Ion distribution measured by electron probe X-ray microanalysis in apoplastic and symplastic pathways in root cells in sunflower plants grown in saline medium

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Little is known about how salinity affects ions distribution in root apoplast and symplast. Using x-ray microanalysis, ions distribution and the relative contribution of apoplastic and symplastic pathways for delivery of ions to root xylem were studied in sunflower plants exposed to moderate salinity (EC=6). Cortical cells provided a considerably extended Na^+ and Cl^- storage facility. Their contents are greater in cytoplasm (root symplast) as compared to those in intercellular spaces (root apoplast). Hence, in this level of salinity, salt damage in sunflower is not dehydration due to extracellular accumulation of sodium and chloride ions, as suggested in the Oertli hypothesis. On the other hand, reduction in calcium content due to salinity in intercellular space is less than reduction in the cytoplasm of cortical cells. It seems that sodium inhibits the radial movement of calcium in symplastic pathway more than in the apoplastic pathway. The cell wall seems to have an important role in providing calcium for the apoplastic pathway. Redistribution of calcium from the cell wall to intercellular space is because of its tendency towards xylem through the apoplastic pathway. This might be a strategy to enhance loading of calcium to xylem elements and to reduce calcium deficiency in young leaves under salinity. This phenomenon may be able to increase salt tolerance in sunflower plants. Supplemental calcium has been found to be effective in reducing radial transport of Na^+ across the root cells and their loading into the xylem, but not sodium absorption. Supplemental calcium enhanced Ca^{2+} uptake and influx into roots and transport to stele.

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1. Introduction

Salt stress is one of the major abiotic stresses limiting crop growth and productivity. NaCl salinity can cause injury to plants due to absorption of toxic levels of sodium and chloride ions by roots and their transport to shoots (Taiz and Zeiger 2006). It can also antagonize the uptake of other essential nutrients, such as nitrogen, phosphorus, potassium and calcium, thereby inducing their deficiency or imbalance in plant organs or cells in spite of their abundance in soil or the growth medium (Tester and Davenport 2003; Wahome 2003). Deleterious effects of NaCl salinity on plant growth and mineral nutrition are, thus, attributed to a decrease in osmotic potential of root growth medium, specific ion toxicity, nutrient imbalance and deficiency as a result of disruption of ion uptake (Kwon *et al.* 2009).

In low salinity, osmotic solutes in plant organs are normally not in sufficient concentration to have major ability for inward water movement in such situations. Some of plants try to accumulate high levels of compatible solutes in plant organs in order to create a water potential gradient for inward water movement (Ebrahimi 2010). Under NaCl salinity, the enhanced rates of Na^+ and Cl^- accumulation in roots and its transport to shoots also alters the activity of various apoplastic enzymes in the constituent cells of plant roots (Philippar and Soll 2007).

Sunflower cultivars vary from being very sensitive to semi-tolerant of salt stress (Ashraf and Tufail 1995; Francois 1996).

Keywords. Apoplastic pathway; calcium; root; sunflower; symplastic pathway

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Sunflower crop grows well on a wide range of soils, such as sandy loam, black and alluvial soil. The ideal soil pH for growth of sunflower is from 6.5 to 8. An understanding of the factors affecting the growth of sunflower plants in degraded lands, such as saline soils, is necessary in order to increase the yield of oilseeds and maintain good oil quality. NaCl salinity (50 and 100 mM) has been reported to affect seedling survival rather than seed germination in sunflower (Delgado and Sanchez-Raya 2007). In addition, sodium and chloride ions generally accumulate more in the vegetative parts as compared to reproductive parts in sunflower plants (Ebrahimi and Bhatla 2011). The differences in their ion concentrations is due to the fact that vegetative parts are exposed to salinity for much longer periods and the reproductive parts being fed by phloem are better protected as well as exposed for relatively shorter durations.

Roots directly suffer from soil salinity. Differences in ion distribution in different subcellular compartments in various cell types in roots may explain differences in plant response to soil salinity. In saline soil conditions, roots must continue to acquire essential nutrients while excluding toxic ions (Davenport 2007). In order to achieve this, roots try to preferentially take up essential ions, such as K^+ and Ca^{2+}, over toxic ions, such as Na^+ and Cl^-, in saline conditions in the growth medium. Enhanced Na^+ exclusion by plant roots is an important trait that increases salt tolerance in many crops growing in saline soils (Yeo 2007). In some crops raised in saline soils, enhanced Cl^- exclusion from crops roots is associated with increased salt tolerance rather than Na^+ exclusion (Storey and Walker 1999). Not much is known about the putative mechanisms that determine Cl^- exclusion except that it may involve restriction of Cl^- uptake and its increased sequestration in root vacuoles (Storey *et al.* 2003).

