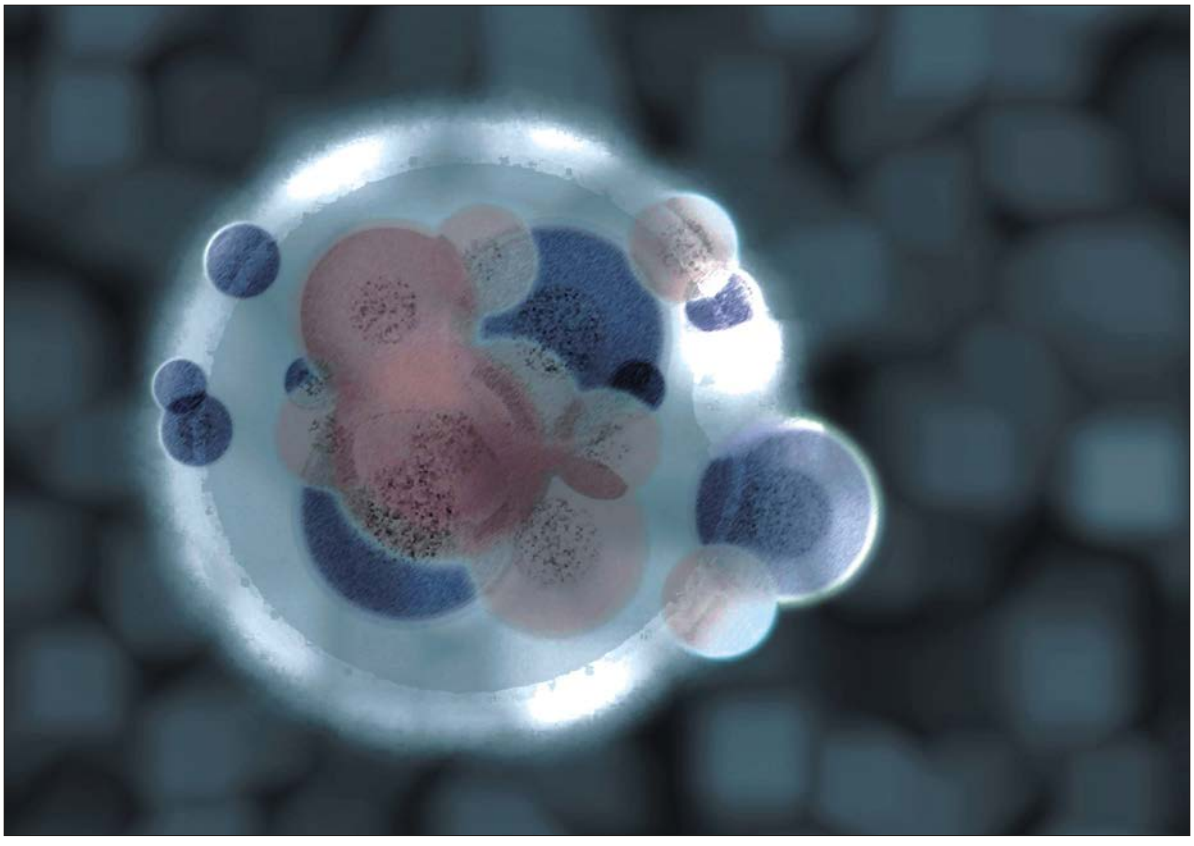

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Role of Glycinebetaine and Ascorbic Acid in the Alleviation of Salt-Stress Induced Micro-Morphological Damages in Sweet Pepper Seedlings

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Abstract: The effects of exogenously applied glycinebetaine or ascorbic acid (pre-soaking or pre-soaking plus spraying) on the salt-stress induced ultrastructural damages in sweet pepper seedlings were investigated. The seedlings grown in hydroponic culture containing nutrient solution for 4 weeks treated with NaCl at (0 and 6000 ppm). Salinity induced ultrastructural damages in leaf, the most notable changes were swelling of thylakoid membrane, disintegration of grana stacking and intergrana lamellae and showed a wavy configuration, increase the number of plastoglobuli and starch grains (number and size), mitochondria swelling, the number of mitochondria cristae was decreased (absent or often very short) and the cristae were observed only on one side of the bowl, but on the other side consisted of two membranes of the envelope and separated by a thin matrix layer. Moreover, the thickness of cell wall and the vacuolar volume or sizes were also increased as well as formation of the Myelin-Figures from plasma membrane. These damages were alleviated by the pretreatment with glycinebetaine or ascorbic acid (pre-soaking or pre-soaking plus spraying) at certain concentrations.

