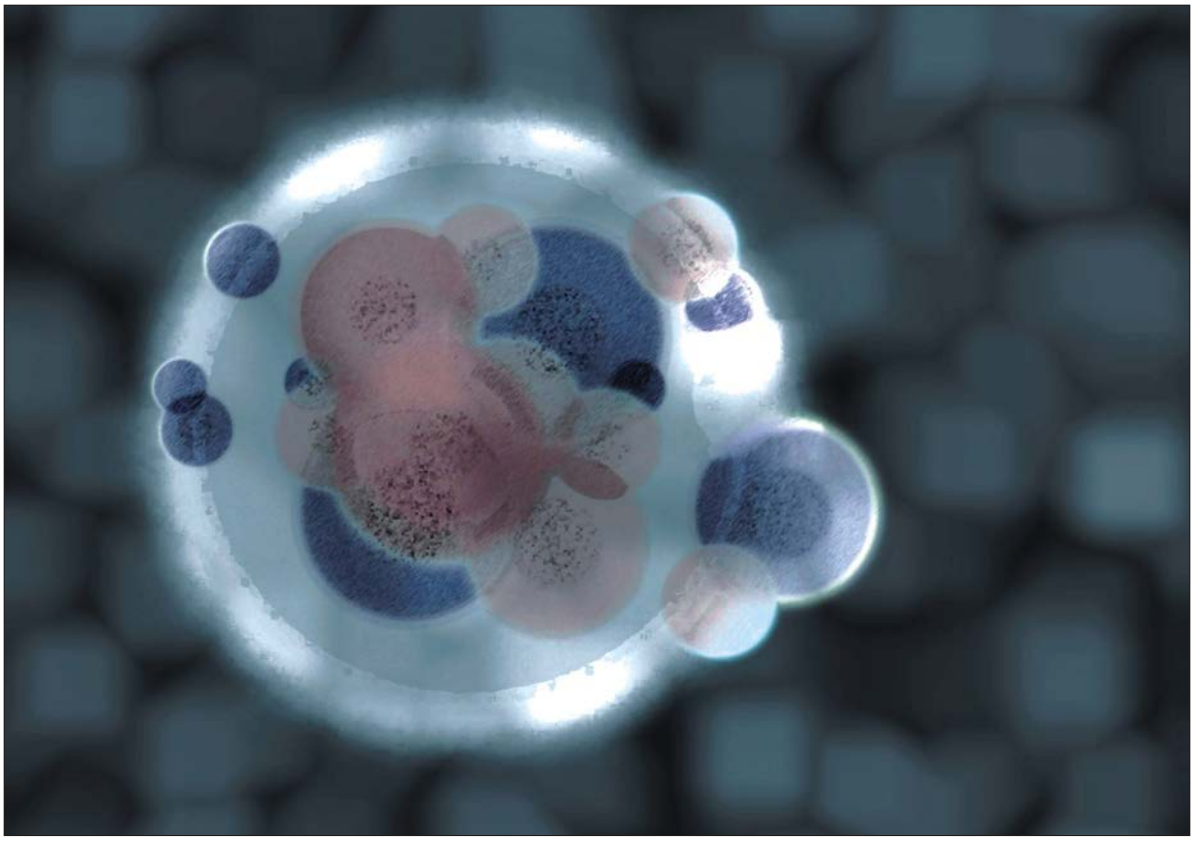

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Foliar Application with Riboflavin (Vitamin B_2) Enhancing the Resistance of *Hibiscus sabdariffa* L. (Deep Red Sepals Variety) to Salinity Stress

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Abstract: The effect of salinity stress alone and with foliar application with 100 ppm riboflavin (vit. B_2) on seedling growth, organic solutes accumulation, ion uptake and the activity of antioxidant enzymes in *Hibiscus sabdariffa* L. was investigated. Analysis of data (ANOVA) revealed that, growth of *Hibiscus sabdariffa* L. seedlings was reduced with increasing NaCl concentration, while water content and dry matter were not changed significantly at the low levels of salinity. Soluble carbohydrate, protein and proline were found to be increased significantly. The content of Na^+ was significantly increased, while K^+ as well as K^+/Na^+ and Ca^{++}/Na^+ ratios were decreased as salinity levels increased. Calcium and total cations were slightly affected. Foliar application with vit. B_2 induced stimulatory effects on almost measured parameters. On the other hand, Na^+ content decreased significantly. Further, salinity induced marked increases in lipid peroxidation and the activity of antioxidant enzymes (CAT, POD, APX and GR), while membrane stability index was significantly decreased. Spraying of salinized seedlings with vit. B_2 induced a marked increase in MSI and antioxidant enzymes, whereas the content of lipid peroxidation was decreased in comparing with salinized seedlings. Results suggest that, vit. B_2 may have a potential role as an effective antioxidant by regulating osmotic and ionic balance and enhancing the resistance of *H. sabdariffa* L. seedlings to salinity stress.

