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Full Length Research Paper

Exogenous ascorbic acid increases resistance to salt of *Silybum marianum* (L.)

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Salinity stress has negative effects on agricultural yield throughout the world, affecting production whether it is for subsistence or economic gain. This study investigates the inductive role of vitamin C and its application mode in mitigating the detrimental effects of irrigation with diluted (10, 20 and 30 %) NaCl + water on *Silybum marianum* L. plants. The results show that 10% of salt water exhibited insignificant changes, while the higher levels impaired growth by reducing seed germination, dry weights of shoot and root, water status and chlorophyll contents. However, irrigation with salt water enhanced carotenoids and antioxidant enzyme activities. The detrimental effects of salt water were ameliorated by application of 100 ppm ascorbic acid (vitamin C). The inductive role of vitamin was associated with the improvement of seed germination, growth, plant water status, carotenoids, endogenous ascorbic acid and antioxidant enzyme activities. Moreover, vitamin C alone or in combination with 30% NaCl water increased the intensity of protein bands as well as synthesized additional new proteins with molecular weights of 205, 87, 84, 65 and 45 kDa. This could increase tolerance mechanisms of treated plants towards water salinity.

Key words: Salinity, stress, vitamin C, antioxidant, NaCl, enzyme.

INTRODUCTION

Soil salinity is a major constraint to food production because it limits crop yield and restricts use of previously uncultivated land. Seed germination, one of the most critical phases in plant life, is greatly affected by salinity (Abo-Kassem, 2007), which either induces a state of dormancy at low levels or completely inhibits germination at higher levels (Iqbal et al., 2006). Pahlavani et al. (2006) proved that genetic information regarding seed germination could help to improve seedling emergence in saline soil through breeding programs. Increasing sodium concentration in plant tissue can increase oxidative stress, which causes deterioration in chloroplast structure and an associate lose in chlorophyll. This leads to a decrease in chlorophyll, while increasing carotenoids content (Khosravinejad and Farboondia, 2008). Furthermore, reactive oxygen species (ROS) like superoxide, hydrogen peroxide and hydroxyl radicals are generated (Wahid et al., 2007). ROS are highly reactive in the absence of any protective mechanism. They can seriously disrupt normal metabolism through oxidative damage to essential membrane lipid, proteins and pigments (Di – Baccio et al., 2004; Çakmak, 2005). To scavenge ROS, Mittler (2002) showed that plants synthesize different types of defense system composed of non-enzymatic antioxidants, such as ascorbic acid and enzymatic antioxidants like catalase (CAT), peroxidase (POD), ascorbate peroxidase (AP) and glutathione reductase (GR). Scavenging system has a potential to quench ROS in stress tolerance plants (Koca et al., 2007; Sairam et al., 2005). Osmotic adjustment is the cellular response to turgor reduction. The cytosolic and organellar machinery of glycophytes and halophytes are equivalently sensitive to Na^+ and Cl^- ; therefore, osmotic adjustment is achieved in these compartments by accumulation of compatible osmolytes and osmo-protectants (Bohnert, 1995; Bohnert and Jensen, 1996). However, Na^+ and Cl^- are energetically efficient osmolytes for osmotic adjustment and are compart-mentalized into the vacuole to minimize cytotoxicity (Bressan et al., 1995). The adaptation to salinity stress is accompanied by alterations

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in the level of protein patterns. Salinity induces the synthesis of salt stress-specific proteins. Some of these proteins were suggested to protect the cell against the adverse effect of salt stress. Vitamins were generally found to affect gene expression. They induced the synthesis and increased the amount of the original proteins which were already present in the control plants, as well as the appearance of additional new bands (Azooz, 2004; Bassuony et al., 2008; Beltagi, 2008). The significant increase in the intensity of the original bands appearing in the control indicates that vitamins have profound effects on the qualitative and quantitative changes in the protein component of these plants, which might be linked with improvement of their growth and productivity.Vitamin C is a small and water-soluble antioxidant molecule that acts as a primary substrate in the cyclical pathway for detoxification and neutralization of superoxide radicals and singlet oxygen (Noctor and Foyer, 1998). Many studies reported that the optimal concentration of vitamin C exhibited beneficial effect on growth and yield of some crop plants grown under saline conditions (Azooz, 2004; Khan et al., 2006; Bassuony et al., 2008). They reported that ascorbic acid (vitamin C) can play an inductive role in alleviating the adverse effect of salinity on plant growth and metabolism in many plant. So, the main objective of this study was to investigate the inductive role of 100 ppm vitamin C solution either before (seed soaking) or after (shoot spraying) cultivation on seed germination, growth, water status, antioxidant enzymes and protein patterns of *Silybum marianum* (L) Gaertner plants under irrigation with diluted NaCl.