Key words: Sweet pepper, salinity, glycinebetaine, ascorbic acid, leaf ultrastructure

INTRODUCTION

Soil salinity is an important agricultural problem especially in Farm lands dependent on irrigation. It causes great losses to agriculture by lowering the yields of various crops. Salinity stress caused various physiological and biological changes in plants. It damaged photosynthetic components, inactivated protein and enzymes, destroyed cell membrane structure and permeability by causing lipid peroxidation and injuries to plant metabolism (Meneguzzo *et al*., 1998). Moreover, plant growth was severely affected by salt through morphological, anatomical, ultrastructural changes (Mitsuya *et al*., 2000).

The cellular organelles such as plasmamembrane, endoplasmic reticulum and mitochondria are known to be severely affected in response to adverse environmental conditions (Čiamprová and Mistrik, 1993).

Salt stress caused swelling the thylakoids, swelling stroma, accumulation of a large starch grains and lipid droplets more than the control plants and distortion of grana stacking (Yamane *et al*., 2003), as well as disintegration of grana stacking and intergrana lamellae in rice plant (Rahman *et al*., 2002). Furthermore, salinity caused detached plasma lemma from the cell wall at various positions, indicating plasmolysis and absent in control plants (Rahman *et al*., 2000). And salinity stress increased the myelin-figures released from the plasma membrane (Rahman *et al*., 2001).

Glycinebetaine is one of the osmoregulators solutes naturally accumulate in the plants (Thomas *et al*., 1992). In this sense, accumulation of glycinebetaine represents a major biochemical adaptation in several plants (Rhodes and Hanson, 1993). In plants that synthesize glycinebetaine, it is accumulated in leaves in response to salt stress and water deficient. In addition, to its role as osmoprotectant, glycinebetaine has been reported to stabilize photosynthetic reactions and the cell membranes (Yang *et al*., 1996). It can protect the oxygen evolving machinery of chloroplasts when exposed to high NaCl concentrations (Murata *et al*., 1992). Exogenous glycinebetaine improves stress tolerance by preventing photoinhibition (Ma *et al*., 2006) and reducing oxidation of lipid membranes (Chen *et al*., 2000; Demiral and Turkan, 2004) in a wide variety of accumulator/non-accumulator plants.

Ascorbic acid is a major primary antioxidant (Nijs and Kelley, 1991), plays an important role in preserving the activity of enzymes (Padh, 1990). Moreover, Ascorbic acid is a natural antioxidant compound may be accumulated in all plants under normal and stress conditions. Sweet pepper (*Capsicum annuum* L.) is among the most

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important crop for the world human nutrition and is a moderately-sensitive to salt stress. The aim of this study contributes to a general understanding of the effect of salinity on mesophyll cell ultrastructure and planned as an attempt to minimize the harmful effects of salinity on sweet pepper plants growing in NFT, through application of glycinebetaine and ascorbic acid as pre-soaking or pre-soaking plus spraying treatments.

MATERIALS AND METHODS

Experiment was carried out in the glasshouse of the Agricultural Botany Department, Faculty of Agriculture, Mansoura University during the winter season 2005.

The following experiment was conducted to study the harmful effects of salinity on sweet pepper plants growing in NFT, through pre-soaking compared to pre-soaking plus spraying sweet pepper plants with osmoregulator (glycinebetaine) at 2000 or 4000 ppm or antioxidant (ascorbic acid) at 50 or 100 ppm under normal or saline conditions.

Plant materials: Sweet pepper (*Capsicum annuum* L.) seeds were secured from the Gohara Co. Egypt.

Chemicals:

- Glycinebetaine was supplied by Sigma Chemical Co., USA and used at the concentration of 2000 or 4000 ppm.
- Ascorbic acid (Vit. C) was obtained from EL-Gomhoria Co., Egypt and was used at the concentration of 50 or 100 ppm.
- Sodium Chloride (NaCl) from EL-Gomhoria Co., Egypt and was used at the concentration of 1500 (4.84 dS m^{-1}), 3000 (7.19 dS m^{-1}) and 6000 ppm (11.88 dS m^{-1}).

Methods of planting: A lot of homogenous sweet pepper seeds were surface-sterilized by soaking in 0.001 $HgCl_2$ for one minute and washed with distilled water. Then divided into 5 groups and each group individually presoaked in distilled water (control), GB (2000 or 4000 ppm) or AsA (50 or 100 ppm). Seeds were germinated in small plastic boxes (5520 cm) containing peat moss and perlite (1:1) as a rooting medium moistured by nutrient cooper solution (Cooper, 1979). Boxes containing the seeds were germinated in an incubator at 25±2°C in the dark.

