic acid buffer for 5 min. Then formic acid buffer–methanol 90:10 for 30 min was used to re-equilibrate. The peak area of the standard was considered for determination of sample concentration. Quantification of phytohormones was calculated based on Ge et al. [20], Ma et al. [17], and Tang et al. [19] methods using the peak areas with identified amounts of IAA, CK, and GA3.

For extraction and evaluation of ABA, 1 g of fresh leaves were ground in 10 mL of 80% methanol including 0.01 g of ascorbic acid and 0.01 g polyvinylpyrrolidone (PVP). The homogenate was stirred overnight at 4 °C. After centrifugation at 4000 g for 15 min, the supernatant was recovered and adjusted to pH 8.0. The aqueous methanol was evaporated under reduced pressure at 35 °C. The residue was dissolved in 5 mL of water. These samples were frozen and thawed for three cycles. After centrifugation at 4000 g for 15 min, the supernatant was recovered and adjusted to pH 2.5 and 10 mL ethyl acetate was added to collect free ABA in ethyl acetate. The ethyl acetate was then evaporated. The resulting dried precipitate was dissolved in 1 mL of 3% methanol containing 0.1 M acetic acid and was filtered through a 0.45 mm membrane filter [21]. Then, 10 µL of extract was injected into a C18 reverse phase column (4.6 × 250 mm Diamonsic C18, 5 µm). It was eluted with a linear gradient of methanol (3–97%) containing 0.01% acetic acid at a flow rate of 4 mL/min. The detection was run at 260 nm with a diode array detector. Quantification was obtained using the peak areas with known amounts of ABA (5–50 ng/ml) based on Li et al. [21].

The extraction and evaluation of polyamines were performed based on Walter and Geuns [22].

Statistical analysis

All experiments were conducted with three replicates, and the differences between treatments with different variables were tested by general linear model followed by Duncan test. Differences were considered as significant at $p < 0.05$. To determine the best target for PBZ and GA_3 and understand their roles under salinity, Principal component analysis (PCA) was applied, and the results were shown. Principal component analysis is a popular method to reduce a set of observations of possibly correlated variables into a set of principal components. In other words, each parameter in PCA analysis receives component score based on the quantified data which contain the same information but orthogonal to each other.

Results

Growth parameters

Increasing NaCl concentration negatively affected the fresh weight, dry weight, stem length, root length, and water content of sweet sorghum (Table 1). The results indicated that these characteristics were significantly altered by GA_3 and PBZ treatments. As a general pattern, GA_3 and PBZ treatments affected fresh weight, dry weight, stem length, and root length. The lengths of stem and roots as well as the fresh and dry weight of stems were significantly

Table 1 Effects of GA_3 and PBZ treatments on some growth parameters of sweet sorghum under salt stress

Salt	Treatment	Stem length (mm)	Root length (mm)	Root/shoot length ratio	Shoot fresh weight (mg)	Shoot dry weight (mg)	Root fresh weight (mg)	Root dry weight (mg)	Root/shoot dry weight ratio	Water content of leaves (mg)
0 (mM)	Control	$220^{b} \pm 6$	$116^{cd} \pm 7$	$0.53^{e} \pm 0.02$	$151^{b} \pm 10$	$15^{b} \pm 1$	$35^{d} \pm 8$	$3.5^{c} \pm 0.2$	$0.22^{e} \pm 0.01$	$136^{b} \pm 10$
	17 μM PBZ	$116^{ef} \pm 6$	$145^{a} \pm 7$	$1.25^{a} \pm 0.13$	$99^{de} \pm 7$	$11^{c} \pm 1$	$58^{b} \pm 9$	$4.9^{ab} \pm 0.5$	$0.43^{b} \pm 0.03$	$87d^{de} \pm 6$
	17 μM GA_3	$253^{a} \pm 10$	$126^{bc} \pm 6$	$0.50^{e} \pm 0.05$	$199^{a} \pm 10$	$20^{a} \pm 1$	$45^{bcd} \pm 3$	$3.9^{c} \pm 0.6$	$0.22^{e} \pm 0.02$	$179^{a} \pm 7$
100 (mM)	Control	$159^{d} \pm 6$	$118^{cd} \pm 7$	$0.75^{d} \pm 0.08$	$116^{cd} \pm 4$	$15^{b} \pm 0.4$	$53^{bc} \pm 9$	$5.5^{a} \pm 0.3$	$0.37^{cd} \pm 0.03$	$101^{cd} \pm 4$
	17 μM PBZ	$102^{f} \pm 11$	$134^{ab} \pm 8$	$1.3^{a} \pm 0.21$	$79^{f} \pm 9$	$11^{c} \pm 1$	$75^{a} \pm 9$	$5.3^{ab} \pm 0.9$	$0.49^{a} \pm 0.06$	$69^{f} \pm 9$
	17 μM GA_3	$196^{c} \pm 12$	$107^{de} \pm 12$	$0.55^{e} \pm 0.03$	$125^{c} \pm 8$	$15^{b} \pm 1$	$38^{cd} \pm 8$	$3.2^{c} \pm 0.5$	$0.22^{e} \pm 0.04$	$110^{c} \pm 7$
200 (mM)	Control	$126^{e} \pm 3$	$110^{d} \pm 5$	$0.87^{c} \pm 0.04$	$85^{ef} \pm 13$	$12^{c} \pm 1$	$45^{bcd} \pm 7$	$3.9^{c} \pm 0.4$	$0.32^{d} \pm 0.01$	$73^{ef} \pm 12$
	17 μM PBZ	$106^{f} \pm 7$	$109^{de} \pm 11$	$1.02^{b} \pm 0.08$	$75^{f} \pm 9$	$11^{c} \pm 1$	$52^{bcd} \pm 10$	$4.5^{b} \pm 0.4$	$0.42^{bc} \pm 0.06$	$64^{f} \pm 8$
	17 μM GA_3	$167^{d} \pm 13$	$98^{e} \pm 6$	$0.59^{e} \pm 0.03$	$102^{de} \pm 14$	$14.8^{b} \pm 2$	$38^{cd} \pm 8$	$3.7^{c} \pm 0.2$	$0.25^{e} \pm 0.02$	$87^{de} \pm 13$