S. marianum (L) Gaertner plant, also called milk thistle, is an annual or biannual plant of the Asteraceae family. This fairly typical thistle has red to purple flowers and shiny pale green leaves. It is used in cases of liver diseases (cirrhosis, jaundice and hepatitis) and gallbladder disease, and is claimed to protect the liver against poisons. Silibinin (syn. silybin, sylimarin I) is a hepatoprotective (anti-hepatotoxic) antioxidant (radical-scavenging agent), thus stabilizing and protecting the membrane lipids of the hepatocytes (liver cells). Silicristin inhibits the enzymes peroxidase and lipoxygenase. Silidianin is a plant growth regulator. A study implemented in 2000 and making such claims by the Agency for Healthcare Research and Quality (AHRQ) concluded that "clinical efficacy of milk thistle is not clearly established". However, a more recent study did show the activity against liver cancers. Cochrane's review in 2005 considered 13 randomized clinical trials which assessed milk thistle in 915 patients with alcoholic and/or hepatitis B or C virus liver diseases.

They questioned the beneficial effects of milk thistle for patients with alcoholic and/or hepatitis B or C virus liver diseases and highlighted the lack of high-quality evidence to support this intervention. Cochrane concluded that better quality of randomized clinical trials on milk thistle versus placebo is needed.

MATERIALS AND METHODS

Plant material growth and treatment condition

This experiment was sown in trays containing vermiculite and daily irrigate with different levels (10, 20 and 30%) of NaCl + water and 100 ppm vitamin solution on seeds of *S. marianum* (L.) Gaertner. Plant transpiration rate was estimated as described by Bozcuk (1975). Relative water content (RWC) of leaves was determined according to Smart (1974).

Photosynthetic pigments

Chlorophyll (chl a and b) and carotenoids contents in leaves were estimated in 80% acetone extracts according to Lichtenthaler and Wellburn (1983).

Analyses of antioxidant enzymes activities

Assay of catalase activities

The reaction mixture 1.5 mM Na- ethylenediaminetetraacetic acid (EDTA) consists of 50 mM phosphate buffer (pH 7.6 0.1 ml 100 mM H_2O_2 and enzyme extract at 340 nm for 1 min established as enzyme activity (Çakmak and Marschner, 1992).

Assay of ascorbate peroxidase activities

Total ascorbate peroxidase activity was assayed according to Nakano and Asada (1981). The reaction mixture (1.5 ml) contained 50 mM phosphate buffer (pH 6.0), 0.1 µM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 and 50 µL enzyme extract. The reaction was started by the addition of H_2O_2 and ascorbate oxidation measured at 290 nm for 1 min. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mM^{-1}) and the results were expressed in µM H_2O_2 $min^{-1}g^{-1}$ dry mass (DM), taking into consideration that 2 mol ascorbate are required for reduction of 1 mol H_2O_2 (McKersie and Leshem, 1994).

Assay of glutathione reductase *(GR) activities*

Total GR activity was assayed as described by Foyer and Halliwell (1976) with minor modification. The reaction mixture (1.0 ml) consisted of 100 mM phosphate buffer (pH 7, 8) 0.1 µM EDTA, 0.05 mM NADPH, 3.0 mM oxidized glutathione (GSSG) and 50 µL enzyme extract. The reaction was started by the addition of GSSG and the NADPH oxidation rate was monitored at 340 nm for 1 min. Enzyme activity was determined using the molar extinction coefficient for NADPH (6.2 mM^{-1} cm^{-1}) and expressed as µmol $NADPHmin^{-1}mg^{-1}DM$.

Assay of superoxide dismutase (SOD) activities

Total SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) as described by Gianopolitis and Reis (1977). The reaction mixture (1.5 ml) contained 50 mM phosphate buffer (pH 7.8), 0.1 µM EDTA, 13 mM methionine, 75 µM NBT, 2 µM riboflavin and 50 µL enzyme extract. Riboflavin was added last and tubes were shaken and illuminated with a two 20-W fluorescent tubes. The reaction was allowed to proceed for 15 min after which the lights were switched off and the tubes covered with a black cloth. Absorbance of the reaction mixture was read at 560 nm. One unit of the defined

amount of enzyme is required to cause 50% inhibition of the NBT photoreduction rate and results were expressed as SOD activity mg^{-1}DM.

Statistical analysis

All the data were statistically analyzed by one-way analysis of variance (ANOVA). The least significant difference (LSD) method was used to test the difference between treatments and $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS packet software

RESULTS AND DISCUSSION

The germination percentage seeds under different levels of NaCl+water irrigation (Figure 1) was unaffected at 10% NaCl. However, a significant decrease at the higher levels was recorded. The maximal germination percentage was 30% NaCl as compared with control. Seeds soaked in 100 ppm vitamin C increased their percentage of germination. It was noticeable that the inhibitory effect imposed by NaCl irrigation was completely alleviated at the mild (20%), while at the highest (30%) NaCl+water level, the maximal germination percentage was 83.3%. The inhibitory effect of NaCl+water on seed germination may be partially osmotic due to declining solute potential or ion toxicity due to accumulation of some ions in the seeds, which can alter some physiological processes such as enzyme activation (Croser et al., 2001; Hajer et al., 2006; Jaleel et al., 2007). Abo-Kassem (2007) reported that high salinity delayed radical emergence and decreased germination percentage. The improvement effect of vitamin C on germination proved the success of using vitamin C as pretreatment of *S. marianum* (L) Gaertner seeds to reduce the inhibitory effect of stress on their germination. These positive results of vitamin C on seeds germination were reported by Shaddad et al. (1990) and Arab and Ehsanpour (2006). Arrigoni and De Tullio (2000) reported that exogenous ascorbic acid increased the level of ascorbic acid NaCl+water uptake by different tissues. The additional vitamin C is associated with the partial inhibition of ROS production (Shalata and Neumann, 2001). So, it can be concluded that, the inductive role of vitamin C in seed germination is attributed to its antioxidant activity.