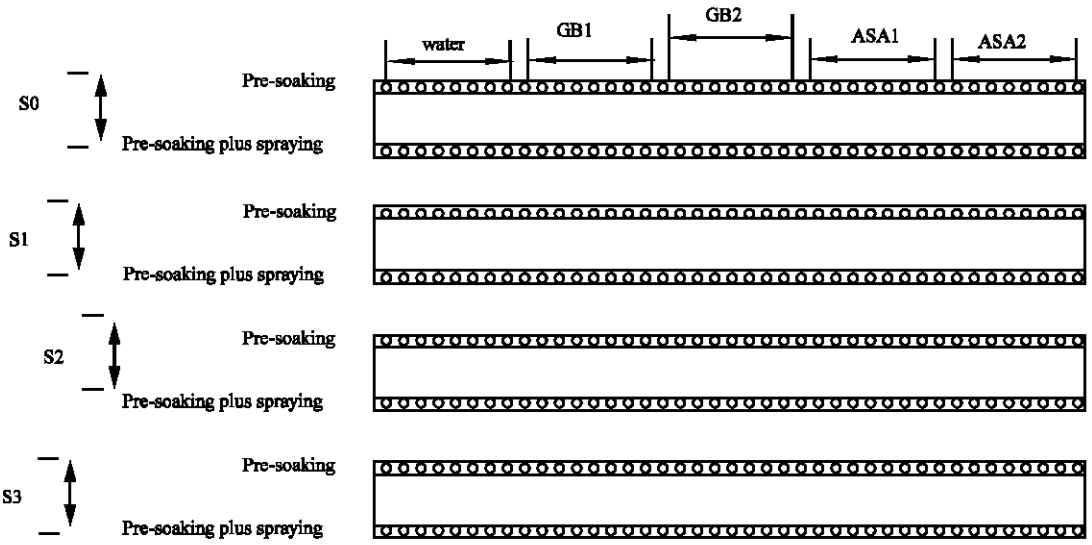
The experiment layout consisted of 8 plastic channels (4 m long and 10 cm diameter). Every two channels were provided by an electric pump representing four groups (0, 1500, 3000 and 6000 ppm). Each channel had 40 pores (6 cm diameter). After 40 days from pre-soaking, 2 uniform seedlings were transferred into 6 cm perforated pots (reticulated) containing peat moss and perlite (1:1) as a rooting medium. Every channel was divided into 5 sets i.e., water, GB1, GB2, AsA1, AsA2. Each set contained 8 seedlings (one seedling/pot) spaced 10 cm representing a Nutrient Film Technique (NFT).

Each group of channels was divided into two subgroups i.e., pre-soaking and pre-soaking plus spraying. The seedlings assigned for pre-soaking plus spraying were sprayed twice (10 and 20 days from transplanting) with the same levels previously applied in the first group (pre-soaking method).

To keep the concentrations of sodium chloride and mineral nutrients constant, the solution was changed every 5 to 7 days and the volume of the solution maintained by adding distilled water as required. A nutrient solution was pumped into the channels at a flow rate one L min^{-1} from a reservoir containing 10 L.

For study the effect of salinity, glycinebetaine, ascorbic acid and their interactions on mesophyll ultrastructure, it was intended to illustrate only the treatment which proved to be more effective in this respect. Small pieces (5 mm^2) from the right midrib region of the second leaf of sweet pepper were taken at the age 30 days after transferring plants.

Double fixation in Glutaraladehyde (2.5%) and osmium tetroxide (1%) was used. The fixative solutions were prepared in 0.01 M phosphate buffer of 6.5. Glutaraladehyde was used first for overnight in the refrigerator and replaced with cold buffer for 15 min and then the buffer was replaced with osmium tetroxide for 1 h. Most of the osmium tetroxide was removed and replaced with two changes of buffer for 15 min each. The materials were passed along the dehydration gradient by substituting the buffer with 50, 70, 80, 95 and 100% acetone, 10-15 min in each except 100% for which two changes of 30 min were made. The pure acetone was replaced gradually with a mixture of acetone and epoxy resin the 100% acetone was replaced with 2:1, 1:1 and 1:2 mixtures of acetone/epoxy resin for 15 min each. The dilute resin was then replaced with pure resin for overnight and was replaced again with fresh resin for 2 h and the materials were thus ready for embedding. Thick sections were made first to select the suitable area for ultrathin section (50-100 μ) using LKB ultratone III microtome. Sections were collected on copper grids and double stained with saturated uranyl acetate in 70% ethanol and Reynolds lead citrate for 15 min each. Sections were viewed, investigated and photographed, using transmission electron microscopy (JEOL 100s TEM).



RESULTS AND DISCUSSION

Chloroplast ultrastructure: The ultrastructure of sweet pepper mesophyll chloroplasts of plants grown under non-salinized condition shows an obvious internal structure. A typical chloroplast in sweet pepper mesophyll cells of the control leaf tissue is shown in Fig. (1a).

The chloroplast possessed typical well-developed grana and intergrana regions. The stroma thylakoids were generally oriented parallel to the long axis of the chloroplasts. The compact stroma contained a few starch grains.

The chloroplast of sweet pepper leaf taken from plants grown under NaCl saline conditions induced clear changes in the ultrastructure of chloroplast.