Values are means (± SD) of three replicates, and similar letters illustrated as superscripts in each column are not significant ($p < 0.05$)

improved by GA_3 application. However, the impact of GA_3 was slightly reduced by increasing salinity in the medium. The stem length increased significantly by GA_3 either with or without salt as compared with control (no PBZ) and PBZ treatments. Though PBZ treatments reduced stem length and fresh and dry weights of stem, these characteristics did not change significantly in the medium supplemented with salt. However, the root length and fresh and dry weights of roots were significantly increased by PBZ, either with or without NaCl, compared to GA_3 treatments (Table 1). In our experiments, root/shoot length ratio and root/shoot dry weight ratio in sweet sorghum improved by increasing salinity. Looking at both ratios, it seemed GA_3 has a more negative effect compared to PBZ. Under salinity, the water content (WC) of sweet sorghum decreased significantly by increasing salinity for both GA_3 and PBZ treatments. Gibberellic acid increased WC in non-saline medium, but PBZ decreased it significantly compared to the control. Under salt stress, the reduction in WC in GA_3-treated plants was higher than PBZ-treated plants. For instance, the water content of plants treated with GA_3 and PBZ in 200 mM NaCl was reduced by 54% and 28%, respectively, compared to plants grown in the medium without salt.

Photosynthetic Pigments

Our results indicated that chlorophyll *a*, chlorophyll *b*, the total chlorophyll as well as the carotenoid contents were increased significantly by higher salinity. Treated plants with PBZ and GA_3 under 200 mM salt increased total chlorophyll about 21, and 43%, respectively, compared with untreated plants. When 100 mM NaCl was added to the medium, chlorophyll content of plants treated with GA_3 decreased compared to PBZ-treated plants either with or without salt. In contrast, PBZ improved chlorophyll and carotenoid contents in response to 100 mM salt compared to the control and GA_3 (Table 2).

Water soluble carbohydrates

The level of water soluble carbohydrates (WSC) in the leaves of sweet sorghum was enhanced by increasing salinity, especially in 200 mM NaCl. Application of PBZ without salt improved WSC dramatically (around 100%) compared to control. However, the impact of PBZ was reduced slightly by increasing salinity in the medium. The highest WSC content was recorded in the medium containing PBZ and 200 mM salt, which was not significantly different from GA_3-treated plants at 200 mM NaCl (Table 2).

Polyamines

The results of GA_3, PBZ, and salinity on endogenous concentration of putrescine (PUT) are shown in Fig. 1A. Using GA_3 as a single (without salinity) treatment increased PUT content 70% more than untreated plants and 100% higher than PBZ treatment. The concentration of PUT in GA_3-treated plants decreased markedly by salinity; down to 60% in 200 mM salt. In contrast, PBZ as well as untreated plants with hormones increased PUT up to 100 mM NaCl and then decreased PUT to the lowest amount in 200 mM NaCl (Fig. 1A). Gibberellic acid and PBZ in combination with salt stress had different impact on spermidine content. The highest spermidine (SPD) content was observed in GA_3 treated plants. However, when NaCl was added to the medium, plants responded differently. For instance, in 100 mM NaCl spermidine concentration decreased in plants irrespective of the treatment (GA_3, PBZ, or control), while in 200 mM NaCl spermidine concentration increased (Fig. 1B). The overall pattern of spermine (SPM) content is shown in Fig. 1C. In this study, under 100 mM salt stress spermine content decreased, but it increased in 200 mM NaCl by PBZ. Salt stress without exogenous PBZ or GA_3 application increased spermine only in 200 mM NaCl. A similar pattern was observed when GA_3 was used.

Because GA_3 content was affected by PBZ and GA_3 treatments, we were interested in knowing how and to what extend GA_3 variation may affect polyamine content. Therefore, we calculated the ratio of total polyamine/GA_3 which showed that the total polyamine/GA_3 in PBZ-treated plants was higher than GA_3 treated plants and untreated plants (control). This ratio was increased in 100 and 200 mM NaCl in PBZ and control plants, whereas in GA_3-treated plants it was increased only in 200 mM NaCl (Fig. 1D).

Phytohormones concentration

To understand the interaction of treatments including GA_3, PBZ, and NaCl, the endogenous concentrations of some key phytohormones were measured. As a general pattern, GA_3 content in plants decreased by increasing salinity. Our results indicated that the level of GA_3 content decreased significantly in 0, 100, and 200 mM NaCl, either with or without GA_3 or PBZ. Although, the plants treated by GA_3 showed higher GA_3 content than PBZ-treated plants, the reduction in GA_3 content was higher in GA_3-treated plants than PBZ in 200 mM NaCl. Our results show that GA_3 in 200 mM salt reduced GA_3 content by about 73%, but PBZ reduced GA_3 content by about 48% (Fig. 2A).

The level of indoleacetic acid (IAA) was much higher in GA_3-treated plants compared to PBZ under salt stress

Table 2 Effect of GA_3 and PBZ on chlorophyll, carotenoid, and water soluble carbohydrate (WSC) of sweet sorghum under salinity

Salt	Treatment	chlorophyll *a* (mg g^{-1}FW)	chlorophyll *b* (mg g^{-1}FW)	Total chlorophyll (mg g^{-1}FW)	Carotenoid (mg g^{-1}FW)	Leaf WSC (mg g^{-1}DW)
0 (mM)	Control	$1.27^{cd} \pm 0.26$	$0.42^{de} \pm 0.07$	$1.91^{cd} \pm 0.1$	$0.48^{cde} \pm 0.03$	$13^{d} \pm 2.5$
	17 µM PBZ	$1.68^{bc} \pm 0.16$	$0.56^{bc} \pm 0.03$	$2.28^{bc} \pm 0.2$	$0.57^{bc} \pm 0.03$	$25^{abc} \pm 2.5$
	17 µM GA_3	$1.13^{d} \pm 0.20$	$0.39^{e} \pm 0.09$	$1.55^{d} \pm 0.3$	$0.41^{e} \pm 0.09$	$14^{cd} \pm 1.5$
100 (mM)	Control	$1.65^{bc} \pm 0.01$	$0.55^{bcd} \pm 0.001$	$2.23^{bc} \pm 0.01$	$0.53^{cd} \pm 0.02$	$20^{bcd} \pm 0.2$
	17 µM PBZ	$2.15^{a} \pm 0.16$	$0.67^{ab} \pm 0.06$	$2.86^{a} \pm 0.2$	$0.67^{ab} \pm 0.04$	$31^{ab} \pm 4.3$
	17 µM GA_3	$1.17^{d} \pm 0.2$	$0.26^{f} \pm 0.08$	$1.62^{d} \pm 0.2$	$0.44^{de} \pm 0.04$	$31^{ab} \pm 3.4$
200 (mM)	Control	$1.97^{ab} \pm 0.17$	$0.59^{abc} \pm 0.07$	$2.63^{ab} \pm 0.2$	$0.72^{a} \pm 0.1$	$26^{ab} \pm 3.3$
	17 µM PBZ	$2.06^{ab} \pm 0.38$	$0.7^{a} \pm 0.01$	$2.76^{ab} \pm 0.5$	$0.67^{ab} \pm 0.08$	$32^{a} \pm 3.2$
	17 µM GA_3	$1.69^{bc} \pm 0.3$	$0.48^{cde} \pm 0.04$	$2.21^{bc} \pm 0.4$	$0.56^{bc} \pm 0.07$	$31^{ab} \pm 3.6$