Changes of protein patterns by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were also analyzed in germinated seeds of *S. marianum* (L) Gaertner (Figure 2), to follow any possible alterations in gene expression in these seeds as a result of seeds treatment with 30% alone, NaCl + water vitamin C alone or both in comparing with control (seeds germinated in tap water). Protein bands indicated the presence of about 14 polypeptides with apparent molecular weights ranging from 6.5 to 205 kDa. Seeds germinated in 30% NaCl+water (lane 2) showed that NaCl+water salinity enhanced the synthesis of most original proteins which were already present in control seeds (lane C), especially, 55, 36, 29, 24, 20, 14 and 6.5 kDa polypeptides as well as synthesis of additional five new proteins with molecular weight of 205, 87, 84, 65 and 45 kDa. Soaking of seeds in vitamin C elevated the levels of proteins in most bands of both seeds germinated either in 0 (control) or 30% NaCl+water. Further, in seeds germinated in 30% NaCl + water, vitamin C (lane 3) also, resulted in appearance of five new proteins with molecular weight of 205, 87, 84, 65 and 45 kDa, respectively. In addition, the protein band 0 which had disappeared in seeds germinated in 0% NaCl+water and vitamin C; reappeared. Similar results were reported by Azooz (2004), Kassim and Dowidar (2006) and Beltagi (2008).

The changes in protein profile may be due to adaptation of *S. marianum* (L) Gaertner seeds to NaCl+water stress. The new bands of proteins in seedlings germinated in NaCl+water or in combination with vitamin C may be due to *de novo* synthesis of new protein (Gopala et al. 1987; Azooz, 2004) . Bassuony et al. (2008) has shown that vitamin treatments induces a significant alterations in the enzymes related to protein metabolism; which indicates that vitamins might act as activators of protein synthesis. The new bands and the significant increase in the intensity of *S. marianum* (L) Gaertner as well as the original bands appearing in the control indicate that vitamin C has stimulatory effect on the protein component, which might be linked with the improvement of seed germination and growth. Therefore, it can be suggested that the new proteins which appeared in seedlings germinated in 30% NaCl+water alone or with vitamin C and did not appear in untreated seedlings (control), may play an inductive role in triggering a special system helping seeds to tolerate NaCl+water stress and increase their capacity to germinate.

Fresh and dry weights of root and shoot (Figures 3a and b), and water status in terms of water content (WC), RWC of leaves and transpiration (Figures 4c and d) of *S. marianum* (L) Gaertner plants, exhibited variations as a result of NaCl+water irrigation, and compared with the control, no significant differences were found in growth parameters (fresh and dry weights of root and shoot) and water status of plants irrigated with 10% NaCl+water. Moreover, stimulation effects on dry weight of shoot (Figure 3d) and relative water content of leaves (Figure 4c) were recorded. However, a significant decrease was observed at the higher NaCl+water levels. The growth parameters yields of tested plants appeared to be positively correlated with their WC and RWC. NaCl+water salinity caused more inhibition in roots growth than in shoots. So, root/shoot ratios (on the basis of fresh weight) were increased with increase of NaCl+water level. Kaya et al. (2003) reported that the root growth was more sensitive and adversely affected as compared to shoot growth under salinity conditions. Reduction in plant growth as a result of NaCl+water stress has also been