Key words: Antioxidant enzymes, ion uptake, lipid peroxidation, membrane stability index (MSI), organic solutes

INTRODUCTION

Salinization of agricultural areas due to intense practices and irrigation is an important feature limiting crop yield and productivity and as a result salinization of the soil has become an increasingly important topic (Flowers and Flowers, 2005).

Salinity stress causes inhibition of plant growth due to a reduction in water availability, sodium ion accumulation and mineral imbalances, leading to cellular and molecular damage. Many of different plant species have developed different mechanisms to cope with salinity stress (Munns, 2002). The osmotic adjustment by net solute accumulation has been considered an important mechanism to salt tolerance in plants. The reduction in osmotic potential in salt stressed plant can be a result of inorganic ions and compatible organic solutes accumulation (Hasegawa *et al*., 2000). Salinity was shown to increase the uptake of Na^+ or decrease the uptake of K^+ and Ca^{++} (Yildirim *et al*., 2006). This was accompanied with liberation of reactive oxygen species (ROS) which consequently increased the malondialdehyde (MDA) content (a product of lipid peroxidation) which in turn inhibited growth and crop yield.

Reactive oxygen species are strong oxidized species that cause oxidative damage to membrane lipids and proteins (Molassiotis *et al*., 2006). Thus, cell membrane stability has widely been utilized to differentiate salt-tolerant and salt-sensitive cultivars (Eraslan *et al*., 2007). The ROS are highly reactive in the absence of any protective mechanism. One of the protection mechanisms is antioxidant system (Foyer *et al*., 1994). So, salt affected glycophytes induced antioxidant enzymes like peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), which might play an important role in scavenging active oxygen species (Koca *et al*., 2007; Athar *et al*., 2008).

Hibiscus sabdariffa L. is well known in Egypt with the name of Karkadeh. It is one of the most important and popular medicinal plants (Malvaceae) and its dye is used in drinks. The present investigation was aimed to enhancing the resistance of *H. sabdariffa* L. seedlings to salt stress, through foliar application with riboflavin (vit. B_2). This may provide further information on the injury effects of salinity and repairing these injurious effects, through the main role of vit. B_2 as coenzymes and probably play other roles in the biochemical processes and antioxidant defense system. The inductive responses of vit. B_2 on seedlings growth, organic solutes accumulation, ion uptake and antioxidant enzymes of *H. sabdariffa* L. seedlings grown under salinity treatments were investigated.

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MATERIALS AND METHODS

Plant materials and treatments: This study was conducted within the period of January to April 2008 in King Faisal University.

Foliar spraying with 100 ppm riboflavin (vit. B_2) solution aiming to enhance the resistance of *Hibiscus sabdariffa* L. seedlings to salt stress was investigated. Homogenous seeds of *Hibiscus sabdariffa* L. (deep red sepals variety) were surface sterilized with 1% sodium hypochlorite for 5 min, rinsed thoroughly three times with distilled water. The washed seeds were planted in plastic pots (12 cm in diameter and 10 cm in height, 15 seeds in each pot) lined with polyethylene bags and filled with soil composed of clay and sand (1:1 by volume) with different concentrations of NaCl (0, 30, 60, 90 and 120 mM NaCl). Six replicates were prepared for each NaCl treatment. Then, the pots were kept in growth chamber maintained at 32/28±2°C day/night (12 h) temperature cycles and relative humidity 60%. When the seedlings were at two-leaf stage, the pots were divided into two sets (3 replicates for each set). Foliar spraying with distilled water was applied to the seedlings of the first set (reference control), while the seedlings of the second set were sprayed with the aqueous solution of 100 ppm riboflavin. Spraying was conducted two times at intervals of 4 days (the soil was covered during spraying). The test seedlings were daily irrigated with water to reach in each treatment to the above desired salinization levels and left to grow for the period of the experiment (15 days).

Harvesting: Seedlings were harvested 15 days after germination and their root and shoot lengths were measured and the seedlings were washed and weighed freshly. To determine the dry matter, the fresh material was dried in an aerated oven at 70°C with successive weighing until weight of each sample became constant. The samples were grinded into fine powder and stored in sealed glass containers at room temperature, for various analytical experiments.

Determination of organic solutes and inorganic ions: Soluble and insoluble carbohydrates were extracted from the plant tissues and quantified by the anthrone sulphuric acid method (Fales, 1951). The soluble and insoluble proteins were determined according to Bradford (1976). Total free amino acids were extracted from plant tissues and determined according to the method of Lee and Takahashi (1966). Proline was determined according to Bates *et al.* (1973). The inorganic ions (sodium, potassium and calcium) were determined by flame photometer method (Williams and Twine, 1960).