Values are means (± SD) of three replicates, and similar letters illustrated as superscripts in each column are not significant ($p < 0.05$)

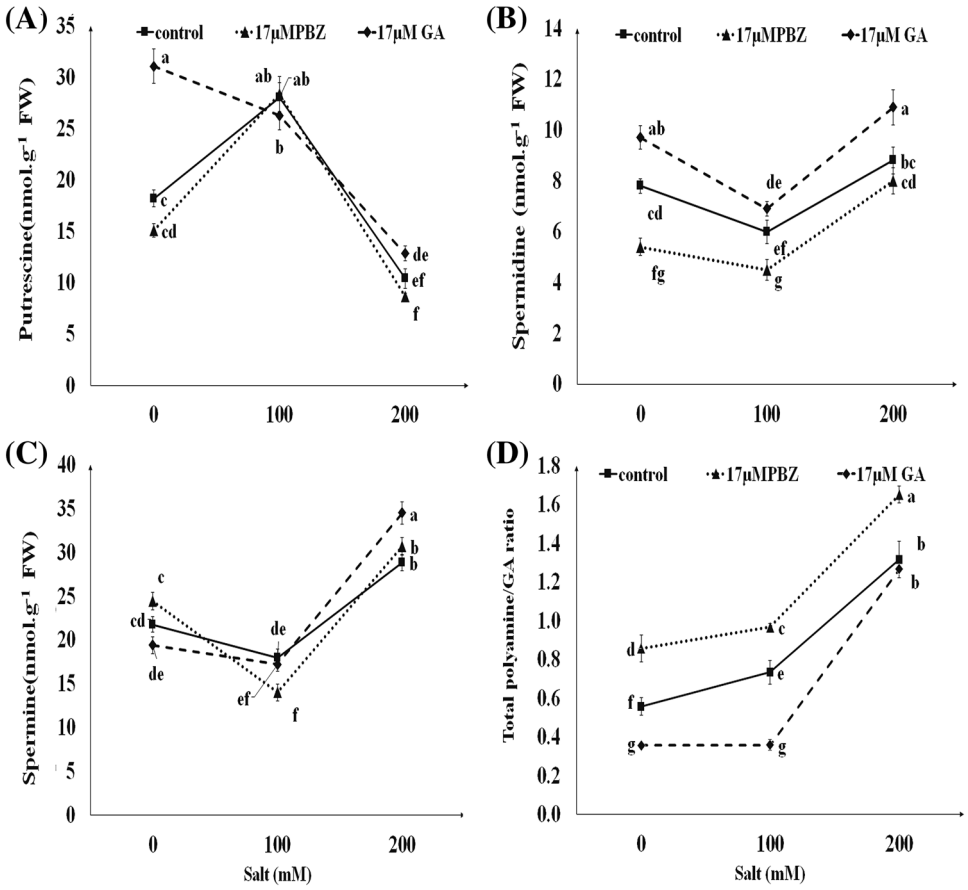


Fig. 1 The effect of GA3 and PBZ treatments on leaf putrescine (**A**), spermidine (**B**), spermine (**C**), and total polyamines/GA_3 ratio (**D**) of sweet sorghum under salinity. The values are means of three replicates, ± standard error. Common letters are not significant ($p < 0.05$)

(Fig. 2B). The levels of IAA decreased in plants with or without GA_3 and PBZ as concentration of NaCl increased in the medium. The amount of IAA in PBZ-treated plants was significantly lower than GA_3-treated and -untreated plants. While large reduction in IAA concentration was

