DISSERTATION

ENVIRONMENTAL STRESS RECOVERY OF HORNED POPPY (*GLAUCIUM* SPP.) USING GROWTH REGULATOR TREATMENTS

Submitted by

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ABSTRACT

ENVIRONMENTAL STRESS RECOVERY OF HORNED POPPY (*GLAUCIUM* SPP.)USING GROWTH REGULATOR TREATMENTS

Plant species and cultivars within a species vary in their recovery from salinity and drought stresses when conditions become more favorable. Water conservation, especially in arid and semiarid regions of the world, is a necessity. Plant species, and cultivars within a species, vary in their salinity and drought tolerance. These variations are the result of variations in genes relating to drought tolerance mechanisms and their interaction with the environment. In order to reduce water usage, it is important to understand the mechanisms of plant adaptation to salinity and drought stresses. Many reports have confirmed the internal modification in growth regulator in terms of types and concentrations under stress conditions. Externally applied growth regulator amendments affect the internal balance of growth regulator and can help the plant to regrow and recover from stress. Horned Poppies (*Glaucium* SPP.) are members of the Poppy family, Papaveraceae, that are native to the Mediterranean and Middle East regions. All horned poppies have blue-green foliage that is deeply pinnatified to pinnatisect and typically grow 30-50 cm long. The leaf have varying degrees of texture from glaucous to villous. All leaves are lyrate to sublyrate shaped and have a rosette growth habit. They have solitary blooms on flower stalks that grow above the foliage. All species have four petals in their corolla and their pistil is completely surrounded by stamens. They all develop long horned-shaped seed siliquiforms with the stigma remaining to cap off the top of the fruit. Species of interest in this study were *G. flavum, G. grandiflorum, G. acutidentatum* and *G. corniculatum*. *G. flavum* Crantz is the most widely spread species in the genus. It's distinguished from other species studied by several characteristics. The

sepals have crisp, pilose hairs on the surface and the petals can be solid yellow, red or reddish mauve. *G. flavum* is most often recognized for the yellow petals and is commonly referred to as the Yellow Horned Poppy. *G. acutidentatum* is the most glabrous species with smooth sepals and ovaries. Although the ovary is smooth, the resulting siliquae is subtorulose. The petals are solid orange-buff in color. *G. grandiflorum* has only one main flower stem while other species have multiple flower stalks growing from the base of the rosette. The sepals have short, stiff hairs making the surface hirsute. The petals are dark orange to crimson red with a black spot at the base of the petal. *G. corniculatum* (L.) J.H. Rudolph has some unique characteristics. Its leaf have a soft, villous texture and its sepals are scabrous to hirsute. The petals are yellow, orange or red.

The objectives of this study were to 1) determine whether applications of Abscisic acid (ABA), salicylic acid (SA), fusicoccin (FC), and ethephon (E) could promote *Glacium* spp. Growth and recovery from salinity stress; 2) determine whether applications of (ABA), (SA), (FC), and (E) could promote *Glacium* spp. growth and recovery from drought stress; 3) determine the most effective concentrations of each growth regulator in the recovery of the stressed plants; 4) evaluate the recovery degree from salinity and drought stresses among the common Horned Poppy species that are available at Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum* and *G. acutidentatum*s ; 5) determine which evaluation criteria are associated with superior recovery rate; 6) confirm selection criteria for evaluation of salinity and drought tolerance in Horned Poppy species; 7) test the change in the concentration of the internal growth regulator under stress conditions and during recovery in those Horned Poppy species studied. Lysimeter columns were used in this study which was replicated twice in the CSU Plant Science greenhouse. Four Growth regulator treatments were used and were applied weekly with irrigation water. Three levels of each regulator were used. Treatments continued for

two months. Data were collected weekly on leaf color (using color chart), leaf size (using Image J software), and the quality and general attractiveness of the plant using personal visual estimation (using a scale of 0 to 9 where 9 is the optimum quality; a rating of 6.0 or higher indicated acceptable quality). Samples were collected for TNC, RSC, proline and tissue Na^+ and K^+ content analysis for each treatment at the end of the experiemnt. Evapotranspiration (ET) measurements were collected every 2 to 3 days during the four month growth period. Five weight readings per pot were made during each measurement and the average value was used for ET calculation. ET was calculated by mass difference and expressed as mm d^{-1}. Internal growth regulator content of plants were assessed before applying the initial treatment and at the end of the experiment. Plant growth regulator concentrations changed over time were quantified using a protocol in which a 50 mg plant material only is needed to quantify most major plant hormones by HPLC–ESI–MS/MS. This method was the best in current study since sampling was done every 2 weeks over the course of the experiment. Sample solutions (50 µl) were injected into the reverse-phase C18 Gemini HPLC column for HPLC–ESI–MS/MS analysis. *G. flavum* was found to recovery more quickly compared to *G. acutidenatum*, *G. grandflorum* and *G. corniculatum*. Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM E and 0.03 mM FC in enhancing salinity stressed *Glaucium* spp. recovery. *Glaucium* spp. under salinity stress exhibited a positive response to growth regulator treatments in terms of improved leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and K^+/Na^+ ratio. *G. flavum* showed greater tendency to recover from salinity stress at all growth regulator treatments when compared to the other species tested. The treatment of 2 mM ABA is recommended to improve the recovery rate of *Glaucium* spp. under salinity stress. On the basis of the number of times in the best statistical category for leaf characteristics, overall plant quality (attractiveness),

water use, TNC, RSC, and proline, *G. flavum* was found to have the greatest recovery rate from drought stress when compared to *G. acutidentatum, G. grandiflorum* and *G. corniculatum.* Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM E and 0.03 mM FC in enhancing drought stressed *Glaucium* spp. recovery. Growth regulator treatments could affect proline accumulation throught their effect on the overall growth of the plant that affect all plant activities especially different growth regulator concentrations and interactions. Comparisons of internal individual growth regulator content among species, growth regulator treatments, sampling dates and their interactions clearly showed significant differences. During the two month course of the recovery, the concentrations of both IAA and IBA increased gradually. There was slight significant increase overtime in IAA and IBA concentration under the control treatment over the course of the two month recovery period. The treatment of 2 mM ABA achieved the highest increase in both IAA and IBA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. There was slight significant increase overtime in GA_3 concentration under the control treatment. The treatment of 2 mM ABA achieved the highest increase in GA_3 in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. The concentrations of zeatin increased gradually in all tested species during recovery. Zeatin concentration increased slightly overtime under the control treatment. The treatment of 2 mM ABA achieved the highest increase in zeatin in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. The highest increase was in *G. flavum*, where zeatin increased from 8.0 to 29.0 ng/g Dwt (263%) under control treatment, while the increase was 2422, 2196, 2050 and 1174% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively. Even under control treatment, there was a slight increase in SA content. The treatment of 2 mM ABA achieved the highest increase in SA

in all tested species, followed by 20 mM ethephon, 0.03 mM Fusiccocin and 2 mM SA. In *G. flavum*, SA increased from 0.4 to 0.9 ng/g Dwt (125%) under control treatment, while the increase was 720,600, 533 and 300% under the treatments of 2 mM ABA, 20 mM ethephon, 0.03 Fusiccocin and 2 mM SA respectively. On the other hand, and during the course of the recovery, the concentrations of the internal ABA decreased gradually over time. Under the control treatment, there was a slight significant decrease overtime in ABA concentration during the recovery period. The treatment of 2 mM ABA achieved the highest decrease in ABA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. In *G. flavum*, ABA decreased from 2.6 to 1.4 ng/g Dwt (-46 %) under control treatment, while the decrease was -88,-85, -76 and -68% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively. In summary, *Glaucium* spp. under salinity and drought stress exhibited a positive response to growth regulator treatments in terms of improving leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and water use efficiency. *G. flavum* showed greater tendency to recover from drought stress at all growth regulator treatments when compared to the other species tested. The treatment of 2 mM ABA is recommended to improve the recovery rate of *Glaucium* spp. under salinity and drought stress. During stress, internal ABA accumulation was evident to cope with stress conditions. During recovery, when the circumstances were favorable for growth, other groups of growth regulator that are needed for accelerated cell division, enlargement and growth such as auxins, gibberellins, and cytokinnins were abundant.

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SUMMARY

Plant species and cultivars within a species vary in their recovery from salinity stress when conditions become more favorable. These variations are associated with genes relating to stress tolerance mechanisms and their interaction with the environment. Horned Poppies (*Glaucium* spp.) are members of the Poppy family, Papaveraceae and are native to the Mediterranean and Middle East. The objectives of this study were to 1) determine whether applications of Abscisic acid (ABA), salicylic acid (SA), fusicoccin (FC), and ethephon (E) could promote *Glacium* spp. growth and recovery from salinity stress; 2) determine the most effective concentrations of each growth regulator in the recovery of the stressed plants; 3) evaluate the recovery degree from salinity stress among the common Horned Poppy species that are available at Denver Botanic Gardens, *G. flavum*, *G. corniculatum*, *G. grandiflorum* and *G. acutidentatums*; 4) determine which evaluation criteria are associated with superior recovery rate; 5) confirm selection criteria for evaluation of salinity tolerance in Horned Poppy species. Lysimeter columns were used in this study which was replicated twice in the CSU Plant Science greenhouse. Four Growth regulator treatments were used and were applied weekly with irrigation water. Three levels of each regulator were used. Treatments continued for two months. Data were collected weekly on leaf color (using color chart), leaf size (using Image J software), and the quality and general attractiveness of the plant using personal visual estimation (using a scale of 0 to 9 where 9 is the optimum quality, with a rating of 6.0 or higher indicating acceptable quality). Samples were collected for TNC, RSC, proline and tissue Na^+ and K^+

content analysis for each treatment at the end of the experiemnt. *G. flavum* was found to have higher recovery rate when compared to *G. acutidenatum*, *G. grandflorum* and *G. corniculatum*. Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM Fusicoccin in enhancing salinity stressed Glaucium spp. recovery. *Glaucium* spp. under salinity stress exhibited a positive response to growth regulator treatments in terms of improving leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and K^+/Na^+ ratio. *G. flavum* showed greater tendency to recover from salinity stress at all growth regulator treatments when compared to the other species tested. The treatment of 2 mM ABA is recommended to improve the recovery rate of Glaucium spp. under salinity stress.

INTRODUCTION

Plant species and cultivars within a species vary in their stress tolerance (Epstein et al., 1980; Pasternark, 1987; Saranga et al., 1992). These variations are due to variations in genes relating to stress tolerance mechanisms and their interaction with the environments (Shanon, 1985; Bohnert et al., 1995; Igartua, 1995; Duncan and Carrow, 1999). Stress-inducible genes can be functional or regulatory. Functional genes control water channels and other transporters, detoxification enzymes, protection molecules such as late embryogenesis abundant (LEA) proteins, key enzymes for osmolyte biosynthesis, or different proteases. Regulatory genes regulate the expression of the functional genes and include, among others, transcription factors, protein kinases and phosphatases, and those involved in abscisic acid biosynthesis (Shinozaki and Yamaguchi-Shinozaki (2007).

Horned Poppies (*Glaucium* spp.) are members of the Poppy family, Papaveraceae and are native to the Mediterranean and Middle East regions. All horned poppies have blue-green foliage that is deeply pinnatified to pinnatisect and typically grow 30-50 cm long. The leaf have varying

degrees of texture from glaucous to villous. All leaf are lyrate to sublyrate shaped and have a rosette growth habit. They have solitary blooms on flower stalks that grow above the foliage. All species have four petals in their corolla and their pistil is completely surrounded by stamens. They all develop long horned-shaped seed siliquiforms with the stigma remaining to cap off the top of the fruit. Species of interest in this study are *G. flavum*, *G. grandiflorum*, *G. acutidentatum* and *G. corniculatum*.

G. flavum Crantz is the most widely distributed species in the genus. It's found in the coasts of Britain and the Atlantic Islands to the coasts of the Mediterranean Basin and the Black Sea (Grey-Wilson, 2000). It grows predominantly on sandy beaches and as a result it is commonly known as the Sea Horned Poppy. This likely indicates that *G. flavum* is salt tolerant. According to Davis (1965), *G. flavum* is distinguished from other species by several characteristics. The sepals have crisp, pilose hairs on the surface and the petals can be solid yellow, red or reddish mauve. *G. flavum* is most often recognized for the yellow petals and is commonly referred to as the Yellow Horned Poppy. The ovary is densely papillose to tuberculate, basically a bumpy surface. The siliquae will retain the papillose to tuberculate texture. In Turkey, *G. flavum* normally flowers from May through the summer and even though it is most often found at sea level, it does grow into river valleys as well (Davis, 1965).

G. grandiflorum Boiss & É. Huet is native to Turkey in the southern part of the Caucasus Mountains but it is also found in Syria, Iran and the Sinai (Grey-Wilson, 2000). *G. grandiflorum* has only one main flower stem while other species have multiple flower stalks growing from the base of the rosette (Davis, 1965). The sepals have short, stiff hairs making the surface hirsute. The petals are dark orange to crimson red with a black spot at the base of the petal. The pedicle of the flower exceeds the subtending leaf, which differs from the other *Glaucium* species. There are two

varieties of *G. grandiflorum* var. *grandiflorum* and var. *torquatum.* *G. grandiflorum* var. *torquatum* has red petals with a black blotch and can be found in calcareous hillsides. *G. grandiflorum* var. *grandiflorum* is found in fields, banks and rocky slopes.

G. acutidentatum is the most glabrous species with smooth sepals and ovaries. Although the ovary is smooth, the resulting siliquae is subtorulose. The petals are solid orange-buff in color. *G. acutidentatum* is found at elevations of 950-1400 m on dry hills (Davis, 1965).

G. corniculatum (L.) J.H. Rudolph is native to the Mediterranean basin, Atlantic Islands, Caucasus Mountains, Bulgaria, Romania, northern Iraq and northwestern Iran (Grey-Wilson, 2000; Davis, 1965). *G. corniculatum* also has some unique characteristics. Its leaf have a soft, villous texture and its sepals are scabrous to hirsute. There is some conflicting information about *G. corniculatum*'s corolla. The petals are yellow, orange or red (Davis, 1965) with a black basal spot (Grey-Wilson, 2000).

Saline environments affect plant growth in different ways including reduction in water uptake, gradual accumulation of ions to toxic levels, and reduction of nutrient accessibility (Rameeh et al., 2012). The detrimental effects of salinity on plant growth include osmotic stress, ion toxicity, nutritional disturbances (Greenway and Munns, 1980; Lauchli, 1986; Cheeseman, 1988), damage to photosynthetic systems by excessive energy (Brugnoli and Bjorkman, 1992), and structural disorganization (Flowers et al., 1985; Delfine et al., 1998; Romero-Aranda et al., 1998). Plants respond to salinity stress through a number of physiological changes including lowered leaf osmotic potential and/or a loss of turgor potential which can cause growth suppression (Levitt, 1980). Salt tolerant plants often mediate stress by osmotic adjustment, therefore minimizing changes in turgor potential which affect plant growth responses linked to carbon dioxide assimilation and cell elongation (Harivandi et al., 1992). Some growth regulator are known

to help in stress tolerance such as abscisic acid. Many of the plant responses to stress occur via chemical signals such as the phytohormone abscisic acid (ABA) (Wilkinson and Davies, 2002) and it is well known that the endogenous levels of ABA in vegetative plant tissues rise in response to stresses that cause plant water deficit (Taylor et al., 2000; Bray, 2002; Zhang et al., 2008). Moreover, a clear relationship between plant ABA content and plant tolerance to water deficit has been described (Kulkarni et al., 2000; Liu et al., 2005). The protective effect of ABA is based on the fact that ABA primarily promotes stomatal closure to minimize transpirational water loss and it mitigates stress damage through the activation of many stress-responsive genes, which collectively increase the plant stress tolerance (Bray, 1997, 2002; Zhang et al., 2008). However, there are no studies dealing with the effects of exogenous ABA on the expression of stress-related genes and on the physiology of plants except for Aroca et al. (2008) who evaluated the influence of exogenous ABA application on plant development, physiology, and expression of several stress related genes after both drought and a recovery period. Their results showed that the application of exogenous ABA had contrasting effects on *Lactuca sativa* plants.

The balance between carbohydrate production and consumption will impact the ability of a plant species to cope with salinity stress (Huang and Fry, 1999; Lee et al., 2008a, 2008b). The decline in salinity tolerance in some species can be associated with reduced carbohydrate availability and reduced effectiveness of Na^+ exclusion and K^+ active uptake and transport (Qian and Fu, 2005; Lee et al., 2007; Shahba, 2010b, Shahba, 2012). Accordingly, fluctuations in these osmoregualtors are expected during the recovery from stress.

Proline accumulates in larger amounts than other amino acids in salt stressed plants (Lee et al., 2008b). Proline accumulation is the first response of plants exposed to salt stress and water-deficit stress and is though to reduce injury to cells (Ashraf and Foolad 2007). Maggio et al. (2002)

suggested that proline may act as a signaling/regulatory molecule able to activate multiple responses that participate in the adaptation process to elevated salinity levels. Rapid accumulation of proline in tissues of many plant species in response to salt, drought or temperature stress has been attributed to enzyme stabilization and/or osmoregulation (Flowers et al., 1977; Levit, 1980). Ahmad et al. (1981) measured proline content fluctuations under high salinity levels in salt tolerant and sensitive ecotypes of creeping bentgrass *(Agrostis stolonifera* L.) and concluded that the salt tolerant ecotype accumulated more proline in response to high salinity levels. Lee et al. (2008b) concluded that proline was the primary organic osmolyte for osmotic adjustment and its accumulation was higher in salt tolerant seashore paspalum genotypes. However, other reports have indicated a negative effect of proline on salinity tolerance. Marcum (2002) has reported that proline accumulates in grasses under salinity stress at insufficient levels to achieve osmotic adjustment. Torello and Rice (1986) concluded that proline accumulation has no significant osmoregulatory role in salt tolerance of five turfgrass species ['Fults' alkaligrass *(Puccinellia distans* L. Parl.), 'Dawson' red fescue *(Festuca rubra* L. var trichophylla Gaud.), 'Jamestown' red fescue *(Festuca rubra* L. var commutata Gaud.), 'Adelphi' and 'Ram I' Kentucky bluegrass *(Poa pratensis* L.)] following their exposure to 170 mM NaC1 salinity stress. Because of these contrasting reports on the role of proline in salt tolerance, its use as a selection criterion for salt tolerance has been questioned (Ashraf and Harris, 2004). Thus proper testing is required before making any conclusion regarding proline role in salinity tolerance in specific species. Accordingly, fluctuations in proline content are expected during the recovery from stress.

Many studies discussed plant responses to stress via internal chemical signals and growth regulator adjustments. However, few studies have dealt with the effects of exogenous growth regulator applications on the expression of stress-related genes and/or on the physiology of

plants under stress such as Aroca et al. (2008) who evaluated the influence of exogenous ABA application on plant development, physiology, and expression of several stress related genes after both drought and recovery. The objectives of this study, therefore, are to (1) determine whether applications of abscisic acid (ABA), salicylic acid (SA), fusicoccin (FC), and ethephon (E) could promote *Glacium* spp. growth and recovery from salinity stress; (2) determine the most effective concentrations of each growth regulator in the recovery of the stressed plants; (3) evaluate the recovery degree from salinity stress among the common Horned Poppy species that are available at Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum* and *G. acutidentatum*s ; (4) determine which evaluation criteria are associated with superior recovery rate; (5) confirm selection criteria for evaluation of salinity tolerance in Horned Poppy species.

MATERIALS AND METHODS

Lysimeter columns were used for these experiments. Columns were placed in the green house. Sixty plants of each species that were planted in 15 cm in diameter and 50 cm long PVC tubes containing potting mix were used. These plants were previously used for salinity tolerance strees screening and already suffered different degrees of stress. Salinity treatments that were previously applied were control (Tap water), EC = 5, EC = 15 and EC = 25 dS m^{-1}. Saline solutions were prepared using instant ocean salt mixture added to the irrigation water. Experimental design was randomized complete Block (RCB). Each block represented one of the replications and contains 48 tubes. Four blocks were used. Used plants in each tube were selected of similar size and height, hold the same number of leaf and suffered similar degree of stress. Four Growth regulator treatments were used and were applied weekly with irrigation water. Three levels of each regulator were used. Evapotranspiration was measured weekly to monitor the change in the evapotranspiration. Four representative tubes for each species were

used as lysimeters and were watered with enough water and left to drain for 2 h, after which the weight of each tube was recorded. Each tube was then re-weighed every 24 hours. The daily changes in weight represent the daily evapotranspiration for each species. Treatments continued for two months.

Data Collection. During the course of the experiment data were collected weekly on plant leaf color (using leaf color chart), leaf size (using Image J software), and general attractiveness of the plant using personal visual estimation (using a scale of 0 to 10 where 10 is the optimum quality, with a rating of 6.0 or higher indicating acceptable quality).

Samples were collected for TNC, RSC, proline and tissue Na^+ and K^+ content analysis for each treatment. Total nonstructural carbohydrate content, RSC, tissue Na^+ and K^+ and proline content were determined at the termination of the experiment. Shoot tissue at the termination of the experiment was harvested and washed with cold distilled water to remove plant debris for carbohydrate analysis. Approximately 5 g samples from the treatments were freeze-dried (Genesis 25 LL Lyophilizer, Virtis, Gardiner, NY). After freeze-drying, samples were ground with a Wiley mill, sieved though a screen with 425 µm openings, and kept in airtight vials at –20 ^{o}C. TNC was measured using the method described by (Chatterton et al. (1987). In brief, 25 mg freeze-dried samples were transferred to 5 ml of 0.1% clarase solution and incubated at 38°C for 24 h. Then, 0.5 ml of hydrochloric acid (50%, v/v) was added to the incubation solution. After the solution was incubated at room temperature for 18 h, the pH value of the solution was adjusted to between 5 and 7 with 10 and 1 N NaOH. This resulting solution was used to determine TNC content using a spectrophotometer at 515 nm wavelength (model DU 640; Beckman).

To measure the free reducing sugar, 25 mg of the freeze dried, ground, and sieved sample was extracted with 10 ml of 0.1 M phosphate buffer (pH = 5.4) for 24 h at room temperature. An extracted aliquot (0.2 mL) was used to determine the reducing sugar content by using the same method as was used to measure TNC.

To measure ion content, about 5 g of shoots were harvested, washed with deionized water, and dried at 70 ^{o}C for 24 h. Dried shoots were ground in a Wiley mill and passed through a screen with 425 µm openings. Approximately 1 g of dried and screened sample was weighed and ashed for 7 h at 500 ^{o}C. Ash was dissolved in 10 ml of 1N HCl and diluted with deionized water. Solution aliquots were analyzed for $Na^{+}$and K^{+} by inductively-coupled plasma atomic emission spectrophotometry (ICP-AES) (Model 975 plasma Atomcomp, Thermo Jarrell Ash Corp., Franklin, Mass.).

Actual proline tissue accumulation levels were determined according to the method of Bates et al. (1973) as modified by Torello and Rice (1986) with approximately 0.5g fresh weight of tissue. Samples were ground with liquid nitrogen in a mortar. Each sample was homogenized in 10 ml of 3% aqueous sulfosalicylic acid followed by agitation for 1h prior to filtration through #2 Whatman filter paper. After filtration 2 ml of extract from each sample was reacted with 2 ml of ninhydrin reagent (1.25 mg of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M H_3PO_4) and 2 ml of glacial acetic acid followed by 1 h of heating at 100 ^{o}C in an enclosed water bath. Samples were then quickly cooled by immersion in an ice bath and total proline was determined spectrophotometrically at 520 nm. Actual proline tissue accumulation levels were determined by subtracting mean control data from growth regulator treatment data for all cultivars during the entire experimental period.

Data analysis. Effects of species, growth regulator, and growth regulator levels and their interactions were determined by analysis of variance (SAS Institute, 2007). Monitored parameters (leaf color, leaf area, plant quality, K^+/Na^+, TNC, RSC and proline contents of shoots) were analyzed on individual measurement dates to examine the differences in the recovery rate among different treatment. Means were separated by least significant difference at the 0.05 level of probability.