Membrane stability index (MSI): It was measured as described by Sairam *et al.* (1997). Leaf discs (0.1 g) were taken in glass beaker containing 10 mL deionized water in two sets. One set was subjected to 40°C for 30 min and its conductivity of solution was measured by a conductivity meter (C_1). Second set was kept in a boiling water bath (100°C) for 10 min and its conductivity was also recorded (C_2). Membrane stability index (MSI) was calculated as follows:

$$MSI = [1- (C_1 /C_2) \times 100]$$

Lipid peroxidation: The level of lipid peroxidation was measured as the content of malondialdehyde (MDA) using the thiobarbioturic method (Zhao *et al.*, 1994). Concentration of MDA formed was calculated by using the extinction coefficient of 155 mM cm^{-1} for MDA at 532 nm.

Assay of some antioxidant enzymes

Preparation of extract: Leaf tissue (0.3 g) was frozen in liquid nitrogen and ground finely to a fine powder and extracted at a ratio 1:4 (w/v) fresh weight in extraction buffer (50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% soluble PVP. The homogenate was centrifuged at 15.000 g for 10 min at 4°C and the resulting supernatant was used for peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) assays.

Assay of peroxidase activity: The activity of peroxidase (EC 1.11.1.7) was determined using guaiacol reaction (Maehly and Chance, 1954). The increase in absorbance due to the formation of tetraguaicol was monitored at 470 nm (Klapheck *et al.*, 1990).

Assay of catalase activity: Catalase activity (EC 1.11.1.6) was assayed as described by Aebi (1984). The activity of catalase enzyme was estimated by the decrease of absorbance at 240 nm as a consequence of H_2O_2 consumption and was expressed according to Havir and McHale (1987).

Assay of ascorbate peroxidase activity: The activity of APX (EC 1.11.1.11) was determined from the decrease in the absorbance at 290 nm for 1 min of ascorbate as ascorbate oxidized (Chen and Asada, 1992).

Assay of glutathione reductase activity: Glutathione reductase (EC 1.6.4.2) was measured depending on the rate of decrease in the absorbance of NADPH at 340 nm (Foyer and Halliwell, 1976).

Statistical analysis: All data were analyzed statistically by one-way ANOVA. Values in the figures indicate the mean values±SD based on three independent determinations (n = 3) and the least significant difference (LSD) was used to test the difference between treatments; $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growth performance of the *Hibiscus sabdariffa* L. seedlings was estimated by measuring the lengths of roots and shoots as well as fresh weight and dry matter yields. These parameters (Fig. 1a-c) were decreased almost linearly with increasing of NaCl concentration. On the other hand, dry matter (Fig. 1d) and water content (Fig. 1e) exhibited a good tolerance up to the level of 60 mM NaCl, thereafter the values were significantly decreased, when compared to the control. Foliar spraying with 100 ppm vit. B_2 induced stimulatory effects on seedling growth and consequently the fresh weight and water content, especially at higher levels of salinity in comparing with unsprayed seedlings.