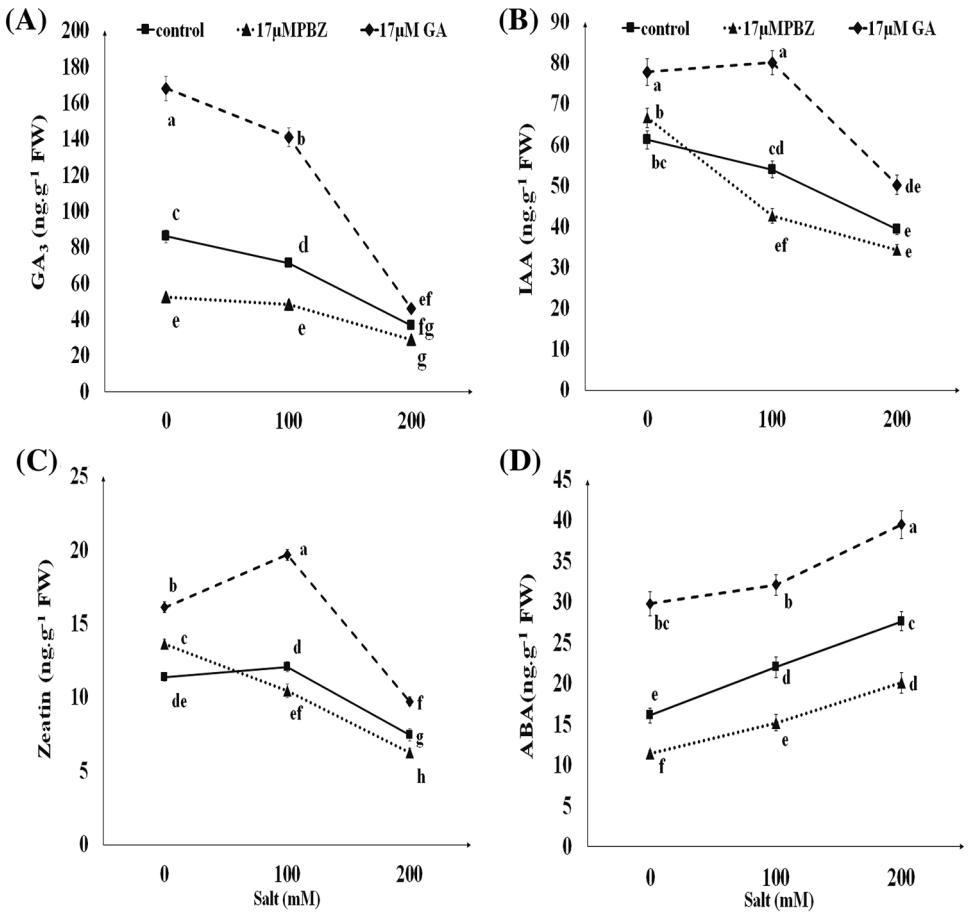


Fig. 2 The effect of GA_3 and PBZ on endogenous concentrations of leaf GA_3 (**A**), IAA (**B**), zeatin (**C**), and ABA (**D**) in sweet sorghum under salinity. The values are means of three replicates, ± standard error. Common letters are not significant ($p < 0.05$)

observed by PBZ, GA_3, and untreated plants at 200 mM NaCl, these differences were not significant (Fig. 2B).

In GA_3-treated plants zeatin, concentration with or without NaCl application was the highest compared to untreated and PBZ-treated plants. Generally, zeatin content increased up to 100 mM NaCl, but significantly declined when salt concentration increased to 200 mM NaCl. Additionally, PBZ-treated plants had the lowest amount of zeatin, especially at 200 mM NaCl (Fig. 2C).

On the other hand, the concentration of ABA increased as the concentration of NaCl increased (Fig. 2D). The highest amount of ABA was observed in GA_3-treated plants with 200 mM NaCl.

To understand the role of different phytohormones in response to salt stress, we calculated their ratio to GA_3. In this regard, the ABA/GA_3 ratio (Fig. 3A) increased sharply in 200 mM NaCl under GA_3 or PBZ application. Interestingly, in severe salt stress (200 mM), this ratio was much higher in GA_3-treated plants than PBZ-treated ones.

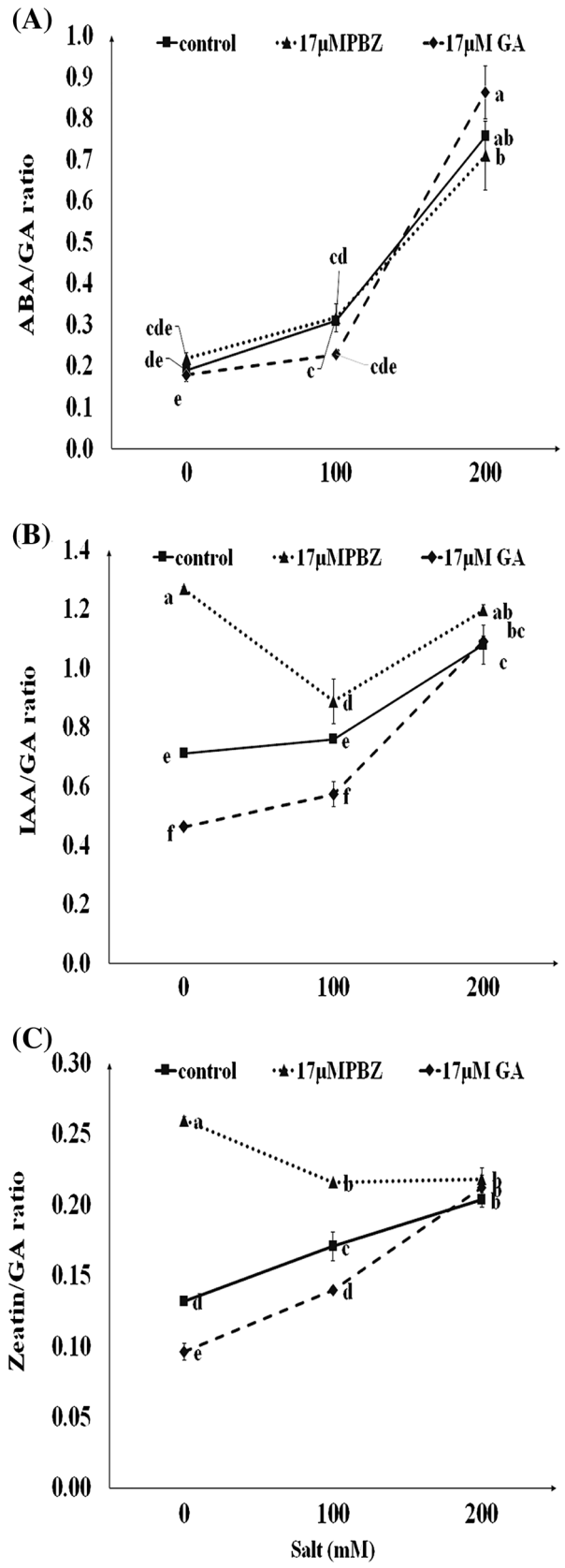
The IAA/GA_3 ratio in PBZ treatments with or without salt was higher than control and GA_3-treated plants. In GA_3-treated or control plants, this ratio was high when plants were exposed to 200 mM salt concentration. In PBZ-treated plants the IAA/GA_3 ratio declined under 100 mM NaCl and then increased when the salt concentration was increased to 200 mM (Fig. 3B).

Generally, the ratio of CK (in this case zeatin) to GA_3 was the highest in PBZ-treated plants irrespective of whether or not the plants were exposed to salinity treatment. When 100 mM NaCl was added to the medium, this ratio decreased slightly and remained constant in 200 mM salt concentration. In contrast, GA_3 treatment in response to salinity increased the CK/GA_3 ratio slightly (Fig. 3C).

Identification of NaCl, GA_3, and PBZ objectives

To identify the best objectives for salinity, GA_3 and PBZ in sweet sorghum, principal component analysis (PCA) was carried out. The principal components 1 and 2 (PCA1 and PCA2, respectively) obtained from the growth parameters as well as physiological and phytohormones parameters subjected to salinity are shown in Fig. 4A. Gibberellic acid and PBZ, as PCA1, described 61.1% and PCA2 described 20.5% (the sum equals to 81.6%) of all measured parameters. In fact, PCA visualized the main objectives of GA_3 and PBZ on sweet sorghum, under salt stress (Fig. 4A, B).