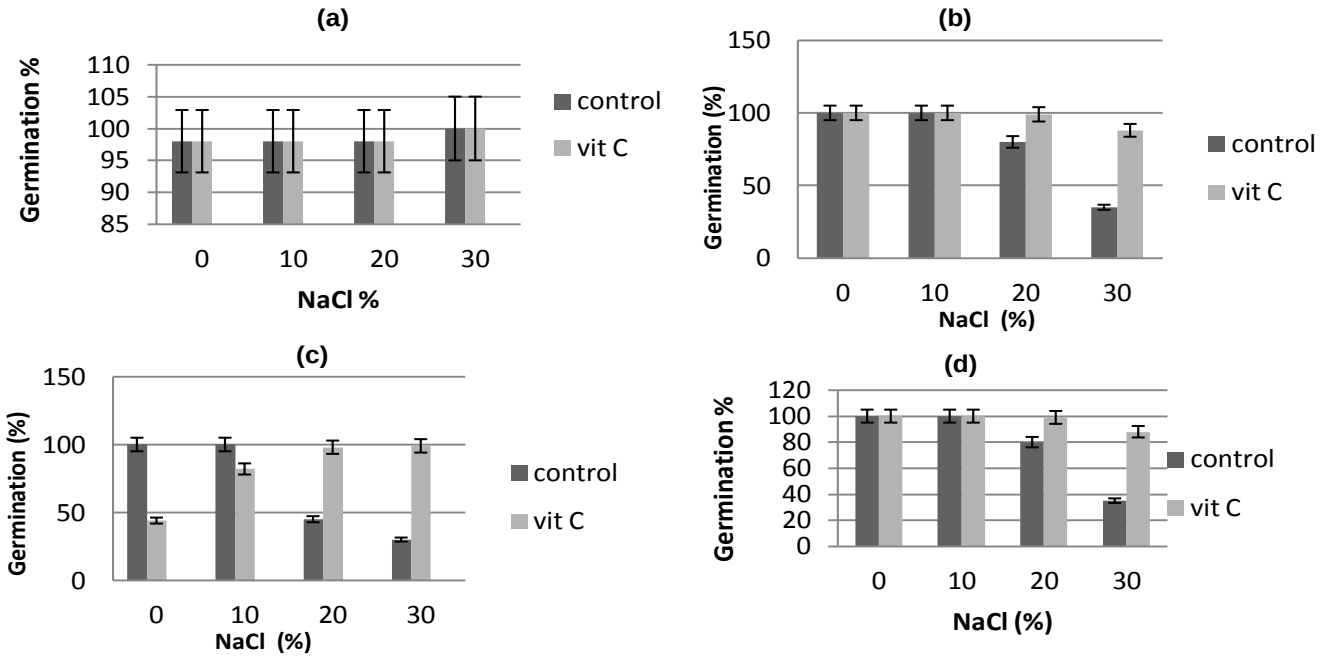

Figure 1. Effect of different NaCl+water levels (%) on percentage germination of *Silybum marianum* (L) Gaertner plants seeds after being soaked for 8 h in 100 ppm vitamin C and air dried. (a) 1st, (b) 2nd, (c) 3rd and (d) 4th week. Vertical bars represent ±SD.

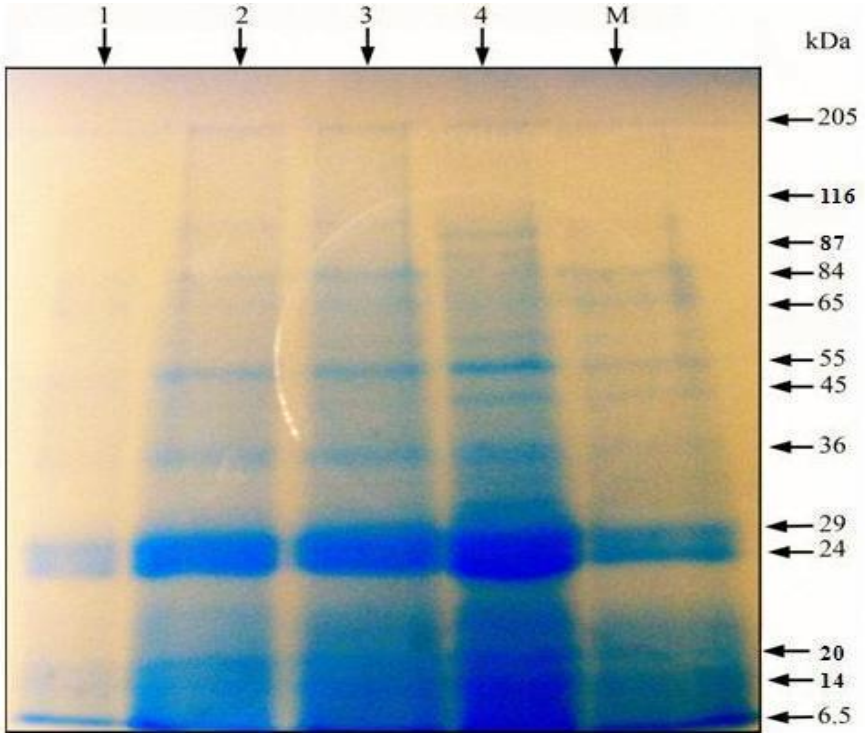

Figure 2. Analysis of protein patterns by one-dimensional SDS-PAGE extracted from germinated seeds of *Silybum Marianum* (L) Gaertner PIants in 30% NaCl + water and/or 100 ppm vitamin C solution. M, Marker protein (6,5 to 205 kDa); lane 1, control (seeds germinated in tap water only); lane 2, seeds germinated in NaCl 30%; lane 3, seeds soaked in 100 ppm vitamin C and seeds germinated in tap water (0.0% NaCl + water); lane 4, seeds soaked in 100 ppm vitamin C and germinated in 30% NaCl + water. Least significant difference (LSD) = 5%. SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