RESULTS AND DISCUSSION

Leaf characteristics:

Leaf color. Comparisons of leaf color among species and growth regulator treatments clearly showed significant differences. Species and growth regulator interaction was also significant (Table 1.1). Comparison of species within each treatment indicated that *G. flavum* had a superior recovery rate (Fig. 1.1). Under control treatment, *G. flavum*, subjected to stress had an acceptable rating of 6.0 and showed reasonable recovery rate. *G. acutidentatum*, *G. grandiflorun*, and *G. corniculatum* leaf color did not recover to the acceptable level by the end of the experiment (Fig. 1.1). The treatment of 2.0 mM ABA resulted in the highest recovery in leaf color followed by 20.0 mM ethephon, 2.0 mM SA and 0.03 mM fusicoccin (Fig. 1.1). *G. conrniculatum* did not show acceptable leaf color ratings under any treatments while *G. grandiflorum* showed acceptable leaf color only under the treatment of 2 mM ABA (Fig.1.1). There was no significant difference among species in the rate of improvement or change compeared with control treatment.

Table 1.1. Analysis of variance with mean squares and treatment significant of leaf color, leaf area, plant quality, shoot K^+/Na^+, total non-structure carbohydrate content (TNC), shoot reducing sugar content (RSC), and proline content in Glaucium spp. during the recovery from salinity stress.

Parameter	Species	PGR	S x G
Leaf color	7.3**	95.8**	55.5*
Leaf area	1.5**	605**	4.6*
Plant quality	7.5**	608**	4.5*
K^+ / Na^+	166.0**	34.0**	155.0*
TNC	1210.0**	1195.0**	1910.0*
RSC.	56.0**	685.0**	412.0*
Proline	1240.0**	1688.0**	1650.0*

*significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$

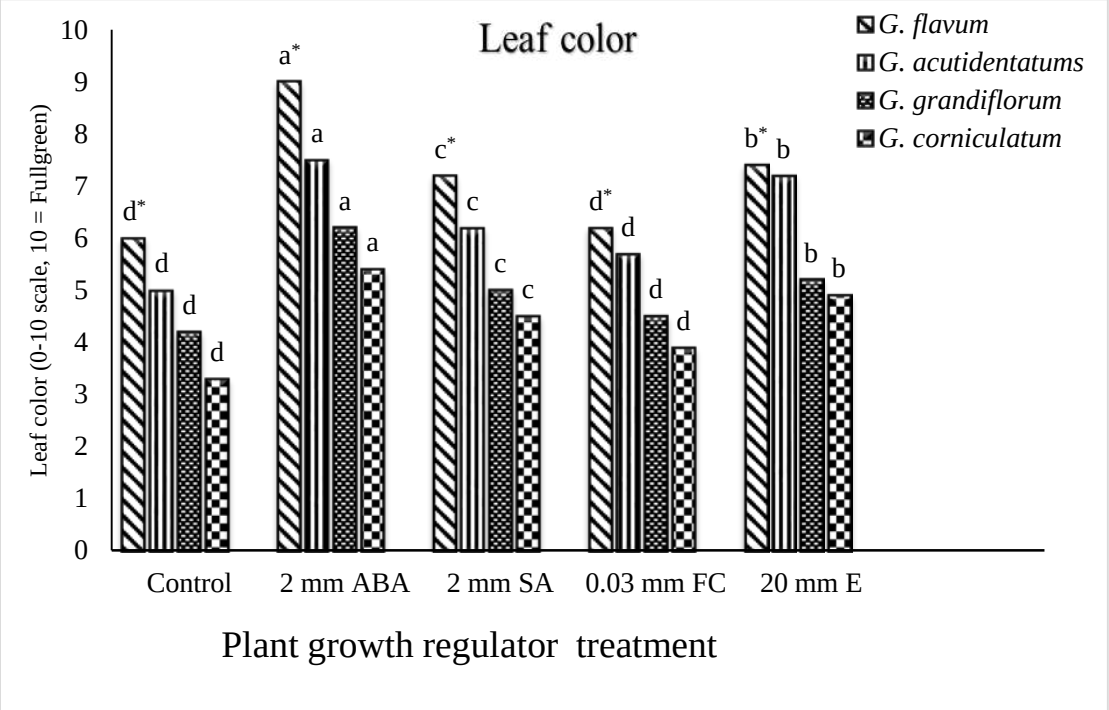

Fig. 1.1. Effect of different growth regulator on leaf color during the recovery from salinity stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$ among growth regulator treatments. Columns labeled with an asterisk are significantly the highest among species within each treatment.

Generally salinity affects leaf color quality to different degrees. As soon as growth conditions improve, the recovery expected at different degress based on the species and growth

conditions. Many studies reported the effect of salinity on leaf color and quality.A decrease in chlorophyll index was reported by Bayat et al. (2012) due to salinity in *Petunia hybrida*. Salinity affected butterhead lettuce leaf differently. Choi and Lee (1999) documented salinity effect on lettuce lower and upper leaf. It is well known that the negative effect of salinity on leaf color is due to the effect on chlorophyll formation processes (Kubis et al., 2004; Murkute et al., 2006; Levitt, 1980; Jaleel et al., 2008; Parida and Das, 2005; El-Desouky and Atawia, 1998; Aggarwal, et al., 2012; Enteshari and Hajbagheri, 2011; Jaleel et al., 2008). Getlawi (2013) indicated a negative effect of salinity on Glaucium spp. leaf color at different degrees. Applying exogenous compounds is one way to reduce destructive effects of abiotic stresses (Yuan and Lin 2008).

Leaf area. Analysis of variance indicated significant differences among species and among growth regulator and their interactions (Table 1.1). Comparison among species indicated that *G.flavum* achieved the highest leaf area under all treatments including control treatment followed by *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum*. The treatment of 2.0 mM ABA resulted in the highest leaf area followed by 20.0 mM ethephon, 2.0 mM SA and 0.03 mM fusicoccin (Fig. 1.2). Under control treatment, *G. flavum* acheived an average leaf area of 9.5 cm^2 while *G. acutidentatum* had an averge leaf area of 7.5 cm^2, *G. grandiflorum* 6.5 cm^2 and *G. corniculatum* 4.5 cm^2 (Fig.1.2). Leaf area increased from 9.5 to 20.5 cm^2 in *G. flavum*, from 7.5 to 19.0, *G. acutidentatum*, from 6.5 to17.5 cm^2 in *G. grandiflorum*, and from 4.5 to 16.0 cm^2 in *G. corniculatum* under the treatment of 2mM ABA (Fig. 1.2). There was no significant difference among species in the rate of improvement or change compeared with control treatment. Leaf area followed a similar trend as leaf color. Leaf that were able to recover healthy color generally have a greater leaf area. Previous work indicated similar salinity effects on leaf area in other species (Abdul Jaleel et al., 2009). Prolonged exposure to high salinity levels

decreased leaf size (Munns et al., 1988; Volkmar et al., 1998, Getlawi, 2013). Usually, leaf area decrease is the first sign of salinity stress (Munns and Termaat, 1986; Chartzoulakis

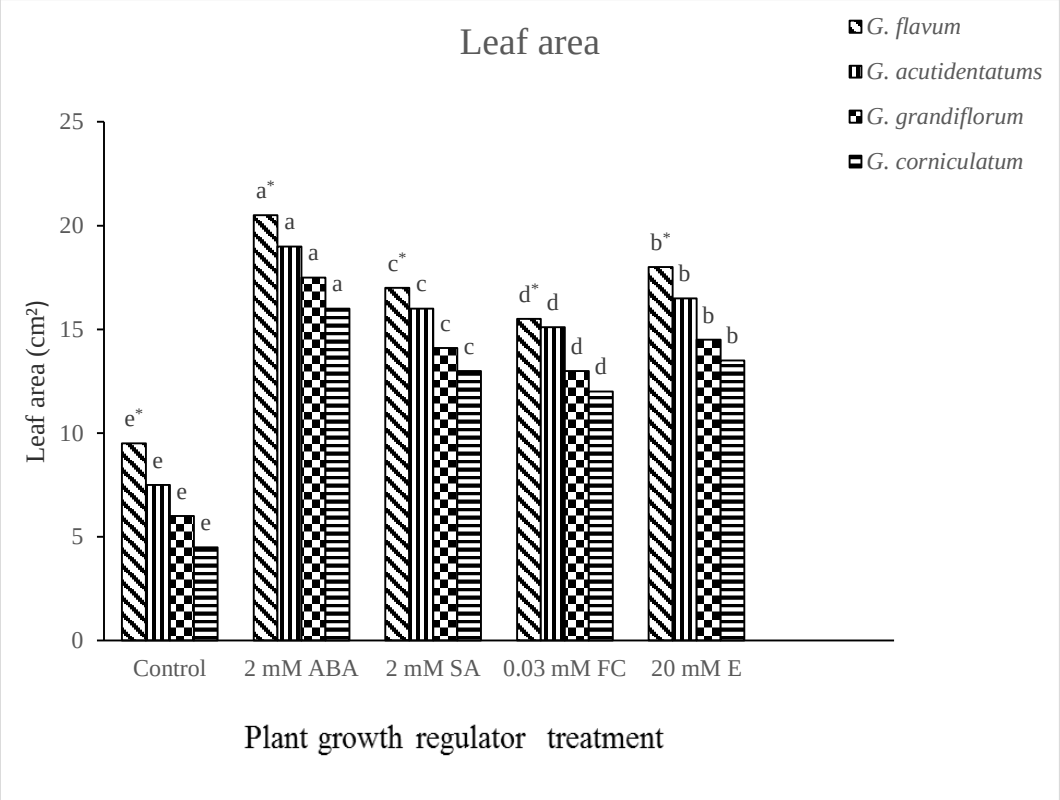

Fig. 1.2. Effect of different growth regulator on leaf area during the recovery from salinity stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$ among growth regulator treatments. Columns labeled with an asterisk are significantly the highest among species within each treatment.

and Klapaki, 2000). Energy saving by reducing leaf area is the first adaptation mechanism to be adopted by plants to cope with salinity stress (Jaleel et al., 2008). This may be a direct effect of salt on rate of cell division, to a slower rate of cell expansion, or a decrease in the duration of cell expansion. If cell division was affected, even if cell growth potential was not affected, final leaf size would be limited due to reduced cell number, (Volkmar et al., 1998). The rapid response to the increase in salinity is mainly osmotic and resulted in inhibition of leaf formation. The long term response is a result of ionic toxicity that accelerates senescence of mature leaf (Munns and

Tester, 2008). Applying growth regulator seemed to enhance *Glaucium* spp. recovery by reducing the negative effects of salinity.

Plant Quality (attractiveness). Plant quality (attractiveness) varied significantly among species and growth regulator treatments. The interaction between species and growth regulator treatments was significant too (Table 1.1). Increasing salinity decreased the attractiveness of all *Glaucium* spp. to different degrees (Fig. 1.3). Without the addition of any growth regulator, *G. flavum* achieved the highest recovery and recorded a quality rate of 5.5, followed by G. *acutidentatum* (4.5), *G. grandiflorum* (3) and *G. corniculatum* (2.2) (Fig. 1.3). The treatment of 2 mM ABA had the most significant recovery effect on all tested species. All species were positively affected at this treatment, where, *G.flavum* had its highest quality of 9, followed by *G. acutidetutum* (7.9), *G. grandiflorum* (6.5) and *G. corniculatum* (5.7) (Fig. 1-3). The treatment of 2mM SA followed the treatment of 2mM ABA and achieved a quality of 7.5 in *G. flavum*, followed by *G. acutidetutum* (6.9), *G. grandiflorum* (5.3) and *G. corniclatum* (4.5) (Fig. 1-3). The effect of 20 mM ethephon was the least among treatment and the treatment of 0.03 mM fucicoccin was intermediate. There was no significant difference among species in the rate of improvement or change compeared with control treatment.

In general, plant growth may be affected by either the absence of or excessive presence of NaCl in the substrate (Downton 1982; Clough 1984; Burchett et al. 1989; Pezeshki et al. 1990; Ball and Pidsley 1995; N. Su´arez and E. Medina, 2005). The ability to limit Na^+ transport into the shoots, and to reduce the Na^+ accumulation in the rapidly growing shoot tissues, is critically important for maintenance of high growth rates and protection of the metabolic procees in elongating cells from the toxic effects of Na^+ (Razmjoo et al., 2008). Many reports have confirmed the internal changes in growth regulator in terms of types and concentrations under

salinity stress (Wilkinson and Davies, 2010; Zhang et al., 2006; Aswath et al., 2005; McCann and Huang, 2008; Qin and Zeevaart, 2002).

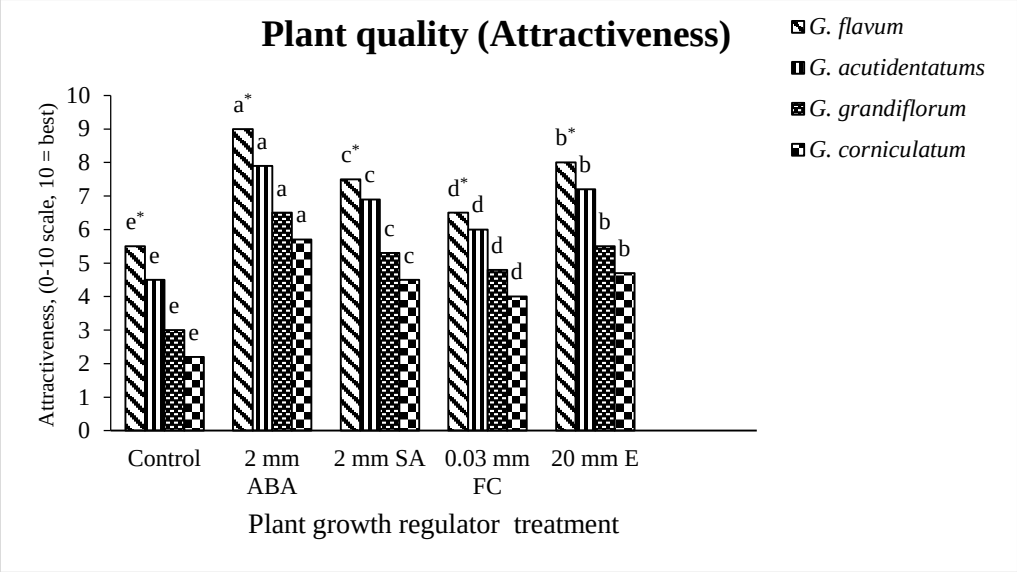

Fig. 1.3. Effect of different growth regulator on plant quality during the recovery from salinity stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$.

Shoot K^+/Na^+ Ratio:

Shoot K^+ and Na^+ varied significantly among species, growth regulator treatments and their interaction (Table 1-1). There was no significant difference among species in the rate of improvement or change compeared with control treatment. Generally, increasing salinity decreased shoot K^+/Na^+ ratio. As salinity increased, Na^+ content increased and K^+ content decreased and as a result plants show salinity stress symptoms. Wyn Jones et al. (1979) suggested a threshold K^+/Na^+ ratio of 1 for normal growth of plants subjected to salinity. Results with horned poppy indicated that K^+/Na^+ ratio was ≥ 1 in all species with the ttreatments of 2 mM ABA, 2 mM SA, and 20 mM ethephon while it was less than 1 in *G. corniculatum* at the treatment of 0.03 mM Fusicoccin.

Table 1.2. Effect of 2mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on K+/Na+ ratio of Glaucium spp. during the recovery from salinity stress.

	K^+/Na^+ ratios				
	PGR treatment				
Species	Control	2 mM ABA	2 mM SA	0.03 mM FC	20 mM E
G. flavum	0.8a†	2.2a*	2.0a	1.2a	1.5a
G. acutidentatum	0.5b	2.0b*	1.7b	1.1b	1.3b
G. grandiflorum	0.4c	1.8c*	1.5c	1.0c	1.2c
G. corniculatum	0.2d	1.4d*	1.2d	0.9d	1.1d

†Values followed by the same letters within a column for each cultivar are not significantly different ($P = 0.05$) based on Fisher's LSD test. Values labeled with an asterisk are significantly the highest ($P = 0.05$) among different growth regulator treatments within each species.

Results indicated that growth regulator treatments influenced K^+/Na^+ ratios during the recovery from salinity stress. Potassium ions and growth regulator play a significant role in stomatal closure, which is necessary for plant survival under stress. Under stress conditions the changes in guard cell ion transport which are responsible for stomatal opening and closure through controlling the turger pressure of guard cells are initiated by the 'drought' hormone abscisic acid (ABA).

Total Nonstructural Carbohydrates (TNC) and Total Reducing Sugar Content (RSC):

Shoot TNC (Table 1.3) and RSC (Tabe 1.4) varied significantly among species, growth regulator treatments and their interaction. Generally, salinity stress decreases shoot TNC and increases RSC of *Glaucium* spp. (Getlawi 2013). Growth regulator treatments significantly affected the recovery rate and TNC vs RSC dynamics. The treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM fusicoccin (Tables 1.3 and 1.4). *G. flavum* achieved the highest recovery rate and as a result the highest level of TNC and the lowest level of RSC (Tables 1. 3 and 1.4). At the treatment of 2 mM ABA, average TNC increased by

92.0, 100.0, 118.0 and 110.0% in *G. flavum*, *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum* respectively (Table 1.3) while RSC decreased by 30, 53, 37.5 and 27.0% in *G. flavum*, *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum* respectively (Table 1.4). An increase in TNC was expected due to observed improvement in leaf color, area and quality that added to the photosynthetic tissues. Recovery from stress escalated the increase in TNC which resulted from the recovery of the leaves.

Table 1.3. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on total non-structural carbohydrates (TNC) in shoots of *Glaucium* spp. measured at the end of the recovery from salinity stress.

	TNC (mg g^{-1} dry weight)				
	PGR treatment				
Species	Control	2 mM ABA	2 mM SA	0.03 mM FC	20 mM E
G. flavum	66.5a†	128.0a	108.5a	88.0a	95.0a
G. acutidentatums	58.5b	119.0bc	101.0b	83.0b	89.0b
G. grandiflorum	48.0c	105.0c	95.0c	74.0c	81.0c
G. corniculatum	42.2d	89.0da	83.0d	68.0d	73.0d

† Values followed by the same letters within a column for each species are not significantly different (P = 0.05) based on a Fisher's LSD test.

Table 1.4. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on total reducing sugar content (RSC) in shoots of *Glaucium* spp. measured at the end of the recovery from salinity stress.

	RSC (mg g^{-1} dry weight)				
	PGR treatment				
Species	Control	2 mM ABA	2 mM SA	0.03 mM FC	20 mM E
G. flavum	45.0a†	15.0d	18.0d	22.0d	20.0d
G. acutidentatums	36.0b	17.0c	20.0c	24.0c	22.0c
G. grandiflorum	32.0c	20.0b	23.0b	26.0b	24.0b
G. corniculatum	22.0d	26.0a	28.0a	30.0a	28.0a

† Values followed by the same letters within a column for each species are not significantly different (P = 0.05) based on a Fisher's test.

As was expected, RSC responded differently (Table 1.4). Reducing sugars in plants mainly consists of glucose and fructose (Ball et al., 2002; Shahba et al., 2003). While nonstructural carbohydrates are energy reserves in plants, soluble reducing sugars are thought to play an

important role in stress tolerance as osmoregulator and as protectants as they prevent cell desiccation (Popp and Smirnoff, 1995). Shahba (2010b) found an increase in RSC and a decrease in TNC when subjected increases in salinity in bermudagrass species (Tifgreen, Tifdwarf and (Tifway) and seashore paspalum cultivars (Shahba et al., 2012). In this study, it was therefore observe the opposite trend during recovery from salinity stress. Carbon reduction during stress could be related to the stress resistance mechanisms that are energy dependent. In general, previous results and our results suggested that carbohydrate availability is a limiting factor for shoot growth under high salinity stress and during recovery from stress.

Growth regulator treatments could affect the dynamics of carbohydrates usage and accumulation throught their effect on the overall growth of the plant that in turn affects all plant activities. TNC serves as the resource for the increased RSC under increased stress conditions i.e. the relationship between TNC and RSC is a source sink relation and this is obvious if we compare their dynamics in Tables (1.3) and (1.4).

Shoot Proline Content:

Shoot proline content varied significantly among species, growth regulator treatments and their interaction (Table 1.5). It is well known that proline content increases in response to salinity (Getlawi 2013; Shahba et al. 2012). During the recovery from salinity stress, the processes of proline accumulation was reversed. Growth regulator treatments significantly affected the recovery rate and Proline content. The treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM Fusicoccin (Table 1.5). *G. flavum* achieved the highest recovery rate and as a result proline content was the lowest in when compared to different growth regulator treatments. At the treatment of 2 mM ABA, proline content decreased by 72.8, 63.5, 61.4 and 57.5 % in *G. flavum, G. acutidentatum, G.*

grandiflorum and *G. corniculatum* respectively (Table 1.5). Although the role of proline accumulation in salinity tolerance is well illustrated in this study, it has been questioned by others (Ashraf and Harris, 2004). These results suggested a positive role for proline in *Glaucium* species salinity tolerance. A positive effect of proline accumulation in salinity tolerance was also reported in *Glaucium* spp. (Getlawi 2013) and in seashore paspalum cultivars (Shahba et al., 2012). Accumulation of proline in plant tissues in response to salinity stress has been attributed to enzyme stabilization and/or osmoregulation (Flowers et al., 1977; Levitt, 1980). The decline of proline content during the recovery of salinity stress indicates its relative importance to coping with stress conditions. It likely enhances membrane stability and mitigates the effect of NaCl on cell membrane disruption and protein structure, act as a sink for carbon and nitrogen for stress recovery, and can buffer cellular redox potential under salinity stress (Ashraf and Foolad, 2007; Maggio et al., 2002). In some cases, the effect of stomatal conductance on water potential is higher than that of proline accumulation. Indeed, proline has been demonstrated to confer abiotic stress tolerance either by increasing the antioxidant system or by increasing osmotic adjustment (Vendruscolo *et al*. 2007; Cvikrová *et al*. 2012; Rai *et al*. 2012). Photosynthetic rate is affected by many factors, including stomatal conduction, CO2 assimilation, photosynthetic enzyme activities, inhibition of PSII activity, and stability of photosynthetic apparatus (Camejo *et al*. 2005; Silva *et al*. 2010).

Table 1.5. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on Proline content (P) in shoots of *Glaucium* spp. measured at the end of the recovery from salinity stress.

Proline content (mg g-1 dry Wight)					
		PGR treatment			
Species	Control	2 mM ABA	2 mM SA	0.03 mM FC	20 mM E
G. flavum	1250.0d†	340.0d	485.0d	590.0d	525.0d
G. acutidentatum	1150.0c	420.0c	595.0c	620.0c	505.0c
G. grandiflorum	1400.0b	540.0b	680.0b	720.0b	610.0b
G. corniculatum	1600.0a	680.0a	740.0a	800.0a	760.0a

† Values followed by the same letters within a column for each species are not significantly different ($P = 0.05$) based on Fisher's LSD test.

The role of abscisic acid in stress physiology has received much attention, and there is now considerable experimental evidence that the physiological effects induced by salinity might be modulated by ABA. It has been shown that saline stress is accompanied by an increased in ABA content (Aspinall and Paleg 1981). In addition, *P. vulgaris* plants adapted to salinity had ABA concentrations substantially higher than those in non-adapted plants (Montero and others 1998). An exogenous ABA treatment reduces leaf abscission and increases salt tolerance in citrus plants (Go´mez-Cardenas et al.2003), but it also decreases total biomass and increases the root to shoot ratio in poplar species (Yin et al. 2004). Abscisic acid (ABA) selectively affects ion transport processes (van Steveninck, 1976). ABA appears to increase the permeability of roots to water and to inhibit excretion of ions into the xylem but not to affect uptake of ions by the root. The effectiveness of ABA may depend on environmental factors such as temperature (Pitman et al., 1974; Pitman and Wellfare, 1978).