The PCA results (Fig. 4) clearly indicated that growth parameters, that is physiological and phytohormones in this



◀**Fig. 3** The effect of GA_3 and PBZ treatments on ABA/GA_3 (**A**), IAA/GA_3 (**B**), and zeatin/GA_3 (**C**) ratios, in leaves of sweet sorghum under salinity. The values are means of three replicates, ± standard error. Common letters are not significant ($p < 0.05$)

study, might be divided into three separate groups. Root length was a group associated with PBZ treatment (Fig. 4A). Whereas, root/shoot length, root/shoot dry weight, root fresh and dry weights, total chlorophyll, carotenoids, WSC, CK/GA_3, and IAA/GA_3 consisted a group linked with NaCl and PBZ + salt (Fig. 4A). Finally, auxin, cytokinin, PUT, SPD, ABA, water content (WC), shoot dry and fresh weights, and shoot length were found on the left side of the biplot strongly related to GA_3 (Fig. 4A). Figure 4B summarizes a simple illustration of the main objectives of the above-mentioned characteristics for GA_3 and PBZ under salinity. We found that the SPM, polyamines/GA_3, and ABA/GA_3 were the main objectives of salinity regulating total chlorophyll, carotenoid, and WS contents in sweet sorghum. In conclusion, the main objectives of PBZ under salinity were ABA/GA_3, CK/GA_3, and IAA/GA_3, while the levels of SPD, ABA, and ABA/GA_3 were the main objectives of GA_3 under salinity (Fig. 4B).

Discussion

As a general response to salt stress, plant growth is affected negatively by a variety of physiological changes, such as osmotic shock, ion toxicity, and nutritional imbalance [23]. This study showed that, although stem length, fresh and dry weight, and water content decreased the adverse effect of NaCl by GA_3 treatment, the impact of GA_3 was reduced slightly with rising salt level. It has been known that, GA_3 enhances plant growth by promoting cell division and elongation [24] under salinity, for instance in arabidopsis [25], wheat [26], and rice [8]. We found that application of GA_3 increased the levels of CK and IAA in leaves, perhaps due to the beneficial effects of hormonal homeostasis of GA_3 and its ion uptake and possibly the photosynthesis process in the salt-stressed plants [8]. Salt stress may reduce carbon movement to shoot [27], yet increase carbon transferring to roots. This will consequently increase the root/shoot ratio, which might be an adaptive response [8]. Previously, it was indicated that the root/shoot ratio was increased in PBZ-treated seedling of rice [28] and the adverse effect was observed by GA_3 [28]. We observed that, PBZ increased root length and subsequently resulted in improved water content under salt stress. The level of CK in sorghum was decreased in PBZ-treated plant. Interestingly, PCA showed that the root/shoot length ratio

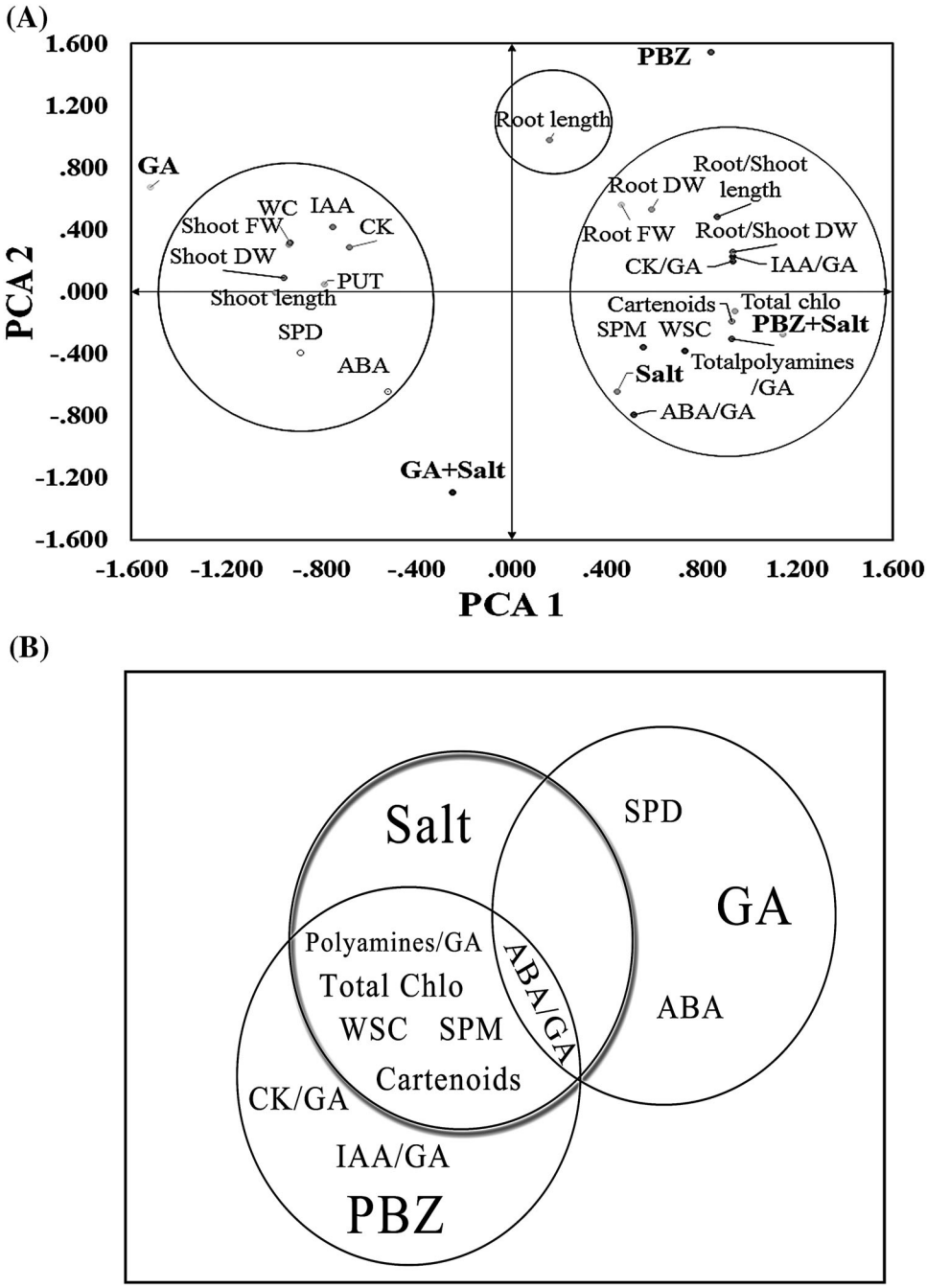


Fig. 4 Result of biplot Principal Components 1 and 2 analysis obtained from phytohormones and physiological parameters in sweet sorghum subjected to PBZ, salinity, GA_3 (**A**), and the main objectives (**B**)