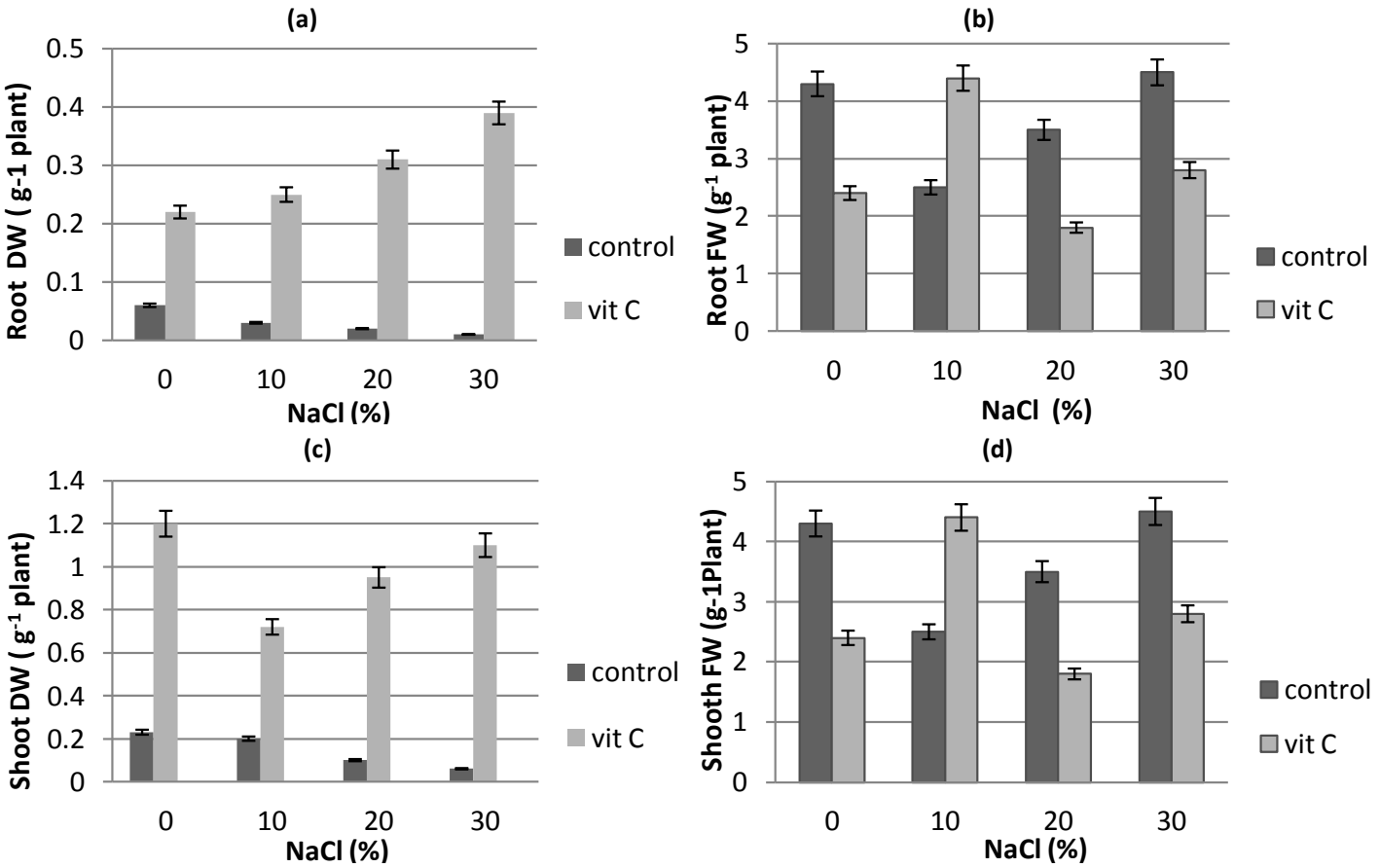

Figure 3. Effect of vitamin C (100 ppm) on seeds after being soaked for 8 h in 100 ppm vitamin C and air dried. (a) Fresh weight and (b) dry weight of root, (c) fresh weight and (d) dry weight of shoot of *Silybum marianum* (L) Gaertner plants grown under different levels of NaCl + water. Vertical bars represent ±SD

reported earlier in several plants (Hajer et al., 2006; Alqurainy, 2007; Long et al., 2008). Increase in NaCl+water level reduced the absorption of water leading to a drop in water content of tested plants. Thus, the inhibitory effect of NaCl+water on growth parameters could be attributed to the osmotic effect of NaCl+water salinity (Salter et al., 2007). In addition, the changes in water status under NaCl+water stress may cause a reduction in meristem activity as well as cell elongation (Shah, 2007).

The adverse effects of NaCl+water salinity on the growth parameters, WC and RWC were mitigated by seed 100 ppm vitamin C. These results are in coincidence with that cited by Azooz (2004), Alqurainy (2007) and Athar et al. (2008). They suggested that vitamin C could accelerated cell division and cell enlargement of treated plants. Shoot spraying with vitamin C was more effective in improving growth parameters of treated plants, which was associated with increasing the WC and RWC of leaves and reduction in transpiration rate. This indicates that shoot spraying probably reflects the efficiency of water uptake and utilization or reduces water loss which consequently causes a concomitant increase in leaf water potential. Hence, it can be concluded that the beneficial effect of vitamin C on growth parameters of *S. marianum* (L) Gaertner has been related to the efficiency of their water uptake and utilization. These suggestions are in a good agreement with present results, which showed that the increase of WC and RWC was associated with a decrease in transpiration rate. Further, it could be suggested that the effectiveness of vitamin C depends on its mode of application, which may enhance the endogenous level of vitamin C and water status of treated plants.In addition, the photosynthetic pigments of *S. Marianum* (L) Gaertner leaves (Figures 5a to c) were substantially affected under NaCl+water irrigation. The content of chl. a and chl. b was more or less unchanged under 10% NaCl+water level, while, at higher levels of Na+water; a significant decrease was observed. On the other hand, the content of carotenoid was increased at low and moderate NaCl+water levels as compared with control. The reduction in chl. b was higher (about 44%) than chl. a, (about 30%) below the control at the highest

