EFFECT OF EXOGENOUS ABSCISIC ACID ON GROWTH AND BIOCHEMICAL CHANGES IN THE HALOPHYTE *SUAEDA MARITIMA*

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ABSTRACT

Different types of phytohormones are being extensively used to alleviate the adverse effect of salinity stress on plant growth. Among those, Abscisic acid (ABA) is a plant stress hormone and one of the most important signaling molecules in plants. Drought and salinity activate De-novo abscisic acid synthesis prevent further water loss by evaporation through stomata, mediated by changes in the guard cell turgor pressure. Under osmotic stress abscisic acid induce the accumulation of protein involved in the biosynthesis of osmolites which increasing the stress tolerance of plant. In addition, exogenous application of ABA enhances the tolerance of plants or plant cells to cold, heat, drought, anoxia and heavy metal stresses. This study was carried out to study the exogenous abscisic (ABA) acid induced regulatory role on the growth, water content, protein content, chlorophyll content, osmolyte accumulation and protein profiling through SDS PAGE in a halophyte, *Suaeda maritima*. The osmolyte accumulation of proline and glycine betaine was found to be more in 50 μM ABA concentrations. The protein profiling through SDS PAGE revealed that~66KDa proteins was not expressed in the control plant and in 10μM ABA treated plants. Interestingly, the ABA treatment induced a new protein of 14.2KDa in 10μM concentration. The ABA treated plants with concentrations 50μM, 100μM and 150μM showed changes in the expression of protein in abundance than the control and 10μM ABA treated plants. The findings in this study indicate that among all the concentrations, 50μM ABA concentration treated plants exhibited higher growth rate.

Keywords: Phytohormones, stress, Abscisic acid, Plant Growth

INTRODUCTION

Halophytes represent salt-tolerant species that thrive in the inhospitable habitats of inland and coastal salt marshes, dunes, beaches, deserts and salt flats. They are adapted to survive under extreme conditions, represented by temperature, (freezing to very hot) salinity (hypo- to hyper-saline) and moisture (drought to water-logging) (**Flowers *et al.,* 1986; W.H.O, 1990**). *Suaeda maritima* is an annual obligate halophyte that has the distinctive feature in that it thrives in both coastal and inland salt marshes. It is a model plant and its salt-tolerance has been extensively investigated (**Flowers *et al.,* 1977; Yeo 1981; Greenway and Munns, 1983; Hajibagheri *et al.,* 1985; Clipson 1987**). It has been well documented that the *Suaeda maritima* plant is an extreme halophyte that thrives in 100-350mM (**Flowers *et al.,* 1977; Yeo and Flowers, 1980**) of particular interest with respect to its potential for exploitation, *Suaeda maritima* has significant salt accumulating properties, indeed, the vacuole can tolerate 500-600mM (**Maathuis *et al.,* 1992**), allowing its cultivation in highly saline lands with the intention, after repeated cropping, of extracting salts from the soil (**Ravindran *et al.,* 2007**). Studies in salinity tolerance on halophytes will provide greater forecast for understanding the impact of salinity stress on plants, and help in monitoring the coastal salt marsh environments sustainability.

Different types of phytohormones are being extensively used to alleviate the adverse effect of salinity stress on plant growth. Among those Abscisic acid (ABA) has been the main focus of most of the plant scientists (**Aldesaquy *et al.,* 1998; Hisamatsu *et al.,* 2000**). Abscisic acid is a plant stress hormone and one of the four most important signaling molecules in plants. Abscisic acid involved in many cellular processes like germination, gravitropism and guard cell mediated stomata opening role (**Levitt, 1980**), Particularly to regulate the water balance and osmotic stress which was well established (**Zhu, 2002; Takahasi *et al.,* 2000**). Under non stress condition, plants retain a low level of abscisic acid, which increase in response to low temperature, drought and osmotic stress (**Levitt, 1980**). Drought and salinity activate De-novo abscisic acid synthesis to prevent further water loss by evaporation through stomata, which is mediated by changes in the guard cell turgor pressure. The mechanism involved fast changes in intracellular Ca²+ concentration and stimulate farther signaling in the cell.