Na^+ may enter xylem stream through many pathways (Tester and Davenport 2003). Some of those pathways have been identified at the molecular and electrophysiological levels (Storey *et al.* 2003). They include different K^+ carriers and non-selective cation channels (Tyerman and Skerrett 1999). In some plants, pathways that bypass membrane transport processes may also be involved in the translocation of Na^+ into the transpiration stream and its translocation to the shoots (Shabala 2007). Cl^- uptake into the cells is facilitated by H^+/Cl^- symporters (Storey *et al.* 2003). At high salinity, Cl^- might also enter the root cells passively through anion channels, although at low salinity, this is reported to be unlikely (Mengel and Kirkby 2001).

With this background information in view, ion contents within the individual root cells from specific regions have been determined by electron probe x-ray microanalysis using frozen specimens. The x-ray microanalysis was restricted to the regions of the primary roots. Thus, the work was primarily confined to a region of the root that accumulates ions from the external medium. Localization of elements in individual root cells or subcellular compartments of plant tissues using x-ray analytical electron microscopy has been successfully applied earlier in a number of investigations (Drew *et al.* 1990; Kosegarten and Koyro 2001; Patrick 2001; Conn, and Gilliham 2010).

On the basis of published data on the organ level in sunflower, 40 mM NaCl in Hoagland solution (EC=6 dS/m) is a critical content of salinity that can cause visible injury in sunflower leaves (Ebrahimi and Bhatla 2011).

Lower concentration does not causes significant impacts on sunflower growth, and at higher salinity levels sunflower plant cannot survive. In order to understand how sunflower roots control Na^+ and Cl^- transport from root to shoot and prefer to accumulate these ions in root cells, a comparative study has been undertaken in the present work with the following aims:

1. Determination of Na^+, K^+, Ca^{2+} and Cl^- contents in epidermis, outer and inner cortical cells and stele
2. Determination of how 40 mM NaCl in the growth medium alters cellular ion distribution in primary root
3. Determining whether there are differences in Na^+, K^+, Ca^{2+} and Cl^- contents in the cytoplasm and apoplast in the cells of primary roots. For this comparative analysis, various cells from roots were analysed for differences in ion accumulation
4. Investigation of whether the ratios of K^+/Na^+ and Ca^{2+}/Na^+ in the cytoplasm differ from those in the apoplast
5. Finally, Examining the effect of 10 mM supplemental calcium on Na^+, K^+, Ca^{2+} and Cl^- loading into xylem and ratios of K^+/Na^+ and Ca^{2+}/Na^+ in root cells under NaCl salinity

2. Materials and methods

2.1 *Plant material and seed germination*

Seeds of sunflower (*Helianthus annuus* L. cv.Morden) were obtained from National Seeds Corporation, India. Uniform-sized seeds were selected and surface-sterilized with 0.1% mercuric chloride for 5 min, thoroughly washed with deionized water and soaked for 12 h in glass distilled water. Seeds were germinated in plastic pots filled with fine silica sand (< 2 mm) and were uniformly irrigated once daily. Emergence of radicle was taken as a sign of germination. The formation and extension of hypocotyls was observed after 48 h. When the cotyledons had fully expanded, three uniform seedlings were retained for growth in each pot.

Potted plants were maintained in a controlled environment room and subjected to 16 h photoperiod diurnally at 25±1°C and 80±5% relative humidity. Throughout the experiment, plants were irrigated daily and uniformly with half-strength Hoagland nutrient solution (pH=6)

containing 40 mM NaCl with or without supplemental calcium. Control plants were not subjected to NaCl treatment.

Plants were harvested after 30 days of growth and their roots were washed with distilled water. Thirty-day-old plants were cut to separate roots. Experiments were conducted in three independent replicates per treatment, each replicate consisting of three plants.