The most notable changes were swelling of thylakoids membrane (Fig. 1b and c). Although, the internal membranes were still intact and disoriented lamellar system with grana and intergrana lamellae becoming swollen and showed a wavy configuration. In addition, distortion or destruction of grana stacking and the stroma contained a large starch grains and increased its number. Moreover, the number of plastoglobuli was also increased (Fig. 1c) and Myelin-Figures were also observed (Fig. 1b).

The chloroplast ultrastructure of sweet pepper leaf taken from plants pre-soaked in ascorbic acid at 100 ppm and combined with NaCl at 6000 ppm showed maintenance the chloroplast structure and the chloroplast containing a small starch grains (number and size) (Fig. 1c) and an increase in the around space of starch grains compared with treatment by NaCl (Fig. 1b). While, pre-soaking in glycinebetaine 4000 ppm and grown under NaCl at 6000 ppm revealed maintenance the chloroplast structure than treatment with ascorbic acid and decreased the number of starch grains as well as space of starch grains. Moreover, the size of the starch grains was increased (Fig. 1d) compared with treatment by ascorbic acid under 6000 ppm NaCl (Fig. 1b-e).

Concerning the effect of pre-soaking plus spraying by ascorbic acid or glycinebetaine on plants growing under NaCl at 6000 ppm, it was observed maintenance the chloroplast structure (Fig. 1f and g) as compared to treatment by NaCl but treatment with glycinebetaine at 4000 ppm under NaCl condition reduced the chloroplast size (Fig. 1f) as compared to control, while treatment by ascorbic acid at 100 ppm with NaCl led to an increase in the chloroplast size (Fig. 1g) as compared to control. Furthermore, plants treated with ascorbic acid at 100 ppm (pre-soaking plus spraying) and grown under NaCl at 6000 ppm maintained the grana stacking in chloroplast than treatment with glycinebetaine at 4000 ppm and showed around shape of the chloroplast compared to control (Fig. 1g). In addition, glycinebetaine at 4000 ppm (pre-soaking plus spraying) combined with NaCl at 6000 ppm reduced number and size of starch grains in chloroplast (Fig. 1f) compared to control, while ascorbic acid at 100 ppm had no effect on the number of starch grains but the size of starch grains was reduced (Fig. 1g).

Moreover, both treatments (pre-soaking plus spraying) by glycinebetaine or ascorbic acid with NaCl reduced the number and size of plastoglobuli in chloroplast (Fig. 1f and g). It could be concluded that pre-soaking plus spraying by glycinebetaine or ascorbic acid was more effective than pre-soaking in overcoming the harmful effect of salinity on chloroplast ultrastructure. The structural changes and swelling of thylakoid caused