Under osmotic stress, abscisic acid induces the accumulation of protein involved in the biosynthesis of osmolites (e.g. Proline, trehalose) which increases the stress tolerance of plant (**Nayyar *et al.,* 2005**). The action of ABA can target particularly guard cells for induction of stomatal closure but may also signal systemically for adjustment towards severe water shortage.

Exogenous application of ABA has been reported to significantly increase in the activities of enzymatic antioxidants; superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APOX) and glutathione reductase (GR) and the contents of non-enzymatic antioxidants; ascorbate, reduced glutathione, tocopherol and carotenoids (**Jiang and Zhang, 2001**). Proline and glycine betaine are compatible solutes involved in cell osmoregulation and protection of proteins during dehydration (**Hose *et al.,* 2001**). It also acts as a free radical scavenger and may be more important in overcoming stress than in acting as a simple osmolyte (**Ramachandra Reddy *et al.,* 2004; Verslues and Bray, 2006**). In addition, exogenous application of ABA enhances the tolerance of plants or plant cells to cold (**Chen *et al.,* 1983**), heat (**Robertson *et al.,* 1994**), drought (**Lu *et al.,* 2009**), anoxia (**Kato-Noguchi, 2000**) and heavy metal stresses (**Hsu and Kao, 2003**). Several studies also showed that ABA pretreatment enhances plant tolerance to salinity. (**Larosa *et al.,* 1985**) was the first to report that ABA pretreatment enhances the adaptation of cultured tobacco cells to salinity. The ultimate aim of the work is to evaluate the effects of different concentration of exogenous exposure of ABA in salt tolerant halophyte plants of *Suaeda maritima* were studied and as a preliminary attempt the investigation has led to predict the threshold concentration.

MATERIALS AND METHODS

Collection of plant seeds and growth condition parameters

The seeds of wild type *Suaeda maritima* were collected from the adjacent banks of Vellar estuary, Portonovo, Tamilnadu, South east coast of India. Generally, the wild plants in sea shores are exposed to natural salt stress which leads to the biosynthesis of endogenous ABA by the stimulation of stress signals by which the plants tolerate the stress. With this state the threshold level of ABA cannot be

measured. Therefore, the non-stressed seeds was collected and maintained in green house condition prior treatment. Proceeding to germination, seeds were shade dried and hand sieved. Seeds of *Suaeda maritima* were immersed thoroughly in 70% (v/v) ethanol for 5 min, sterilized by 0.1% mercuric chloride and washed with sterile distilled water. Seeds were stored in 100ml of distilled water at 27°C for 24hrs. After 24hrs, seeds were sown in plastic cups containing vermiculite moistened soil with half strength Murashige and Skoog (½ MS) medium and irrigated daily with distilled water (Fig. 1a)

Figure 1(a) left - Plants under greenhouse condition
Figure 1(b) right - Hydroponic condition of growth chamber

About six (6) weeks old seedling were then transferred to hydroponic media and maintained in a culture room with a dark/light cycle of 8/16 h at 37°C for 5 days (Fig. 1b). In every one day interval, the nutrient solution was replaced in order to avoid depletion of nutrients. After 5 days of adaptation, these plants were randomly divided into five groups and then treated with different concentration of ABA (Himedia) along with a control (only with nutrient solution). The treatments were given as follows, 10µMABA, 50µM ABA, 100µM ABA and 150µM ABA. After 5 days of treatment, the plants were harvested and washed quickly with distilled water and blotted dried on filter paper and then stored with liquid nitrogen at -70°C for future analysis.