2.2 *Sample preparation for x-ray microanalysis of ions*

Samples were processed according to Zhao *et al.* (2005). Briefly, root segments of 5 mm×5 mm were cut from near the root tip from 30-day-old plants. The tissue samples were put into small cages of aluminium gauze and plunged into liquid N_2–cooled iso-pentane and propane (v/v 1/3) for rapid freezing and freeze-dried at −80°C in the lyophilizing chamber. Modified T-shaped valves were used for infiltration with diethyl ether under vacuum at 25°C for 24 h after freeze-drying. The samples were then cut into small pieces for infiltration with styrene-methacrylate.

They were then transferred into gelatine capsules and polymerized at 60°C for 7 days. The embedded materials were cut using a LKB Nova ultramicrotome to obtain 70–90 nm thick samples, using a diamond knife. The sections were pasted on the stub and coated with gold for observation by transmission electron microscope, with emphasis on cortical cells in roots. The energy dispersive x-ray microanalytical studies were carried out under standardized conditions, as described in Bucking and Heyser (2000), using a Philips 420 electron microscope fitted with PV9100 EDAX system.

Element distribution was documented as a peak-to-background ratio (P:B) in order to diminish the effects of surface irregularities of the sections during analysis. Due to standardization problems with x-ray microanalysis, P:B ratio was used as a semi-quantitative measurement of the element levels in different cellular compartments. X-ray microanalysis of ion contents in roots was also done by field emission scanning electron microscope (FESEM) coupled with EDAX (Vesk *et al.* 2000).

The sections were examined by HITACHI–H 800 Field Emission Scanning Electron Microscope fitted with EDAX- 910 energy disperses x-ray micro analyser. The accelerating voltage was 12 kV with a takeoff angle of 25°. The counting time for all analysis was 60 s (spectra were collected for 60 s) and the data were expressed as counts per second (cps) of an element peak after subtraction of the background. Six measurements per section were carried out for each tissue compartment. Specimen drift was minimized by keeping the specimen/stage temperature at a constant –145°C.

3. Results

The validity of ion content measurements in subcellular compartments depends more on the preparative procedure more than on the microanalysis itself. The aim of cryopreservation in this work was to freeze root samples rapidly so that damage is not caused by the formation of ice crystals. As a preparative technique to fix solutes in their natural compartments, rapid freezing is more versatile than chemical precipitation for both scanning and transmission electron microscopy (Flowers 2007). There was a minimum gap (<15 s) between cutting and freezing in this work. Once frozen, there would be no opportunity for diffusion to take place during sub-sampling of small pieces of roots used for freeze substitution. In this work, a scanning beam of 13×14 μm was found suitable for probing the sections (inner contents of cells). This technique provided useful information about ion distribution in apoplastic and symplastic pathway in salinized and non-salinized root cells in sunflower plants. Through this method, clear distinctions were made from cell to cell in roots.

3.1 *Anatomy of root and its changes*

After freezing the root sections in liquid nitrogen, structural preservation of the root cells was found to be satisfactory. Freezing in iso-pentane+propane and rapid transfer into liquid N_2 gave improved results. Epidermis of roots is characterized by the absence of cuticle and stomata. Cortical cells are considerably homogenous in shape and well developed. Cortex is a major component of the ground tissue in sunflower root and is composed of several layers of thin-walled, loosely arranged parenchymatous cells with intercellular spaces.

3.2 *Energy spectra*

Differences in ionic contents of root cells in control and NaCl-treated plants were revealed by analysing energy spectra on the basis of peak area and P:B ratio of elements as a percentage of the mass fraction analysed. In each EDAX spectrum of elements, the y-axis showed counts per second (cps) and x-axis showed energy in kilovolts. Energy spectra of sodium, potassium, calcium and chloride ions in root cells, measured by x-ray technique, showed significant differences in response to salinity although some relocation of diffusible elements, such as Na^+ and K^+, during the embedding procedure cannot be completely excluded. Data are expressed as counts per second (cps) of an element peak after subtraction of the background. Six measurements per section were carried out for each compartment. X-ray counts were typically in the range from 0.11 to 11.82 cps and the dwell time was 60 s. Peak emissions for carbon and oxygen were also detected but have not been discussed in this study.