Khadri et al. (2006) studied the effect of abscisic acid (ABA) and 100 mM NaCl on common bean (*Phaseolus vulgaris* var. Coco) growth, nitrogenase activity, and nodule metabolism in a controlled environmental chamber. Results revealed that plant dry weight, nodule dry weight, nitrogen fixation, and most enzymes of ammonium and ureides metabolism

were affected by both ABA and NaCl. The addition of one l M ABA to the nutrient solution before the exposure to salt stress reduced the negative effect of NaCl. They suggested that ABA application improves the response of *Phaseolus vulgaris* symbiosis under saline stress conditions, including the nitrogen fixation process and enzymes of ammonium assimilation and purine catabolism. The exogenous application of ABA caused an alteration of zeatin riboside (ZR) content in lucerne under different stress conditions (Dobra *et al*. 2010).

Jung and Luttge (1980) mentioned that fusicoccin (FC) inhibited net excretion of Cl by the glands of the pitchers of the carnivorous plant *Nepenthes hookeriana* ; of Na^+ and Cl^- by the salt glands of the halophytes *Limonium vulgare* and *L. pectinatum* and of K^+ in the nectar of *Acer platanoides* flowers. It had no effect on K^+ elimination with nectar of *Impatiens walleriana* (extrafloral nectaries) and *Abutilón striatum*, Abscisic acid (ABA) stimulated net excretion of K^+ and Cl^- in *Nepenthes* and of Na^+ and CI^- in *Limonium* but had no effects on K^+ in nectar. Thus, FC and ABA had opposing effects on ion excretion by the salt eliminating glands of *Limonium* and *Nepenthes*. Both compounds, however, had similar effects on sugar secretion of nectary glands which was either inhibited or unaffected by FC and ABA. It is suggested that the effects of FC and ABA on ion excretion by gland cells could be reconciled with literature showing FC-stimulation and possible ABA-inhibition of proton pumps at the plasmalemma of plant cells.

Fusicoccin was initially suggested to activate the plasma membrane H^+-ATPase by direct interaction with the enzyme (Marrè, 1979). Later, fusicoccin was demonstrated to bind to a "receptor" belonging to a family of proteins designated 14–3–3 proteins (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). The 14–3–3 proteins constitute a highly conserved family of eukaryotic proteins with multiple regulatory functions (Aitken, 1996). Recently, it was shown that 14–3–3 proteins bind directly to the C-terminal region of the H^+-

ATPase and that fusicoccin stabilizes the H^+-ATPase/14–3–3 complex formed, rendering the association irreversible (Jahn et al., 1997;Oecking et al., 1997;Baunsgaard et al., 1998). This explains earlier observations that plasma membranes isolated from fusicoccin-treated material contained several times more 14–3–3 than plasma membranes isolated from the corresponding controls (Korthout and de Boer, 1994; Oecking et al., 1994). The strong interaction induced by fusicoccin also allowed the H^+-ATPase/14–3–3 complex to be solubilized and purified from isolated plasma membranes (Jahn et al., 1997; Oecking et al., 1997). Binding of 14–3–3 proteins to the C-terminal region of the H^+-ATPase was also shown to occur in the absence of fusicoccin, and it was suggested that 14–3–3 proteins are natural ligands of the H^+-ATPase, regulating H^+ pumping by displacing the autoinhibitory domain of the enzyme (Jahn et al., 1997; Oecking et al., 1997; Baunsgaard et al., 1998). It is known to stimulate the proton pump at the plasma-lemma of cells in a large variety of plant materials; a multiplicity of other effects on transport processes and cell physiology seem to be secondary consequences thereof (Marré, 1977). FC and ABA have antagonistic effects on movements of stomatal guard cells. FC causes stomata to open and prevents closure; this is probably due to enhanced H^+ extrusion from the guard cells (Marré, 1977), which then affects malate and K^+ accumulation, thus providing the basis for turgor increase and stomatal opening at least in some cases (Raschke, 1975, 1977; Hsiao, 1976).

Bayat et al. (2012) evaluated the effects of SA on growth and ornamental characteristics of Persian petunia under salt stress and concluded that foliar application of SA improved growth and ornamental characteristics of Persian petunia under saline and non-saline conditions. Salicylic acid controls photosynthesis system, photosynthesis amount, pigment content and stomatal conductivity and regulates these procedures for appropriate growth and development (Popova *et al.*, 2009, Steven *et al.*, 2006, El-Tayeb, 2005, Kormkaz *et al.*, 2007). Environmental

stresses, such as cold, heat, salinity, and drought, induce ethylene production and oxidative stress and cause damage in plants. Ethylene is produced either chemically through the incomplete combustion of hydrocarbons or biologically by almost all living organisms (Wang et al. 2002, Pech et al. 2004). There is a lot of evidence showing that ethylene is an essential component of a wide range of responses to biotic and abiotic environmental stresses Shinozaki et al. 1999, Wang et al. 2002, Guo and Ecker 2004, El-Tayeb 2005). Further, many of these stress responses integrate ethylene signaling into more complex circuitry involving salicylate and jasmonate signaling (Wang et al. 2002). The effects of ethylene on plants are regulated both at the level of its synthesis and perception of the hormone (Caren 2007, Wang et al. 2002). Tirani et al. (2013) studied the effects of ethylene on chlorophyll (Chl), carotenoid (Car), anthocyanin, flavonoids, ascorbic acid, dehydroascorbic acid, total ascorbate, lipid peroxidation, and ethylene production in leaf of canola pretreated with SA. Their results showed that the ethylene treatments induced lipid peroxidation, lowered significantly Chl and Carotenoids contents and anthocyanin accumulation.

On the basis of the number of times in the best statistical category for leaf characteristics, overall plant quality (attractiveness), TNC, RSC, proline content and K^+/Na^+ ratio, *G. flavum* was found to have higher recovery rate when compared to *G. acutidenatum*, *G. grandflorum* and *G. corniculatum*. Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM Fusicoccin in enhancing salinity stressed Glaucium SPP. recovery. In summary, *Glaucium* spp. under salinity stress exhibited a positive response to growth regulator treatments in terms of improving leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and K^+/Na^+ ratio. *G. flavum* showed a greater tendency to recover from salinity stress at all growth regulator treatments when compared to the other species tested. The

treatment of 2 mM ABA is recommended to improve the recovery rate of *Glaucium* spp. under salinity stress.

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CHAPTER 2

RECOVERY OF *GLAUCIUM* SPP. FROM DROUGHT STRESS USING GROWTH REGULATOR TREATMENTS

SUMMARY

Water conservation especially in arid and semiarid regions of the world is a necessity. Plant species, and cultivars within a species, vary in their drought tolerance. These variations are the result of variations in genes relating to drought tolerance mechanisms and their interaction with the environment. In order to reduce water usage, it is important to understand the mechanisms of plant adaptation to drought stress. Horned Poppies (*Glaucium* spp.) are members of the Poppy family, Papaveraceae, that are native to the Mediterranean and Middle East regions. The objectives of this study were to (1) determine whether applications of Abscisic acid (ABA), salicylic acid (SA), fusicoccin, and ethephon could promote *Glacium* spp. growth and recovery from drought stress; (2) determine the most effective concentrations of each growth regulator in the recovery of the stressed plants; (3) evaluate the recovery degree from drought stress among the common Horned Poppy species that are available at Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum* and *G. acutidentatum*s; (4) determine which evaluation criteria are associated with superior recovery rate; (5) confirm selection criteria for evaluation of drought tolerance in Horned Poppy species. Lysimeter columns were used in this study which was replicated twice in the CSU Plant Science greenhouse. Four Growth regulator treatments were used and were applied weekly with irrigation water. Three levels of each regulator were used. Treatments continued for two months. Data were collected weekly on leaf color (using color chart), leaf size (using Image J software), and the quality and general attractiveness of the plant using personal visual estimation (using a scale of 0 to 9 where 9 is the optimum quality, with a rating of 6.0 or higher indicating

acceptable quality). Samples were collected for TNC, RSC and proline content analysis for each treatment at the end of the experiemnt. Evapotranspiration (ET) measurements were collected every 2 to 3 days during the four month growth period. Five weight readings per pot were made during each measurement and the average value was used for ET calculation. ET was calculated by mass difference and expressed as mm d^{-1}. On the basis of the number of times in the best statistical category for leaf characteristics, overall plant quality (attractiveness), water use efficiency, TNC, RSC, and proline, *G. flavum* was found to have the greatest recovery rate from drought stress when compared to *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum.* Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM fusicoccin in enhancing drought stressed *Glaucium* spp. recovery. Growth regulator treatments could affect proline accumulation through their effect on the overall growth of the plant that affect all plant activities especially different growth regulator concentrations and interactions as it will be indicated in the next Chapter. In summary, *Glaucium* spp. under drought stress exhibited a positive response to growth regulator treatments in terms of improving leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and water use efficiency. *G. flavum* showed greater tendency to recover from drought stress at all growth regulator treatments when compared to the other species tested. The treatment of 2 mM ABA is recommended to improve the recovery rate of *Glaucium* spp. under salinity stress.

INTRODUCTION

Water is a limiting factor in arid and semiarid regions and as a result of drastic water conservation methods are greatly needed. Because of the immense water usage and the diminishing water resources, many arid states have implemented water conservation programs (Soeder and Kappel, 2009). The demand for water has led to an inadequate water supply for landscapes and as

a result negative impacts on the aesthetics and functionality. Therefore, the development of efficient irrigation management programs as well as the selection and improvement of drought tolerant landscape plants has become extremely important in order to maintain landscapes at acceptable quality.

Plant species and cultivars within a species, vary in their drought tolerance. These variations are mainly genetic and could be due to environmental adaptations (Duncan and Carrow, 1999). Usually evaluations for drought tolerance of plants depend on shoot growth, as reported in crop yield response curves proposed by Maas and Hoffman (Igartua, 1995; Maas and Hoffman, 1977).

In arid and semiarid regions, climate and soil can make it difficult for many ornamental plants to grow. Therefore, nurseries are always interested in new plants that will survive well in such climates and satisfy the customer's desire for new beautiful plants. Horned Poppies (*Glaucium* spp.) are members of the Poppy family, Papaveraceae. *Glaucium* spps. are species that have originated in the Mediterranean and Middle East regions. Some species have a wider distribution than others. Horned poppies require full sun and well-drained soils for optimum growth. They should be spaced between 30 and 60 cm apart, and are best grown by seeding in the fall where they are to bloom and thinning to the desired spacing as they germinate in the spring. The roots of the horned poppy are considered poisonous.

All horned poppies have blue-green foliage that is deeply pinnatifid to pinnatisect and typically grow 30-50 cm long. The leaf have varying degrees of texture from glaucous to villous. All leaf are lyrate to sublyrate shaped and have a rosette growth habit. They have solitary blooms on flower stalks that grow above the foliage. All species have four petals in their corolla and their pistil is completely surrounded by stamens. They all develop long horned-shaped seed siliquiforms

with the stigma remaining to cap off the top of the fruit. Species of interest in this study were *G. flavum*, *G. grandiflorum*, *G. acutidentatum* and *G. corniculatum*.

Glaucium flavum Crantz is the most widely spread species in the genus. It is found from the coasts of Britain and the Atlantic Islands to the coasts of the Mediterranean Basin and the Black Sea (Grey-Wilson, 2000). It grows predominantly on sandy beaches and as a result it is commonly known as the Sea Horned Poppy. According to Davis (1965), *G. flavum* is distinguished from other species by several characteristics. The sepals have crisp, pilose hairs on the surface and the petals can be solid yellow, red or reddish mauve. *Glaucium flavum* is most often recognized for the yellow petals and is commonly referred to as the Yellow Horned Poppy. The ovary is densely papillose to tuberculate, basically a bumpy surface. The siliquae will retain the papillose to tuberculate texture.

Glaucium grandiflorum Boiss & É. Huet is native to the southern part of the Caucasus Mountains but it is also found in Syria, Iran and the Sinai (Grey-Wilson, 2000). Turkey is situated between the Mediterranean Sea and the Black Sea, where the precipitation ranges from 580 to 1300 mm/year. However, in the mountain ranges of the country there are great differences in climate changes with harsh winters and drier conditions with low precipitation of 400 mm/year. *Glaucium grandiflorum* has features that distinguish it from other *Glaucium* species. It has only one main flower stem while other species have multiple flower stalks growing from the base of the rosette (Davis, 1965). The sepals have short, stiff hairs making the surface hirsute. The petals are dark orange to crimson red with a black spot at the base of the petal. The pedicle of the flower exceeds the subtending leaf, which differs from the other *Glaucium* species. There are two varieties of *G. grandiflorum*: var. *grandiflorum* and var. *torquatum. Glaucium grandiflorum* var. *torquatum*

has red petals with a black blotch and can be found in calcareous hillsides. *Glaucium grandiflorum* var. *grandiflorum* is found in fields, banks and rocky slopes.

Glaucium acutidentatum Hausskn & Bornm is endemic to Turkey where it is found on dry hillslopes and rocky places (Grey-Wilson, 2000). *G. acutidentatum* is the most glabrous species with smooth sepals and ovaries. Although the ovary is smooth, the resulting siliquae is subtorulose. The petals are solid orange-buff color. *G. acutidentatum* is found at elevations of 950-1400 m on dry hills (Davis, 1965).

Glaucium corniculatum (L.) J.H. Rudolph is native to the Mediterranean basin, Atlantic islands, Caucasus Mountains, Bulgaria, Romania, northern Iraq and northwestern Iran (Grey-Wilson, 2000; Davis, 1965). *G. corniculatum* also has some unique characteristics. Its leaf have a soft, villous texture and its sepals are scabrous to hirsute. The petals are yellow, orange or red (Davis, 1965) with a black basal spot (Grey-Wilson, 2000).

In order to reduce water usage, it is important to understand the mechanisms of plant adaptation to drought stress. Drought resistance includes a range of mechanisms employed by plants to withstand periods of drought (Beard, 1989). Strategic mechanisms include drought escape, drought avoidance, and drought tolerance (Turner, 1986). The significance of each of these strategies is related to drought duration and severity in addition to the plant species. These mechanisms are associated with anatomical, morphological, physiological, and biochemical changes. The reduction in the evapotranspiration (ET) rate and the ability of a species to maintain transpiration as the soil dries are example of drought tolerance mechanisms as the reduction in ET indicates a better water use efficiency. Changes in leaf that facilitate drought tolerance include reduced leaf growth and area, increased pubescence, rolling or folding, and fewer stomates (Duncan and Carrow, 1999). The balance between carbohydrate production and consumption will

impact the ability of plant species to cope with stresses (Huang and Fry, 1999; Lee et al., 2008a, 2008b; Shahba, 2010b, Shahba et al., 2012). Amino acids, especially proline, accumulate in larger amounts to cope with increasing stress in plants (Lee et al., 2008b). Proline accumulation is one of the first responses of plants exposed to water-deficit stress and serves to reduce injury to cells (Ashraf and Foolad 2007). Rapid accumulation of proline in tissues of many plant species in response to drought, salt or temperature stresses has been attributed to osmoregulation (Flowers et al., 1977; Levitt, 1980). However, because of contrasting reports related to proline accumulation effect on stress tolerance (Marcum, 2002; Torello and Rice, 1986), its use as selection criterion for stress tolerance has been questioned (Ashraf and Harris, 2004). Thus it is critical that tests be made before making any conclusion regarding the role of proline in stress tolerance of any specific species.

In a previous study (Getlawi 2013), it was shown that drought tolerance of *Glaucium* spp. is dependent on the internal osmoregulator content. Many studies discussed the plant responses to stress via internal chemical signals and growth regulator adjustments. However, few studies dealt with the effects of exogenous growth regulator applications on the physiology of plants under stress such as Aroca et al. (2008) who evaluated the influence of exogenous ABA application on plant development, physiology, and expression of several stress related genes after both drought and a recovery period. The objectives of this study, therefore, are to (1) determine whether applications of Abscisic acid (ABA), salicylic acid (SA), fusicoccin, and ethephon could promote *Glacium* spp. growth and recovery from drought stress; (2) determine the most effective concentrations of each growth regulator in the recovery of the stressed plants; (3) evaluate the recovery degree from drought stress among the common Horned Poppy species that are available at Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum* and *G. acutidentatum*s;

(4) determine which evaluation criteria are associated with superior recovery rate; (5) confirm selection criteria for evaluation of drought tolerance in Horned Poppy species.

MATERIALS AND METHODS

Lysimeter columns were used for these experiments. Columns were placed in the green house. Sixty plants of each species that were planted in 15 cm in diameter and 50 cm long PVC tube containing potting mix were used. These plants were previously used for drought tolerance strees screening and already suffered different degrees of stress. Stress treatments that were applied included control (100% of the total evapotranspiration), 75%, 50 % and 25% of the total ET. Experimental design was randomized complete Block (RCB). Each block represented one of the replications and contains sixty tubes. Four blocks were used. Used plants in each tube were selected of similar size and height, hold the same number of leaf and suffered similar degree of stress. Four Growth regulator treatments were used and were applied weekly with irrigation water. Three levels of each regulator were used. Evapotranspiration was measured weekly to monitor the change in the evapotranspiration. Four representative tubes for each species were used as lysimeters and were watered with enough water and left to drain for 2 h, after which the weight of each tube was recorded. Each tube was then re-weighed every 24 hours. The daily changes in weight represent the daily evapotranspiration for each species. Treatments continued for two months.

Data Collection. During the course of the experiment data were collected weekly on plant leaf color (using leaf color chart), leaf size (using Image J software), and general attractiveness of the plant using personal visual estimation (using a scale of 0 to 9 where 9 is the optimum quality, with a rating of 6.0 or higher indicating acceptable quality).

ET measurements were collected every 2 to 3 days during the four month growth period. Five weight readings per pot were made during each measurement and the average value was used for ET calculation. ET was calculated by mass difference and expressed as mm d-1.

Samples were collected for TNC, RSC, proline and tissue Na^+ and K^+ content analysis for each treatment. Total nonstructural carbohydrate content, RSC, tissue Na^+ and K^+ and proline content were determined at the termination of the experiment. Shoot tissue at the termination of the experiment was harvested and washed with cold distilled water to remove plant debris for carbohydrate analysis. Approximately 5 g samples from the treatments were freeze-dried (Genesis 25 LL Lyophilizer, Virtis, Gardiner, NY). After freeze-drying, samples were ground with a Wiley mill, sieved thought a screen with 425 µm openings, and kept in airtight vials at – 20 ^oC. TNC was measured using the method described by Chatterton et al. (1987). In brief, 25 mg freeze-dried samples were transferred to 5 ml of 0.1% clarase solution and incubated at 38°C for 24 h. Then, 0.5 ml of hydrochloric acid (50%, v/v) was added to the incubation solution. After the solution was incubated at room temperature for 18 h, the pH value of the solution was adjusted to between 5 and 7 with 10 and 1 N NaOH. This resulting solution was used to determine TNC content using a spectrophotometer at 515 nm wavelength (model DU640; Beckman).

To measure the free reducing sugar, 25 mg of the freeze dried, ground, and sieved sample was extracted with 10 ml of 0.1 M phosphate buffer (pH = 5.4) for 24 h at room temperature. An extracted aliquot (0.2 mL) was used to determine the reducing sugar content by using the same method as was used to measure TNC.

Actual proline tissue accumulation levels were determined according to the method of Bates et al. (1973) as modified by Torello and Rice (1986) with approximately 0.5g fresh weight

of tissue. Samples were ground with liquid nitrogen in a mortar. Each sample was homogenized in 10 ml of 3% aqueous sulfosalicylic acid followed by agitation for 1h prior to filtration through #2 Whatman filter paper. After filtration 2 ml of extract from each sample was reacted with 2 ml of ninhydrin reagent (1.25 mg of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M H3PO4) and 2 ml of glacial acetic acid followed by 1 h of heating at 100 ^{o}C in an enclosed water bath. Samples were then quickly cooled by immersion in an ice bath and total proline was determined spectrophotometrically at 520 nm. Actual proline tissue accumulation levels were determined by subtracting mean control data from growth regulator treatment data for all cultivars during the entire experimental period.

Data analysis. Effects of species, growth regulator, and growth regulator levels and their interactions were determined by analysis of variance (SAS Institute, 2007). Monitored parameters (leaf color, leaf area, plant quality, K^{+}/Na^{+}, TNC, RSC and Proline contents of shoots) were analyzed on individual measurement dates to examine the differences in the recovery rate among different treatment. Means will be separated by least significant difference at the 0.05 level of probability.

RESULTS AND DISCUSSION

Leaf characteristics:

Leaf color. Comparisons of leaf color among species and growth regulator treatments clearly showed significant differences. Species and growth regulator interaction was also significant (Table 2.1). Comparison of species within each treatment indicated that *G. flavum* had a superior recovery rate (Fig. 2.1). Even under control treatment, *G. flavum* leaf color was not below the acceptable rating of 6.0 and showed reasonable recovery rate. *G. acutidentatum* showed acceptable color under the treatments of 2.0 mM ABA, 2.0 mM SA, and 20.0 mM ethephon and

was below 6 only at the treatment of 0.03 mM fusicoccin. *G. grandiflorun* showed acceptable quality only with treatment of 2.0 mM ABA. *G. corniculatum* leaf color did not recover to the acceptable level at the end of the experiment with any treatment. The treatment of 2.0 mM ABA resulted in the highest recovery in leaf color followed by 20.0 mM ethephon, 2.0 mM SA and 0.03 mM fusicoccin (Fig. 2.1). There was no significant difference among species in the rate of improvement or change compeared with control treatment.

Generally drought affects leaf color quality in different degress. As soon as growth conditions improve, recovery expected at different degress too based on the species and growth conditions. Many studies reported the effect of drought on leaf color and quality. Leaf greenness decreased under drought conditions almond genotypes (Yadollahia, 2011). Flexas and Medrano (2002) reported a reduction in leaf greenness in C3 plant leaf under water stress and associated that to degradation in chlorophyll content. The retention of leaf or the observation of 'stay green' under water stress conditions has been reported in some cassava lines and has correlated well with drought tolerance and improved yields (Lenis et al. 2006). The decrease in relative greenness of the leaf under drought conditions is likely due to a decrease in chlorophyll content (Gibon et al., 2000). There was a 38% reduction in chlorophyll content when compared to full irrigation of plants (Din et al., 2011). Increasing water stress reduced the (Chl a) and the (Chl a: b) significantly (Liu, et al, 2011). The pigment content generally decreased due to low synthesis rate and rapid degradation under water stress (Mihailovie et al., 1997; Lei et al., 2006; Yadollahia, 2011). Getlawi (2013) indicated a negative effect of drought on *Glaucium* spp. leave color at different degrees. Applying exogenous compounds is one way to reduce destructive effects of abiotic stresses (Yuan and Lin 2008). Growth regulator act simulatenously in direct and idirect ways to improve the leaf color during recovery.

Table 2.1. Analysis of variance with mean squares and treatment significant of leaf color, leaf area, plant quality, shoot K^+/Na^+, total non-structure carbohydrate content (TNC), shoot reducing sugar content (RSC), and Proline contents in *Glaucium* spp. during the recovery from salinity stress.