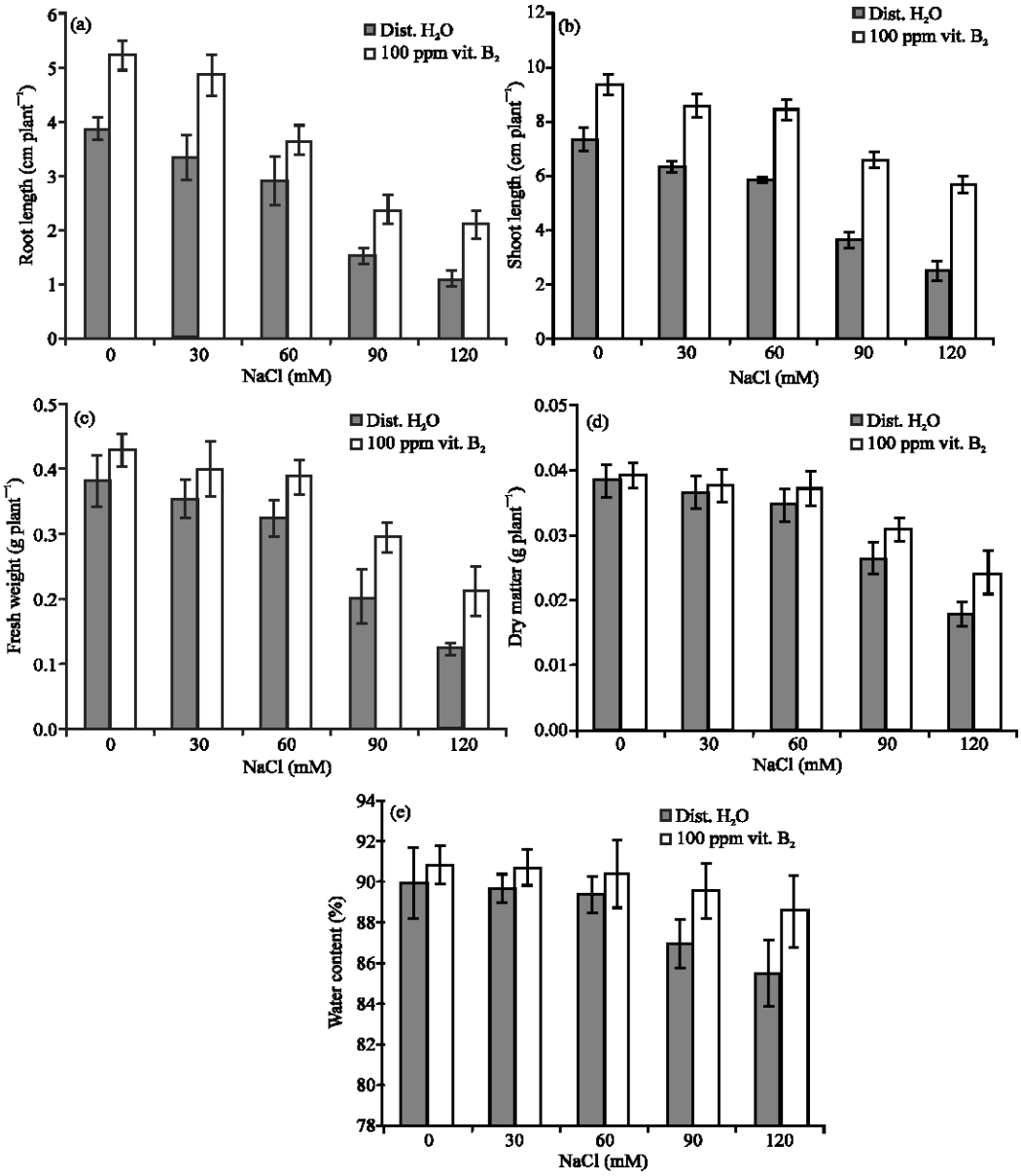

Fig. 1: (a-e) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on root length (a), shoot length (b), fresh weight (c), dry matter (d) and water content % (e) of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

Plant growth is one of the most important agricultural indices of salt tolerance (Munns, 2002; Ruiz *et al*., 2005). The non-significantly changes in dry matter and water content up to 60 mM NaCl were adjusted by the seedlings osmotically, leading to the maintenance of water content (Meloni *et al*., 2004), while the significant decrease in growth parameters at higher salinity levels may be attributed to the osmotic effects (Salter *et al*., 2007). Reduced rate of new cell production may make additional contributions to inhibition of growth (Boscaiu *et al*., 2005). The promotional effects of foliar application of vit. B_2 on growth parameters were associated with an improvement of water content. This probably reflects the efficiency of water uptake and utilization or depression of excessive loss of water by *Hibiscus sabdariffa* L. seedlings as a result of vitamin B_2 treatment, which can be considered as an adaptive response to salinity. Hence, it can be concluded that the beneficial effect of vit. B_2 on seedling growth has been related to the efficiency of their water uptake and utilization.

The contents of soluble and insoluble carbohydrates as well as soluble protein (Fig. 2a-c) were markedly increased as a result of salinity stress. The increase in soluble protein was at the expense of insoluble ones which were significantly reduced (Fig. 2d). Such increase in carbohydrate and protein contents was recorded by Azooz *et al*. (2004) and López *et al*. (2008). Higher accumulation of soluble carbohydrate and protein might play an important role in osmotic adjustment (Shaddad *et al*., 1990). Spraying of salinized seedlings with vit. B_2 exhibited a favourable effect on the production of carbohydrate and protein, especially the soluble ones. Furthermore, vit. B_2 treatment ameliorated the inhibitory effects of salinity on the contents of insoluble protein at most of the salinity levels used. The stimulation effect of riboflavin (vit. B_2) on the biosynthesis of carbohydrate and protein may be taken as a further evidence of the role played by the vitamin in plant adaptation mechanisms. The accumulation of carbohydrate due to vitamin treatment might be attributed to the increase in green area, which consequently leads to increase in photosynthetic activity and plant productivity. Athar *et al*. (2008) has found that, exogenous application of vit. C enhanced photosynthetic rate in wheat cultivars under salinity conditions.