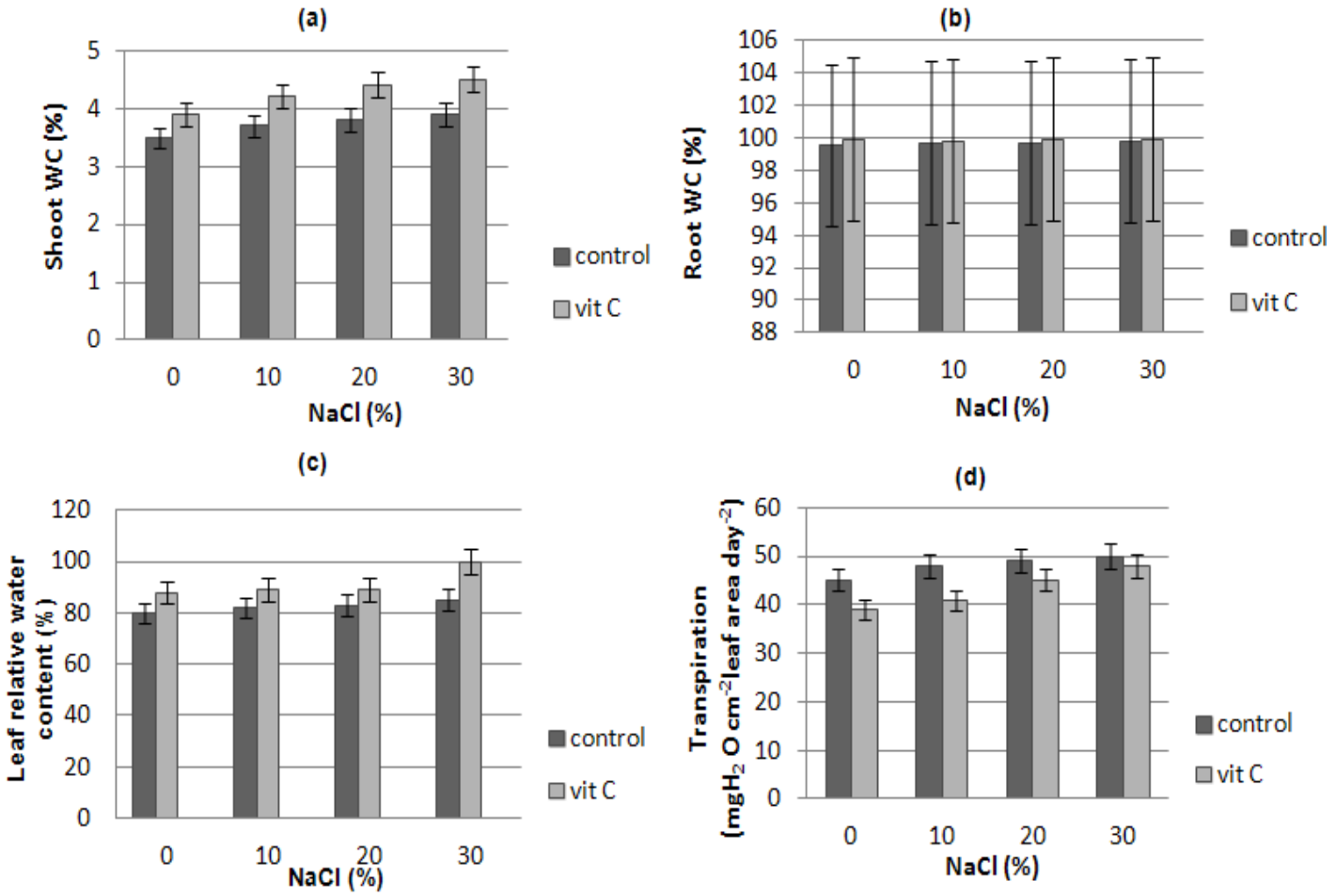

Figure 4. Effect of vitamin C (100 ppm) treatments either by seed soaking on water content (%) of (a) root and (b) shoot, (c) leaf relative water content (%) and (d) transpiration rate of *Silybum marianum* (L) Gaertn plants grown under different levels of sea water. Vertical bars represent ±SD.