Water Content (WC)

The leaf and root were taken and immediately weighed for its fresh weight (FW). The dry weight (DW) was obtained after drying the leaf and root in an oven at 70 °C for 72 h. Then RWC was calculated as given below (**Silveira *et al.,* 2003**).

[Fresh Weight – Dry Weight / Fresh weight] X 100

Estimation of chlorophyll content

The Chlorophyll-a, Chlorophyll-b and total chlorophyll content assays was performed (**Arnon, 1949**). About 200 mg fresh leaves of *S.maritima* was homogenized in 8 ml 80% acetone with homogenizer. Homogenates were then centrifuged at 4° C for 15 min (3000 rpm). Supernatants were used for the analysis of pigments. Absorbance was determined at 645nm, 652nm and 663nm respectively and the following equations were used for calculations (**Litchtenthaler and Wellburn, 1983**).

Total Chl: $A652 \times 27.8 \times 20$ /mg leaf weight
Chl-a: $(11.75 \times A663 - 2.35 \times A645) \times 20$ /mg leaf weight
Chl-b: $(18.61 \times A645 - 3.96 \times A663) \times 20$ /mg leaf weight

Estimation of proline content

The Proline assay was done according to the method of **Bates *et al.,* (1973)**. Five hundred (500mg) of plant tissue was homogenized in 5 ml of 3 per cent aqueous sulphosalicylic acid. The homogenate was centrifuged at 1000Xg for 10min. About 1ml of the extract was mixed with two (2) ml of acid ninhydrin (1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) and 2 ml of glacial acetic acid in a test tube and was heated for an hour at 100°C. The reaction mixture was extracted with 4 ml of toluene and mixed vigorously by using a vortex mixture for 15-20 seconds. The chromosphere containing toluene was aspirated from the aqueous phase. The absorbance of the toluene layer was measured in a spectrophotometer at 520 nm using toluene as blank.

Estimation of Glycine betaine content

The accumulation of Glycine betaine (GB) was determined (**Grieve and Grattan, 1983**). Extract prepared by finely ground dry plant leaf material (0.5 g) was mechanically shaken with 20 ml of deionized water for 24 h at 25°C. The samples were filtered and the filtrates were diluted in 1:1 ratio with 2 N sulphuric acid. Aliquot was kept in centrifuge tubes and then cooled in ice water for 1 h. Cold KI-I2 reagent was added and the mixture was gently mixed with a vortex mixture. The samples were stored at 0-4°C for 16 h. After 16 hours the samples were centrifuged at 10,000 g for 15 minutes at 0°C. The supernatant was carefully aspirated with a one (1) ml micropipette. The periodite crystals were dissolved in 9 ml of 1, 2-dichloro ethane. After two (2) hours, the absorbance was measured at

365 nm using GB as standard.

Total Protein extraction and estimation

The total protein was extracted by phenolic method (**Wang *et al.,* 2007**) with some modifications using BPP (borax/ phenol/polyvinylpyrolidine). About 1g of frozen leaf tissue from *S. maritima* was powdered and re-suspended in 3 mL of ice-cold extraction buffer containing 100mMTris (pH 8.0), 100mM EDTA, 50 mM borax, 50 mM vitamin C, 1% PVPP w/v, 1% triton X-100 v/v, 2% β-mercaptoethanol v/v , 30% sucrose w/v and vortexed for 5min at room temperature. Two volumes of tris saturated phenol (pH 8.0) was added and further vortexed for 10min. The sample was centrifuged at 4°C for 15min at 15, 000g and the upper phase was transferred to a new centrifuge tube. Equal volume of extraction buffer was added to the mixture, vortexed for 10 min, followed by centrifugation to the above mentioned condition. Proteins from the supernatant were precipitated by adding five volumes of ammonium acetate saturated-methanol, and incubated at -80°C for 8 h. After centrifugation as described above, the protein pellet was re-suspended and rinsed twice with ice-cold methanol followed by ice-cold acetone at 15,000g for 15 min at 40C. Finally, the washed pellet was air-dried, recovered with lysis buffer (9M urea, 2% CHAPS, 13mM DTT, 1% IPG buffer) and stored at -80°C for further studies.