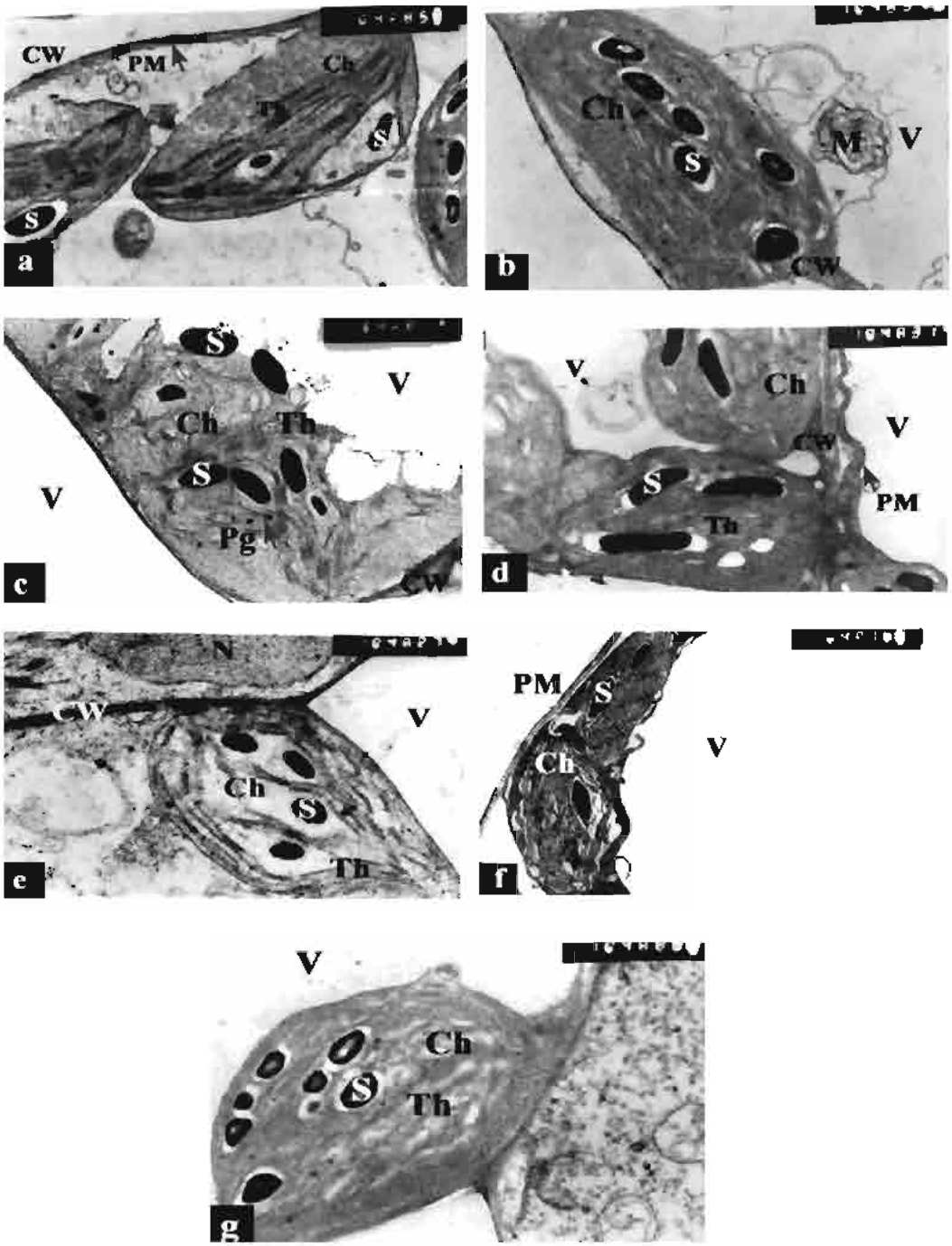


Fig. 1: Transmission electron micrographs of mesophyll cells of sweet pepper showing chloroplast ultrastructural changes, a = Control, b; c = 6000 ppm NaCl, d = Pre-soaking in GB 4000 ppm + NaCl 6000 ppm, e = Pre-soaking in AsA 100 ppm + NaCl 6000 ppm, f = Pre-soaking plus spraying with GB 4000 ppm + NaCl 6000 ppm, g = Pre-soaking plus spraying with AsA 100 ppm + NaCl 6000 ppm, Ch = Chloroplast, CW = Cell Wall, MV = Membrane vesicles, Mt = Mitochondria, N = Nucleus, Nu = Nucleolus, Pd = Plasmodesmata, Pg = Plastoglobuli, PM = Plasma membrane, S = Starch grain, V = Vacuole, Th = Thylakoid

by the treatment with NaCl in the present study are probably due to change in the ionic composition of the stroma liquid (Yamane *et al*., 2003).

Since the permeability of the thylakoid membranes to NaCl is considered to be low (Ball *et al*., 1994) and mono- or divalent cations lead to stacking of adjacent membranes in grana. Thus, swelling of the thylakoids membranes is probably due to a change in the ionic composition of the stroma liquid, rather than an effect of sodium ion on stacking of membranes.

Also, Hernández *et al*. (1995) reported that the degradation of the inner membranes of the chloroplast is due to the effect of salt induced oxidative-stress on foliar ultrastructure. The other interesting point in chloroplast structure under salinity conditions was that the chloroplast stroma contained large starch grains (Fig. 1b).

Rahman *et al*. (2000) suggested that the increased accumulation of starch in the chloroplast under salinity may be attributed to either the damage of the sucrose-phosphate synthesis in the cytosol, leading the triose phosphate pathway towards starch synthesis, or, to the damage of enzymes involved in starch degradation via changes in the ionic composition in the chloroplast. In chloroplasts, H_2O_2 is scavenged by ascorbate peroxidase using ascorbic acid as an electron donor in the ascorbate glutathione cycle (Asada, 1999). Under normal conditions, plants effectively scavenge AOS, but the balance between the production of AOS and antioxidant systems is upset by senescence and abiotic stresses such as drought, salt, low-and high temperature, air pollutants and heavy metals, which often result in oxidative damage (Langebartels *et al*., 2002).

Mitochondria ultrastructure: In control plants, mesophyll cells appeared to have normal mitochondria with dense cytoplasm, mitochondria with clear double membranes and normal distribution of cristae (Fig. 2a).

NaCl at 6000 ppm increased the number of mitochondria in sweet pepper leaf (Fig. 2b), damaged the mitochondria at various stages and became swelling. But, the number of mitochondria cristae was decreased (absent or often very short).

The cristae were observed only on one side of the bowl, but on the other side consisted of two membranes of the envelope and separated by a thin matrix layer (Fig. 2b). Moreover, the matrix appeared pale or clear and some mitochondria appeared devoid cristae (Fig. 3d). Pre-soaking sweet pepper seeds in ascorbic acid at 100 ppm or glycinebetaine at 400 ppm failed to reduce the harmful effect of salinity on mitochondria ultrastructure (Fig. 2c and d). Furthermore, pre-soaking plus spraying by ascorbic acid or glycinebetaine on plants growing under NaCl at 6000 ppm, increased the size of mitochondria to the long axis (Fig. 2e-f). The damaged elicited by NaCl treatment in mitochondria may result from at least one or more of the following responses (a) a large accumulation of sodium and /or chloride ions in leaf cells (Rahman *et al*., 2002); (b) a difference in ion compartmentation, the deleterious ion (s) having greater access to the mitochondria in plants and/or © a greater sensitivity of the mitochondria to the presence of the ions in the cells or the culture medium (Smith *et al*., 1982).