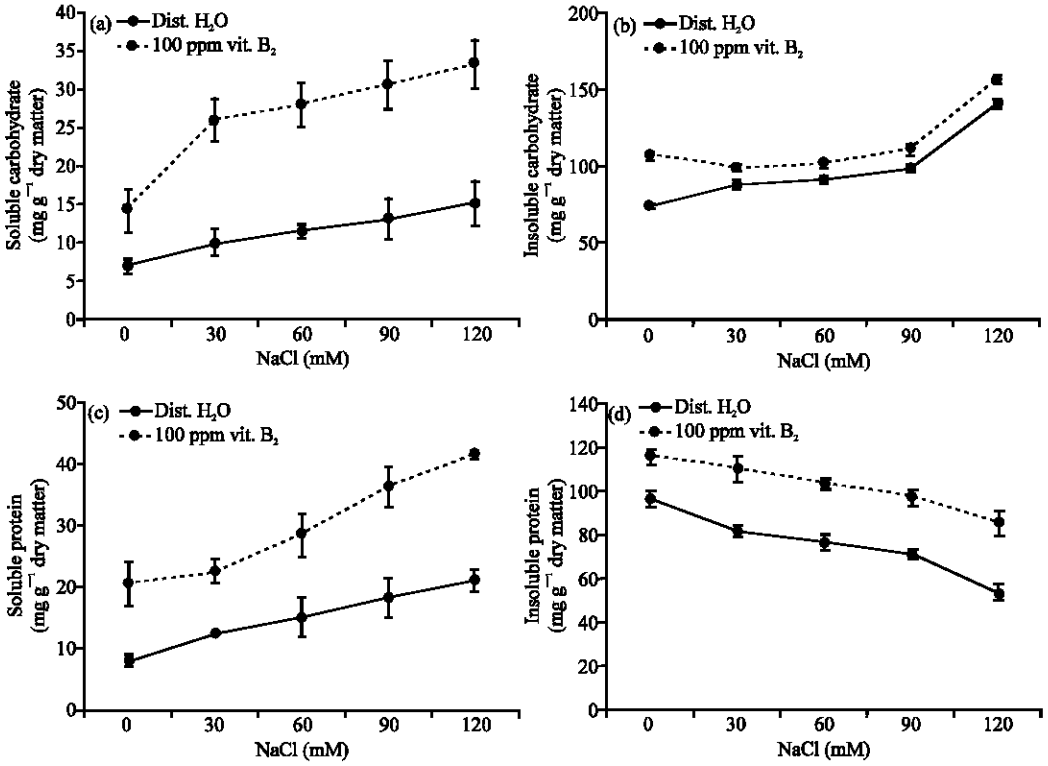

Fig. 2: (a-d) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on the contents of soluble carbohydrate (a), insoluble carbohydrate (b), soluble protein (c) and insoluble protein (d) of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

Salinity exhibited a non significant change on the content of total free amino acids, while stimulatory effect was observed on the accumulation of proline (Fig. 3a, b). The accumulation of proline was more obvious (about 3-fold) at the highest level of salinity as compared to the control (0.0 NaCl). Proline is one of the most accumulated osmolytes and acts as osmoprotectant in plants under salinity stress (Tripathi *et al*., 2007). It can also functions as a hydroxyl radical scavenger, stabilizer cell membranes by interacting with phospholipids (Jain *et al*., 2001). Present results, together with the other reports, suggest that the accumulation of proline observed in *H. sabdariffa* L. might function as a source of solute for intracellular osmotic adjustment and stabilizer cell membranes in salt stress response. Foliar spraying with 100 ppm vit. B_2 solution exhibited stimulatory effect on the accumulation of proline, while the contents of total free amino acids were reduced. This indicates that, the increase in proline could be at the expense of other free amino acids. This may be due to the promotion effect of vitamin B_2 in the conversion of the free amino acid into proline. Further, the reduction of total free amino acids as a result of vitamin B_2 treatment might be due to the role of vitamin in enhancing the incorporation of free amino acids into protein or iso-enzymes in order to increase salt tolerance. The accumulations of organic solutes (soluble carbohydrate, protein and proline) have been proved to be helpful in osmoregulation in plant species, playing an important role in tolerance to salinity stress (Bartels and Sunkar, 2005), these conclusions are confirmed with the results in the present study.

The obtained results (Fig. 4a, b, e and f) showed that under salinity stress Na^+ was sharply accumulated, while K^+ concentration as well as K^+/Na^+ and Ca^{++}/Na^+ ratios were significantly decreased as salinity levels increased. The inhibitory effects of salinity in the ratios of K^+/Na^+ and Ca^{++}/Na^+ were about 5 and 4-fold, respectively at the highest (120 mM NaCl) salinity level when compared with control. On the other hand, Ca^{++} and total cations remained relatively constant at most of the salinity levels used (Fig. 4c, d). These results are in conformity with the results obtained by Yildirim *et al*. (2006), Roussos *et al*. (2007) and López *et al*. (2008). They found that under salinity stress the uptake of Na^+ was increased, while the contrary was observed with respect to K^+. The increase of Na^+ and the reduction in K^+ content has been interpreted as resulting from competition between K^+ and Na^+. The antagonistic relation between Na^+ and K^+ indicated that, the high levels of Na^+ generated a kind of competition on the level of sites of K^+ absorption and thus limited the absorption of K^+ (Rejili *et al*., 2007). The high content of Na^+ could disrupt the nutrient balance, thereby causing specific ion toxicity despite disturbing osmotic regulation, while the reduction in K^+ concentration could inhibit growth by reducing the capacity for osmotic adjustment and turger maintenance or by adversely affecting metabolic functions (Ashraf and Harris, 2004; López *et al*., 2008). In respect to the slightly effects of salt stress on the contents of Ca^{++} and total cations; there are many reports on different plant species that the concentration of some basic elements was not affected by salinity (Chen *et al*., 2001). Foliar spraying with vit. B_2 resulted in reducing Na^+ and K^+ contents, while favouring Ca^{++} and consequently the Ca^{++}/Na^+ ratio became comparable with untreated salinized seedlings. It suggests a differential regulation of ion transport by foliar application of vitamin. The influence of riboflavin (vit. B_2) on the mechanism of uptake of ions may be related to its effect on membrane permeability and rate of ion entry through the membrane. Further, increasing the content of