Quantification of Proteins

The Protein concentration was determined for leaf, shoot and root of *S. maritima* by bicinchoninic acid protein assay kit (Sigma aldrich) following the manufacturer's manual using the LAMBDA 25 UV/Vis Spectrophotometer (Perkin Elmer) was used as the standard (1µg/µl) and the absorbance was measured at 562nm.

Protein profiling through SDS-PAGE

The Proteins were resolved in SDS–Polyacrylamide gel (**Laemmli, 1970**) as described by **Ausubel *et al.,* (1989)**. Routinely, 1.5 mm thick gel was casted using a separating gel (10 %) and a stacking gel (5%). Aliquots of protein samples of equal concentration were analyzed. The precipitated proteins were recovered by centrifugation in a microfuge for 10min. The supernatant was carefully aspirated and the pellet was dissolved in 15µL of 0.1N NaOH and 5µL of 4X sample buffer. The samples were heated to 60°C for 15min, loaded into the slots and the slots were filled with 1X electrophoresis buffer and a constant current of 21mA/gel was applied.

Statistical analysis

The statistical analysis of variance was used to evaluate the data and significant differences among means were determined by one-way ANOVA ($P<0.05$). Statistical calculation was performed with SPSS 16.0 for windows.

RESULTS AND DISCUSSION

The halophyte plant species are well adapted to saline habitats and domestication of those species as new crops could in the future help to sustain food production in many regions of the world (**Colmer *et al.,* 2005**). Salinity affects almost every aspect of plant physiology and biochemistry. Since the presence of salt in the root medium reduces external osmotic potential and thus compromises water absorption, halophyte plant species are exposed in their natural habitats to both ion toxicities and to physiological drought (**Flowers and Colmer, 2008**). The plant performance in the harsh environment appeared to be directly limited by their ability to synthesize this plant growth regulator. Environmental and developmental signals are operating in the regulation of ABA synthesis in plant tissues. A different approach was used to study the implication of ABA in the mechanism of stress-tolerance. The present data show that ABA is assuming key functions in *Suaeda maritima*. In the present study, different concentration of ABA was supplied exogenously to study the threshold concentration of ABA. ABA is considered to be a stress hormone and relates closely with the adaptation of plants to stressful conditions and therefore protects plants against salinity and water stress (**Bano and Aziz, 2003; Zhu, 2003; Gurmani *et al.,* 2007**). Dry mass, water content, the leaf area and photosynthetic pigments regarded as a growth parameter increased generally with the exogenous ABA treatment under salinity stress according to the level of ABA treatment.

Growth rate

The leaf growth was estimated by measuring the fresh weight (FW) and dry weight (DW). The Leaf fresh weight significantly increased in all concentrations of ABA treated plants (Fig. 2) than the control plants, while exogenous ABA had no impact on leaf DW (Fig.3). Higher root fresh (Fig. 4) and dry weights (Fig .5) was recorded in all concentration of ABA treatment than the control. ABA controls longer-term root growth responses through regulation of gene expression

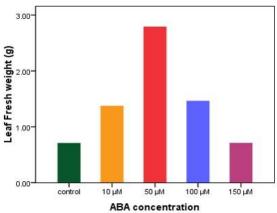that favors maintenance of root growth, which optimizes water uptakes (**Zhang *et al*., 2006**).