In addition, Pareek *et al*. (1997) recorded that salinity stress caused a dilated cristae and increased density, these changes in the ultrastructure of mitochondria are probably indication of salt associated alterations in mitochondrial energy status resulting in decline of ATP levels. Moreover, Rahman *et al*. (2002) suggested that the number of mitochondria in the rice cells was increased by the treatment with NaCl or NaCl plus glycinebetaine.

Cell wall and plasma membrane ultrastructure: In control plants, the cell wall of the mesophyll cells was thin (Fig. 3a), but in salt treated plants it was thick (Fig. 3c) and the plasma membrane was shrinked and partly detached of the cell wall (Fig. 3b). Furthermore, salinity increased cytoplasmic vesiculation and plasmolysis of the cell membrane (Fig. 3d), which were absent in control plants (Fig. 3a). In addition, cytoplasm contained smaller to larger membrane vesicles from the plasmalemma and fragmentation or degradation of tonoplast (Fig. 3d) while the control cells had no membrane vesicles.

Moreover, the thickness of cell wall and the vacuolar volume or size were also increased (Fig. 3b and c) compared to control. Furthermore, the Myelin-Figures were also found from plasma membrane (Fig. 3b), which were absent in control plants (Fig. 3a) and these was an accumulation of lipid droplets in cytoplasm (Fig. 3c).

Pre-soaking seeds of sweet pepper in ascorbic acid at 100 ppm and grown under NaCl at 6000 ppm, maintained the cell wall structure and reduced the vacuolar volume, but was not effective to reduce the shrinkage and detachment of plasma membrane (Fig. 3f). In addition, the membrane vesicles were absent compared to NaCl treatment. While, pre-soaking in glycinebetaine 4000 ppm combined with NaCl at 6000 ppm was more effective to maintain the ultrastructure of cell wall and plasma membrane than pre-soaking in ascorbic acid (Fig. 3e).

The application of ascorbic acid or glycinebetaine (pre-soaking plus spraying) on plants grown under NaCl at 6000 ppm showed the similar effect of pre-soaking on reducing the harmful effect of salinity on cell wall ultrastructure (Fig. 3h and g) but it is less effect to reduce the detachment of plasma membrane from the cell wall (Fig. 3e and f).