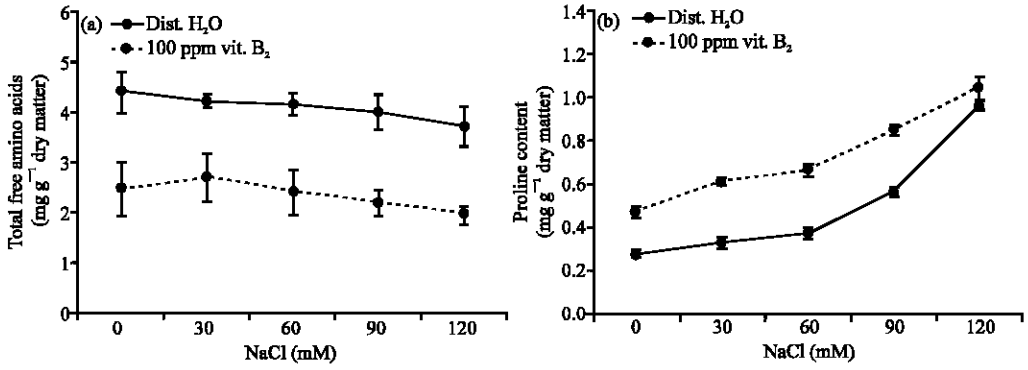

Fig. 3: (a, b) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on total free amino acids (a) and proline (b) contents of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