Figure 2 Effect of different concentration of exogenous ABA on leaf fresh of *Suaeda maritima.*
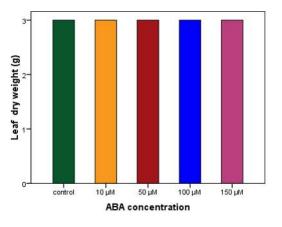

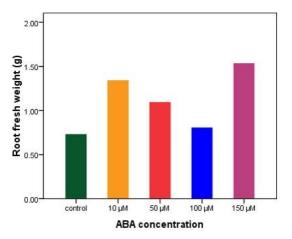**Figure 3** Effect of different concentration of exogenous ABA on leaf dry weight of *Suaeda maritima.*

Figure 4 Effect of different concentration of exogenous ABA on Root fresh weight of *Suaeda maritima.*
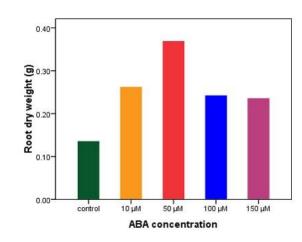

Figure 5 Effect of different concentration of exogenous ABA on Root dry weight of *Suaeda maritima.*

Water content

The leaf water content (LWC) was found slightly increased in 50μM ABA treated plant (Fig.6) than the other treated and control plants. But, the Root water content (RWC) was found decreased in 50μM ABA (Fig.7). However, there was no significant difference observed in this water relation attribute. **Mattioni *et al.,* (1997)**, reported that more proline and free amino acids accumulation was recorded in lower values of LWC potential more than RWC percent which recorded accumulation of lesser proline and free amino acid content.
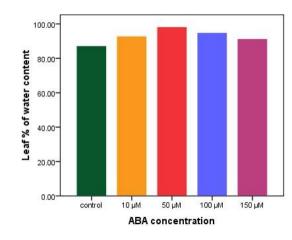

Figure 6 Effect of different concentration of exogenous ABA on Leaf % of water content
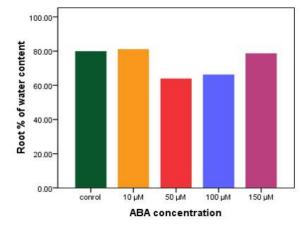

Figure 7 Effect of different concentration of exogenous ABA on Root % of water content

High concentrations of salt (i.e., ions, mostly Na^+) in the soil solution can impair water and nutrient uptake, reduce growth and photosynthetic activity. Furthermore high salinity can lead to an unfavorable Ca^{2+} or K^+ to Na^+ ratio, toxic intracellular Na^+ concentrations and peroxidation of membrane lipids (Levitt 1980). The reduced ability of the plant to take up water induces water

deficit effects comparable to drought stress. This includes ABA biosynthesis and accumulation, which regulate measures against water loss such as closure of stomata and increased production of compatible osmoprotectants and antioxidants.

Estimation of protein content

The changes in the protein content of the leaf in response to different concentration of ABA are given in (Fig.8). The protein content increased in all ABA treated groups of *Suaeda maritima* than the control. Protein content in the tissues of many plants may have declined under drought or salinity stress, because of proteolysis and decreased protein synthesis (**Joshi and Misra, 2000**).
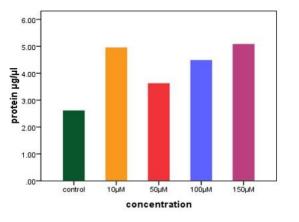

Figure 8 Effect of different concentration of exogenous ABA on protein content (µg/g tissue) of *Suaeda maritima.*

Estimation of chlorophyll

An increasing trend in total chlorophyll content of the leaf was noticed (Fig.9) in concentration of 50µM ABA and thereafter, it steadily declined. The chlorophyll "a" (Fig.10) and chlorophyll "b" (Fig.11) content of *Suaeda maritima* was found to increase with the increasing ABA concentration up to 50µM and at higher concentrations of ABA there was a decrease in the chlorophyll content. **Travagila *et al*., ((2007)** reported that exogenous ABA exposure to wheat plants resulted in the increases in chlorophyll and carotenoid contents compared to control. **Basak *et al.,* (2012)** showe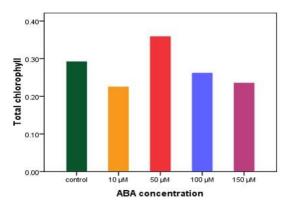d that 50µM ABA content caused increasing of pigment content compared to control in tomato cultivars while 100µM affects the pigment content.