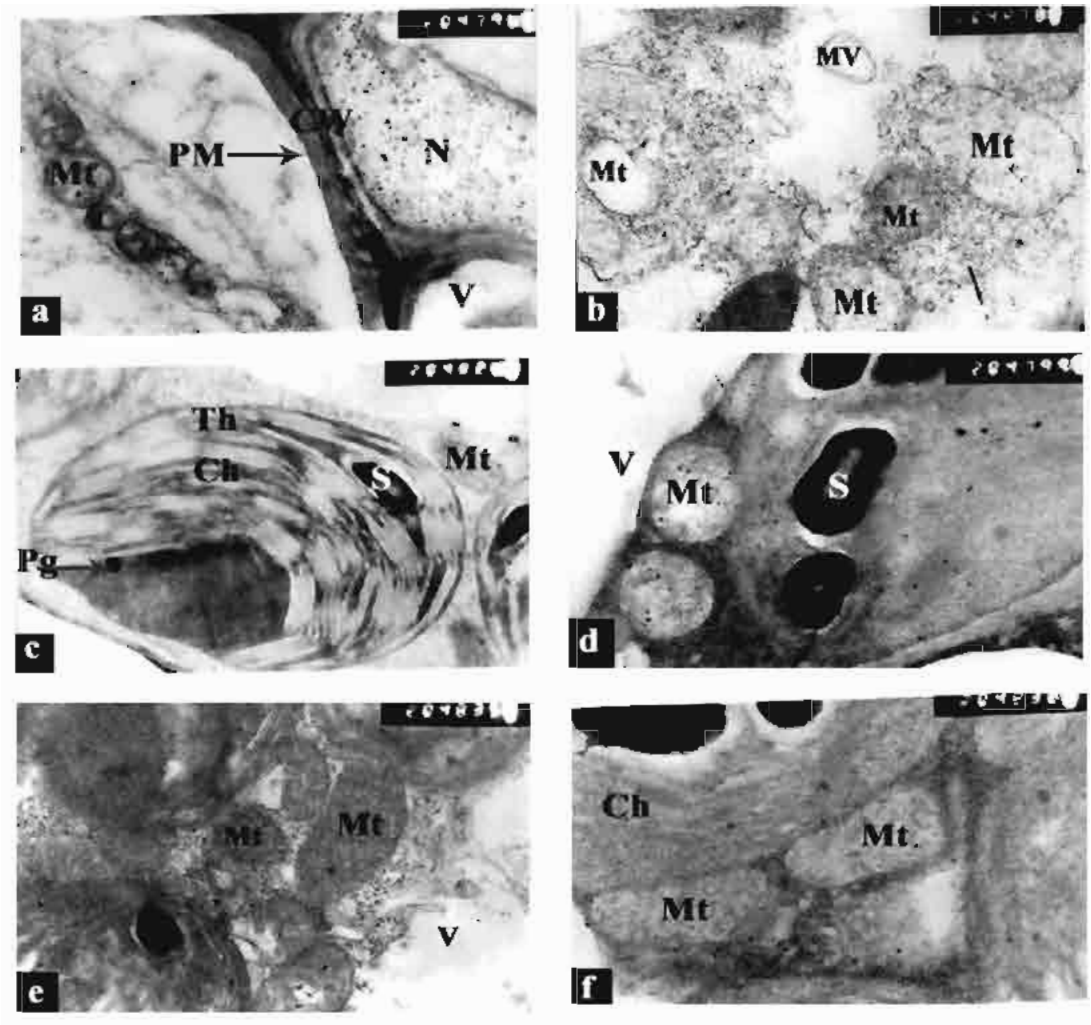


Fig. 2: Transmission electron micrographs of mesophyll cells of sweet pepper showing mitochondria ultrastructural changes, a = Control, b = 6000 ppm NaCl, c = Pre-soaking in GB 4000 ppm + NaCl 6000 ppm, d = Pre-soaking in AsA 100 ppm + NaCl 6000 ppm, e = Pre-soaking plus spraying with GB 4000 ppm + NaCl 6000 ppm, f = Pre-soaking plus spraying with AsA 100 ppm + NaCl 6000 ppm

In this study, the most frequently observed ultrastructural alterations due to NaCl treatment was the formation of many small to large vacuoles and membrane vesicles in the leaf mesophyll cells. Cachorro *et al.* (1993) suggested that these vesicles might act to store the toxic ions thus avoiding their injurious action in the cytoplasm. This vacuolation is an adaptive response to compartmentalize sodium ions away from the cytosol (Matoh *et al.*, 1987). Furthermore, Koryo (1997) pointed out that a multiplicity of small vacuoles possesses larger surface than one big vacuole, hence a higher exchange capacity (Na versus K). This system enables a plant cell to avoid ion toxicity, imbalance and interactions between substances in the cytoplasm (Rahman *et al.*, 2001).

The accumulation of lipid droplets is considered as a reserve of energy to be used by the cell to cover the increased demand in metabolic energy required to tolerant salinity in selected cells (Rahman *et al.*, 2000). Exogenous glycinebetaine improves stress tolerance by preventing photoinhibition (Ma *et al.*, 2006) and reducing oxidation