Parameter	Species (S)	PGR	S xPGR
Leaf color (0-10)	6.5**	82.0**	70.5*
Leaf Area (cm^2)	2.2**	4.9**	3.1*
plant quality (0-10 scale)	6.7**	6.9**	6.4*
TNC (mg g^{-1} dry Wight)	990.0**	1010.0**	750.0*
RSC (mg g^{-1} dry Wight)	44.0**	540.0**	33.0*
Proline content (mg g^{-1} dry Wight)	980.0**	1211.0**	1050.0*
Total ET (mmd^{-1})	1.2**	3.0**	2.5*

*significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$

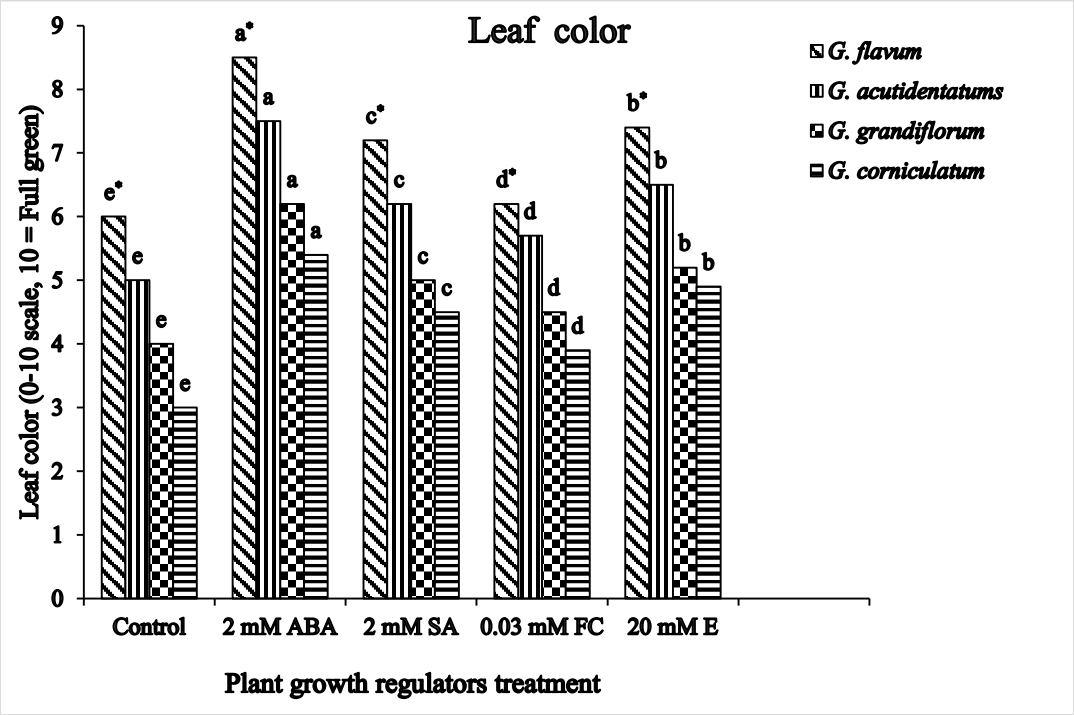

Fig. 2.1. Effect of different growth regulator on leaf color during the recovery from drought stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$ among growth regulator treatments. Columns labeled with an asterisk are significantly the highest among species within each treatment.

Leaf area. Analysis of variance indicated significant differences among species and among growth regulator and their interactions (Fig. 2.2). Comparison among species indicated that *G.flavum* achieved the highest leaf area under all treatments including control treatment followed

by *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum*. The treatment of 2.0 mM ABA resulted in the highest leaf area followed by 20.0 mM ethephon, 2.0 mM SA and 0.03 mM fusicoccin (Fig. 2.2). Under control treatment, *G. flavum* acheived an average leaf area of 9.5 cm^2 while *G. acutidentatum* achieved an averge leaf area of 7.5 cm^2, *G. grandiflorum* achieved 6.0 cm^2 and *G. corniculatum* achieved 4.5 cm^2 (Fig. 2.2). Leaf area increased from 9.5 to 20.5 cm^2 in *G. flavum*, from 7.5 to 19.0 in *G. acutidentatum*, from 6.0 to17.5 cm^2 in *G. grandiflorum*, and from 4.5 to 16.0 cm^2 in *G. corniculatum* under the treatment of 2mM ABA (Fig. 2.2). Leaf area followed the similar trend of leaf color. Leaf that were able to recover healthy color generally have a greater leaf area. There was no significant difference among species in the rate of improvement or change compeared with control treatment.

It is logic that the leaf area followed the trend of leaf color since healthy leaf should have a greater leaf area. Although there was considerable decrease in overall leaf area in *G.flavum*, it appeared to be the most drought tolerant species. Water stress is one of the most common environmental factors affecting plant growth and productivity. Reduced water availability induces numerous physiological and biochemical changes in all plant organs. Gas exchange in leaf is limited, which in turn reduces carbon assimilation. Changes in the distribution of photo-assimilates can reduce vegetative growth (Boyer, 1970; Gehrmann, 1985; Singer et al., 2003) as well. The reduction of leaf area is principally explained by a lower leaf unfolding rate which results in smaller leaf size (Lecoeur et al., 1995; Lecoeur and Guilioni, 1998). The reduction in leaf area could be an adapting mechanism to water stress. The mechanism, by which plant leaf area is reduced under water stress, is thought to be the reduction of cell elongation, which leads to reduction of cell size and therefore a reduction of leaf area (Schuppler et al., 1998). Prolonged exposure to drought decreased leaf size (Munns et al., 1986; Volkmar et al., 1998, Getlawi, 2013).

Getlawi (2013) indicated a negative effect of drought on *Glaucium* spp. leave area. Applying growth regulator seemed to enhance *Glaucium* spp. recovery by reducing the destructive effects of drought and the enhancement of cell division and enlargement due to reestablishing the balance between different types of growth regulator.

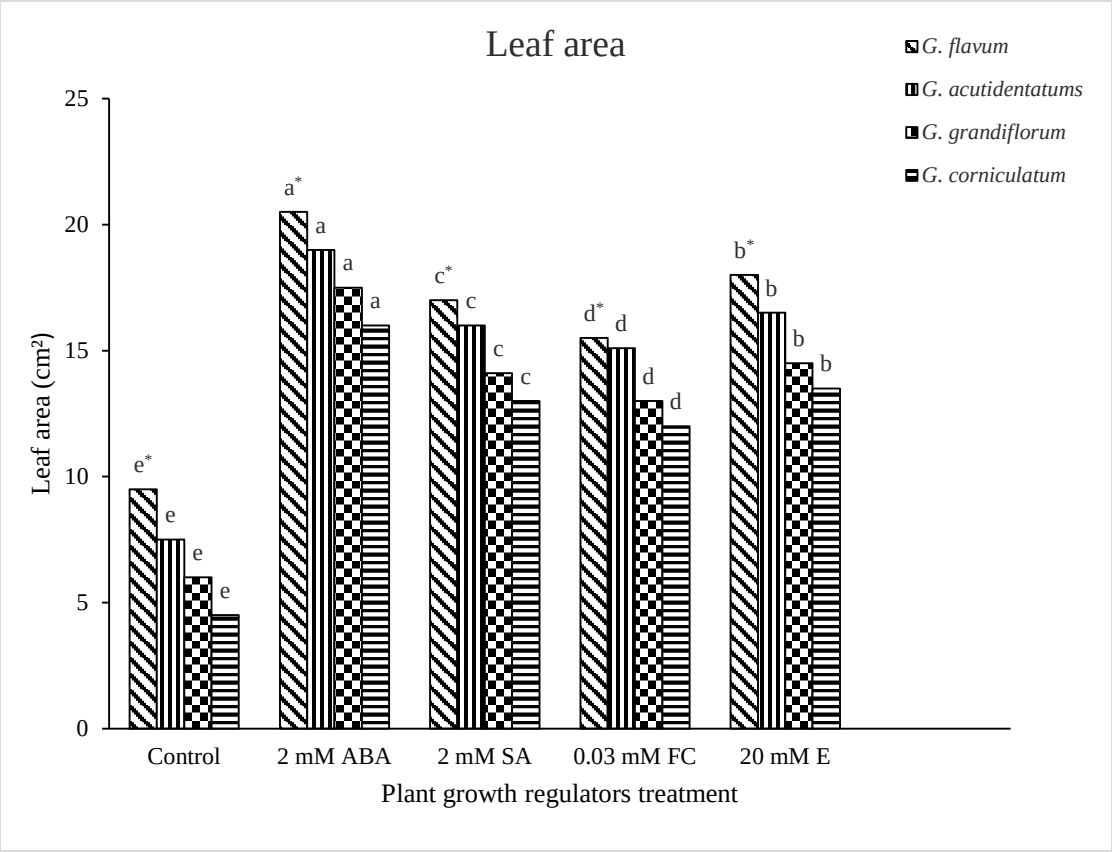

Fig. 2.2. Effect of different growth regulator on leaf area during the recovery from drought stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$ among growth regulator treatments. Columns labeled with an asterisk are significantly the highest among species within each treatment.

Plant Quality (attractiveness):

Plant quality (attractiveness) varied significantly among species and growth regulator treatments. The interaction between species and growth regulator treatments was significant too (Fig 2.3). Drought decreased the attractiveness of all *Glaucium* spp. at different degrees (Fig.

2.3). Without the addition of any growth regulator, *G. flavum* achieved the highest recovery and recorded a quality rate of 5.5, followed by *G. acutidentatum* (4.5), *G. grandiflorum* (3) and *G. corniculatum* (2.2) (Fig. 1.3). The treatment of 2 mM ABA had the most significant recovery effect on all tested species. At 2 mM ABA *Glaucim flavum* had its highest quality of 9, followed

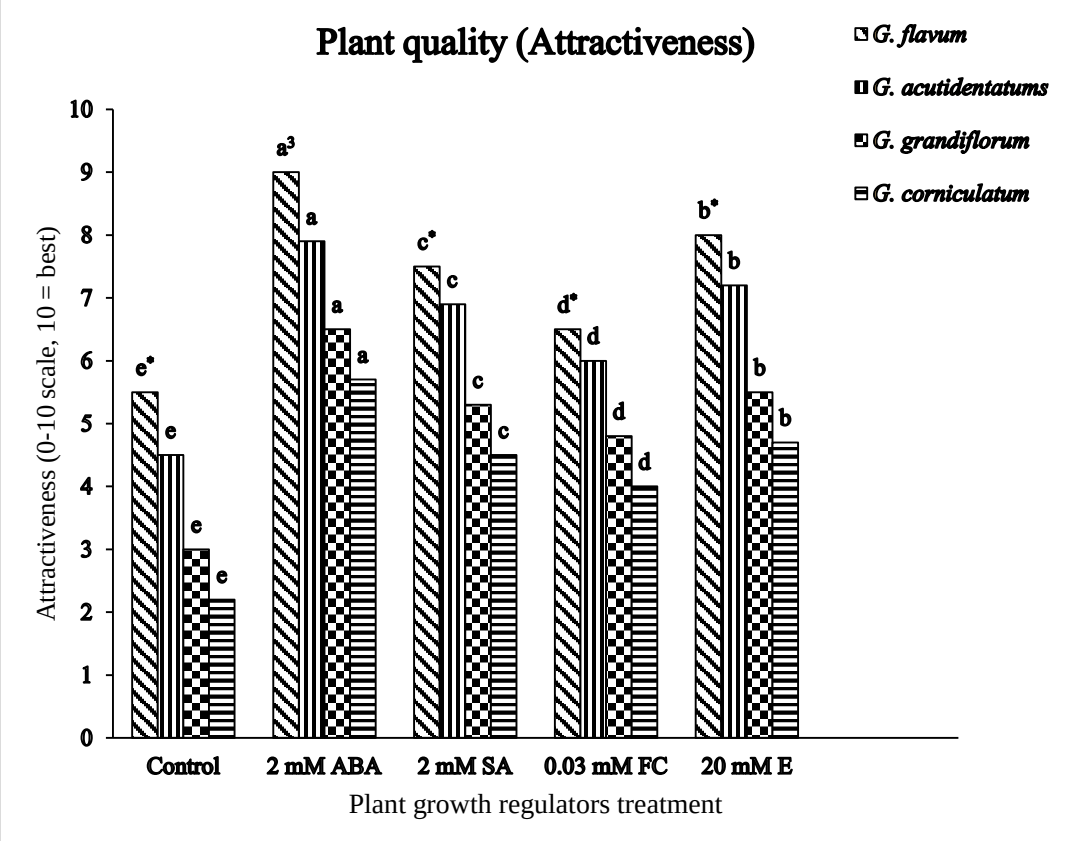

Fig. 2.3. Effect of different growth regulator on plant quality during the recovery from drought stress of four *Glacium* spp. Columns labeled with different letters are significantly different at $P = 0.05$.

by *G. acutidetutum* (7.9), *G. grandiflorum* (6.5) and *G. corniclatum* (5.7) (Fig. 2-3). The treatment of 20 mM ethephon achieved a quality of 8 in *G. flavum,* followed by *G. acutidetutum* (7.2), *G. grandiflorum* (5.5) and *G. corniclatum* (4.7) (Fig. 2.3). The effect of 0.03 mM

fucicoccin was the least among treatments. There was no significant difference among species in the rate of improvement or change compeared with control treatment.

Strategies that include both morphological and physiological modifications. These modifications may affect plant leaf greenness, leaf size and shape, plant height (shoot to root ratio) and flowering quality. Water stress has been shown to significantly reduce plant size (Champolivier and Merrien, 1996). Studies have also shown that drought stress can affect the growth of plant organs differently (Spollen et al., 1993) which may result in the alteration of morphology (French and Turner, 1991). Putievsky et al. (1990) reported that water stress had a negative impact on green tissue yield of *Geranium*. Drought caused reduction in all growth parameters of *Matricaria chamomile* (Razmjoo et al., 2008). Furthermore, a study by Flexas and Medrano (2002) showed that moisture deficiency affects various physiological and metabolic responses such as stomatal closure, decline in growth rate and photosynthesis. Also, Baher et al. (2002) showed that greater soil water stress decreased plant height and total fresh and dry weight of *Satureja hortensis*. Colom and Vazzana (2002) showed that the number of branches per plant and total plant dry weight was negatively affected by water stress in *Eragrostis curvula*. The range of drought in which the plant is able to survive varies according to the species (Ball 1988). The ability to limit Na^+ transport into the shoots, and to reduce the Na^+ accumulation in the rapidly growing shoot tissues, is critically important for maintenance of high growth rates and protection of the metabolic process in elongating cells from the toxic effects of Na^+ (Razmjoo et al., 2008) which is a process that requires sufficient water in plant cells. Also, drought may directly or indirectly inhibit cell division and enlargement and finally the growth of the whole plant. Drought caused a decline in the quality of bermudagrass cultivars (Shahba, 2010b) and seashore paspalum cultivars (Shahba et al., 2014). In addition, elevated drought may adversely affect photosynthesis

and as a result adversely affect plant biomass production through reduced accumulation of carbon products (Munns and Termaat, 1986). The reduction in the number of flowers usually is more drastic than other growth parameters under high drought as it is a cumulative effect (Razmjoo et al., 2008). Fewer flowers and reduced size of flowers adversely affect the attractiveness of landscape plants.

Many reports confirmed the internal modification in growth regulator in termes of types and concentrations under drought stress (Wilkinson and Davies, 2010; Zhang et al., 2008; Aswath et al., 2005; McCann and Huang, 2008; Qin and Zeevaart, 2002). Externally growth regulatots amendments affect the internal balance of growth regulator that can help the plant to regrow and recover from stress (Yuan and Lin 2008).

Water use:

Drought avoidance is an important drought resistance strategy. Drought avoidance can be achieved through the reduction in water use or water loss through the canopy and increasing water uptake of roots from deeper soils. ET is a measure of water use and is an indicator of plant vigor. ET varied significantly ($P < 0.05$) among species, among growth regulator and their interaction (Table 2.1). The decline in ET rate under drought stress was expected. *G. flavum* showed lower ET rates under drought conditions compared to *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum*. *G. corniculatum* had the highest ET rates at the control treatment (Table 2.2). The treatment of 2.0 mM ABA resulted in the highest recovery rate and as a result the highest ET rate in all species followed by, 2.0 mM SA, 20.0 mM ethephon and 0.03 mM fusicoccin. *G. flavum* showed the highest ET rates under all growth regulator treatments compared to *G. acutidentatum*, *G. grandiflorum* and *G. corniculatum*. (Table 2.2).

Many species have shown considerable interspecific diversity for various environmental stresses, including drought (Duncan and Carrow, 1999; Trenholm et al., 1999; Lee et al., 2004c). Kim and Beard (1988) found that species/cultivar differences in ET rates under non-limiting soil moisture conditions were associated with canopy resistance and total leaf area. High canopy resistance and/or a low leaf area resulted in lower ET. Arunyanark et al. (2008) reported a reduction in transpiration rate as a result of drought while the transpiration efficiency, as indicated by total dry matter production, was increased in peanut (*Arachis hypogaea* L.). Baranyiova et al. (2014) concluded that, the use of growth regulator is accompanied with a number of positive effects, especially in the conditions of water deficit. By applying growth regulator we can reach a partial elimination of environmental stress effect. Growth regulator can improve water use efficiency. They also have influence on increase of roots: above ground biomass ratio and can also influence the accumulation of antioxidants that protect plants. According to their results, growth regulator treatments increased the CO2 assimilation rate and stomatal conductance in winter wheat under drought conditions (Baranyiova et al. 2014).

Table 2.2. Effect of different growth regulator treatment Total ET (mmd^{-1}) on *Glacium* spp. during the recovery from drought stress. Linear regression of different total ET (mmd-1) of measured at the end of the experiment vs. growth regulator treatment (control, 2 mM Abscsic acid, ABA, 2 mM salicylic acid, SA, 0.03 mM fusicoccin, FC, and 20 Mm ethephon, E.

	Total ET (mmd^{1})					
	PGR treatment					
Species	Control	2 mM ABA	2 mM SA	0.03 mM	FC	20 mM E
G. flavum	1.1d†	4.5a	4.0a	3.5a		3.6a
G. acutidentatum	1.6c	4.0b	3.6b	3.2b		3.3b
G. grandiflorum	2.1b	3.5c	3.3c	3.0b		3.2b
G. corniculatum	2.4a	3.0d	2.8d	2.5c		2.7c

† Values followed by the same letters within a column for each cultivar are not significantly different ($P = 0.05$) based on a Fisher's test.

Osmotic adjustment:

Osmotic adjustment facilitates water uptake and limits water loss from cells. Thus tissues may sustain metabolic and physiological functions under drought stress in addition to the stability of cell membrane. Tested osmotic adjustment parameters included shoot total nonstructural carbohydrates, total reducing sugar content and shoot proline content. Osmoregulator accumulation is a way of coping with drought stress. At favorable conditions, when plants start to recover, osmoregulator content decrease.

Total Nonstructural Carbohydrates and Total Reducing Sugar Content:

Shoot TNC and RSC varied significantly among species, growth regulator treatments and their interaction (Table 2.1). Generally, drought stress decreases shoot TNC and increases RSC of *Glaucium* spp. (Getlawi 2013). Growth regulator treatments significantly affected the recovery rate and the dynamic balance between TNC and RSC. The treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM Fusicoccin (Tables 2.3 and 2.4). *G. flavum* achieved the highest recovery rate and as a result the highest level of TNC and the lowest level of RSC (Tables 2.3 and 4). At the treatment of 2 mM ABA, average TNC increased by 109.9, 142.4, 138.5 and 164.5% in *G. flavum, G. acutidentatum, G. grandiflorum* and *G. corniculatum* respectively (Table 2.3) while RSC decreased by 72.7, 55.6, 32.6 and 6.7% in *G. flavum, G. acutidentatum, G. grandiflorum* and *G. corniculatum* respectively (Table 2.4). An increase in TNC was expected due to continued improvement in leaf color, area and quality that add to the photosynthetic tissues. Reovery from stress escalated the increase in TNC which resulted from the recovery of the shoot system.

Reducing sugars in plants mainly consists of glucose and fructose (Ball et al., 2002; Shahba et al., 2003). While nonstructural carbohydrates are energy reserves in plants, soluble

reducing sugars are thought to play an important role in drought tolerance as osmoregulator (Popp and Smirnoff, 1995).

Table 2.3. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on total non-structural carbohydrates (TNC) in shoots of *Glaucium* spp. measured at the end of the recovery from drought stress.

	TNC (mg g^{-1} dry weight)				
	PGR treatment				
Species	Control	2mM ABA	2mM SA	0.03mM FC	20mM E
G. flavum	55.5a†	116.5a	112.4a	98.0a	104.0a
G. acutidentatums	46.0b	111.5b	107.0b	92.0b	95.0b
G. grandiflorum	39.0c	93.0c	88.0c	82.0c	84.0c
G. corniculatum	31.0d	82.0d	76.0d	68.0d	71.0d

† Values followed by the same letters within a column for each cultivar are not significantly different (P = 0.05) based on a Fisher LSD test.

Table 2.4. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on total reducing sugar content (RSC) in shoots of *Glaucium* spp. measured at the end of the recovery from drought stress.

	RSC (mg g^{-1} dry weight)				
	PGRtreatment				
Species	Control	2mM ABA	2mM SA	0.03mM FC	20mM E
G. flavum	44.0a†	12.0d	15.0d	19.0d	17.0d
G. acutidentatums	36.0b	16.0c	19.0c	23.0c	20.0c
G. grandiflorum	27.0c	18.2b	22.0b	26.0b	24.0b
G. corniculatum	24.4d	22.4a	25.0a	29.0a	27.0a

† Values followed by the same letters within a column for each cultivar are not significantly different (P = 0.05) based on a Fisher LSD test.

Carbon reduction could be related to the drought resistance mechanisms that are energy dependent. The results suggested that carbohydrate availability was a limiting factor for shoot growth under high drought stress. Shahba (2010b) found an increase in RSC and a decrease in TNC with drought increase in bermudagrass species (Tifgreen, Tifdwarf and Tifway) and seashore paspalum cultivars (Shahba et al., 2012, 2014).

Soluble carbohydrates may interact with membrane phospholipids and proteins to stabilize their structures and prevent desiccation under drought stress (Popp and Smirnoff, 1995). TNC serves as the resource for the increased RSC under drought conditions. The balance between carbohydrate production and consumption impacts the ability of plants to cope with stresses (Huang and Fry, 1999; Lee et al., 2008a, 2008b; Shahba, 2010b, Shahba et al., 2012).

In this study, it was logic to obtain an opposite trend during recovery from drought stress. In general, previous results and our results suggested that carbohydrate availability is a limiting factor for shoot growth under drought stress and during recovery from stress.

Growth regulator treatments affected the dynamics of carbohydrates usage and accumulation throught their effect on the overall growth of the plant that affect all plant activities. TNC serves as the resource for the increased RSC under increased stress conditions i.e. the relationship between TNC and RSC is a source sink relation and this is obvious if we compare their dynamics in Tables (2.3) and (2.4).

Shoot Proline Content:

Shoot proline content varied significantly among species, growth regulator treatments and their interaction (Table 2.5). It is well known that Proline content increases in response to drought (Getlawi 2013; Shahba et al. 2014). During the recovery from drought stress, the processes of Proline accumulation was reversed. Growth regulator treatments significantly

affected the recovery rate and Proline content. The treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM fusicoccin (Table 2.5). *G. flavum* achieved the highest recovery rate and as a result proline content was the lowest in response to different growth regulator treatments. At the treatment of 2 mM ABA, Proline content decreased by 59.5, 50.0, 36.9 and 19.7 % in *G. flavum, G. acutidentatum, G. grandiflorum* and *G. corniculatum* respectively (Table 2.5). Zhang et al. (2007) recorded that a gradual degradation of ABA was observed when drought stress was removed.

Table 2.5. Effect of 2 mM Abscsic acid (ABA), 2 mM salicylic acid (SA), 0.03 mM fusicoccin (FC), and 20 mM ethephon (E) on Proline content (P) in shoots of *Glaucium* spp. measured at the end of the recovery from drought stress.

Proline content (mg g^{-1} dry weight)					
		PGR treatment			
Species	Control	2mM ABA	2mM SA	0.03mM FC	20mM E
G. flavum	1110.0a†	450.0d	510.0d	580.0d	530.0d
G. acutidentatum	950.0b	475.0c	565.0c	620.0c	590.0c
G. grandiflorum	840.0c	530.0b	587.0b	672.0b	620.0b
G. corniculatum	710.0d	570.0a	620.0a	700.0a	655.0a

† Values followed by the same letters within a column for each cultivar are not significantly different ($P = 0.05$) based on Fisher's test.

Although the role of proline accumulation in drought tolerance was proven in this study, it has been questioned by others (Ashraf and Harris, 2004). These results suggested a positive role for proline in *Glaucium* species salinity tolerance. A positive effect of proline accumulation in drought tolerance was also reported in *Glaucium* spp. (Getlawi 2013) and in seashore paspalum cultivars (Shahba et al., 2014). Accumulation of proline in plant tissues in response to salinity stress has been attributed to enzyme stabilization and/or osmoregulation (Flowers et al., 1977; Levitt, 1980; Vendruscolo *et al*. 2007; Cvikrová *et al*. 2012; Rai *et al*. 2012). The decline of proline content during the recovery of drought stress indicates its importance to cope with the stress conditions. It likely enhances membrane stability and act as a sink for carbon and nitrogen for stress recovery, and can buffer cellular redox potential under stress conditions (Ashraf and

Foolad, 2007; Maggio et al., 2002). Photosynthetic rate is affected by many factors, including stomatal conduction, CO2 assimilation, photosynthetic enzyme activities, inhibition of PSII activity, and stability of photosynthetic apparatus (Camejo *et al*. 2005; Silva *et al*. 2010).