Figure 9 Effect of different concentration of exogenous ABA on total chlorophyll (g g-1 fr. wt.) of *Suaeda maritima.*
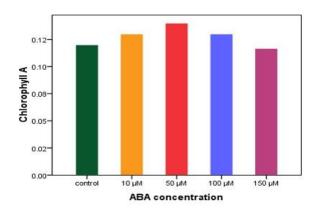

Figure 10 Effect of different concentration of exogenous ABA on chlorophyll "a" (g g-1 fr. wt.) of *Suaeda maritima.*
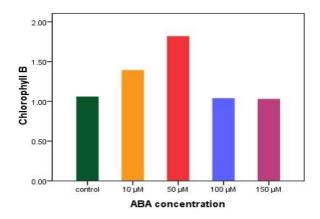

Figure 11 Effect of different concentration of exogenous ABA on chlorophyll "b" (g g-1 fr. wt.) of *Suaeda maritima.*

Effect of salinity on proline content

The effects of different concentration of exogenous ABA on the proline content in the leaf is shown in Fig.12. There was a gradual rise in the level of proline with increasing concentration of ABA the control. **Gurmani *et al*., (2011)**, reported that ABA increased proline accumulation significantly in the shoot of rice in hydroponic condition and in the field experiments. An increasing accumulation of proline was found with increasing salt tolerance. Salinity tolerance has been associated with the capacity of a species to accumulate proline and it acts as an intracellular osmoticum. Proline is believed to function as compatible solute in balancing cytoplasmic and vacuolar water potentials (**Hassine *et al*., 2008**). The leaf always had more proline than the stem and root. ABA treated plant leaves possessed comparatively higher amounts of proline, providing evidence for an efficient role of these metabolites as osmoprotectants under salinity stress. ABA induces the accumulation of protein involved in the biosynthesis of osmolytes (e.g., proline, trehalose), which increases the stress tolerance of plants (**Nayyar *et al*., 2005**). The data in the present study demonstrated that among all the concentration of ABA, 50µM ABA pretreated leaves exhibited higher proline accumulation when compared to control.
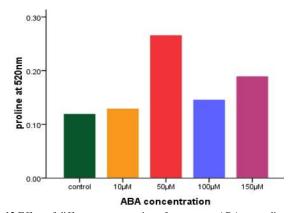

Figure 12 Effect of different concentration of exogenous ABA on proline content (µM /g tissue) of *Suaeda maritima.*

Estimation of Glycine betaine

The accumulation of glycine betaine (GB) in the leaves at various ABA concentrations is presented (Fig.13). There was a steady increase in the GB accumulation with increasing ABA up to 50µM in *Suaeda maritima* at higher concentrations. GB is considered a good indicator of salt tolerance. The presence of higher amounts of GB in the plant organs indicated a higher degree of salt tolerance. Even though the accumulation of GB was quite high, the osmoprotective mechanism in combination with other mechanisms, such as antiport, may produce plants with even higher levels of salt tolerance **(Daniell *et al*., 2001)**. The accumulation of GB may function as an alternative defense mechanism to saline environments **(Jageendorf and Takabe, 2001)**. Conflicting date are, however, Available in the literature concerning the putative influence of ABA on proline and glycine betaine synthesis **(Colmer *et al*., 2005)**.
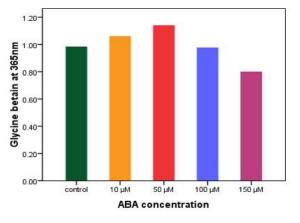
Figure 13 Effect of different concentration of exogenous ABA on glycine betaine (µg/g tissue) of *Suaeda maritima*.