was positively correlated with CK/GA_3 and IAA/GA_3 ratios. In our study, shoot fresh weight of sorghum was correlated with changes in plant height as reported by Yim et al. [28]. However, we showed that shoot fresh weight was negatively associated with total polyamines/GA_3 ratio. Moreover, various studies have shown that high polyamines level enhances cell division, root initiation, and early growth [29]. Therefore, altered level of GA_3 resulting from PBZ and GA_3 treatment in sorghum plants leads to different allocation patterns. Consequently, it seems that, root/shoot ratio is regulated by CK/GA_3, IAA/GA_3, and total polyamines/GA_3 ratios.

Abiotic stress normally changes phytohormones level and photosynthesis process. It has been reported that chlorophyll content is decreased in salt susceptible plants such as tomato and pea [30], but it is increased or unchanged in relatively salt tolerant plants such as pearl millet, sugar beet, and cabbage [31]. Our data indicated that chlorophyll and carotenoid contents were increased significantly with salinity. This finding might be due to the nature of sorghum variety used in this study, which is a

relatively salt tolerant sweet sorghum cultivar Sofra. However, the chlorophyll content in leaves of some varieties of sorghum reduced as a result of higher NaCl levels [32], indicating a genotype dependent response of sorghum cultivars to salinity [33]. It should be kept in mind that, chlorophyll content as a photosynthesis response of sorghum is also dependent on NaCl concentration. The positive effect of PBZ on chlorophyll accumulation was reported in some plant species [34], and a similar observation was seen in our study. This finding might be either due to decrease in leaf area by PBZ and condensation of chlorophyll [35] or increase in cytokinin levels in leaves [13]. In contrast, the decrease in chlorophyll resulted by GA_3 treatment might be due to the expansion of the leaves and dilution of chlorophyll [36]. It has also been shown that polyamines are involved in the delayed loss of chlorophyll [37]. Furthermore, the result of PCA analysis indicated that, the total chlorophyll and carotenoids contents were strongly correlated with the total polyamines/GA_3 ratio in sweet sorghum. Since the total amount of polyamines/GA_3 ratio was significantly enhanced by PBZ and salinity, it might be concluded that PBZ and salinity regulated chlorophyll and carotenoid accumulation by total polyamines/GA_3 ratio.

Some variations have been reported in soluble carbohydrates content in response to salinity in different varieties of sorghum [3, 38, 39]. For instance, salinity decreased the amount of glucose and fructose in sorghum cv. Keller, while increased them in cv. Sofra [38]. In this study, WSC was increased in response to salinity, but PBZ was effective only under non-salinity condition. In wheat, however, PBZ increased WSC in the leaves under salinity. On the other hand, in sorghum plants treated with salinity supplemented with either GA_3 or PBZ, WSC content correlated positively with the total amount of polyamines/GA_3 and ABA/GA_3 ratios. Interestingly, ABA/GA_3 is a common target of GA_3 and PBZ under salinity. Additionally, the increase in the total polyamines/GA_3 ratio led to improved chlorophyll content and consequently resulted in an increase in WSC in sorghum plants treated with PBZ.

Our results indicated that putrescine (PUT) levels decreased, while spermine (SPM) levels increased by salinity. Accumulation of SPM by raising salinity was reported by Maiale et al. [10] in rice. Chai et al. [40] provided strong evidence indicating that SPM improved growth of sweet sorghum under salinity. It can be suggested that SPM may act as a compatible solute, like proline, for homeostasis of the cells under NaCl treatment. Iqbal and Ashraf [26] reported that GA_3 affects plant ability to synthesize polyamines based on the concentration of GA_3 under salinity. Triazole compounds, including PBZ, reduce ethylene formation by blocking amino cyclopropane carboxylic acid (ACC) oxidase leading to an increase in polyamines level [13]. Low levels of putrescine detected in our study might be linked to the ABA biosynthesis and the inhibition of gibberellic acid biosynthesis [41]. Furthermore, accumulation of SPD in sweet sorghum is linked with ABA and is negatively correlated with IAA/GA_3 and CK/GA_3 ratios. Therefore, it seems that the reduction in GA_3 levels by PBZ together with the GA_3 addition to the medium regulates PUT concentration under salinity [26]. Seemingly, the alternation of PUT content might be modulated by ABA, IAA/GA, and CK/GA levels in sweet sorghum.

Application of paclobutrazol reduced GA_3 content in sorghum plants, possibly via the inhibition of monooxygenases that catalyze oxidative steps from *ent*-kaurene to *ent*-kaurenoic [13]. Accumulation of DELLA protein in GA signaling pathway or GA degradation in arabidopsis plants might be other reasons for sorghum plants lack of response to GA_3 as reported [24]. Interestingly, an increased level of DELLA expression was observed when sorghum treated with exogenous PBZ [42]. Therefore, the difference in sorghum plants response to GA_3 and PBZ treatments under salinity might be related to hormonal hemostasis, GA_3 metabolism, and the stability of DELLA proteins.

It was reported that auxin influences GA biosynthesis and interacts positively with GA in plant signaling [7]. For instance, increasing GA_3 levels leads to significant increase in IAA content [43]. This effect may be due to either increased IAA biosynthesis or increase in auxin transport from apical meristem. Björklund et al. [43] showed that, increasing IAA content in *Populus* sp. treated with GA_3 was correlated with auxin transport from apical meristem. Also, it is shown that the application of GA_3 during salt stress in rice improves auxin content, which is similar to our findings [44]. There is conflicting reports on PBZ interaction with auxin in plants under stress conditions. Some reports indicated that triazoles such as PBZ have no significant impact on auxin levels [13], but other investigations have reported that the IAA levels reduced by PBZ under abiotic stress [45]. In the present study, severe salinity dramatically reduced IAA, CK, and some polyamines levels in sorghum, as was reported by Björklund et al. [43] and Shao et al. [46] in other plant species. Salinity increased IAA/GA_3 ratio, which was also markedly increased by PBZ, a GA_3 antagonist. Björklund et al. [43] suggested that IAA/GA_3 ratio is established by complex cross-talk and self-control mechanisms and possibly by the expression of genes involved in auxin transport and GA_3 metabolism.