NaCl+water level, resulting in a higher chl. a/chl. b . The inhibitory effect of NaCl+water stress on photosynthetic pigments was completely alleviated as a result of vitamin C treatments. Moreover, the values of pigments were higher than those of control plants at most NaCl+water levels used. These results reinforce the results obtained by Shah (2007) and Beltagi (2008). The reduction observed in chlorophyll content under NaCl+water irrigation could be as a result of inhibition of chlorophyll biosynthesis or increased of its degradation (Khan et al., 2006). Furthermore, under NaCl+water stress, an oxidative stress could result, which causes deterioration in chloroplast structure. This leads to a decrease in chlorophyll content, while carotenoid content increased (Khosravinejad and Farboondia, 2008). Carotenoids are known to act as efficient quenchers of free radical caused by ROS. Thus, increasing carotenoids in plants treated with NaCl+water and/or vitamin C could enhance the capacity of these plants to minimize the damage caused by ROS. Therefore, chlorophyll content of plants treated with vitamin C was increased, which could result from the protection effect of vitamin C and carotenoids to the photosynthetic apparatus from NaCl+water induced oxidative stress (Khan et al. , 2006) .An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, and in turn, these radicals can initiate chain reactions. When the chain reaction occurs in a cell, it can cause damage or death. Moreover, when the chain reaction occurs in a purified monomer, it produces a polymer resin, such as a plastic, a synthetic fiber, or an oil paint film. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E, as well as

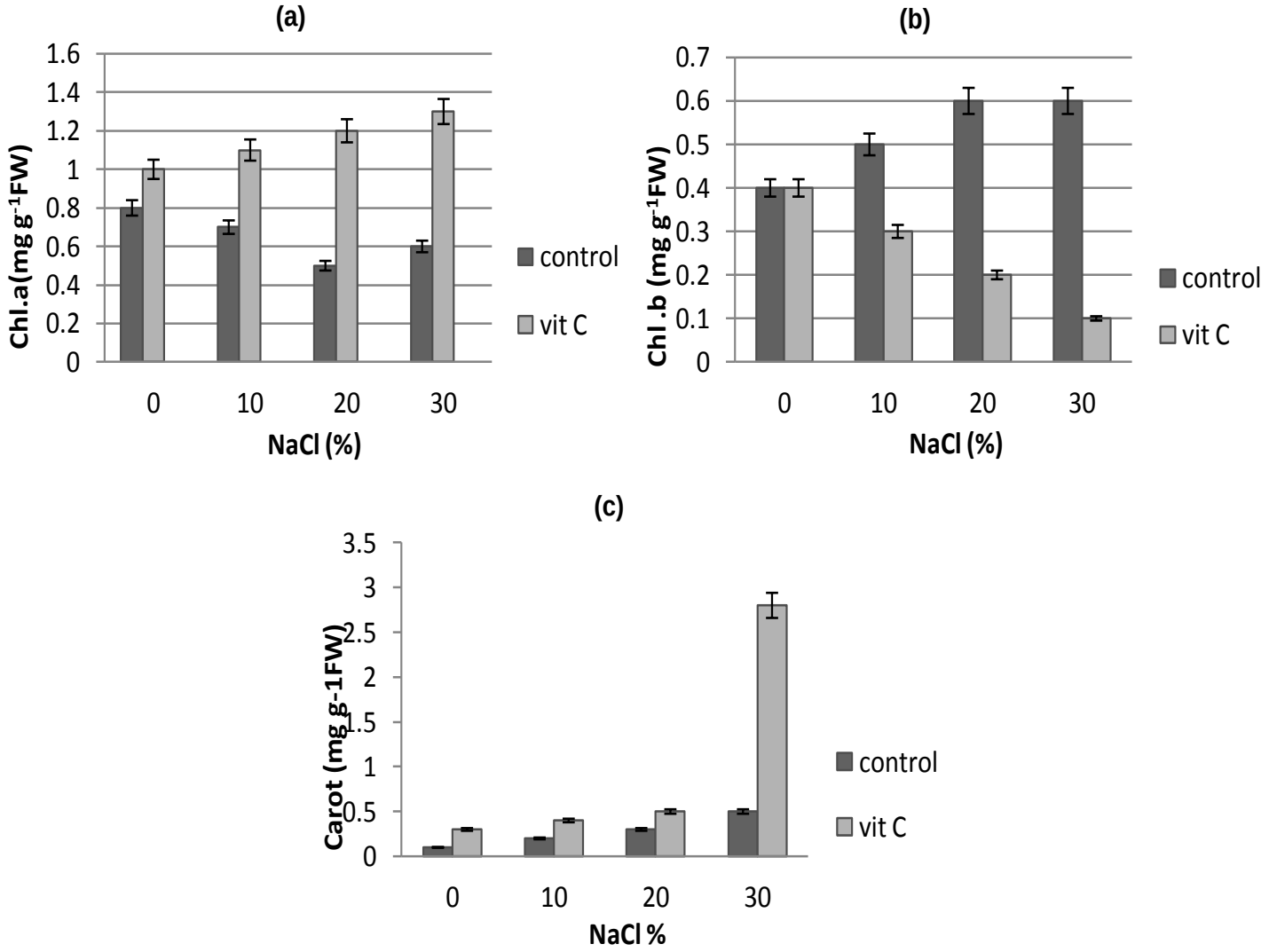

Figure 5. Effect of vitamin C (100 ppm) treatments either by seed soaking or shoot spraying on (a) chl a, (b) chl b, and (c) carotenoids of *Silybum marianum* (L.) Gaertn plants grown under different levels of NaCl + water. Vertical bars represent ± SD.

enzymes such as catalase, superoxide dismutase and various peroxidases (Figure 6). Low levels of antioxidants or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

Ascorbic acid or "vitamin C" is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. Since one of the enzymes needed to make ascorbic acid has been lost by mutation during primate evolution, humans must obtain it from the diet; it is therefore a vitamin.

