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Antioxidant and anatomical responses in shoot culture of the apple rootstock MM 106 treated with NaCl, KCl, mannitol or sorbitol

A.N. MOLASSIOTIS*,[1], T. SOTIROPOULOS**, G. TANOU*, G. KOFIDIS***, G. DIAMANTIDIS* and I. THERIOS*

*School of Agriculture, Aristotle University of Thessaloniki, GR-54124 Thessaloniki, Greece**
*NAGREF, Pomology Institute, POB 122, Naousa GR-59200, Greece***
*Department of Botany, School of Biology, Aristotle University, GR-54124, Thessaloniki, Greece****

Abstract

To determine whether the major influence of high salinity is caused by the osmotic component or by salinity-induced specific ion toxicity, we compared the effects of mannitol, sorbitol, NaCl and KCl (all in concentratuions corresponded to osmotic potential -1.0 MPa) on the antioxidant and anatomical responses of the apple rootstock MM 106 explants grown in the Murashige and Skoog (MS) medium. All the compounds had a significant influence on explant's mineral composition and reduced the leaf water content, whereas mannitol and salts decreased chlorophyll (Chl) content and increased proline content. Superoxide dismutase (SOD), peroxidase (POD) and non-enzymatic antioxidant activities as well as H_2O_2 content were increased in the leaves and stems. In addition, in the leaves of explants exposed to NaCl an additional Mn-SOD isoform was revealed, while specific POD isoforms were detected in the leaves and stems treated with NaCl or KCl. However, catalase activity was depressed in the salt-treated leaves. At the ultrastructural level, the NaCl-treated leaves had the thickest lamina, due to an extensive increase of the size of epidermal and mesophyll cells. Also, an increase of the relative volume of the intercellular spaces in response to NaCl was observed. The results suggest that Na accumulation is the first candidate for the distinct antioxidant and anatomical responses between saline and osmotically generated stress in the MM 106 explants.

Additional key words: catalase, chlorophyll, *Malus domestica*, peroxidase, polyacrylamide gel electrophoresis, proline, superoxide dismutase.

Introduction

Salinity is a significant environmental factor limiting productivity and allocation of crops, throughout the world. The resulting damage to plants exposed to salinity has been ascribed to ion toxicity, nutrient imbalance and osmotic stress (Grattan and Grieve 1998, Zhu 2001). Although many studies have tried to determine whether plant damage is primarily caused by the osmotic effect or by specific ion toxicity, this point is still controversial (Bahaji *et al.* 2002).

In addition to these interrelated and coexisting impacts, salinity is secondary expressed as oxidative stress (Lee *et al.* 2001, Zhu 2001, Panda and Upadhyay 2004) due to rapid and transient accumulation of reactive oxygen species (ROS), like superoxide radical, hydroxyl radical and singlet oxygen. ROS interact with a wide range of molecules causing pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and DNA mutation (Mittler 2002). To mitigate and repair the damage initiated by ROS, plants have evolved specific protective mechanisms. Antioxidants can be divided into two classes including *a*) the low molecular mass non-enzymatic free radical scavengers and *b*) enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) (Xiong and Zhu 2002). SOD is a major scavenger of $^{\cdot}O_2^-$ and its enzymatic action results in the formation of H_2O_2 and O_2. POD decomposes H_2O_2, by oxidation of co-substrates, such as phenolic

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Abbreviations: CAT - catalase; Chl - chlorophyll; FRAP - ferric reducing antioxidant power; MS medium - Murashige and Skoog medium; PAGE - polyacrylamide gel electrophoresis; POD - peroxidase; SOD - superoxide dismutase; WC - water content.
[1] Author for correspondence; fax: (+32) 310 402705, e-mail: amolasio@agro.auth.gr

compounds and/or antioxidants, whereas CAT breaks down H_2O_2 into water and molecular oxygen (Mittler 2002).

Plants grown under salinity also accumulate compatible solutes, such as sorbitol, mannitol and proline (Xiong and Zhu 2002). These substances, referred as osmolytes, were originally thought to function as osmotic buffers lowering the cellular osmotic potential to sustain water absorption from saline solutions and to restore intracellular ion homeostasis (Zhu 2001). Recently, it has been demonstrated that these substances exhibit also ROS scavenging properties although the underlying mechanism is not clear (Xiong and Zhu 2002). Apart from these responses, salinized plants display also morphological and developmental adjustments, which at cellular level imply several anatomical disarrangements (Shannon *et al.* 1994, Romero-Aranda *et al.* 1998, Bahaji *et al.* 2002, Sam *et al.* 2003).