Since GA_3 can induce salt tolerance in plants by increasing CK and IAA, it seems that sorghum is able to survive under salinity with less damage through changes to CK and IAA levels. Moreover, triazole compounds can

stimulate CK accumulation [13]. We found that the root/shoot ratio in PBZ-treated sorghum increased in response to NaCl. The decrease in CK content under salt stress might be due to the movement of CK from roots to shoots.

ABA has a critical role in abiotic and biotic stress and is a key hormone in response to salinity stress [47]. The detected trend of ABA accumulation under salinity in this study was consistent with many other works showing that either an improved ABA biosynthesis or an enhanced translocation from the roots to the shoots may happen, after a particular stress threshold, in response to salinity [48]. The antagonistic roles between ABA and GA_3 are presumed for numerous plant developmental processes such as germination and flowering [7]. However, there are conflicting data about the effect of GA_3 on ABA level. Although pretreatments of wheat seeds with GA_3 reduced ABA accumulation in response to seawater [49], Iqbal and Ashraf [26] reported that GA_3 improved the levels of free ABA in leaves in salt tolerant wheat cultivars, while reverse trends were observed for the salt intolerant cultivars. The PCA results indicated that ABA/GA_3 was a common target of GA_3 and PBZ under salinity. Therefore, the ABA/GA_3 ratio plays a pivotal role in response to salinity, explaining the levels of ABA in GA_3 and PBZ treatments.

Finally, our findings indicate that GA_3 can reduce the negative effects of salt by increasing some phytohormones in moderate salt stress. However, the main objectives of GA_3 under salinity were ABA and SPD contents as well as ABA/GA_3 ratio. Though GA_3 and PBZ have different roles against salt stress, ABA/GA_3 ratio was a similar target of GA_3 and PBZ. This study suggests that altered levels of GA_3 resulting from PBZ- and GA_3-treated plants cause different allocation patterns in sweet sorghum by regulation of CK/GA_3, IAA/GA_3, and total polyamines/GA_3 ratio. Also, accumulation of chlorophyll pigments, carotenoids, and water soluble carbohydrates in sorghum plants under salinity, that is regulated by the total polyamines/GA_3 and ABA/GA_3 ratios, is positively correlated with PBZ application.

Acknowledgments Authors thank University of Isfahan and University of Payame Noor for their support. The authors are grateful to Dr Mahmoud-Reza Hemami for statistical analysis and Professor Acram Taji and Dr Mehdi Layeghifard for their critical review of the manuscript. Support of Plant Antioxidants Center of Excellence in University of Isfahan is gratefully acknowledged.

References

1. Horie T, Karahara I, Katsuhara M (2012) Salinity tolerance mechanisms in glycophytes: an overview with the central focus on rice plants. Rice 5:11
2. Ji H, Pardo JM, Batelli G, Van Oosten MJ, Bressan RA, Li X (2013) The Salt Overly Sensitive (SOS) pathway: established and emerging roles. Mol Plant 6:275–286
3. Tari I, Laskay G, Takacs Z, Poor P (2013) Response of Sorghum to abiotic stresses: a review. J Agron Crop Sci 199:264–274
4. Kafi M, Shariat Jafari M, Moayedi A (2013) The sensitivity of grain Sorghum (*Sorghum bicolor* L.) developmental stages to salinity stress: an integrated approach. J Agric Sci Technol 15:723–736
5. Netondo GW, Onyango JC, Beck E (2004) Sorghum and salinity: I. Response of growth, water relations, and ion accumulation to NaCl salinity. Crop Sci 44:797
6. Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B (2012) Identification and profiling of salinity stress-responsive proteins in *Sorghum bicolor* seedlings. J Proteomics 75:4139–4150
7. Weiss D, Ori N (2007) Mechanisms of cross talk between gibberellin and other hormones. Plant Physiol 144:1240–1246
8. Khan NA, Nazar R, Iqbal N, Anjum NA (2012) Phytohormones and abiotic stress tolerance in plants. Springer, Berlin
9. Javid MG, Sorooshzadeh A, Moradi F, Sanavy SAMM, Allahdadi I (2011) The role of phytohormones in alleviating salt stress in crop plants. AJCS 5:726
10. Maiale S, Sánchez DH, Guirado A, Vidal A, Ruiz OA (2004) Spermine accumulation under salt stress. J Plant Physiol 161:35–42
11. Fletcher R, Gilley A, Sankhla N, Davis TD (1999) Triazoles as plant growth regulators and stress protectants. Hortic Rev 24:55–138
12. Jaleel CA, Gopi R, Manivannan P, Kishorekumar A, Gomathinayagam M, Vam RP (2007) Changes in biochemical constituents and induction of early sprouting by triadimefon treatment in white yam (*Dioscorea rotundata* Poir.) tubers during storage. J Zhejiang Univ Sci B 8:283–288
13. Rademacher W (2000) Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Annu Rev Plant Biol 51:501–531
14. Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circ Calif Agric Exp Sta 347:1–32
15. Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochem Soc Trans 11:591–592
16. Dubois M, Gilles KA, Hamilton JK, Rebers P, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
17. Ma Z, Ge L, Lee AS, Yong JWH, Tan SN, Ong ES (2008) Simultaneous analysis of different classes of phytohormones in coconut (*Cocos nucifera* L.) water using high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry after solid-phase extraction. Anal Chim Acta 610:274–281
18. Shindy WW, Smith OE (1975) Identification of plant hormones from cotton ovules. Plant Physiol 55:550–554
19. Tang Y, Wang L, Ma C, Liu J, Liu B, Li H (2011) The use of HPLC in determination of endogenous hormones in anthers of bitter melon. J Life Sci 5:139–142
20. Ge L, Yong JWH, Tan SN, Yang XH, Ong ES (2004) Analysis of some cytokinins in coconut (*Cocos nucifera* L.) water by micellar electrokinetic capillary chromatography after solid-phase extraction. J Chromatogr 1048:119–126
21. Li X-J, Yang M-F, Chen H, Qu L-Q, Chen F, Shen S-H (2010) Abscisic acid pretreatment enhances salt tolerance of rice seedlings: proteomic evidence. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1804:929–940
22. Walter HJ-P, Geuns JM (1987) High speed HPLC analysis of polyamines in plant tissues. Plant Physiol 83:232–234