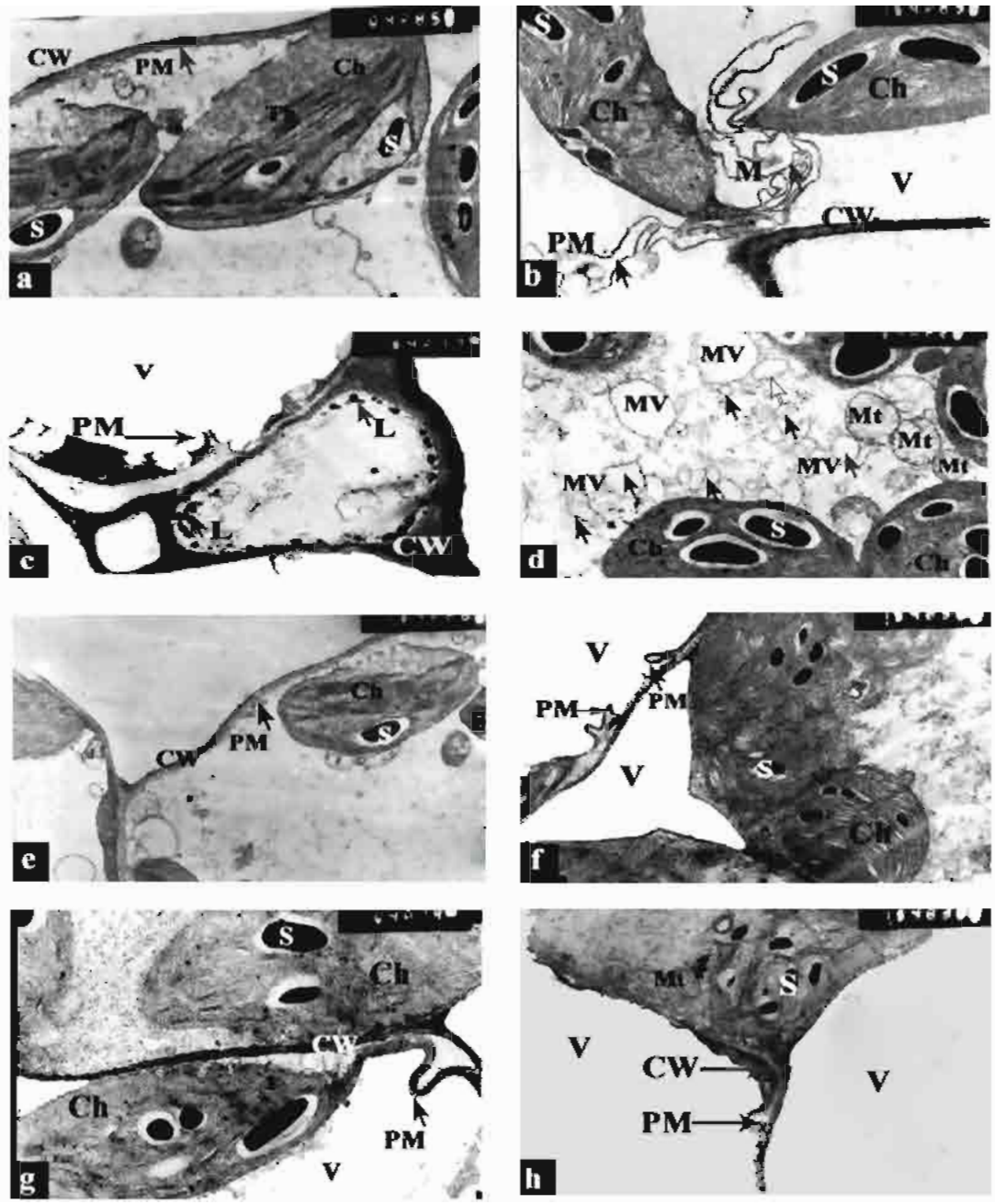


Fig. 3: Transmission electron micrographs of mesophyll cells of sweet pepper showing cell wall and plasma membrane ultrastructural changes, a = Control, b; c = 6000 ppm NaCl, d = Pre-soaking in GB 4000 ppm + NaCl 6000 ppm, e = Pre-soaking in AsA 100 ppm + NaCl 6000 ppm, f = Pre-soaking plus spraying with GB 4000 ppm + NaCl 6000 ppm, g = Pre-soaking plus spraying with AsA 100 ppm + NaCl 6000 ppm, Ch = Chloroplast, CW = Cell Wall, L = lipid droplets, M = Myelin-Figures, MV = Membrane vesicles, Mt = Mitochondria, N = Nucleus, Nu = Nucleolus, Pd = Plasmodesmata, Pg = Plastoglobuli, PM = Plasma membrane, S = Starch grain, V = Vacuole, Th = Thylakoid

of lipid membranes (Chen *et al*., 2000; Demiral and Turkan, 2004) in a wide variety of accumulator/non-accumulator plants.

In this study, the plasma membrane was detached from the cell wall at several positions indicating plasmolysis which might be caused by excess ions or osmotic stress (Pareek *et al*., 1997). In addition, salt stress is known to result in extensive lipid peroxidation, which has often been used as indicator of salt induced oxidative damage in membranes (Hernández and Almansa, 2002).

Ascorbic acid can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduced H_2O_2 to water via ascorbate peroxidase reaction and is the major antioxidant that scavenges H_2O_2 (Chen and Gallie, 2004). Chun *et al.* (2004) showed that application of ascorbic acid (AsA) enhanced the ability of active oxygen scavenging in chloroplast of rice plants growing under saline condition and protection of thylakoid membrane lipids against oxidations compared with control.

Glycinebetaine is a one of osmoprotectant and it occurs in many drought-and salt tolerant angiosperms. It is likely that betaine is involved in the protection of macro component of plant cell, such as protein complex and membranes under stress conditions (Sakamoto and Murata, 2000).

It could be concluded that exogenous application of glycinebetaine on sweet pepper plants led to reducing the harmful effect of salinity on the ultrastructure mesophyll cells compared to plants grown under NaCl salinity. However, results on tomato indicated that chloroplast area and number of plastoglobuli were not affected by glycinebetaine application, whereas the relative area of starch grains increased slightly in salt stressed compared with control. Moreover, salinity induced ultrastructural damages in leaf which were largely prevented by pretreatment with glycinebetaine. These damages may be due to maintaining chloroplast volume (Rajasekaran *et al*., 1997) and/or protect membranes from lipid peroxidation and protect the integrity and stability of membrane under salt stress (Fang *et al*., 2004).

The obtained results showed that the ultrastructural injuries caused by NaCl salinity could be alleviated by application of glycinebetaine or ascorbic acid as pre-soaking or pre-soaking plus spraying and the effect varied according to the application methods and was a concentration dependent. However, ascorbic acid applied as pre-soaking plus spraying tended to be more effective in this respect.

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