Representative energy dispersive x-ray spectra for the cytoplasm, intercellular space and cell wall of cortical cells demonstrated clear peaks for K^+ and Ca^{2+} under non-saline conditions (figure 1A) or for Na^+ and Cl^- in saline conditions (figure 1B). Na^+ peak in the cytoplasm of outer cortical cells was in the range of detection limit (Na^+=0.11) in non-saline conditions. In some cases, sodium content was not detectable after focusing the electron excitation beam onto the cytoplasm of epidermal cell in this condition. Figure 1 also shows the energy spectra of the characteristic x-ray analysis of ions present in the cell wall of cortical cells in sunflower plant roots after growing in non-saline medium. In this region, sodium was detectable (=0.65 cps). Na^+ and Cl^- enrichment in cortical cells in saline condition, as compared to control, was confirmed by the relative percentage of Na^+ and Cl^- in comparison with the distribution of K^+ and Ca^{2+}. Furthermore, the peak for potassium ions was clearly larger than that for calcium in the cytoplasm, cell wall and intercellular space of cortical cells in non-saline conditions (figure 1A).

The x-ray measurement analysis of ions in roots grown in saline medium revealed a distinct Na^+ signal in root cells and it could be detected in the cytoplasm, cell wall and in intercellular space. The peak area for chloride was generally bigger than that of sodium in saline conditions (figure 1B).

3.3 *Profile of Na^+ and Cl^-*

In sunflower plants grown in non-saline conditions, content of sodium ions in root cells was very low and that of chloride ions a little higher than sodium. In the roots of control plants, distribution of Na^+ and Cl^- followed a different pattern. The highest mean contents of sodium and chloride ions were in the cytoplasm of inner and outer cortical cells, respectively, and lowest contents were observed in the cytoplasm of epidermal cells for both of ions.

Under 40 mM NaCl salinity, chloride content in root cells was more than of other ions, and sodium content was more than potassium and calcium. In this condition, the highest mean content for sodium and chloride ions was in the cytoplasm in cortical cells and lowest for Na^+ in xylem elements and for Cl^- in epidermal cells. In terms of absolute quantities, most of the Na^+ and Cl^- were in the vacuoles that occupied a large volume of the cell cytoplasm in saline conditions.

In non-saline conditions, Na^+ content in roots was mainly detectable in the cytoplasm of inner cortical cells, whereas the levels in the cytoplasm of outer cortical cells were near the x-ray microanalytical limit of detection. In a few cases, Na^+ was not detectable in the cytoplasm of epidermal and outermost cortical cells. Sodium content increased slightly

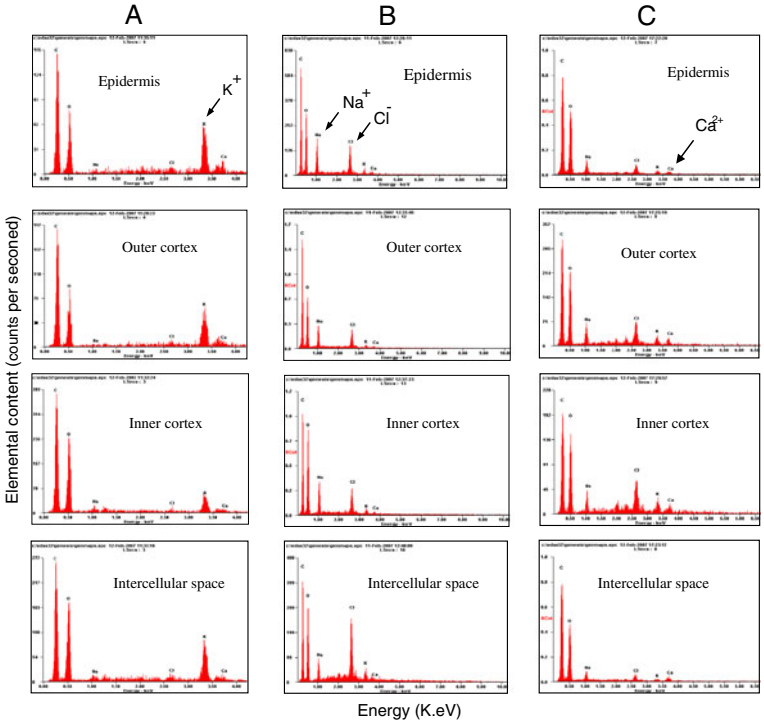

Figure 1. X-ray spectra of the element content (counts per second) in root cells (A: Half-strength Hoagland Solution (HHS), B : HHS + 40 mM NaCl, C: HHS + 40 mM NaCl + 10 mM $CaSO_4$).

from the outermost to innermost cortical cells in non-saline conditions. It was lowest in the cytoplasm of epidermal cells, higher in the intercellular space and highest in the cytoplasm of innermost cortical cells in roots grown under non-saline conditions.