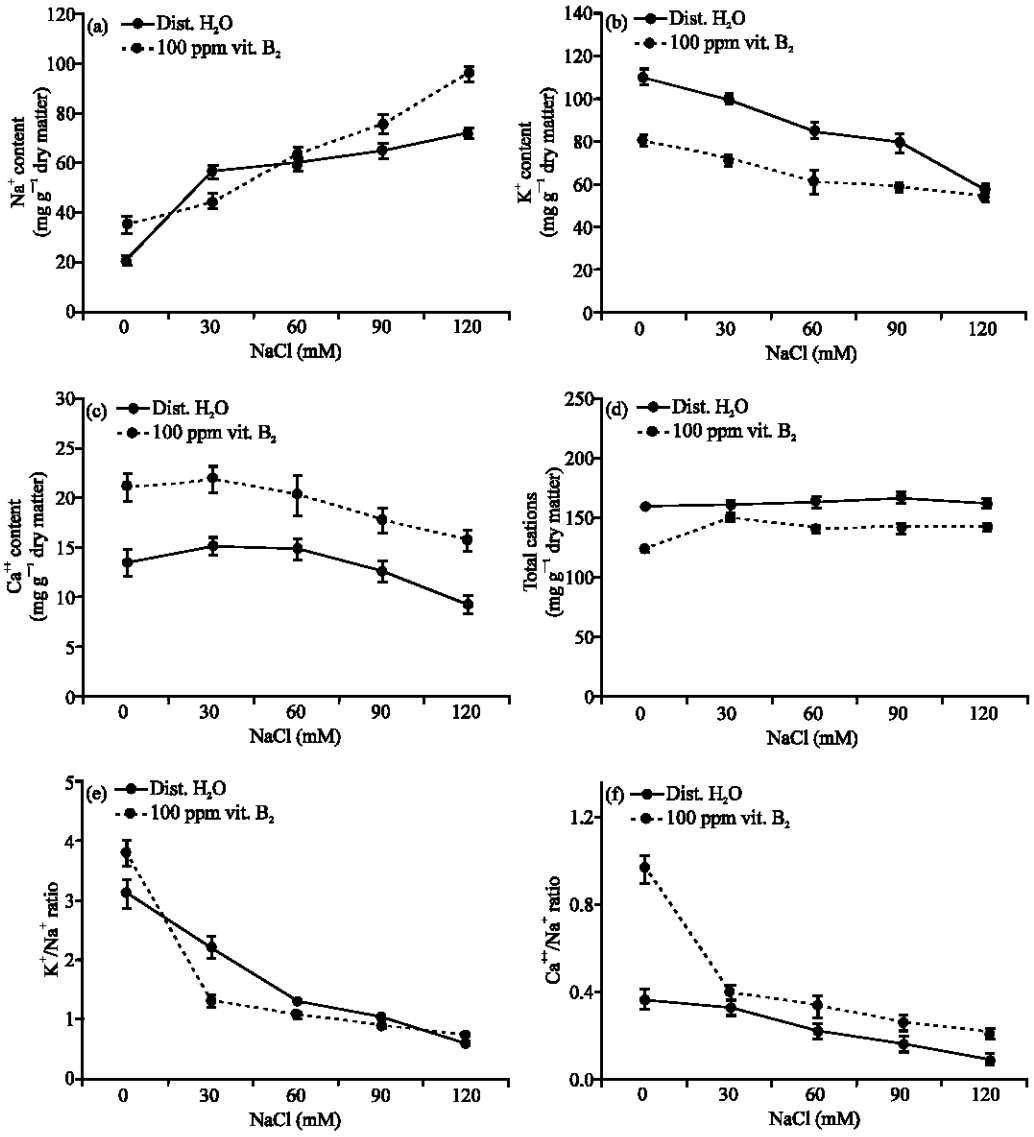

Fig. 4: (a-f) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on the contents of Na^+ (a), K^+ (b), Ca^{++} (c), total cations (d), the ratio of K^+/Na^+ (e) and Ca^{++}/Na^+ (f) of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

Ca^{++} that governs the tissue extensibility by maintaining the turgor of seedling tissues under stress conditions. So, it may be concluded that an increase in the concentration of Ca^{++} could ameliorate the deleterious effects of salinity on growth and yield (Sivritepe *et al*., 2003). The contents of total cations were slightly decreased, but they remained the same in salinized seedlings, when sprayed with vit.B_2. This leads to assume that an ameliorative function of riboflavin enrichment over NaCl toxicity and vit. B_2 may be involved in the maintaining of these cations in adequate amounts to enhance the metabolic process (Azooz *et al*., 2002).

Lipid peroxidation which measured as the content of malondialdehyde (MDA), has often been used as indicator of salt-induced oxidative damage in membranes by Hernández and Almansa, 2002. Membrane stability index has been used to assess tolerance of various plant species (Sudhakar *et al*., 2001). The present results (Fig. 5a, b) are parallel to these observations. The results showed that, there were no significant differences in MDA content and membrane stability index (MSI) which was estimated as electrolyte leakage at low and moderate levels of salinity while a significant increase in MDA and decrease in MSI were observed at higher salinity levels,

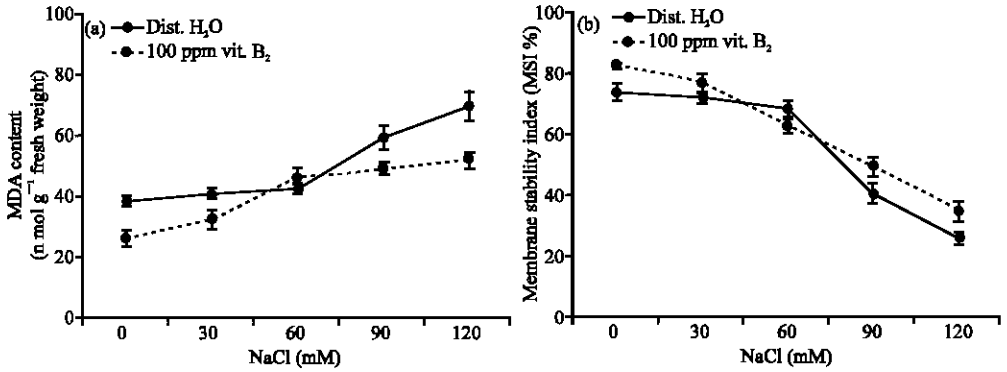

Fig. 5: (a, b) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on malondialdehyde (MDA) content (a) and the percentage of membrane stability index (MSI%) (b) of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