Leaf protein profiling

The effect of exogenous ABA in the level of protein expressed in the leaves of *S. maritima* was identified by performing different concentration of treatments with ABA. SDS-PAGE analysis was performed and the protein profile was outlined. On 10% SDS-PAGE gel, distinct protein bands were observed with varying expression levels and molecular weight. Plants treated with 10µM ABA showed differential expression of protein at 14.2 kDa which was analyzed through alpha-imager against standard protein marker (Fig.14). The 29 KDa protein did not get expressed only in 10µM ABA. Approximately 66 KDa proteins were not expressed in control and in 10µM ABA treated plants. Protein extracted from the leaf of *S. maritima* with concentrations of 50µM, 100µM and 150µM ABA showed changes in the expression of protein in abundance when compared with non- stressed control and in 10µM ABA plants, which can be due to the concentration increase which makes the plant more compatible to survive as salt tolerant.
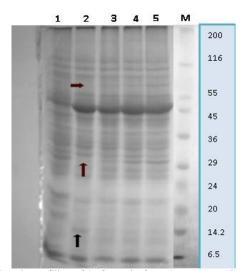
Figure 14 Protein profiling of leaf protein from *S. maritima* 10% SDS-PAGE showed differential expression after treatment with different concentration of ABA. Control (Lane1); 10µM (Lane 2); 50 µM (Lane 3); 100 µM (4); 150 µM (5); Marker (M). All lanes were loaded with 20µg protein. Arrows indicates the increased and decreased polypeptide expression between treated and normal plants.

CONCLUSION

Salinity is a major stress, limiting the increase in the demand for food crops. More than 20% of cultivated land worldwide (~ about 45 hectares) is affected by salt stress and the amount is increasing day by day. Plants on the basis of adaptive evolution can be classified roughly into two major types: the halophytes (that can withstand salinity) and the glycophytes (that cannot withstand salinity and eventually die). Majority of major crop species belong to this second category. Thus salinity is one of the most brutal environmental stresses that hamper crop productivity worldwide **(Flowers, 2004; Munns and Tester, 2008)**. The concern for halophytes starts with the fact that they exhibit multiple mechanisms to adapt with stress. The physiology of Halophytes pave way for discovering the mechanism in which various plants gets and may get adapted to survive in extreme conditions. Endogenous ABA levels in plants gets increased under various abiotic stress conditions such as water, drought, salinity and cold. The effect of exogenous application on the capacity of plants to overcome the stress has been shown under these conditions. The exogenous ABA applications have decreased the negative effects caused by temperature, drought, salinity and cold stresses in the plants and this effect of ABA is related to the ABA induced antioxidant systems **(Zhang *et al*., 2005; Khadri *et al*., 2006; Travagila *et al*., 2007; Yang *et al*., 2010)**. **(Swamy and Smith, 1999)** reported that the endogenous ABA production under stress conditions in positively related to the stress resistance and exogenous ABA plays an important role in the adaptation of plants to stress. All ABA concentrations caused increase of all growth parameters in plants than the control in our study. When changes in root-leaf water content, pigment content, protein content and osmolyte accumulation were evaluated, the ABA applications caused increase in growth rate, protein, pigment content and osmolyte accumulation. However 50µM ABA applications caused increase in growth rate, pigment and osmolyte accumulation than the control and in higher concentrations. The results of the present study highlights that the ABA can impose positive effects in 50µM concentration. Thus, the present investigations convey that the effect of exogenously applied 50µM ABA on the above mentioned parameters were highly significant. This confirms that that the positive impact of exogenous 50µM ABA among the treated plants was associated with an increase in the leaf and root total ABA concentration. It is therefore, suggested that optimal application of 50µM ABA can benefit plant growth on stress tolerance studies. This study will pave way for a solid foundation necessary for further investigation of the ABA mediated salt tolerance networks.

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