The mean content of Na^+ in root cells increased due to 40 mM NaCl salinity. Under saline conditions, Na^+ content in all root cells was always more than that in non-saline conditions. Accumulation of these ions in the symplastic pathway in cortical cells was always more than that in the apoplastic pathway. The increase in sodium and chloride ions contents in root cells appeared to be larger in the presence of 2 mM calcium (control) as compared to 10 mM calcium in the saline growth medium. Sodium content in the cytoplasm of the inner cortical cells was higher than that in the cytoplasm of epidermal cells. Sodium content in the intercellular space was less than that in the cytoplasm in cortical cells in presence of NaCl salinity. Sodium content in root cells under saline conditions was significantly different with 2 or 10 mM calcium treatments. It decreased in salinized root cells in the presence of 10 mM calcium as compared to 2 mM calcium in the growth medium. The maximum and minimum reduction in sodium content in root cells due to 10 mM supplemental calcium was observed in xylem and epidermal cells, respectively.

Under non-saline conditions, chloride content in the cytoplasm of cortical cells was higher than that in the cytoplasm of epidermal cells. Ch#loride content in the cytoplasm of outer cortical cells and in cell wall was almost similar to that in control root cells (figure 2). It was lower in intercellular spaces in comparison with the cytoplasm of inner cortical cells under non-saline conditions. It was least in the cytoplasm of epidermal cells. NaCl salinity increased chloride content in all root cells significantly. Maximum increase in chloride content was observed in the epidermal cells due to NaCl salinity (tenfold increase as compared to that in controls). However, chloride content in epidermal cells was still less than in other cells under saline conditions. Chloride content was the highest in the cytoplasm of inner cortical cells among root cells grown under 40 mM NaCl salinity (sixfold increase as compared to that in controls). Chloride content decreased due to 10 mM supplemental calcium sulphate in root cells in the presence of 40 mM NaCl. The minimum and maximum reduction in chloride content was observed in the cytoplasm of outer cortical cells and in xylem elements due to supplemental calcium sulphate, respectively.

3.4 *Profile of K^+ and Ca^{2+}*

The content of potassium and calcium ions was considerably higher than that of sodium and chloride ions in root cells in non-saline conditions. In general, among cations, potassium content in root cells was more than others in sunflower plants grown under non-saline conditions. The highest mean content of K^+ was in the cytoplasm of cortical cells and lowest in the cytoplasm of epidermal cells (figure 2). In roots grown under non-saline conditions, K^+ content in the cytoplasm of cortical cells was twofold higher than that in the intercellular space of these cells. In non-saline conditions, the potassium gradient in root cells was much steeper than that in salinized root cells. In non-salinized roots, the chemical gradient of potassium decreased substantially across the cortex and this gradient was probably steeper than the potassium gradient across the salinized roots.

NaCl salinity (40 mM) induced a lowering of K^+ content in the apoplast and symplast of cortical cells in roots. In salt-treated plants, the profiles of K^+ and Na^+ showed a significant decrease in potassium and an increase in sodium content in root cells. Minimum potassium content was detected in cell wall under NaCl salinity. It seems that NaCl salinity decreases both the absolute amount of K^+ in root and the ratio between that transmitted to the stele and that retained by the root cells themselves. However, potassium content was almost constant across the root cells under salinity treatment (figure 2). The salt-induced reduction in K^+ content in root cells was larger in the presence of 2 mM calcium (control) than 10 mM calcium in the growth medium (figure 2).

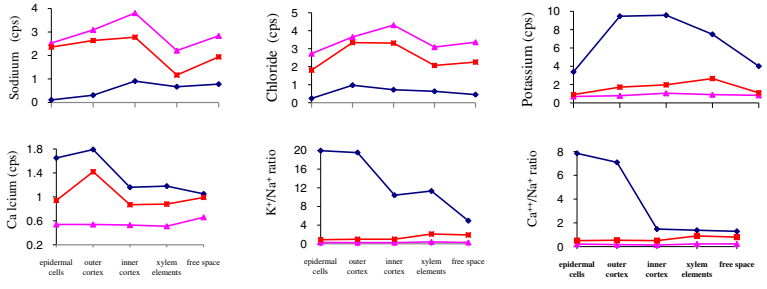

Figure 2. Ions content and ratio in different root cells in sunflower plants irrigated with Hoagland solution (–◆–) or plus 40 mM NaCl (–▲–) or plus 40 mM NaCl + 10 mM $CaSO_4$ (–■–) for 30 days in sand culture medium.