23. Flowers T, Hajibagherp M, Yeo A (1991) Ion accumulation in the cell walls of rice plants growing under saline conditions: evidence for the Oertli hypothesis. Plant Cell Environ 14:319–325
24. Colebrook EH, Thomas SG, Phillips AL, Hedden P (2014) The role of gibberellin signalling in plant responses to abiotic stress. J Exp Biol 217:67–75
25. Fahad S, Hussain S, Matloob A, Khan FA, Khaliq A, Saud S, Hassan S, Shan D, Khan F, Ullah N (2015) Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul 75:391–404
26. Iqbal M, Ashraf M (2013) Gibberellic acid mediated induction of salt tolerance in wheat plants: growth, ionic partitioning, photosynthesis, yield and hormonal homeostasis. Environ Exp Bot 86:76–85. https://doi.org/10.1016/j.envexpbot.2010.06.002
27. de Lacerda CF, Cambraia J, Oliva MA, Ruiz HA (2005) Changes in growth and in solute concentrations in sorghum leaves and roots during salt stress recovery. Environ Exp Bot 54:69–76
28. Yim K-O, Kwon Y, Bayer D (1997) Growth responses and allocation of assimilates of rice seedlings by paclobutrazol and gibberellin treatment. J Plant Growth Regul 16:35–41
29. Bais HP, Ravishankar G (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tiss Org Cult 69:1–34
30. Lapina L, Popov B (1970) The effect of sodium chloride on the photosynthetic apparatus of tomatoes. Russ J Plant Physl 17:580–584
31. Jamil M, Rehman S, Rha E (2007) Salinity effect on plant growth, PSII photochemistry and chlorophyll content in sugar beet (*Beta Vulgaris* L.) and cabbage (*Brassica Oleracea Capitata* L.). Pakistan J Bot 39:753–760
32. Nawaz K, Talat A, Hussain K, Majeed A (2010) Induction of salt tolerance in two cultivars of sorghum (*Sorghum bicolo*r L.) by exogenous application of proline at seedling stage. WASJ 10:93–99
33. Sun L, Zhou Y, Li F, Xiao M, Tao Y, Xu W, Huang R (2012) Impacts of salt stress on characteristics of photosynthesis and chlorophyll fluorescence of sorghum seedlings. Sci Agric Sinica 45:3265–3272
34. Sharma DK, Dubey A, Srivastav M, Singh A, Sairam R, Pandey R, Dahuja A, Kaur C (2011) Effect of putrescine and paclobutrazol on growth, physiochemical parameters, and nutrient acquisition of salt-sensitive citrus rootstock Karna khatta (*Citrus karna* Raf.) under NaCl stress. J Plant Growth Regul 30:301–311
35. Misra A, Sahu S, Misra M, Singh P, Meera I, Das N, Kar M, Sahu P (1997) Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars. Biol Plant 39:257–262
36. Abbaspour J, Ehsanpour AA, Amini F (2012) The role of Gibberellic acid on some physiological responses of transgenic tobacco (*Nicotiana tabacum* L.) plant carrying Ri T-DNA. JCMR 3:75–80
37. Shu S, Guo S-R, Yuan L-Y (2012) A review: polyamines and photosynthesis advances in photosynthesis-fundamental aspects. In Mohammad Najafpour (ed) Tech. p 439–464
38. Almodares A, Hadi M, Ahmadpour H (2008) Sorghum stem yield and soluble carbohydrates under different salinity levels. Afr J Biotechnol 7:4051–4055
39. de Lacerda CF, Cambraia J, Oliva MA, Ruiz HA, Prisco JT (2003) Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. Environ Exp Bot 49:107–120
40. Chai Y, Jiang C, Shi L, Shi T, Gu W (2010) Effects of exogenous spermine on sweet sorghum during germination under salinity. Biol Plant 54:145–148
41. Anwar R, Mattoo AK, Handa AK (2015) Polyamine interactions with plant hormones: crosstalk at several levels. In: Kusano T, Suzuki H (eds) Polyamines: a universal molecular nexus for growth, survival, and specialized metabolism. Springer, Berlin, p 267–302
42. Gao S, Xie X, Yang S, Chen Z, Wang X (2012) The changes of GA level and signaling are involved in the regulation of mesocotyl elongation during blue light mediated de-etiolation in *Sorghum bicolor*. Mol Biol Rep 39:4091–4100
43. Björklund S, Antti H, Uddestrand I, Moritz T, Sundberg B (2007) Cross-talk between gibberellin and auxin in development of Populus wood: gibberellin stimulates polar auxin transport and has a common transcriptome with auxin. Plant J 52:499–511
44. Kaya C, Tuna A, Yokaş I (2009) The role of plant hormones in plants under salinity stress. In: Ashraf M, Ozturk M, Athar H (eds) Salinity and water stress. Springer, Berlin, p 45–50
45. Aly AA, Latif HH (2011) Differential effects of paclobutrazol on water stress alleviation through electrolyte leakage, phytohormones, reduced glutathione and lipid peroxidation in some wheat genotypes (*Triticum aestivum* L.) grown in vitro. Rom Biotech Lett 6:6710–6721
46. Shao T, Li L, Wu Y, Chen M, Long X, Shao H, Liu Z, Rengel Z (2016) Balance between salt stress and endogenous hormones influence dry matter accumulation in Jerusalem artichoke. Sci Total Environ 568:891–898
47. Tran L-SP, Pal S (2014) Phytohormones: a window to metabolism, signaling and biotechnological applications. Springer, Berlin
48. Maggio A, Barbieri G, Raimondi G, De Pascale S (2010) Contrasting effects of GA3 treatments on tomato plants exposed to increasing salinity. J Plant Growth Regul 29:63–72
49. Aldesuquy H, Ibrahim A (2001) Interactive effect of seawater and growth bioregulators on water relations, abscisic acid concentration and yield of wheat plants. J Agron Crop Sci 187:185–193