Osmoregulation is one of drought resistance mechanisms of plants (Izadi *et al.*, 2009). Applying exogenous compounds is one way to reduce destructive effects of abiotic stresses (Yuan and Lin 2008). The role of abscisic acid in stress physiology has received much attention, and there is now considerable experimental evidence that the physiological effects induced by drought might be modulated by ABA. Abscisic acid (ABA) selectively affects ion transport processes (van Steveninck, 1976). ABA appears to increase the permeability of roots to water and to inhibit excretion of ions into the xylem but not to affect uptake of ions by the root. The effectiveness of ABA may depend on environmental factors such as temperature (Pitman et al., 1974; Pitman and Wellfare, 1978). In water stress conditions the changes in guard cell ion transport which are responsible are initiated by the 'drought' hormone abscisic acid (ABA). The effect of ABA on cytoplasm and vacuole is more important in stomatal closure where ions are released across the tonoplast, from vacuole to cytoplasm (Hetherington, 2001; Schroeder et al., 2001; Fan et al., 2004; Roelfsema & Hedrich, 2005). Increased ABA accumulation was consistent with a putative role in regulation of proline accumulation in the leaf of Cassava (*Manihot esculenta)* and tobacco in response to drought conditions (Alves and Setter 2004; Dobra *et al*. 2010). Proline accumulation under drought was related to an increase in ABA content and the subsequent reduction in Proline during rehydration was related to a decrease in ABA content (Trotel-Aziz *et al*. 2000). An et al. (2014) tested the effects of exogenous application of abscisic acid on membrane stability, osmotic adjustment, photosynthesis and hormonal status of two lucerne (*Medicago sativa* L.) genotypes under high temperature stress

and drought stress and indicated that foliar application of ABA to stressed plants significantly decreased electrolyte leakage and stomatal conductance, and increased recovery in growth and leaf water potential in the two genotypes under drought conditions. Dobra *et al*. (2010) found that combined heat and drought stress resulted in a decrease in ABA in upper leaf of tobacco, accompanied by more serious damage than in lower and middle leaf and contributed to Zeatin Riboside (ZR) accumulations in the roots of drought-stressed tobacco plants to its higher stress tolerance. It has been proposed that ABA exerts a regulatory effect on Proline accumulation and its subsequent mobilization in response to environmental stresses (Trotel-Aziz *et al*. 2000; Nayyar and Walia 2004; Gomes *et al*. 2009).

Salicylic acid (SA) is a messenger molecule which plays a nonenzymatic anti-oxidant role in regulating plant physiological mechanisms during stress occurrence (Arfan *et al.,* 2007). Fresh and dry weight of root and shoot, stem diameter and leaf number of cucumber plant increased by spraying salicylic acid at drought condition (Bayat *et al.,* 2012; Yildirim *et al.,* 2008). Morphological characteristics like leaf area, plant height, root and shoot dry weight, biomass, flower number and diameter and primary and secondary shoot numbers enhanced by applying SA compared to no SA application, at drought condition (Martin-Mexand and Larqué-Saavedra, 2001). Salicylic acid controls photosynthesis system, photosynthesis amount, pigment content and stomatal conductivity and regulates these procedures for appropriate growth and development (Popova *et al.,* 2009, Steven *et al.,* 2006, El-Tayeb, 2005, Kormkaz *et al.,* 2007). Zarghami et al. (2014) investigated the effect of salicylic acid in enhancing stress tolerance in *Petunia* plants and found a reduction in drought negative effects of drought stress on *Petunia*.

Also, morphological and ornamental characteristics of flowers improved by higher doses of salicylic acid and electrolyte leakage decreased using 2 mM of salicylic acid.

Environmental stresses, such as cold, heat, salinity, and drought, induce ethylene production and oxidative stress and cause damage in plants. Ethylene is produced either chemically through the incomplete combustion of hydrocarbons or biologically by almost all living organisms (Wang et al. 2002, Pech et al. 2005). There is a lot of evidence showing that ethylene is an essential component of a wide range of responses to biotic and abiotic environmental stresses (Shinozaki et al. 1999, Wang et al. 2002, Guo and Ecker 2004, El-Tayeb 2005). Further, many of these stress responses integrate ethylene signaling into more complex circuitry involving salicylate and jasmonate signaling (Wang et al. 2002). The effects of ethylene on plants are regulated both at the level of its synthesis and perception of the hormone (Caren et al. 2007, Wang et al. 2002). Tirani et al. (2013) studied the effects of ethylene on chlorophyll (Chl), carotenoid (Car), anthocyanin, flavonoids, ascorbic acid, dehydroascorbic acid, total ascorbate, lipid peroxidation, and ethylene production in leaf of canola pretreated with SA. Their results showed that the ethylene treatments induced lipid peroxidation, lowered significantly Chl and Carotenoids contents and anthocyanin accumulation.

Jung and Luttge (1980) mentioned that fusicoccin (FC) inhibited net excretion of Cl by the glands of the pitchers of the carnivorous plant *Nepenthes hookeriana* ; of Na^+ and Cl^- by the salt glands of the halophytes *Limonium vulgare* and *L. pectinatum* and of K^+ in the nectar of *Acer platanoides* flowers. It had no effect on K^+ elimination with nectar of *Impatiens walleriana* (extrafloral nectaries) and *Abutilón striatum*, Abscisic acid (ABA) stimulated net excretion of K^+ and Cl^- in *Nepenthes* and of Na^+ and CI^- in *Limonium* but had no effects on K^+ in nectar. Thus, FC and ABA had opposing effects on ion excretion by the salt eliminating glands of *Limonium*

and Nepenthes. Both compounds, however, had similar effects on sugar secretion of nectary glands which was either inhibited or unaffected by FC and ABA. It is suggested that the effects of FC and ABA on ion excretion by gland cells could be reconciled with literature showing FC-stimulation and possible ABA-inhibition of proton pumps at the plasmalemma of plant cells.

Fusicoccin was initially suggested to activate the plasma membrane H^+-ATPase by direct interaction with the enzyme (Marrè, 1979). Later, fusicoccin was demonstrated to bind to a "receptor" belonging to a family of proteins designated 14–3–3 proteins (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). These proteins constitute a highly conserved family of eukaryotic proteins with multiple regulatory functions (Aitken, 1996). Recently, it was shown that 14–3–3 proteins bind directly to the C-terminal region of the H^+-ATPase and that fusicoccin stabilizes the H^+-ATPase/14–3–3 complex formed, rendering the association irreversible (Jahn et al., 1997; Oecking et al., 1997; Baunsgaard et al., 1998). It is known to stimulate the proton pump at the plasma- lemma of cells in a large variety of plant materials; a multiplicity of other effects on transport processes and cell physiology seem to be secondary consequences thereof (Marré, 1977). FC and ABA have antago- nistic effects on movements of stomatal guard cells. FC causes stomata to open and prevents closure; this is probably due to enhanced H^+ extrusion from the guard cells (Marré, 1977), which then affects malate and K^+ accumulation, thus providing the basis for turgor increase and stomatal opening at least in some cases (Raschke, 1975, 1977; Hsiao, 1976). Moreover, Clint & Blatt (1989) indicated that the fusicoccin-induced increase in K^+ influx should be attributed to energy-linked transport, and this would predict cytoplasmic acidification by fusicoccin in *Vicia*, with consequent inhibition of the efflux at the plasmalemma. It is also worth noting that comparison of the effects of fusicoccin on

cation and anion influx in *C. communis* suggests malate rather than chloride as the balancing anion in the stimulated influx (Clint, 1987).

On the basis of the number of times in the best statistical category for leaf characteristics, overall plant quality (attractiveness), water use efficiency, TNC, RSC, and proline, *G. flavum* was found to have the greatest recovery rate from drought stress when compared to *G. acutidentatum, G. grandiflorum* and *G. corniculatum.* Also, the treatment of 2 mM ABA was the most effective followed by 2 mM SA, 20 mM ethephon and 0.03 mM fusicoccin in enhancing drought stressed *Glaucium* spp. recovery. Growth regulator treatments could affect proline accumulation throught their effect on the overall growth of the plant that affect all plant activities especially different growth regulator concentratiions and interactions as it will be indicated in the next Chapter. In summary, *Glaucium* spp. under drought stress exhibited a positive response to growth regulator treatments in terms of improving leaf characteristics, plant height, overall plant quality (attractiveness), TNC, and water use efficiency. *G. flavum* showed greater tendency to recover from drought stress at all growth regulator treatments when compared to the other species tested. The treatment of 2 mM ABA is recommended to improve the recovery rate of *Glaucium* spp. under drought stress.

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CHAPTER 3

GROWTH REGULATOR DYNAMICS DURING THE RECOVERY OF *GLAUCIUM* SPP. FROM SALINITY AND DROUGHT STRESSES

SUMMARY

Many reports confirmed the internal modification in growth regulator in terms of types and concentrations under stress conditions. Externally growth regulator amendments affect the internal balance of growth regulator that can help the plant to regrow and recover from stress. The objective of this study was to test the change in the concentration of the internal growth regulator under stress conditions and during recovery in the common Horned Poppy species that were available from Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum and G. acutidentatums*. Internal growth regulator content of plants were assessed before applying first treatment and at the end of the experiment. Plant growth regulator concentrations change over time were quantified using a protocol in which a 50 mg plant material only is needed to quantify most major plant hormones by HPLC–ESI–MS/MS. This method was the best in current study since sampling was done every 2 weeks over the course of the experiment. Samples solutions (50 µl) were injected into the reverse-phase C18 Gemini HPLC column for HPLC–ESI–MS/MS analysis. Comparisons of internal individual growth regulator content among species, growth regulator treatments, sampling dates and their interactions clearly showed significant differences. During the two month course of the recovery, the concentrations of both IAA and IBA increased gradually. There was slight significant increase overtime in IAA and IBA concentration under the control treatment over the course of the two month recovery period. The treatment of 2 mM ABA achieved the highest increase in both IAA and IBA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. There was slight

significant increase overtime in GA_3 concentration under the control treatment. The treatment of 2 mM ABA achieved the highest increase in GA_3 in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. The concentrations of zeatin increased gradually in all tested species during recovery. Zeatin concentration increased slightly overtime under the control treatment. The treatment of 2 mM ABA achieved the highest increase in zeatin in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. The highest increase was in *G. flavum*, where zeatin increased from 8.0 to 29.0 ng/g Dwt (263%) under control treatment, while the increase was 2422, 2196, 2050 and 1174% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively. Even under control treatment, there was a slight increase in SA content. The treatment of 2 mM ABA achieved the highest increase in SA in all tested species, followed by 20 mM ethephon, 0.03 mM Fusiccocin and 2 mM SA. In *G. flavum*, SA increased from 0.4 to 0.9 ng/g Dwt (125%) under control treatment, while the increase was 720,600, 533 and 300% under the treatments of 2 mM ABA, 20 mM ethephon, 0.03 Fusiccocin and 2 mM SA respectively. On the other hand, and during the course of the recovery, the concentrations of the internal ABA decreased gradually over time. Under the control treatment, there was a slight significant decrease overtime in ABA concentration during the recovery period. The treatment of 2 mM ABA achieved the highest decrease in ABA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. In *G. flavum*, ABA decreased from 2.6 to 1.4 ng/g Dwt (-46 %) under control treatment, while the decrease was -88,-85, -76 and -68% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively. During stress, internal ABA accumulation was evident to cope with stress conditions. During recovery, when the circumstances were favorable for growth, other groups of growth regulator that are needed for

accelerated cell division, enlargement and growth such as auxins, gibberellins, and cytokinnins were abundant.

INTRODUCTION

Many reports confirmed the internal modification in growth regulator in termes of types and concentrations under stress conditions (Wilkinson and Davies, 2010; Zhang et al., 2007; Aswath et al., 2005; McCann and Huang, 2008; Qin and Zeevaart, 2002). External growth regulator amendments affect the internal balance of growth regulator that can help the plant to regrow and recover from stress. Water deficit due to either drought or salinity results in various physiological and bio-chemical changes in plants (Farooq et al. 2009). Responses of the plants to water deficit stress include changes in osmolyte accumulation, stomatal conductance, growth, and expression of specific genes. Accumulation of the osmolytes under water stress contributes to osmotic adjustment by maintaining cell turgor (Mahajan and Tuteja 2005; Seki et al. 2007). For plant defense against various stresses, some growth regulator play a role in the signaling pathways (Overmyer et al. 2003). Also, growth regulator can stabilize cell membranes against stress conditions by interacting with membrane phospholipids (Guschina et al. 2002).

Plant species and cultivars within a species vary in their drought and salinity response and tolerance (Epstein et al., 1980; Pasternark, 1987; Saranga et al., 1992). These variations probably result from the genetic variations especially in genes relating to stress tolerance mechanisms and their interaction with environments (Shannon, 1985; Bohnert et al., 1995; Igartua, 1995; Duncan and Carrow, 1999). Usually evaluations for drought and salt tolerance of plants depend on shoot (aboveground) growth, as reported in crop yield response curves proposed by Maas and Hoffman (Igartua, 1995; Maas, 1987; Maas and Hoffman, 1977).

Plant hormones play a major role in plant growth, development and response to biotic and abiotic stresses (Davies 1995; Crozier et al. 2000). Different hormones play different characteristic biological effects. To understand plant growth and development as affected by plant hormones, accurate and efficient measurements of each of these hormones at different levels (whole plant, organ, cellular and sub-cellular) are required. Simultaneous quantitative profiling of multiple classes of hormones provides a basis for defining additive, synergistic or antagonistic hormone activities and identifying hormone networks regulating plant functions in addition to their dynamics under different environmental conditions (Aloni et al., 2006; Ho et al., 2003; Nemhauser, et al., 2006; Gazzarina and McCourt, 2001). In most analysis methods, crude plant extracts are fractionated and purified by solid-phase extraction, liquid–liquid extraction, gas or liquid chromatography to increase hormone concentration, and the plant hormones are detected by radioimmunoassay, enzyme-linked immunosorbent assays (ELISAs), flame ionization, UV, fluorescence or electrochemical detection (River and Crozier, 1987; Hedden, 1993; Brenner, 1981; Reeve and Crozier, 1980; Weiler, 1984; Reinhold et al., 1981; Davis, 1987; Linskens and Jackson, 1986; Pan and Wang, 2009; Birkemeyer, 2003; Müller; 2002). These procedures usually require significant amounts of solvent, time and labor and not highly sensitive or specific. The sensitivity and specificity of high-performance liquid chromatography (HPLC) with electrochemical detection can limit its application for the measurement of a variety of endogenous plant hormones at physiological levels in plant samples (River and Crozier, 1987; Reinhold et al., 1981; Davis, 1987; Linskens and Jackson, 1986). The use of a mass spectrometer (MS) as a detector for profiling and quantification of plant hormones and metabolites provides high sensitivity and selectivity (Birkemeyer et al., 2003; Müller et al., 2002; Kowalczyk and Sandberg, 2001; weber et al., 2001). Gas chromatography–mass spectrometry (GC–MS) has

been used successfully to specifically identify and quantify plant hormones at high sensitivity. For quantitative measurement of endogenous plant hormones in crude plant extracts, HPLC–MS/MS provides high sensitivity, specificity, accuracy and reproducibility (Gomez-Cadenas et al., 2002; Durgbanshi et al.2005; Chiwocha et al., 2003; Lopez-Carbonell and Jauegui, 2005; Matsuda et al., 2005, Ross et al., 2004; Wilbert, 1998; Zhou et al., 2003).

In the previous chapters, it was shown that salinity and drought tolerance of *Glaucium* spp. are dependent on the internal osmoregulator content. Many studies discussed the plant responses to stress via internal chemical signals and growth regulator adjustments. However, there is no published information that addresses the dynamics of internal growth regulator during recovery from salinity and drought stresses. The objective of this study was to test the change in the concentration of the internal growth regulator under stress conditions and during recovery in the common Horned Poppy species that were available from Denver Botanic Gardens, *G. flavum, G. corniculatum, G. grandiflorum and G. acutidentatums*.

MATERIALS AND METHODS

Data Collection. Internal growth regulator content of plants were assessed before applying first treatment and at the end of the experiment.Plant growth regulator concentrations change over time were quantified using the protocol adopted by Pan et al. (2010) and adopted Liu et al. (2013), in which a 50 mg plant material only is needed to quantify most major plant hormones by HPLC–ESI–MS/MS. This method was the best in current study since sampling was done every 2 weeks over the course of the experiment. Leaf were collected randomly from different heights and sides of plants. Samples were frozen with liquid nitrogen in mortar, ground into powder. Each sample (50 mg) was transferred to 2 ml screw-cap tubes and kept in liquid nitrogen. To each 2 ml tube containing the frozen plant materials, 50 μl of the working solution of internal

standards was added. Also, 500 µl extraction solvent, 2-propanol/H2O/concentrated HCl (2:1:0.002, vol/vol/vol) were added to each tube. Tubes were shaken at a speed of 100 r.p.m. for 30 min at 4 °C, then 1 ml dichloromethane was added to each sample and shaken for additional 30 min in a cold room at 4 °C. Samples were put into a refrigerated microcentrifuge at 4°C and centrifuged at 13,000*g* for 5 min. After centrifugation, two phases were formed with some plant debris between the two layers. About 900 µl of the solvent from the lower phase was transferred using a Pasteur pipette into a screw-cap vial and concentrated (not completely dry) using a nitrogen evaporator with nitrogen flow. The samples were redissolved in 0.1 ml methanol. Samples solutions (50 µl) were injected into the reverse-phase C18 Gemini HPLC column for HPLC–ESI–MS/MS analysis. The MS conditions that was used, using a 4000 QTRAP MS, were: turbo spray; ion polarity: negative or positive; needle voltage: − 4,500V or 5,500 V; source temperature: 500 °C; gas: nitrogen; curtain gas: 25 psi; nebulizing gas (GS1): 45 psi; focusing gas (GS2): 30 psi; interface heater: on; collision activated dissociation gas pressure: medium; scan type: MRM; Q1 resolution: unit; Q3 resolution: unit. Tuning and routine maintenance of the 4000 QTRAP liquid chromatography (LC)–MS/MS, as well as the specific use of the Analyst 1.5 software, was performed in accordance with the instructions in the manufacturer's operation manual. To optimize the MS for quantitative analysis, authentic compounds in 50% (vol/vol) methanol with 0.1% (vol/vol) formic acid at 0.2 ng ml^{-1}, prepared by fivefold dilution of working solutions, were directly infused into the electrospray source of a triple quadrupole MS using a syringe pump at 1.2 ml h^{-1}. The needle temperature in the 4000 QTRAP LC–MS/MS was 350 °C.

Quantitative analysis of each plant hormone was performed. The molar amounts of plant hormones in the samples were determined by calculating the 'correction factor' (i.e., response factor) of each authentic plant hormone in comparison with its corresponding internal standard. Correction factors are the ratio of the signal intensity ratio of the internal standard to the corresponding authentic plant hormone on a mole-for-mole basis, as determined by analysis of the calibration standards. The molar amount of each authentic plant hormone = (signal of the plant hormone × the molar amount of corresponding internal standard) × (correction factor) / (signal of the corresponding internal standard in that sample). The hormone amounts were then normalized to the mass of fresh plant tissue determined by weighing before extraction. The calibration curves for each of the plant hormones and internal standards were linear over a 1,000-fold concentration range with linear regression-correlation coefficients more than 0.9. The variations of the retention times were typically ± 0.3 min. When measuring plant hormones in relation to the mass spectral signals of corresponding isotopically labeled internal standards, the mole-for-mole signal intensities of hormones and internal standards were nearly identical, i.e., the correction factors were very close to 1.0.

Reagents and equipments setup. For extraction solvent mixture preparation, 100 µl of concentrated hydrochloric acid was added to 100 ml of 2-propanol and 50 ml of distilled H2O to make the extraction solvent, i.e., 2-propanol: H2O: concentrated HCl (2:1:0.002, vol/vol/vol). The stock solutions were stable for about 3 months. The stock solutions of authentic plant hormones and internal standards were prepared as 50 µg ml^{-1} stock solution by dissolving 2 mg of each authentic plant hormone and internal standard (weighed in powder form) in a final volume of 40 ml methanol at room temperature (25 °C), then were stored at -20 °C. The stock solutions were also stable for about 3 months. The working solution of internal standards was

prepared by combining the stock solutions of the compounds designated as internal standards and diluting the combined stocks with methanol. The final working solution of internal standards contains 1μg ml $^{-1}$ (1 ng μl $^{-1}$) of each internal standard compound. When 50 μl of the working internal standard solution is added to each tissue sample, the amount of each internal standard in the sample is 50 ng. Working solution of authentic plant hormones was prepared by combining the stock solutions of these compounds and diluting the mixture with methanol. The final working solution of authentic plant hormone standards contains 1 μg ml $^{-1}$ (1 ng μl $^{-1}$) of each plant hormone. Samples were calibrated with authentic plant hormones and internal standards to determine the 'correction factor' (i.e., response factor) for each authentic compound in comparison with its internal standard and to assess the linearity of the analysis. For determination of the correction factor, calibration samples containing three concentrations (10, 100 and 500 ng ml $^{-1}$ each of hormones and internal standards; were prepared in triplicate: (a) 10 μl each of working solutions of authentic plant hormones and internal standards plus 980 μl methanol; (b) 100 μl each of working solutions of authentic plant hormones and internal standards plus 800 μl methanol; and (c) 500 μl working solution of authentic plant hormones plus 500 μl working solution of internal standards. For determination of the linearity of the analysis, calibration samples at five concentrations (0, 1, 10, 100 and 500 ng ml $^{-1}$ hormones with a constant concentration of 50 ng ml $^{-1}$ internal standards; a–e) were prepared in triplicate: (a) 50 μl of the internal standard working solution plus 950 μl methanol; (b) 1 μl of the working solution of authentic plant hormones and 50 μl of the internal standard working solution plus 949 μl methanol; (c) 10 μl of the working solution of the authentic plant hormones and 50 μl of the internal standard working solution plus 940 μl methanol; (d) 100 μl of the working solution of the authentic plant hormones and 50 μl of the internal standard working solution plus 850 μl

methanol; and (e) 500 µl working solution of authentic plant hormones plus 50 µl of the internal standard working solution plus 450 µl methanol. The binary solvent system used water with 0.1% (vol/vol) formic acid (A) and methanol with 0.1% (vol/vol) formic acid (B) as mobile phases.

Reverse-phase (RP-C18) HPLC Set up an HPLC was set by setting the oven (holding HPLC column) temperature to 40 °C and the solvent flow rate to 0.3 ml min $^{-1}$. The settings for MS/MS multiple reaction monitoring (MRM) should be optimized on each user's instrument. The MS conditions that were used, using a 4000 QTRAP MS, were as follows: source: turbo spray; ion polarity: negative or positive; needle voltage: − 4,500V or 5,500 V; source temperature: 500 °C; gas: nitrogen; curtain gas: 25 psi; nebulizing gas (GS1): 45 psi; focusing gas (GS2): 30 psi; interface heater: on; collision activated dissociation gas pressure: medium; scan type: MRM; Q1 resolution: unit; Q3 resolution: unit. Tuning and routine maintenance of the 4000 QTRAP liquid chromatography (LC)–MS/MS, as well as the specific use of the Analyst 1.5 software, is performed in accordance with the instructions in the manufacturer's operation manual. To optimize the MS for quantitative analysis, authentic compounds in 50% (vol/vol) methanol with 0.1% (vol/vol) formic acid at 0.2 ng ml^{-1}, prepared by five-fold dilution of working solutions, were directly infused into the electrospray source of a triple quadrupole MS using a syringe pump at 1.2 ml h^{-1}. The needle temperature in the 4000 QTRAP LC–MS/MS was 350 °C; this would need to be optimized in other triple quadrupole MSs.