Supplemental calcium improved K^+ content in cortical cells and xylem elements in sunflower roots in saline conditions. It was significantly higher in the xylem elements in roots subjected to 10 mM calcium, in comparison with similar cells from controls. Potassium content in free space of cortical cells was similar with and without supplemental calcium in saline medium (figure 2).

Calcium content was considerably less than potassium content in all root cells in sunflower plants under non-saline conditions. Calcium content in the cytoplasm of outer cortical cells was more than that in the cytoplasm of inner cortical cells (figure 2). Under non-saline conditions, calcium content in the cytoplasm of cortical cells was more than that in the intercellular space of cortical cells. The highest Ca^{2+} content was detected in the cell wall of cortical cells. Among the cell wall, cytoplasm and intercellular space of cortical cells, the minimum content of calcium was observed in intercellular space in non-saline conditions.

In the presence of 40 mM NaCl in Hoagland solution (with 2 mM Ca^{2+}), the mean Ca^{2+} content in root cells decreased as compared with that in roots of control plants. In roots exposed to salinity, the symplastic and apoplastic distribution of Ca^{2+} was almost uniform but the relative reduction of calcium due to salinity in the symplastic pathway was more than in the apoplastic pathway. A markedly higher calcium level was detected in the outer cortical cells of roots treated with 10 mM of calcium as compared to roots grown in presence of 2 mM calcium treatment in saline medium (figure 2).

3.5 *Profile of K^+/ Na^+ and Ca^{2+}/Na^+*

The K^+/ Na^+ ratio in the cytoplasm of cortical cells of roots decreased from the outer cortex to the inner cortex under non-saline conditions (figure 2). This ratio was similar in the epidermal cells and cytoplasm of outer cortical cells and was threefold greater than that of the inner cortex under non-saline conditions. In the presence of 40 mM NaCl in the growth medium, K^+/Na^+ ratio was almost uniform in all root cells. NaCl salinity (40 mM) significantly reduced this ratio in root cells as compared to non-saline conditions. Maximum reduction was observed in outer cortical cells. It decreased 100-fold as compared to that in non-saline conditions. Supplemental calcium improved this ratio slightly. K^+/Na^+ ratio was slightly higher in 10 mM calcium in comparison with 2 mM calcium in all root cells in saline conditions. The minimum and maximum improvement was observed in epidermal cells and xylem elements, respectively, but the improvement was not significant in any of these cells.

The Ca^{2+}/Na^+ ratio was, however, lesser than the K^+/Na^+ ratio in all root cells in non-saline and saline conditions. This ratio decreased from outer to inner cortical cells in sunflower roots grown under non-saline conditions (figure 2). There were significant differences in Ca^{2+}/Na^+ ratio in root cells in control plants. It was maximum in epidermal cells and minimum in intercellular space of cortical cells in roots grown under non-saline conditions. NaCl salinity decreased this ratio in all root cells as compared to control. In the presence of salinity this ratio was similar in all cells in root. The maximum reduction was observed in epidermal and outermost cortical cells. Supplemental calcium improved this ratio slightly in all root cells, and in the cell wall it was more than others.

4. Discussion

4.1 *Ion distribution in apoplastic and symplastic pathways in root cells is dependent on their long-distance transport and roles in specific zones*

Calcium is taken up by plant roots either by the apoplastic or the symplastic pathway (White 2001). The relative contribution of these two pathways for delivery of calcium to the xylem in different plants is not known (Cholewa 2000). The results obtained in this work showed that salinity affects both pathways in sunflower roots. The content of Ca^{2+} in the cytoplasm is normally very low and is regulated by the activity of membrane-bound transporters. Intracellular level of calcium must be maintained as low as 0.1–0.6 µM within the cytosol in order to avoid phosphate precipitation (Mengel and Kirkby 2001).