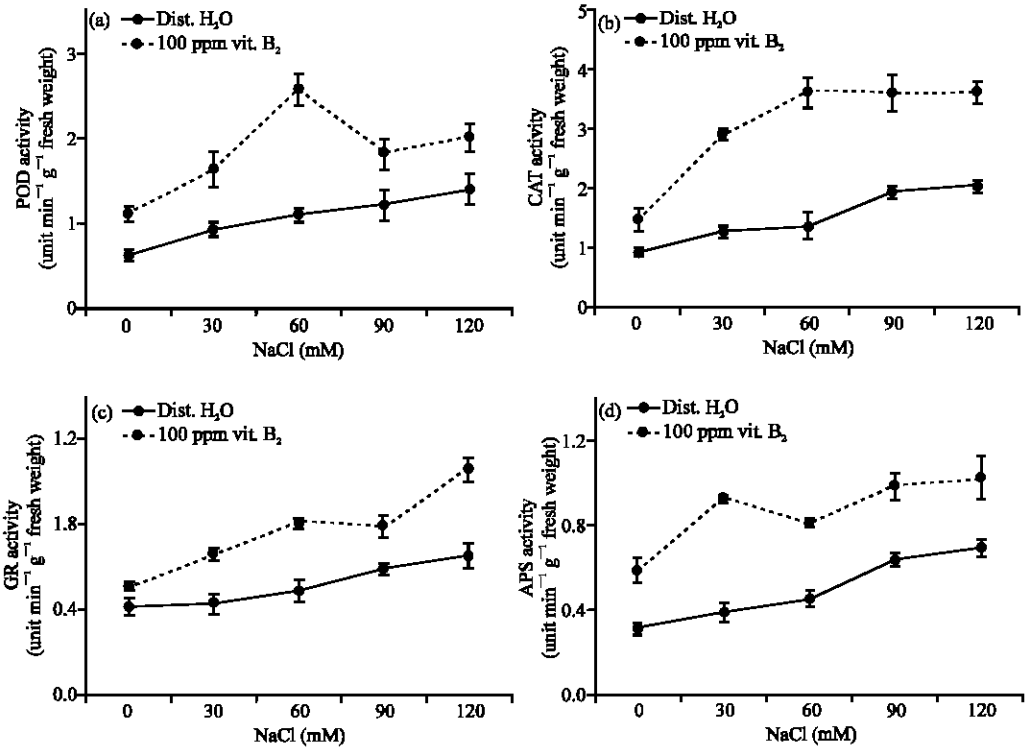

Fig. 6: (a-d) Effect of salinity treatments alone and with foliar application of 100 ppm riboflavin (vit. B_2), on the activities of peroxidase (POD) (a), catalase (CAT) (b), glutathione reductase (GR) (c) and ascorbic peroxidase (APX) (d) of *Hibiscus sabdariffa* L. seedlings. Each value is the mean of three replicates. Vertical bars represent ±SD

as compared with unsalinized plants (0.0 NaCl). These results indicated that *H. sabdariffa* L. seedlings had adapted to salt stress at low and moderate salinity levels. On the other hand, membrane stability index was considerably increased, while the content of MDA was significantly decreased, as a result of foliar application of riboflavine. These increases in MSI might be related to induction of antioxidant responses that protect the plant from oxidative damage, which also coincided with a decrease in the level of MDA.

Salt-stressed seedlings (Fig. 6a-d) showed higher activities of peroxidase (POD) and catalase (CAT) at all salinity levels used than those of non-stressed seedlings. On the other hand, no significant changes were observed

on the activity of glutathione reductase (GR) and ascorbic peroxidase (APX) at low and moderate salinity levels, while their activities were significantly increased at higher levels of salinity. The activity of antioxidant enzymes was reported to increase under salinity in rice (Lee *et al*., 2001), sesame (Koca *et al*., 2007) and wheat (Athar *et al*., 2008). Foliar application of 100 ppm vit. B_2, significantly increased the activity of these enzymes as compared with untreated seedlings subjected only to salinity stress.

The observed increase in the activity of antioxidant enzymes of *H. sabdariffa* L. seedlings could increase their abilities to scavenge O_2^- radicals, which could cause membrane damage, while at higher levels of salinity, it seems that such resistance to oxidative stress may be overcome leading to growth reduction (Agarwal and Pandey, 2004). In the present study, salinity induced enhancement of CAT and POD activity. This increase is supposed to be an adaptive trait possibly helping to overcome the damage of tissue metabolism by reducing toxic level of H_2O_2 produced during cell metabolism and protection against oxidative stress (Agarwal and Pandey, 2004; Koca *et al*., 2007). The increase in GR activity might enhance tolerance to oxidative stress and maintain a high ratio of glutathione reduced (GSH)/ glutathione oxidized (GSSG) which is required for the regulation of ascorbate level and activation of several CO_2 fixing enzymes (Noctor and Foyer, 1998; Verma and Mishra, 2005). Ascorbic peroxidase plays a crucial role in detoxification of cellular H_2O_2 (Tsai *et al*., 2005). Thus, the increase in APS at the higher concentrations of salinity might decrease the level of H_2O_2 in *H. sabdariffa* L. seedlings (Sairam *et al*., 2005). Both constitutive and salt induced increase in antioxidant enzyme activities is important for providing protection against ROS and the salt stress induced increase in oxidative stress actually decides the tolerance level of a plant.

CONCLUSION

The results of this investigation demonstrated that salinity stress especially at higher levels led to a severe oxidative stress on osmotic imbalance of *H. sabdariffa* L. seedlings. Foliar application of riboflavin (vit. B_2) improved the resistance of *H. sabdariffa* L. seedlings to salt stress and the improvement was associated with increasing the activity of antioxidant enzymes, membrane stability index (MSI) and accumulation of osmotically soluble sugar, protein and proline. These protective mechanisms had a cumulative effect in protecting membranes and maintenance of cell form and structure from the harmful effects of salt stress. So, it can be suggested that, *H. sabdariffa* L. seedlings can be grown under mild salt stress along with foliar application of 100 ppm riboflavin (vit. B_2).

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