Data Analysis:

Internal growth regulator contents were analyzed on individual measurement dates to examine the effect of different species, different growth regulator treatments and sampling date during the recovery of four *Glacium* spp. from salinity and drought on internal growth regulator

dynamics. The data of both salinity and drought were subjected to ANOVA to test the stress type (salinity vs drought) effect. There was no significant different between the internal growth regulator data during the recovery from drought and salinity stresses. Therefore, data were pooled over both of them to test the effects of recovery on the internal growth regulator contents. Means separation were performed at $P = 0.05$ by Fisher's LSD test when significant differences were found (SAS Institute, 2007).

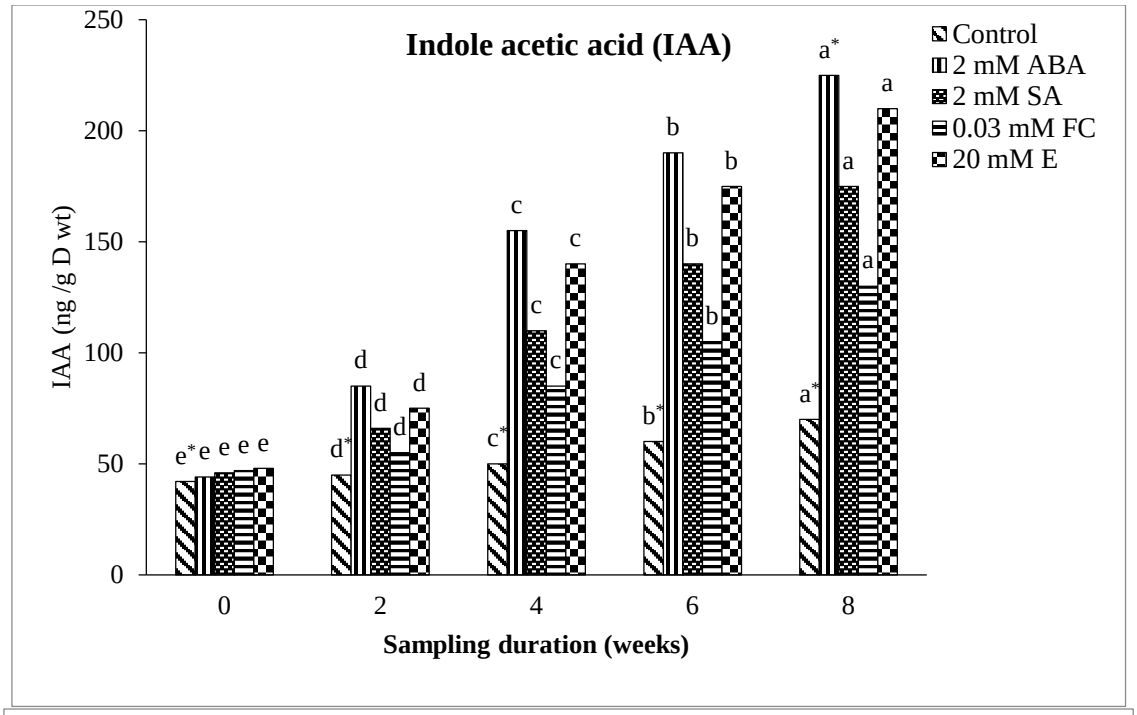
RESULTS AND DISCUSSION

Plant growth regulator are used to modify the rate or pattern of growth, or both, of its response to the internal and external factors that govern development from germination through vegetative growth, reproductive development, maturity, and senescence or aging. Abscisic acid (ABA), is well known to inhibit fruit ripening, responsible for seed dormancy by inhibiting cell growth, inhibits seed germination, down regulates enzymes needed for photosynthesis, stimulates the closure of stomata, inhibits shoot growth but will not have as much effect on roots or may even promote growth of roots, has some effect on induction and maintenance of dormancy and induce common responses such as enhancement of plant hormones. ABA is considered a plant stress hormone. It regulates several important aspects of plant growth and development. Recent studies have demonstrated a pivotal role for ABA in modulation at the gene level of adaptative responses for plants in adverse environmental conditions. Salt stress, drought stress, mechanical leaf injury and wounds resulted in an increase in ABA levels. Exogenous application of ABA was able to increase plant adaptive response to various environmental conditions. Most reports have demonstrated that the application of exogenous ABA provides tolerance to various stress conditions. However, endogenously produced ABA may not show the

same effects as elicited by exogenously applied hormones; and, of course, correlated variations may not reflect the cause.

It is logical to expect an increase in stress hormones during stress time and an increase in other growth regulator during the recovery from stress. Comparisons of internal individual growth regulator content among species, growth regulator treatments, sampling dates and their interactions clearly showed significant differences.

Auxins. Auxins are compounds that stimulate cell elongation, stimulate cell division, stimulate differentiation, stimulate root initiation, delay leaf senescence, can induce fruit setting and growth in some plants and promotes flowering. Auxins tested were Indole Acetic Acid (IAA) and Indole Butyric Acid (IBA). During the two month course of recovery, the concentrations of both IAA and IBA increased gradually (Figures 3.1, 3.2, 3.3, and 3.4). There was slight significant increase overtime in IAA and IBA concentration under the control treatment over the course of the two month recovery period. The treatment of 2 mM ABA achieved the highest increase in both IAA and IBA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. In *G. flavum*, IAA increased from 40 to 60 ng /g Dwt (50%) under control treatment, while the increase was 400, 377, 280 and 177% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.1 top). IBA increased from 40 to 52 ng /g Dwt (30%) under control treatment, while the increase was 388, 311, 242 and 207% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.1 bottom).



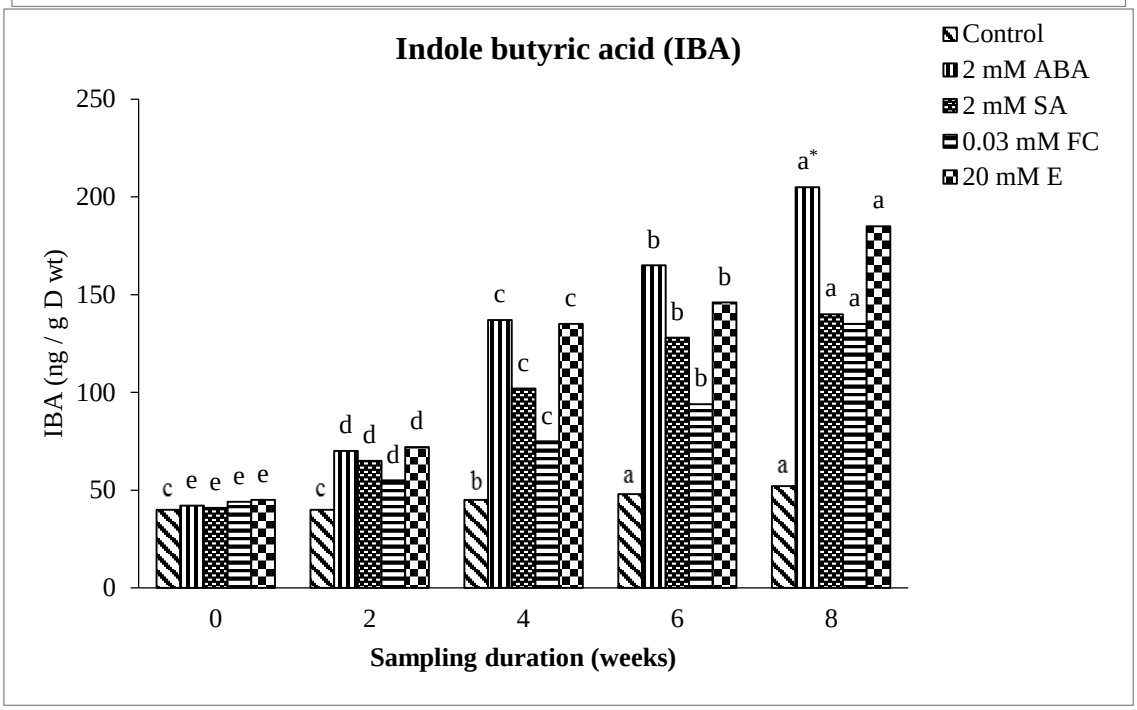


Fig. 3. 1. Effect of recovery duration on the internal contents of IAA (top) and IBA (bottom) during the recovery of *G. flavum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

In *G. acutidentatum*, IAA increased from 40 to 65 ng/g Dwt (63%) under control treatment, while the increase was 396, 346, 268 and 160% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.2 top). IBA increased from 40 to 47 ng/g Dwt (18%) under control treatment, while the increase was 361, 277, 213 and 188% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.2 bottom). In *G. grandiflorum*, IAA increased from 38 to 55 ng/g Dwt (45%) under control treatment, while the increase was 372, 297, 224 and 105% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.3 top). IBA increased from 32 to 46 ng/g Dwt (44%) under control treatment, while the increase was 391, 309, 135 and 100% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.3 bottom). In *G. corniculatum*, IAA increased from 34 to 49 ng/g Dwt (44%) under control treatment, while the increase was 361, 340, 221 and 78% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.4 top). IBA increased from 31 to 41 ng/g Dwt (32%) under control treatment, while the increase was 372, 281, 112 and 59% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.4 bottom).

Gibberellins. Gibberellins (GA_3) are compounds that stimulate stem elongation by stimulating cell division and elongation, stimulate bolting/flowering in response to long days, stimulate enzyme production (a-amylase) in germinating cereal grains for mobilization of seed reserves, can delay senescence in leaf and fruits, promote extra length and fast growth of cells between the plant's nodes and in the leaf and drive the plant rapidly upwards. During the two month course of the recovery, the concentrations of GA_3 increased gradually (Figures 3.5, 3.6, 3.7 and 3.8). There was slight significant increase overtime in GA_3 concentration under the control

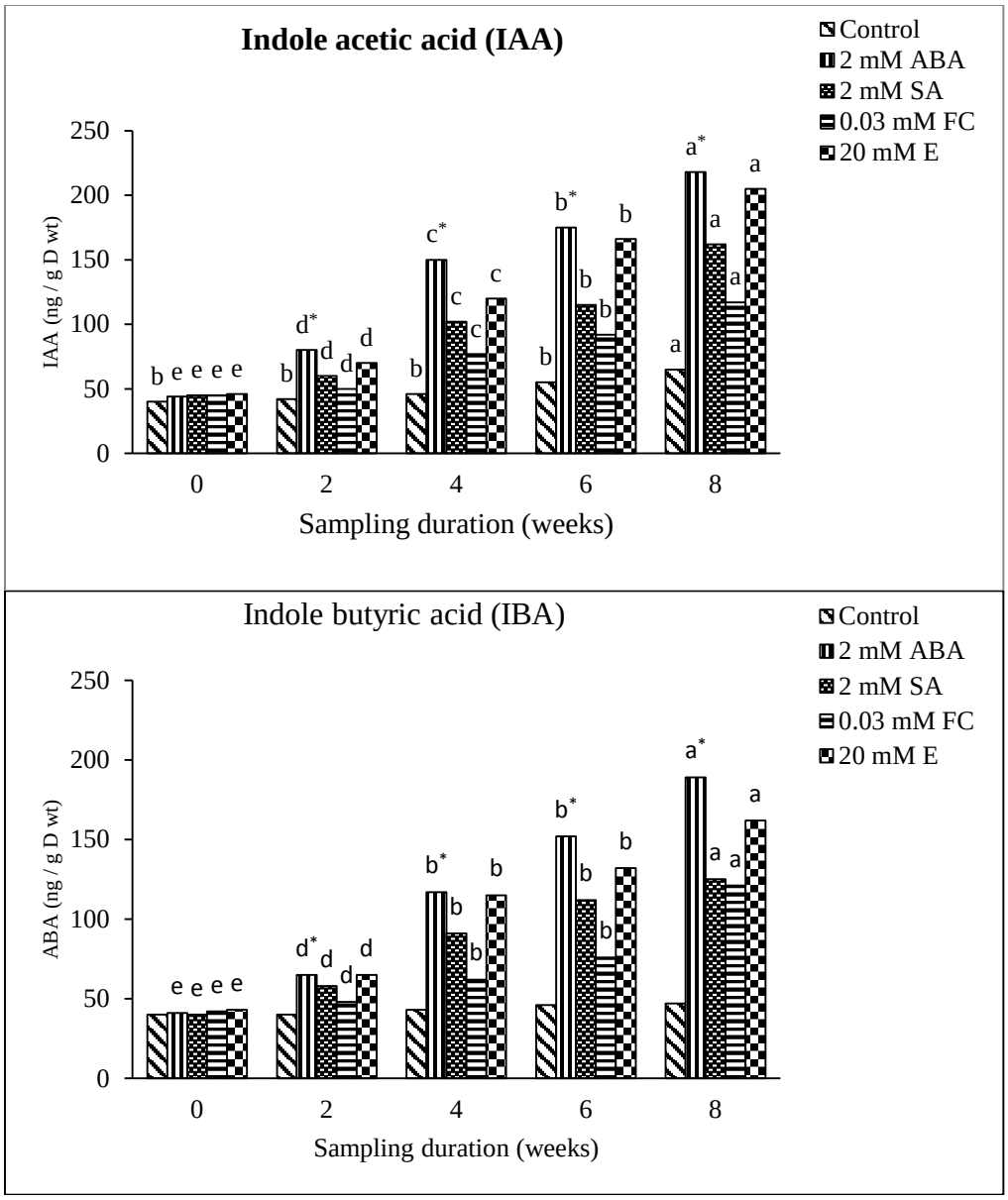


Fig. 3. 2. Effect of recovery duration on the internal contents of IAA (top) and IBA (bottom) during the recovery of *G. acutidentatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

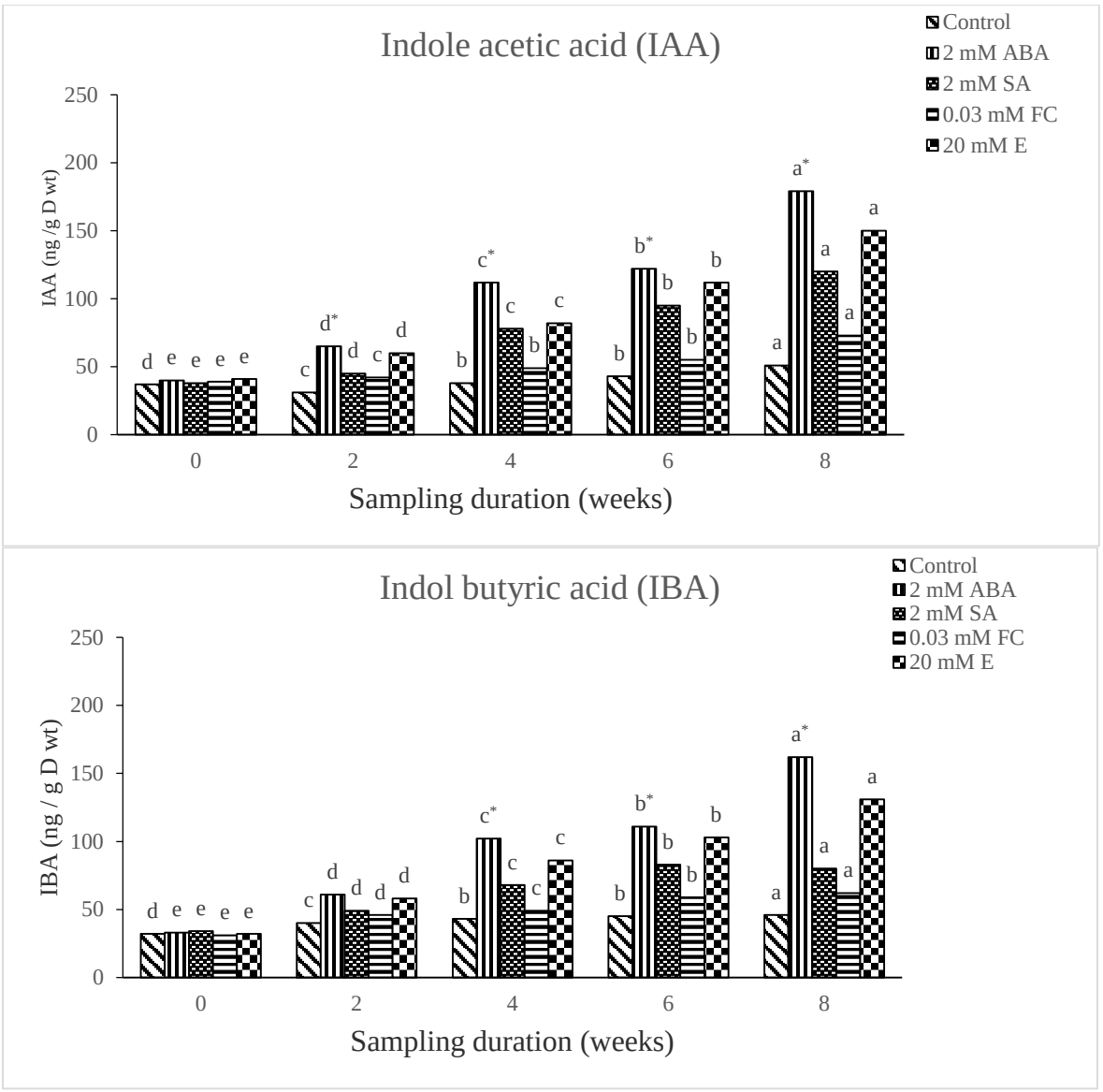


Fig. 3. 3. Effect of recovery duration on the internal contents of IAA (top) and IBA (bottom) during the recovery of *G. grandiflorum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

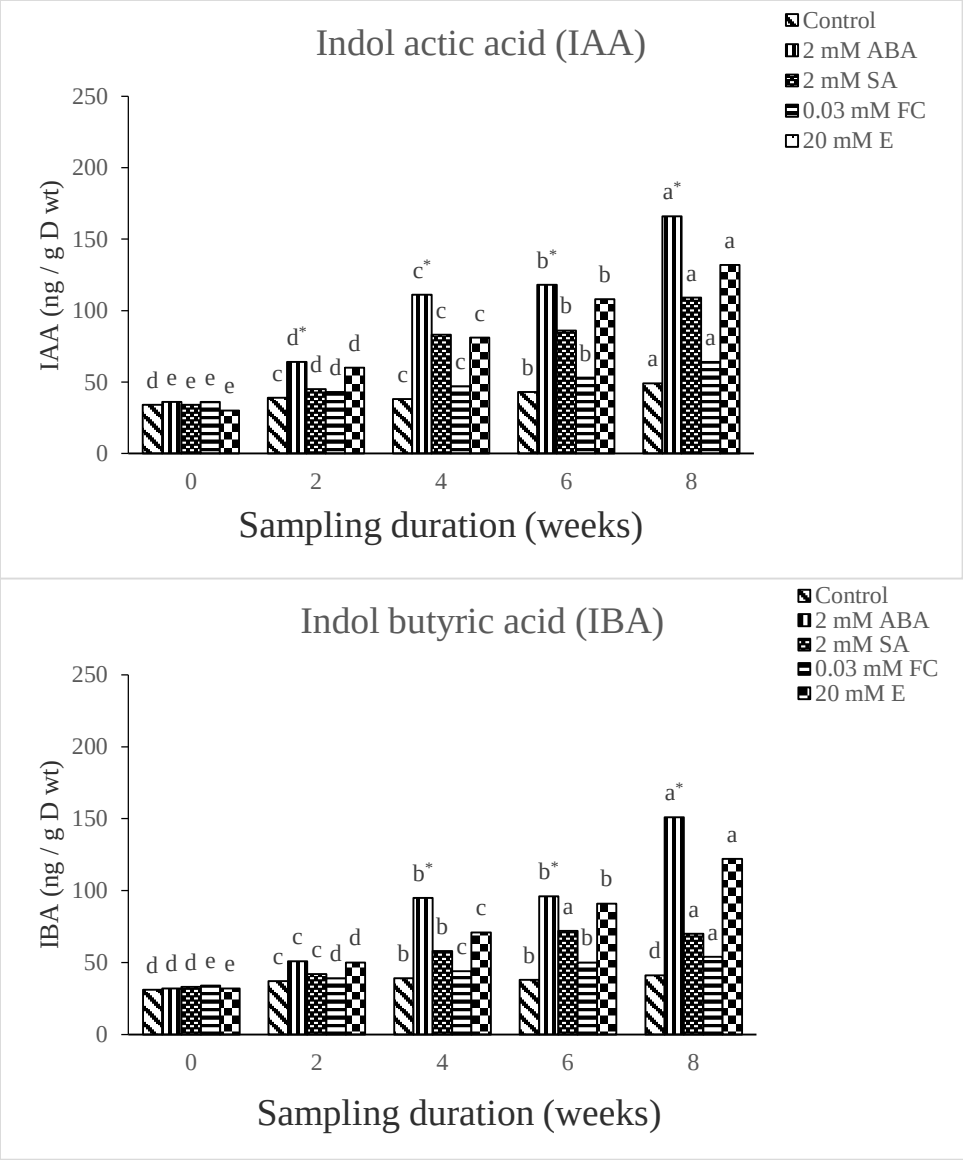


Fig. 3. 4. Effect of recovery duration on the internal contents of IAA (top) and IBA (bottom) during the recovery of *G. corniculatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

treatment over the course of the two month recovery period. The treatment of 2 mM ABA achieved the highest increase in GA_3 in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. In *G. flavum*, GA_3 increased from 1.0 to 2.2 ng/g Dwt (120%) under control treatment, while the increase was 2445,1900, 1567 and 1500% under the

treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.5). In *G. acutidentatum*, GA_3 increased from 0.9 to 2 ng /g Dwt (122%) under control treatment, while the increase was 2300, 1800, 1316 and 1233% under the treatments of 2 mM ABA, 20 mM ethephon,

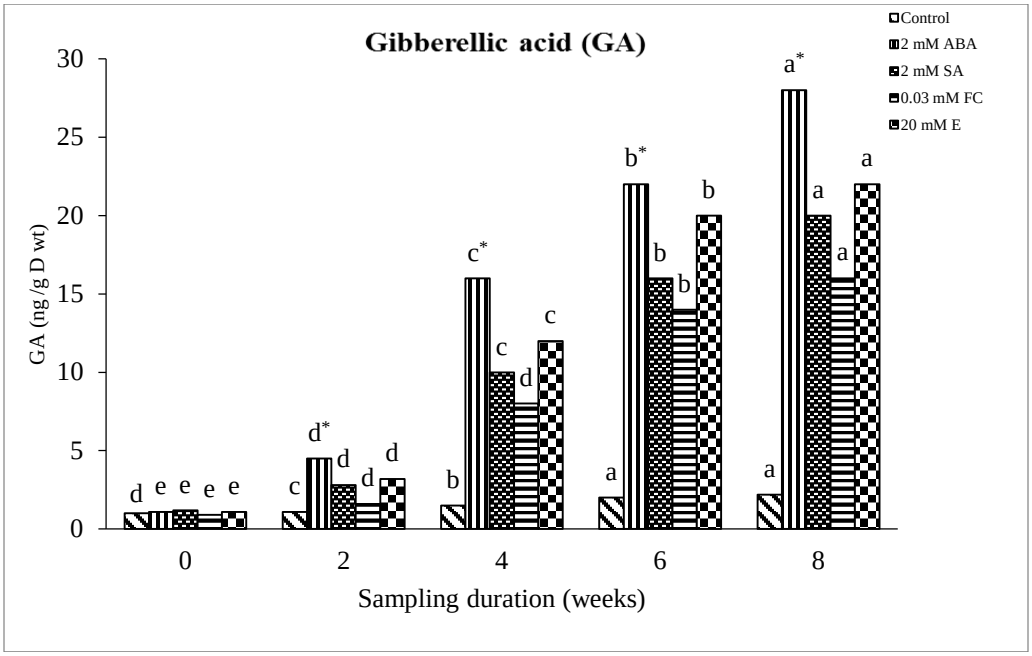


Fig. 3. 5. Effect of recovery duration on the internal contents of Gibberellic acid (GA) during the recovery of *G. flavum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.6). In *G. grandiflorum*, GA_3 increased from 0.7 to 1.6 ng/g Dwt (129%) under control treatment, while the increase was 2000, 1566, 1344 and 1150% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.7). In *G. corniculatum*, GA_3 increased from 0.6 to 0.9 ng/g Dwt (50%) under control treatment, while the increase was 1890, 1471, 1043 and 650% under the

treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.8).