Ion distribution within the root cells is dependent on their long-distance transport to the shoot and their biochemical/osmotic roles (Storey *et al.* 2003). There is a variation in the distribution of Na^+, K^+ and Ca^{2+} in the sunflower root cells which could be correlated with their spatial position within the root. These results suggest that various ions may adopt different uptake pathways in roots. Whereas K^+, Cl^- and Ca^{2+} are predominant elements in the cytoplasm of root cells under non-saline conditions, Na^+ is predominant under NaCl salinity in root cells. Under saline conditions, Na^+ and Cl^- contents are greater in the cytoplasm of cortical cells as compared to intercellular spaces of cortical cells. For plant roots grown under non-saline conditions, a similar analysis reveals that the cytoplasm of root cells contain very little sodium because for growth and development of sunflower, K^+, Ca^{2+} and Cl^- are recognized as essential nutrients while the role of Na^+ remains debatable (Rajendra 2007). It seems that sodium ions in low concentration can substitute for potassium in raising cell turgor in sunflower (Ebrahimi and Bhatla 2011). Most plants growing in normal soils contain very little sodium in root cells (Mengel and Kirkby 2001). Chloride content in cells of sunflower roots is generally higher than sodium. Their concentrations are, however, equal in salty irrigation water in sand culture medium. Uptake rate of chloride ions by root cells in sunflower is higher than that of

sodium ions (Ebrahimi and Bhatla 2011), as also reported earlier in some other plants (Mengel and Kirkby 2001).

The noteworthy observation in the present work is that the total ion content in the cytoplasm is greater than that in the intercellular space of cortical cells. This forms strong evidence that an important factor in salt damage in sunflower plants is not dehydration due to extracellular accumulation of sodium and chloride ions, as also suggested in the Oertli hypothesis (Oertli 1968; Flowers *et al.* 1991). Previous work has shown differential Na^+ accumulation in sunflower leaves as a property of roots (Quintero *et al.* 2007 and 2008). In the region of roots analysed in this work (cortex), it was observed that Na^+ content mirrored the Na^+ content in shoots in low salinity but not in high salinity. It seems that maximum chloride ions get accumulated in cytoplasm (in vacuoles) of cortical cells, as reported by other workers as well (Storey *et al.* 2003).

4.2 *Salinity reduces potassium content in the cytoplasm of cortical cells more than in the intercellular space*

Potassium is involved in many metabolic processes in plant cells. In the cell cytoplasm, K^+ is involved in the neutralization of soluble and macromolecular anions, and it contributes to the osmotic potential of plant cells (Mengel and Kirkby 2001). A disorder in potassium nutrition in root cells has been observed to be one of the nutritional problems induced by NaCl salinity in sunflower (present work). There is a strong competition between sodium and potassium ions for uptake by root hairs and plant cells. Both are monovalent cations and have slight differences in their ionic radii. Therefore, Na^+ can easily substitute for K^+ in less specific processes, such as raising cell turgor. Under saline conditions, the cell wall seems to have an important role in providing potassium for cell cytoplasm in roots, and potassium from the apoplastic pathway gets redistributed to the symplastic pathway because the radial transport of K^+ in plant roots is symplastic (Drew *et al.* 1990).

4.3 *Cell wall provides calcium for other cell compartments in roots under salinity*

Calcium is an important constituent of the middle lamella and cell walls, preventing membrane damage and leakage, strengthening wall structure and increasing the cohesion of cell walls (Fageria 2009). It also plays an important role in the regulation of growth and developmental processes and acts as an intracellular signal molecule. Current investigations reveal that in sunflower roots, calcium content of the cell wall is considerably reduced by NaCl salinity. The mean cytoplasmic Ca^{2+} content in root cells of sunflower plants grown in the presence of 40 mM NaCl is still sufficient to meet the aforementioned biochemical requirements, but the cell wall–associated calcium in cortical cells of roots grown in similar saline conditions is not physiologically sufficient (Flowers *et al.* 1991). Displacement of cell wall–associated calcium by sodium and magnesium ions is likely to increase membrane permeability and loss of Ca^{2+}/Na^+ selectivity. Reduction in calcium content in the free space of cortical cells due to salinity is less than that in their cytoplasm. This might be a strategy to enhance loading of calcium to xylem elements and to reduce calcium deficiency in young leaves under saline conditions because calcium usually reaches the xylem through the apoplastic pathway in plants (Mengel and Kirkby 2001). It seems that sodium ions inhibit the radial movement of calcium from external solution to xylem elements in sunflower roots.