Most other animals are able to produce this compound in their bodies and do not require it in their diets. Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. In other cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins.

Ascorbic acid is redox catalyst which can reduce, and thereby neutralize reactive oxygen species such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the redox enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants. Ascorbic acids present at high levels in all parts of plants and can reach concentrations of 20 millimolar in chloroplasts.Finally, it could be concluded that our results explain the inductive role played by vitamin C in overcoming the detrimental effects of NaCl+water and enhancing the capacity of treated plants to scavenge the free radicals produced as a result of NaCl+water stress. This was associated by improvement of plant growth, water status, carotenoids, endogenous vitamin C and antioxidant enzymes activities, especially AP and GR. Furthermore, vitamin C increases protein synthesis in germinated seeds, including *de novo* synthesis of new proteins and accumulation of certain existing proteins.

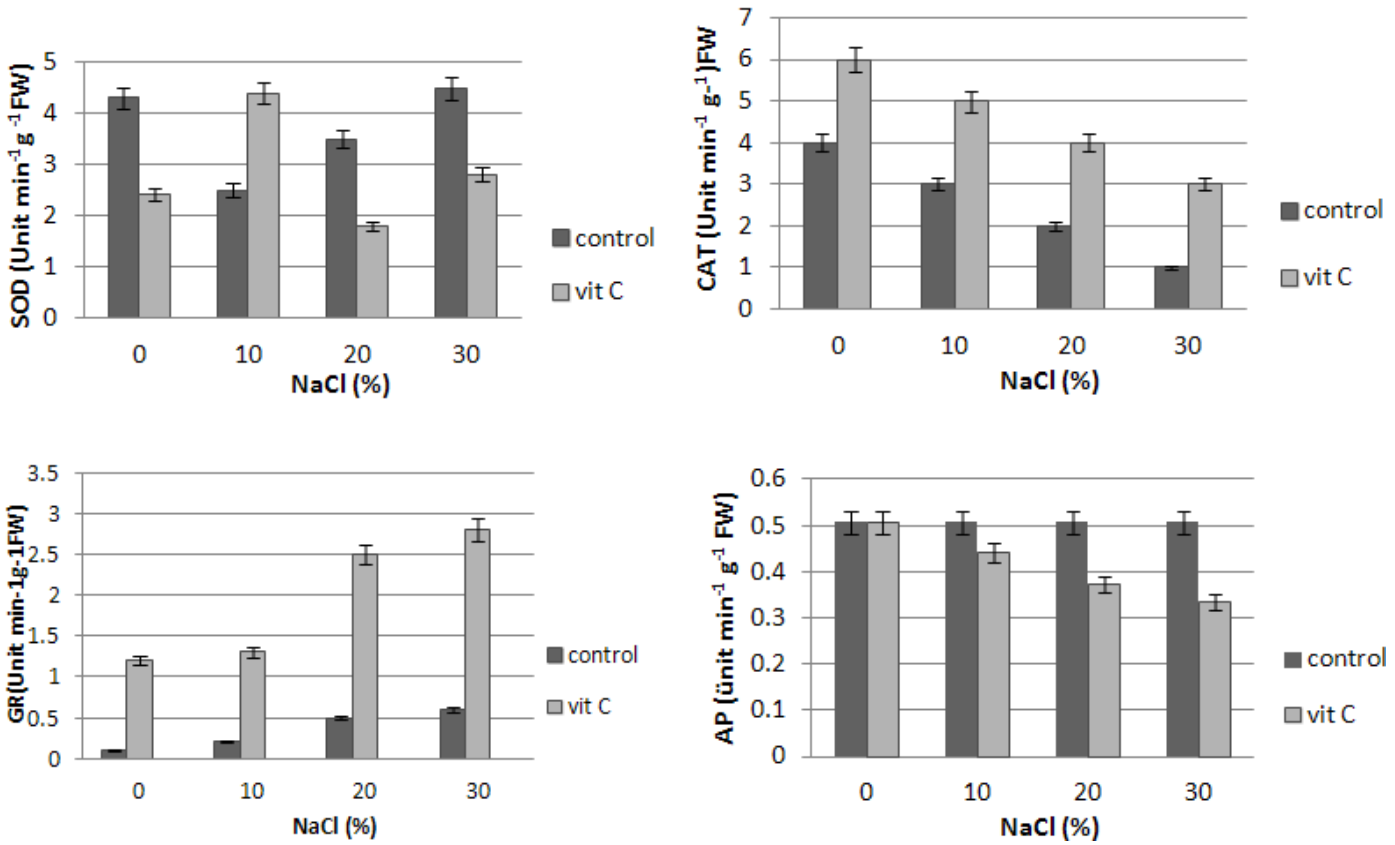

Figure 6. Effect of vitamin C (100 ppm) treatments either by seed soaking or shoot spraying on (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) glutathione reductase (GR) and (d) ascorbate peroxidase (AP) of *Silybum marianum* (L.) Gartner plants grown under different levels of NaCl + water. Vertical bars represent ±SD.

These findings indicate that plants treatment with vitamin C trigger some unknown physiological processes which subsequently lead to improvement of seed germination, growth and development of treated plants.

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