Cytokinins. Cytokinins stimulate cell division, morphogenesis, the growth of lateral buds-release of apical dominance, leaf expansion resulting from cell enlargement, and may enhance stomatal opening in some species and promotes the conversion of etioplasts into chloroplasts via stimulation of chlorophyll synthesis. We tested zeatin as a representative of cytokinins. During recovery, the concentrations of zeatin increased gradually in all tested species (Figures 3.9, 3.10,

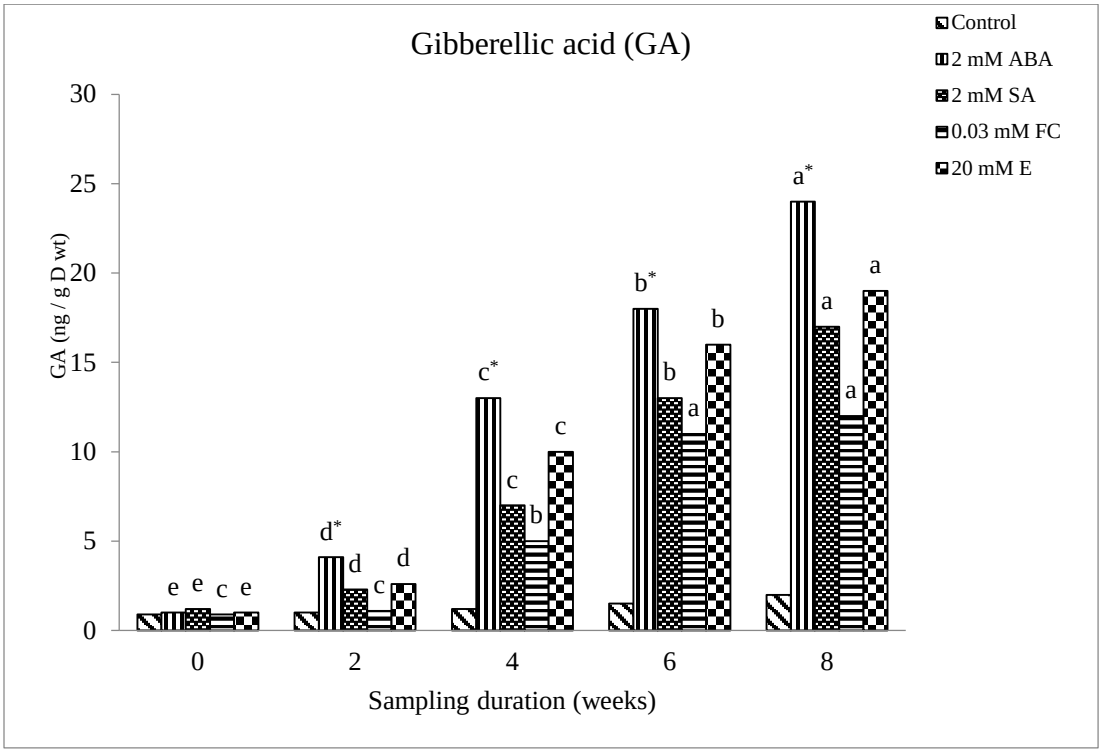


Fig. 3. 6. Effect of recovery duration on the internal contents of Gibberellic acid (GA) during the recovery of *G. acutidentatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

3.11 and 3.12). Zeatin concentration increased slightly overtime under the control treatment during recovery period. The treatment of 2 mM ABA achieved the highest increase in zeatin in

all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. The highest increase was in *G. flavum*, where zeatin increased from 8.0 to 29.0 ng/g Dwt (263%) under control treatment, while the increase was 2422, 2196, 2050 and 1174% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.9). In *G. acutidentatum*, zeatin increased from 8 to 25 ng/g Dwt (213%) under control treatment, while the increase was 2244, 2077, 1900 and 1072% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.10). In *G. grandiflorum*, zeatin increased from 6.0 to 22.0 ng/g Dwt (267%) under control treatment, while the increase was 2329, 2069, 1546

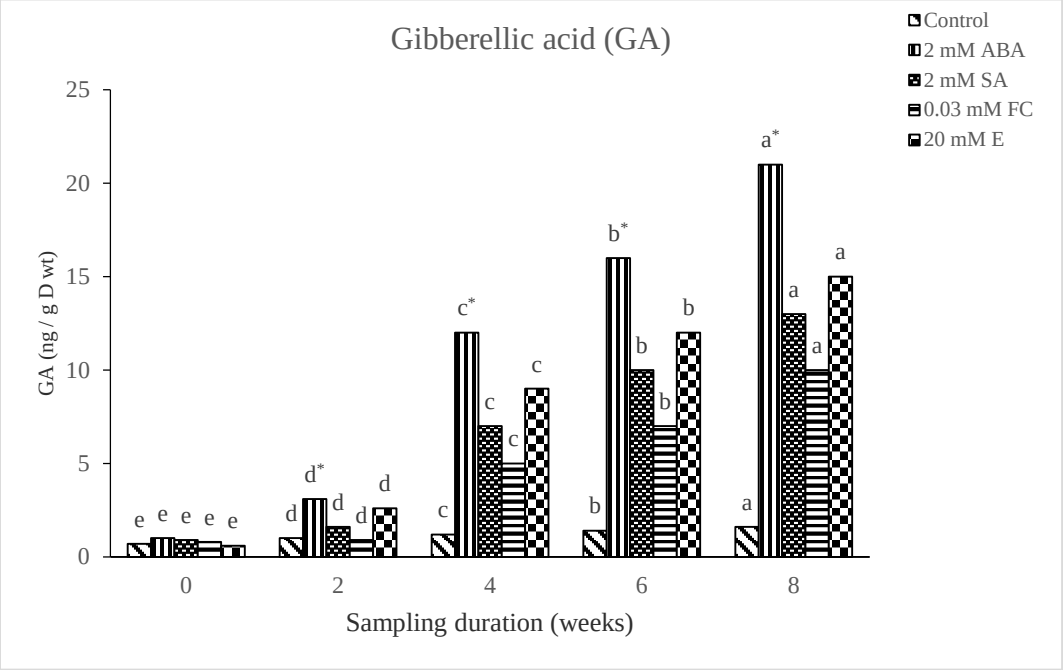


Fig. 3. 7. Effect of recovery duration on the internal contents of Gibberellic acid (GA) during the recovery of *G. grandiflora* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

and 957% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.11). Zeatin increased in *G. corniculatum* from 5 to 18 ng/g Dwt (260%) under control treatment, while the increase was 2617, 1977, 1423 and 843% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.12).

Internal salicylic acid (SA) dynamics. SA enhances cell division and plant growth indirectly by prohibiting auxin and cytokinin losses in plants. During recovery, the concentrations of SA increased gradually (Figures 3.13, 3.14, 3.15 and 3.16). Even under control treatment, there was a slight increase in SA content. The treatment of 2 mM ABA achieved the highest increase in SA in all tested species, followed by 20 mM ethephon, 0.03 mM Fusiccocin and 2 mM SA. In *G. flavum*, SA increased from 0.4 to 0.9 ng/g Dwt (125%) under control treatment, while the increase was 720,600, 533 and 300% under the treatments of 2 mM ABA, 20 mM ethephon, 0.03

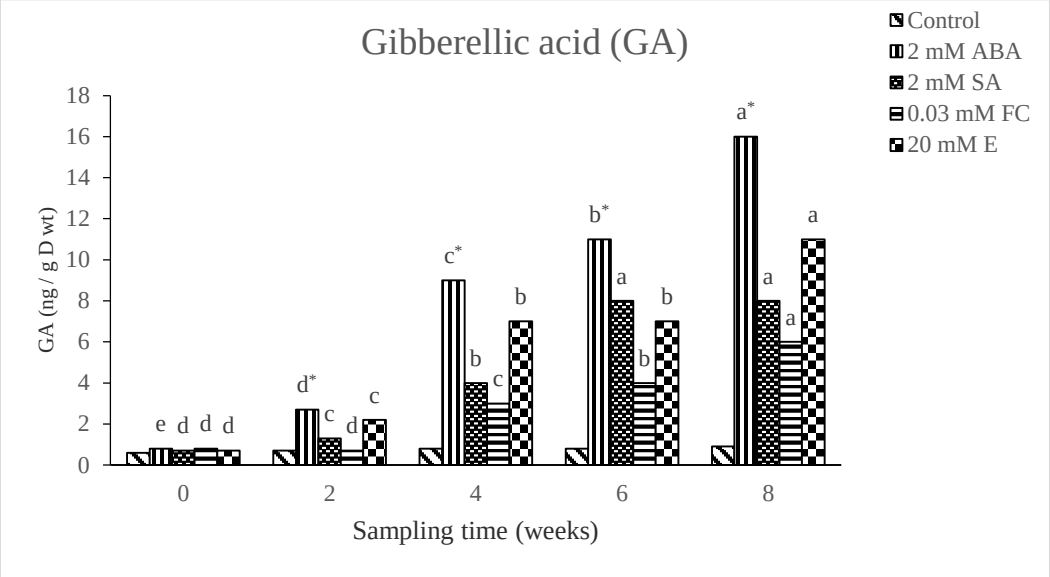


Fig. 3. 8. Effect of recovery duration on the internal contents of Gibberellic acid (GA) during the recovery of *G. corniculatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

Fusiccocin and 2 mM SA respectively (Fig. 3.13). In *G. acutidentatum*, 20 mM ethephon treatment was more effective than 2 mM ABA that had similar effect to 0.03 Fusiccocin. Internal SA content increased from 0.4 to 0.8 ng/g Dwt (100%) under control treatment, while the increase was 650, 600, 600 and 300% under the treatments of 20 mM ethephon, 2 mM ABA, 0.03 Fusiccocin, and 2 mM SA respectively (Fig. 3.14). In *G. grandiflorum*, SA increased from 0.3 to 0.6 ng/g Dwt (100%) under control treatment, while the increase was 700, 500, 433 and 200% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.15). In *G. corniculatum*, SA increased from 0.2 to 0.5 ng/g Dwt (150%) under control treatment, while the increase was 867, 500, 233 and 50% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.16).

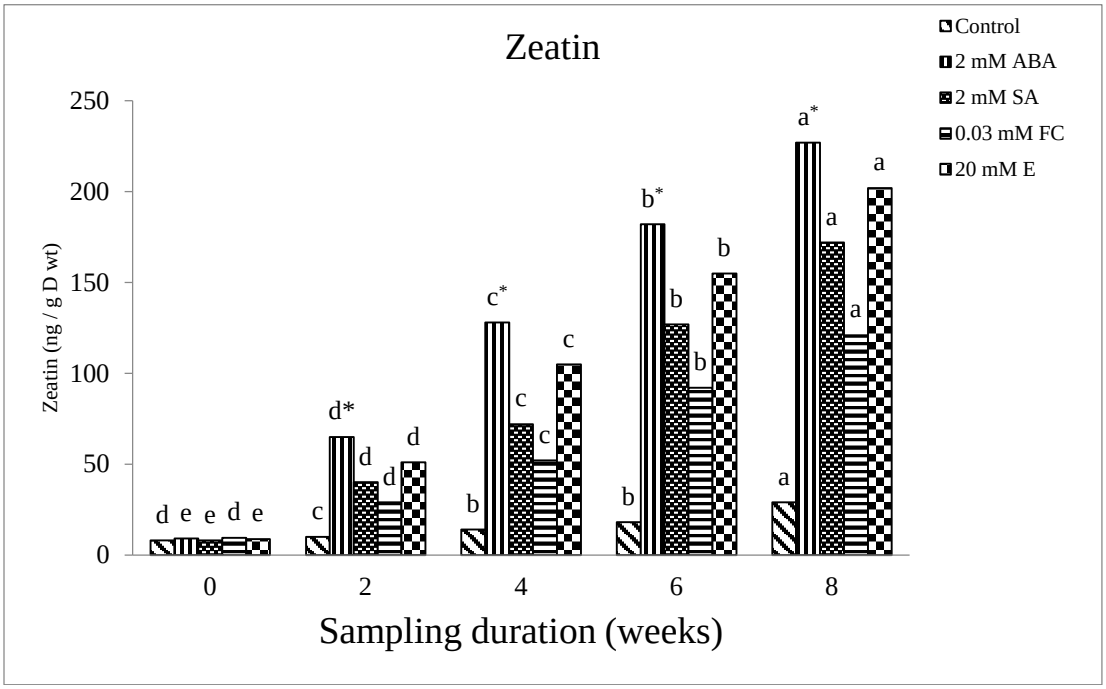


Fig. 3. 9. Effect of recovery duration on the internal contents of Zeatin during the recovery of *G. flavum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

Internal abscisic acid (ABA) dynamics. It is well known that endogenous ABA content increased rapidly under stress conditions and improves stress tolerance in plants. During the course of the recovery, the concentrations of the internal ABA decreased gradually (Figures 3.17, 3.18, 3.19 and 3.20). Under the control treatment, there was a slight significant decrease overtime in ABA concentration during the recovery period. The treatment of 2 mM ABA achieved the highest decrease in ABA in all tested species, followed by 20 mM ethephon, 2 mM SA and 0.03 mM fusiccocin. In *G. flavum*, ABA decreased from 2.6 to 1.4 ng/g Dwt (-46 %) under control treatment, while the decrease was -88,-85, -76 and -68% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.17). In *G. acutidentatum*, ABA decreased from 2.7 to 1.6 ng/g Dwt (-41%) under control treatment, while the decrease was -79, -78, -67 and -62 under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.18). In *G. grandiflorum*, ABA decreased from

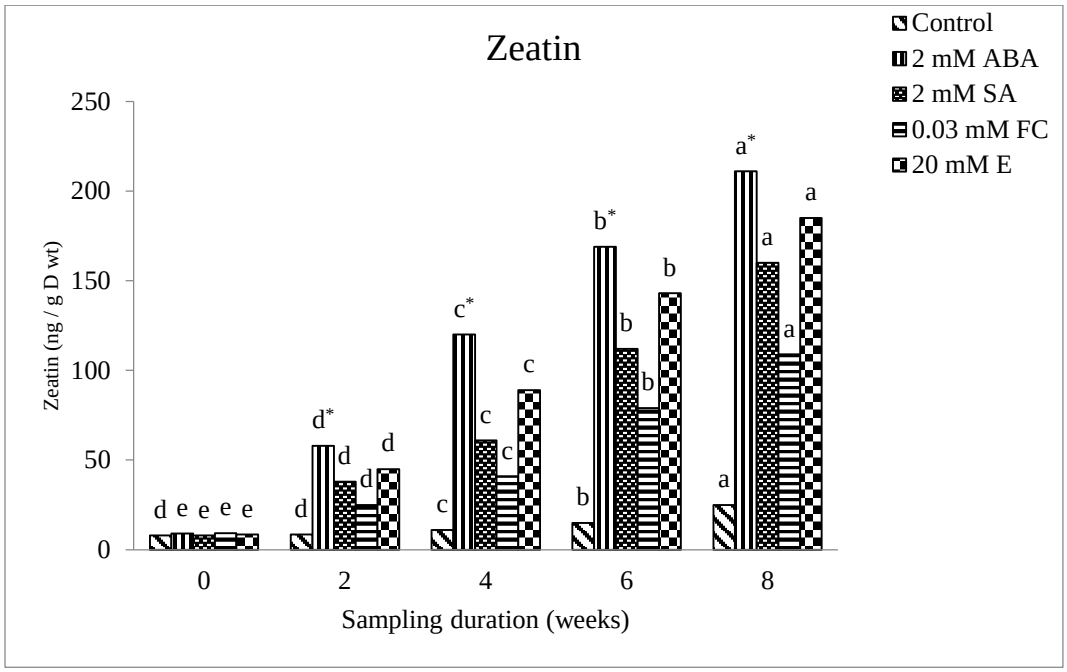


Fig. 3. 10. Effect of recovery duration on the internal contents of Zeatin during therecovery of *G. acutidentatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

2.8 to 1.1 ng/g Dwt (129%) under control treatment, while the decrease was -72, -68, -58 and -50% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.19). In *G. corniculatum*, ABA decreased from 3 to 1.6 ng/g Dwt (-47%) under control treatment, while the decrease was -82, -78, -72 and -66% under the treatments of 2 mM ABA, 20 mM ethephon, 2 mM SA and 0.03 Fusiccocin respectively (Fig. 3.20).

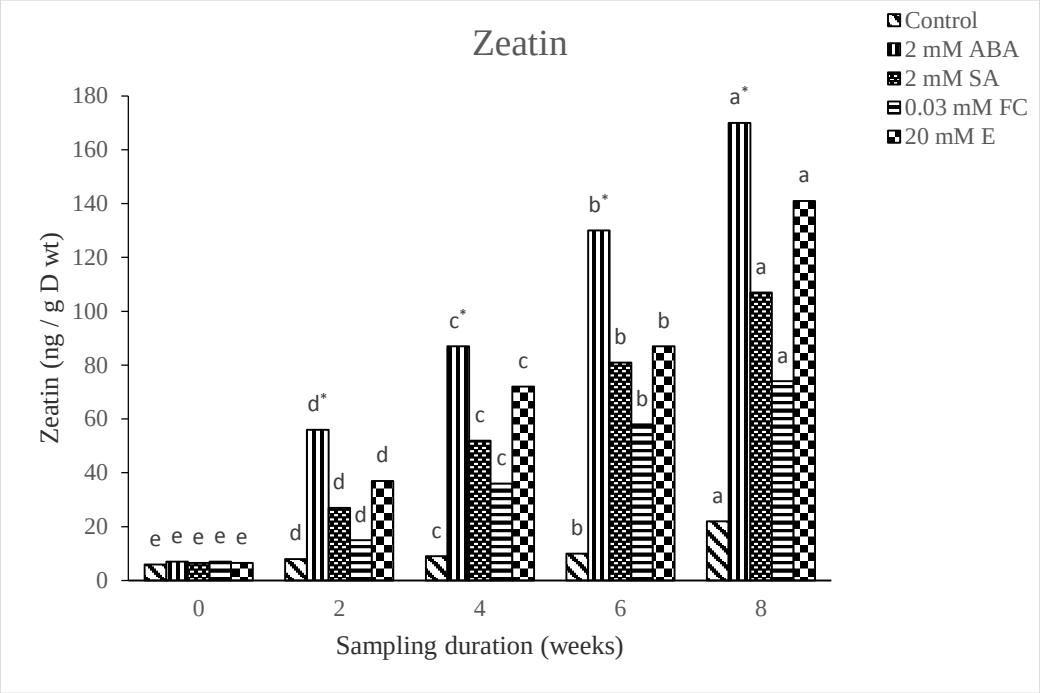


Fig. 3. 11. Effect of recovery duration on the internal contents of Zeatin during the recovery of *G. grandiflorum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

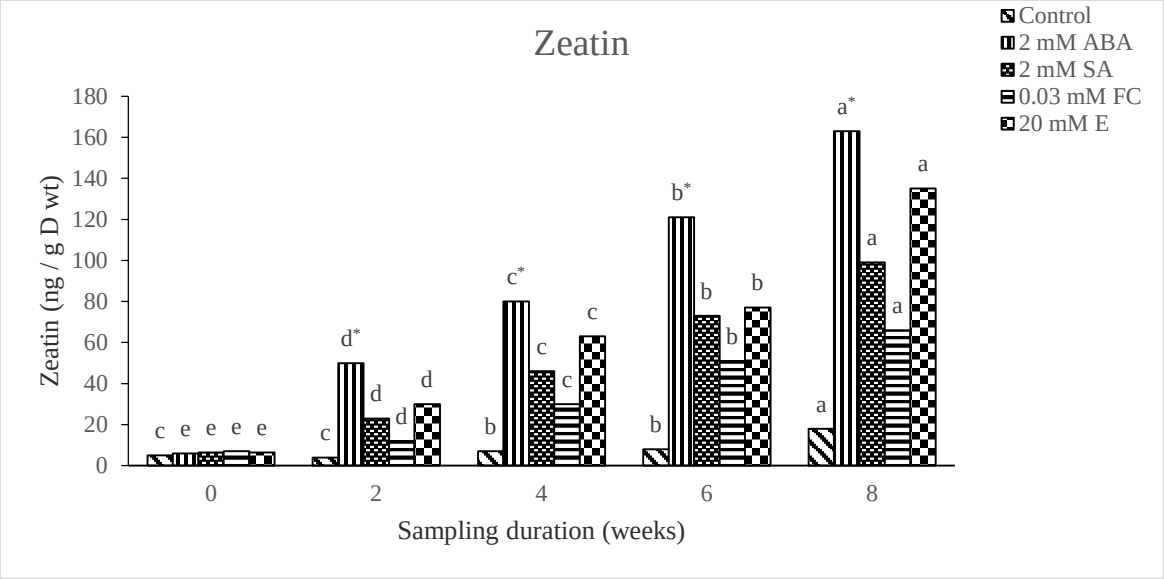


Fig. 3. 12. Effect of recovery duration on the internal contents of Zeatin during the recovery of *G. corniculatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

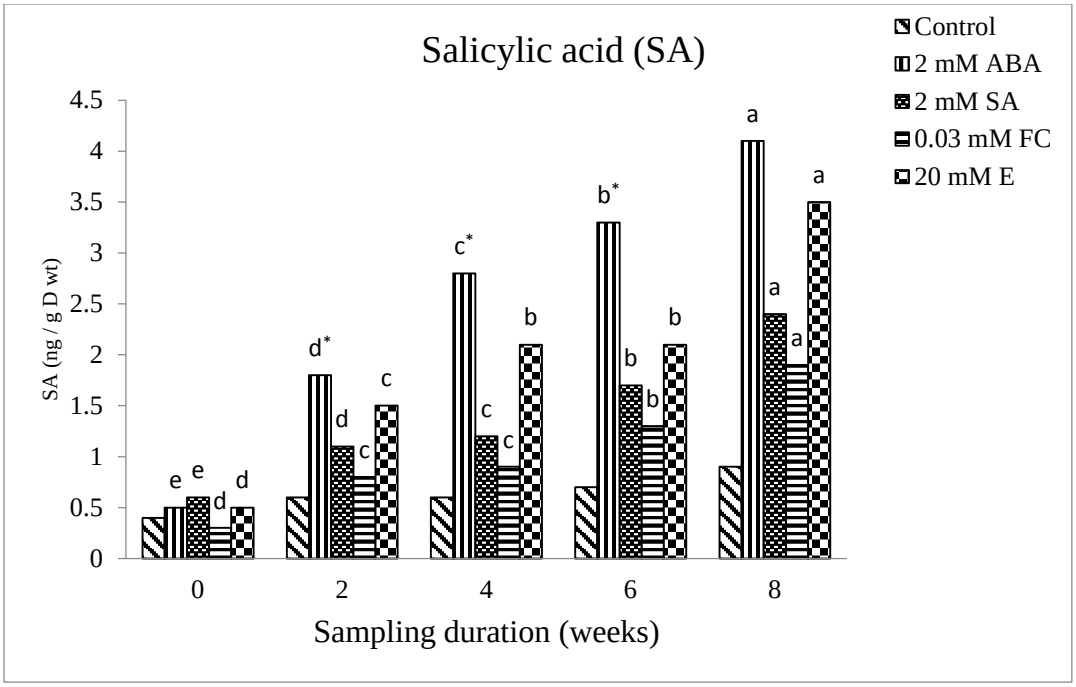


Fig. 3. 13. Effect of recovery duration on the internal contents of Salicylic acid (SA) during the recovery of *G. flavum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