4.4 *Supplemental calcium seems to control sodium loading into xylem but not sodium absorption by root from growth medium*

Some beneficial role of supplemental calcium on improvement of calcium content in the cell wall during NaCl salinity has been observed in sunflower plants, as also reported earlier for some other plants (Kwon *et al.* 2009). Supplemental calcium seems to control sodium loading into the xylem. This appears to be the key for young sunflower plants to tolerate NaCl salinity. Leaves show NaCl toxicity symptoms earlier than root. That is why roots try to accumulate Na^+ and Cl^-, instead of loading them into the xylem for transport to the shoot. In the present work, supplemental calcium (in the form of $CaSO_4$) has been found to be effective in reducing the radial transport of both Na^+ and Cl^- in sunflower roots and long-distance transport to the xylem. This phenomenon thereby reduces leaf injury in sunflower. Sodium content in epidermal cell cytoplasm in roots grown in saline conditions with 2 or 10 mM calcium was almost similar. It seems that supplemental calcium in saline medium cannot reduce the sodium uptake by roots. Supplemental calcium seems to reduce sodium loading to xylem elements but not sodium absorption by roots. More studies are, however, required to explore the exact mechanism of supplemental Ca^{2+} in reducing Na^+ transport and loading to the xylem and enhancing Ca^{2+} uptake and influx to roots in sunflower.

Apoplastic sodium gradient may play a role in the K^+/Na^+ gradient across the cortical cells in sunflower roots. The K^+/Na^+ ratio increases in cortical cell and xylem elements due to supplemental calcium sulphate in saline conditions. Zaman *et al.* (2002) reported that K^+/Na^+ ratio improved due to sulphur application in sunflower.

4.5 *Role of roots in salinity tolerance and nutrient balance*

Sunflower is a semi-tolerant plant to NaCl salinity as a result of accumulation of Na^+ and Cl^- in roots as the main osmotic regulators. Under moderate salinity (EC=6 dS/m), about

65% of Na^+ and Cl^- are restricted in sunflower roots (Ebrahimi and Bhatla 2011). The effect of 10 mM supplemental Ca^{2+} treatment is significant in reducing Na^+ transport across the root cells because of an enhancement of Ca^{2+} uptake and Ca^{2+} influx into roots, transport of Ca^{2+} to stele and alleviation of growth inhibition in roots caused by salinity in sunflower 'cv. Morden', but a little alleviation of calcium content in cell wall. The cell wall seems to provide calcium for other regions of the cell in roots under moderate salinity in sunflower. Extracellular accumulation of Na^+ and Cl^- is not the primary factor for salinity damage in sunflower roots. An increase in solute concentration in root cells is sufficient to balance the salinity of the external medium and is evidence for osmotic adjustment of the cells in sunflower roots. Generally, ion uptake by roots shows marked selectivity for potassium over sodium, but since this is of limited value in excluding sodium or maintaining adequate potassium content in the root cytoplasm, additional regulatory mechanisms are required to minimize this problem in sunflower root.

Cortical cells in sunflower roots act as accumulators of Na^+ and Cl^-. Subcellular distribution of these ions under saline conditions mainly occurs in the cytoplasm of inner cortical cells, with vacuoles having the highest content. As a consequence of large number and volume of cortical cells, they could provide a considerably extended Na^+ and Cl^- storage facility in sunflower roots. In this context, it is interesting to note that the mean ion concentrations in roots, as derived from x-ray microanalysis of the cortical cells, is comparable to that obtained by flame photometry or atomic absorption of whole-root samples (Ebrahimi and Bhatla 2011).

This would seem to indicate retention of Na^+ and Cl^- in the cortex, with transmission of K^+ to the stele, from where it can be transported to the shoots. On the basis of maximum reduction in potassium and calcium content in the cell wall due to salinity, it seems that the cell wall of cortical cells has an important role in saline conditions to provide potassium and calcium for the cytoplasm. This phenomenon may be able to increase salt tolerance in sunflower roots exposed to salinity. Na^+ and Cl^- absorbed by root hairs are partly translocated to the stele and transferred to the shoots, which can cause an inhibition of metabolic processes in the leaves in sunflower plants grown in saline soils.

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