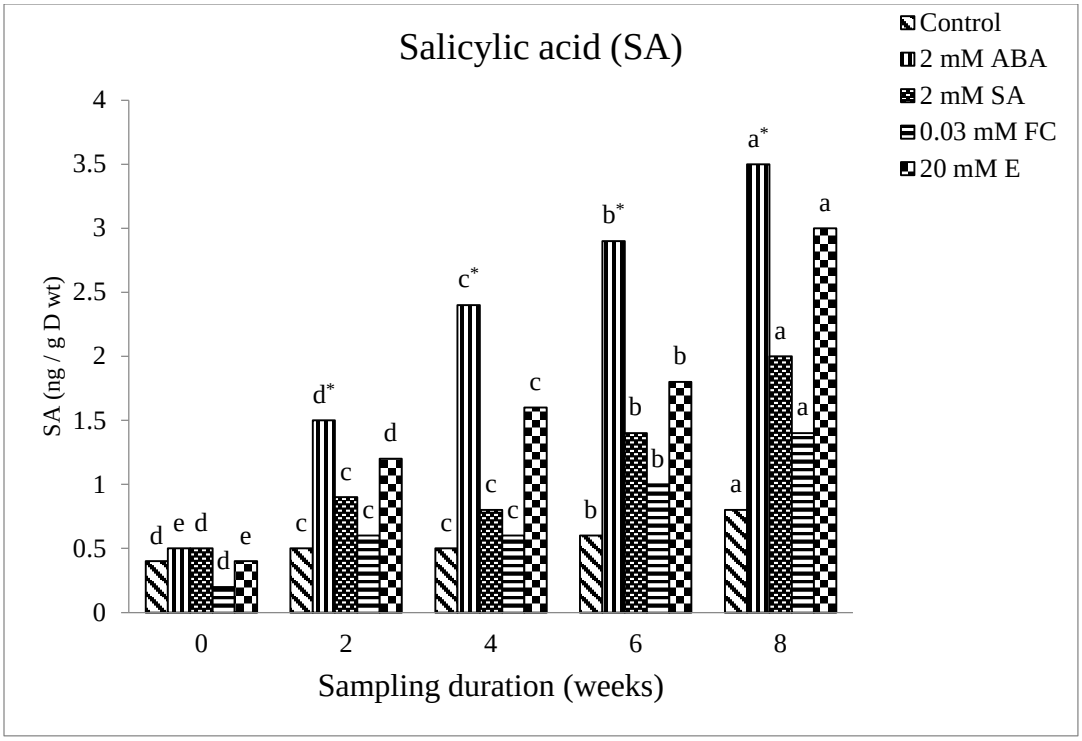


Fig. 3. 14. Effect of recovery duration on the internal contents of Salicylic acid (SA) during the recovery of *G. acutidentatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

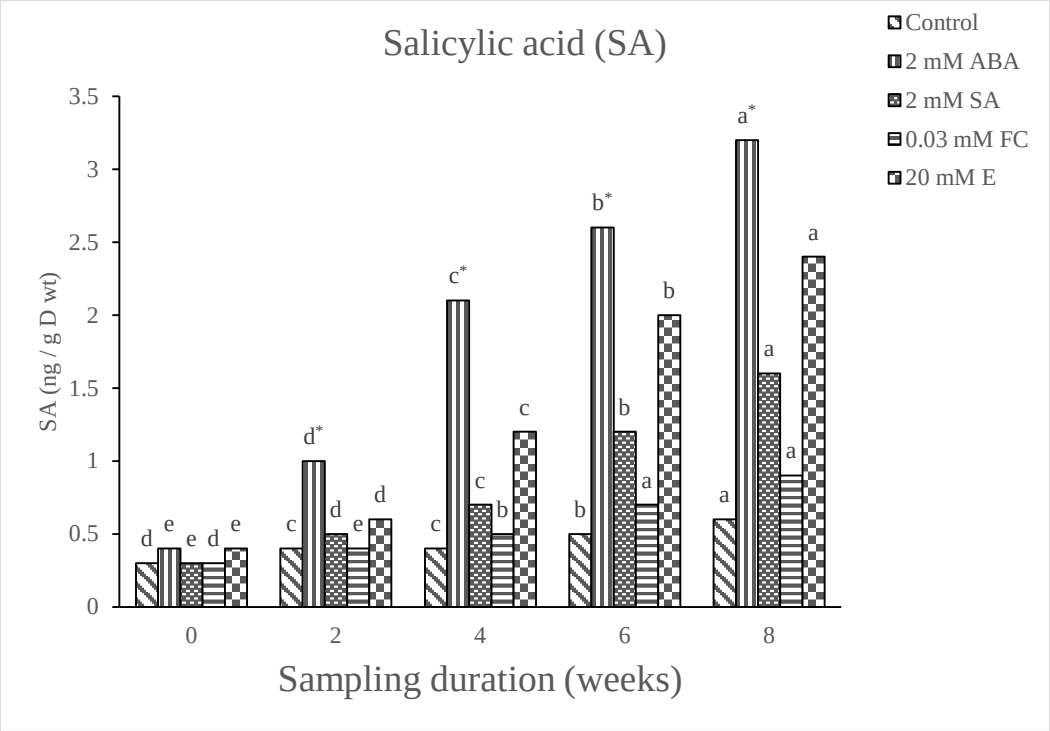


Fig. 3. 15. Effect of recovery duration on the internal contents of Salicylic acid (SA) during the recovery of *G. grandiflorum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

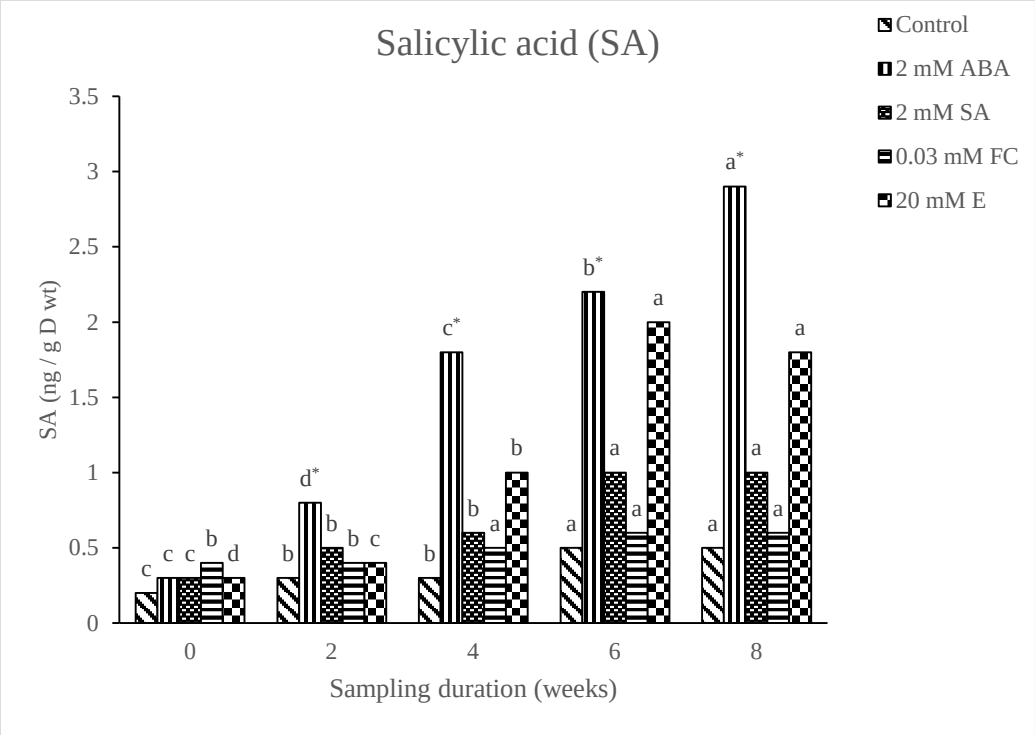


Fig. 3. 16. Effect of recovery duration on the internal contents of Salicylic acid (SA) during the recovery of *G. corniculatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

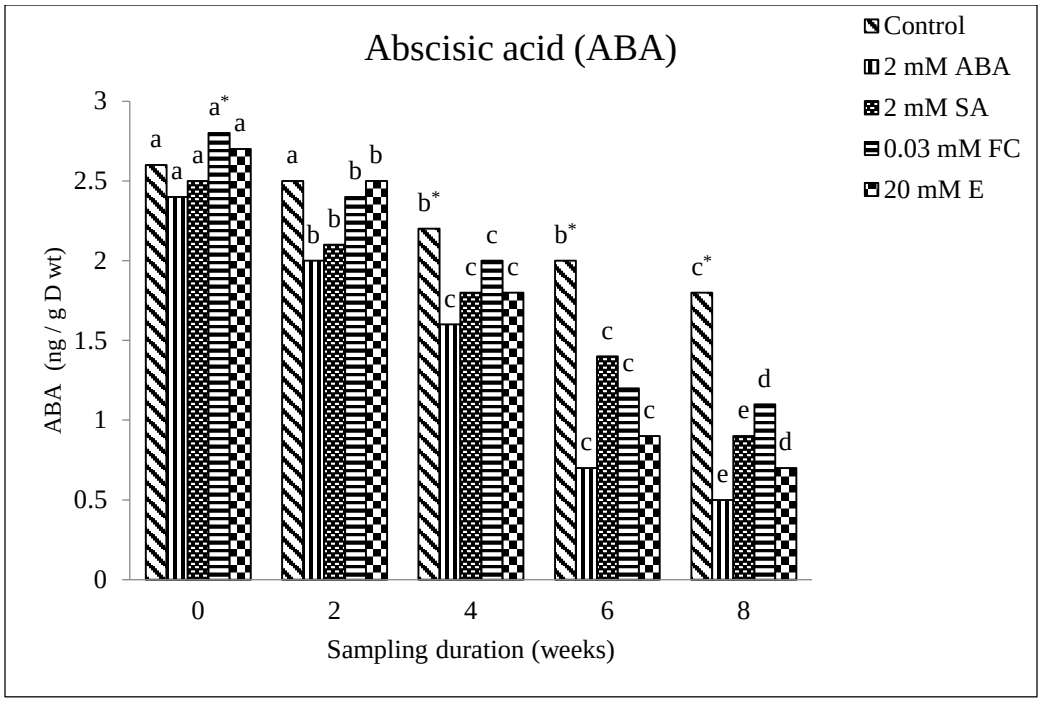


Fig. 3. 17. Effect of recovery duration on the internal contents of Abscisic acid (ABA) during the recovery of *G. flavum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

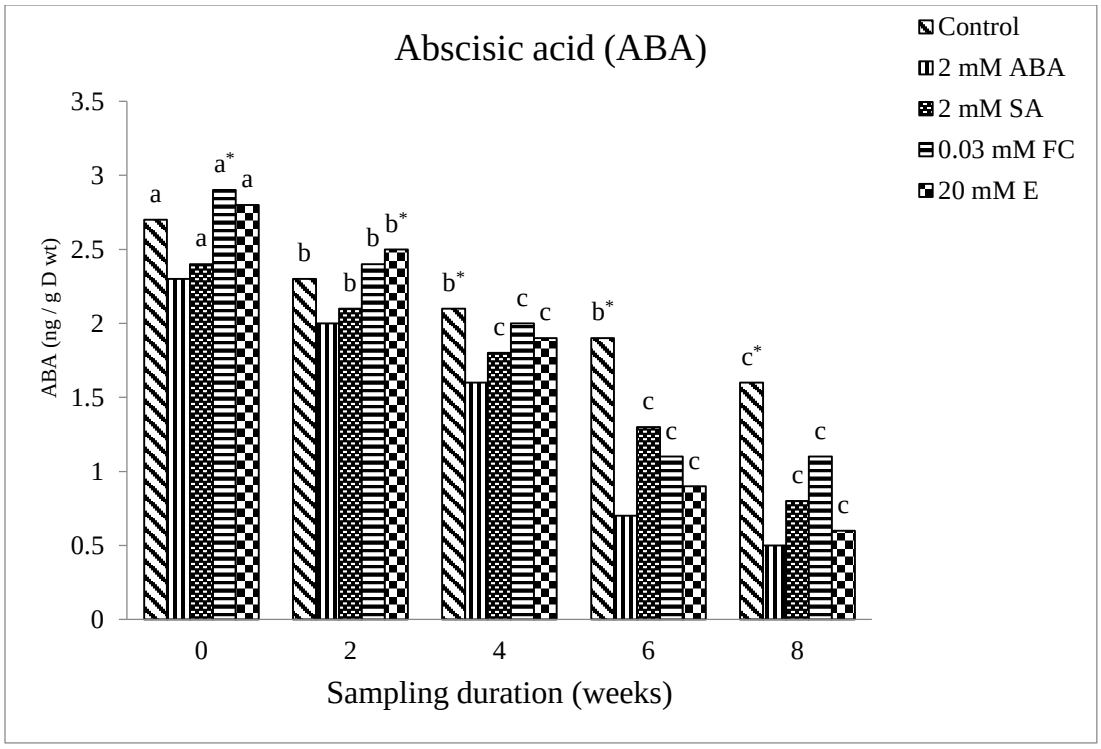


Fig. 3. 18. Effect of recovery duration on the internal contents of Abscisic acid (ABA) during the recovery of *G. acutidentatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

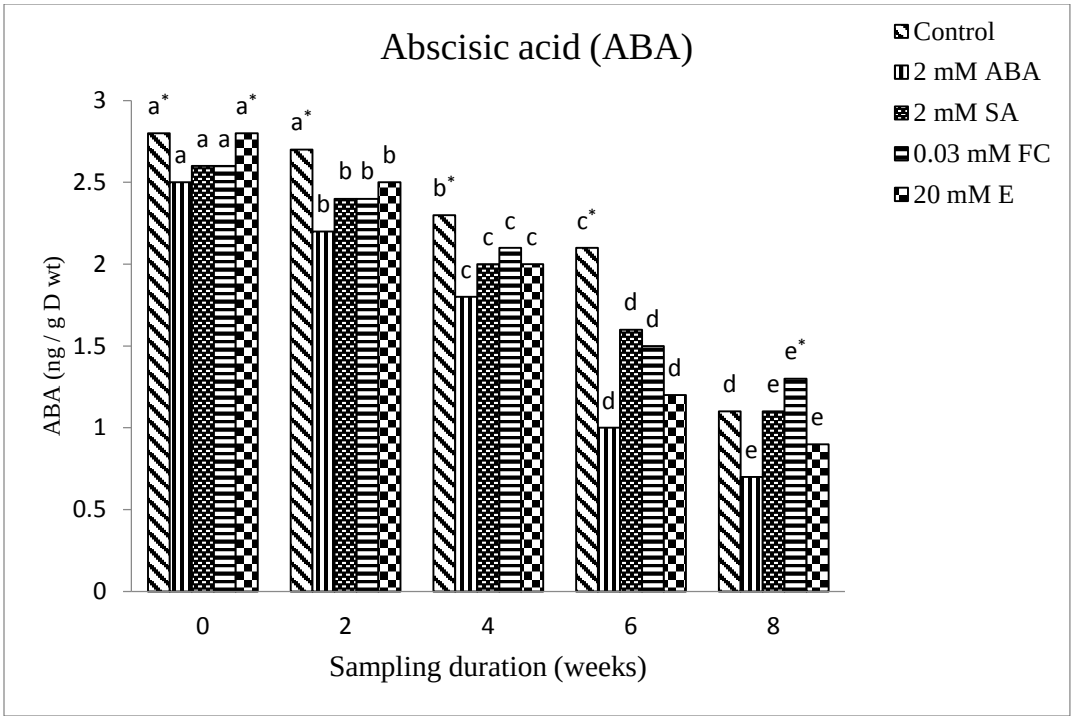


Fig. 3. 19. Effect of recovery duration on the internal contents of Abscisic acid (ABA) during the recovery of *G. grandiflorum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

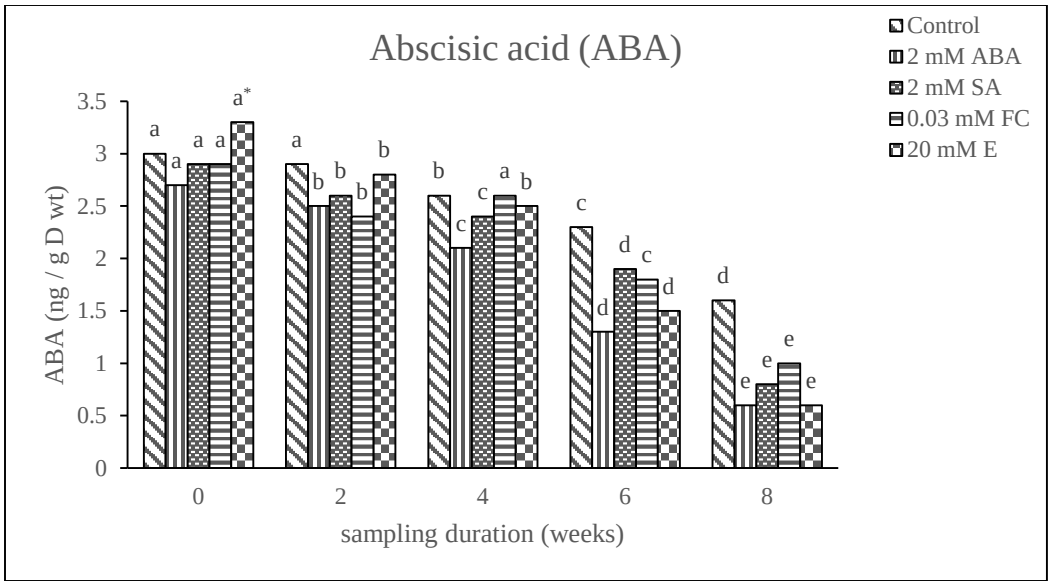


Fig. 3. 20. Effect of recovery duration on the internal contents of Abscisic acid (ABA) during the recovery of *G. corniculatum* from salinity and drought stresses. Columns labeled with different letters are significantly different at $P = 0.05$ for comparison among different sampling dates within each growth regulator treatment. Columns labeled with an asterisk are significantly the highest among different treatments within each sampling date.

Exogenous application of plant growth regulator or fertilizers is considered to induce abiotic stress tolerance in some plants (Khan et al., 2006; Waseem et al., 2006; Hamdia and Shaddad, 2010). Plants exposed to environmental stresses can accumulate various metabolites to cope with the stressed conditions. Again, protection against drought is provided by ABA, which is accepted as a major phytohormone that participates in the responses of plants to abiotic stresses (Mahajan and Tuteja, 2005). Osmotic stress raised the content of ABA in the leaf of maize seedlings, while other growth homone types went down in their concentrations and activities. As expected, at favorable conditions, recovery from stress took place and hormone concentrations started to change to cope with the newly required biological functions and needs.

Many studies showed that endogenous ABA content increased rapidly under water stress, which improved drought tolerance in plants (Zhang et al., 2006). The role of abscisic acid in

stress physiology has received much attention, and there is now considerable experimental evidence that the physiological effects induced by salinity might be modulated by ABA. It has been shown that saline stress is accompanied by an increased in ABA content (Aspinall and Paleg, 1981). In addition, *P. vulgaris* plants adapted to salinity had ABA concentrations substantially higher than those in non-adapted plants (Montero et al., 1998). An exogenous ABA treatment reduces leaf abscission and increases salt tolerance in citrus plants (Go´mez-Cardenas et al., 2002), but it also decreases total biomass and increases the root to shoot ratio in poplar species (Yin et al., 2004). Abscisic acid (ABA) selectively affects ion transport processes (van Steveninck, 1976). ABA appears to increase the permeability of roots to water and to inhibit excretion of ions into the xylem but not to affect uptake of ions by the root. The effectiveness of ABA may depend on environmental factors such as temperature (Pitman et al., 1974; Pitman and Wellfare, 1978). Khadri et al. (2007) suggested that ABA application improves the response of *Phaseolus vulgaris* symbiosis under saline stress conditions, including the nitrogen fixation process and enzymes of ammonium assimilation and purine catabolism. The exogenous application of ABA caused an alteration of Zeatin Riboside (ZR) content in lucerne under different stress conditions (Dobra *et al*., 2010).

Salicylic acid prohibits auxin and cytokinin loss in plants and thus enhances cell division and plant growth. SA keeps photosynthetic aspects like chlorophyll content, at proper level and thus helps plants to well growth and develop (Hayat *et al.*, 2010). Morphological characteristics like leaf area, plant height, root and shoot dry weight, biomass, flower number and diameter and primary and secondary shoot numbers enhanced by applying SA under drought condition (Martin-Mexand and Larqué-Saavedra, 2005). Applying foliar spray of SA resulted in higher root and shoot fresh weight, root and shoot dry weight, stem diameter and leaf number of

cucumber (Yildirim *et al.,* 2008) and maize (Moussa and Khodary, 2003) under saline conditions. Stem diameter, biomass, plant height and leaf number of cucumber enhanced using SA at drought condition (Bayat *et al.,* 2012). SA regulate plant growth and cell division via other hormones like auxin, cytokinin, gibberellin and ABA. SA results in higher cell division in meristems and enhances root length (Shakirova *et al.,* 2007). Salicylic acid controls photosynthesis system, photosynthesis amount, pigment content and stomatal conductivity and regulates these procedures for appropriate growth and development (Popova *et al.,* 2009, Steven *et al.,* 2006, El-Tayeb, 2005, Kormkaz *et al.,* 2007).

Jung and Luttge (1980) mentioned that Fusicoccin (FC) inhibited net excretion of Cl by the glands of the pitchers of the carnivorous plant *Nepenthes hookeriana*; of Na^+ and Cl^- by the salt glands of the halophytes *Limonium vulgare* and *L. pectinatum* and of K^+ in the nectar of *Acer platanoides* flowers. It is suggested that the effects of FC and ABA on ion excretion by gland cells could be reconciled with literature showing FC-stimulation and possible ABA-inhibition of proton pumps at the plasmalemma of plant cells. Fusicoccin was initially suggested to activate the plasma membrane H^+-ATPase by direct interaction with the enzyme (Marrè, 1979). Later, fusicoccin was demonstrated to bind to a "receptor" belonging to a certain family of proteins. (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). FC and ABA have antago- nistic effects on movements of stomatal guard cells. FC causes stomata to open and prevents closure; this is probably due to enhanced H^+ extrusion from the guard cells (Marré, 1979), which then affects malate and K^+ accumulation, thus providing the basis for turgor increase and stomatal opening at least in some cases (Raschke, 1976, 1977; Hsiao, 1976). Moreover, Clint & Blatt (1989) indicated that the fusicoccin-induced increase in K^+ influx should be attributed to energy-linked transport, and this would predict cytoplasmic acidification

by fusicoccin in *Vicia*, with consequent inhibition of the efflux at the plasmalemma. It is also worth noting that comparison of the effects of fusicoccin on cation and anion influx in *C. communis* suggests malate rather than chloride as the balancing anion in the stimulated influx (Clint, 1987).

Bayat et al. (2012) evaluated the effects of SA on growth and ornamental characteristics of Persian petunia under salt stress and concluded that foliar application of SA improved growth and ornamental characteristics of Persian petunia under saline and non-saline conditions. Salicylic acid controls photosynthesis system, photosynthesis amount, pigment content and stomatal conductivity and regulates these procedures for appropriate growth and development (Popova *et al.,* 2009, Steven *et al.,* 2006, El-Tayeb, 2005, Kormkaz *et al.,* 2007).

Ethylene is known to stimulate the release of dormancy, stimulates shoot and root growth and differentiation, stimulates flower induction, stimulates flower opening and may have a role in adventitious root formation. Environmental stresses, such as cold, heat, salinity, and drought, induce ethylene production and oxidative stress and cause damage in plants. Ethylene is produced either chemically through the incomplete combustion of hydrocarbons or biologically by almost all living organisms (Wang et al. 2002). There is a lot of evidence showing that ethylene is an essential component of a wide range of responses to biotic and abiotic environmental stresses Wang et al. 2002, Guo and Ecker 2004, El-Tayeb 2005). Further, many of these stress responses integrate ethylene signaling into more complex circuitry involving salicylate and jasmonate signaling (Wang et al. 2002). The effects of ethylene on plants are regulated both at the level of its synthesis and perception of the hormone (Wang et al. 2002). Tirani et al. (2013) studied the effects of ethylene on chlorophyll (Chl), carotenoid (Car), anthocyanin, flavonoids, ascorbic acid, dehydroascorbic acid, total ascorbate, lipid peroxidation,

and ethylene production in leaf of canola pretreated with SA. Their results showed that the ethylene treatments induced lipid peroxidation, lowered significantly Chl and Carotenoids contents and anthocyanin accumulation.

In conclusion, during stress, internal ABA accumulation was evident to cope with stress conditions. During recovery, when the circumstances were favorable for growth, other groups of growth regulator that are needed for accelerated cell division, enlargement and growth such as auxins, gibberellins, and cytokinnins were abundant, and stress signal hormones dissapeared.

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