In view of the above information, an attempt was made to evaluate and to differentiate the specific antioxidant and anatomical effects of salinity, from the pure osmotic impacts inflicted also by other causes. Using the high commercial value MM 106 apple rootstock as plant material we imposed iso-osmotic stress (-1.0 MPa) by the inclusion of mannitol, sorbitol, NaCl, and KCl in the Murashige and Skoog (1962, MS) medium. We used these types of polyols to induce osmotic stress since it is known that in Rosaceous fruit trees, such as apple, osmotic adjustment is facilitated mainly by sorbitol and mannitol accumulation (Wang and Stutte 1992). Research was performed *in vitro* to allow better control of the stress conditions and environmental parameters.

Materials and methods

Plants, culture conditions and treatments: Shoot tips (approximately 20 mm in length) of the apple (*Malus domestica* Borkh) rootstock MM 106 were used as explants. Each explant was cultured onto 25 × 100 mm glass test tube containing 10 cm^3 of the MS medium supplemented with 30 g dm^{-3} sucrose, 1.5 mg dm^{-3} benzyladenine (BA) and 0.2 mg dm^{-3} gibberellic acid (GA_3). The stress imposition was inflicted adjusting the osmotic potential of MS culture medium to -1.0 MPa, (25 °C) by the addition of 576 mM mannitol, 562.5 mM sorbitol, 240 mM NaCl and 220 mM KCl, while the MS medium was referred as the control (-0.21 MPa). The osmotic potential of each medium was evaluated prior to agar addition with a *Wescor* osmometer (*HR 33-T Dew Point Microvoltmeter*, Logan, USA). The pH of the media was adjusted to 5.6 before autoclaving at 121 °C for 15 min. The explants were incubated in a growth chamber at 22 ± 1 °C with 16-h photoperiod and irradiance of 55 μmol m^{-2} s^{-1} for 15 d.

Measurements of water and mineral content: The leaf water content (WC) was calculated from the equation WC [%] = (f.m. - d.m.)/f.m. × 100, measuring both fresh (f.m.) and dry mass (d.m.). For the mineral concentration measurements the explants were dry ashed in a muffle furnace at 500 °C for 6 h. The ash then dissolved in 0.1 M HCl. Calcium, K, Mg, Na, Zn, Fe and Mn concentrations were measured by atomic absorption spectrometry (*Perkin-Elmer 2380*, USA) whereas Cl concentration by potentiometric titration with 0.01 M $AgNO_3$.

Determination of chlorophyll, proline and H_2O_2 content: The leaf chlorophyll (Chl) content was estimated by extraction of leaf disks (0.77 cm^2) with 96 % ethanol in water bath at 78 °C, until complete discoloration of the disks. The absorbance of the extracts was measured at 665 and 649 nm. Total Chl (*a*+*b*) content was calculated by the equations given by Wintermans and Mots (1965) and expressed on the basis of dry mass. Proline content in the leaves and stems of explants was measured as described by Bates *et al.* (1973). The H_2O_2 content was estimated according to Pazdzioch-Czochra and Widenska (2002) with a *Shimadzu RF-500* spectrofluorimeter (*Shimadzu*, Kyoto, Japan).

Preparation of extracts: For POD and CAT assay the leaves and stems of explants were homogenized in a cold mortar with a pestle in a three-fold volume of 50 mM Na-phosphate buffer (pH 6.5) containing 2.0 % (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and 1 M NaCl. To prepare crude extracts for SOD assay the plant material was homogenized in an extraction buffer consisting of 50 mM Tris (pH 7.8), 2.0 % (m/v) PVPP and 1 mM EDTA. The homogenates were centrifuged (12 000 *g* for 20 min at 4 °C) and the supernatants were used for enzyme assays. For the determination of the non-enzymatic antioxidant activity, the leaves and stems of explants were extracted according to Kuo *et al.* (1999). Protein was estimated by the method of Bradford (1976), using bovine serum albumin as a standard.

Antioxidant activity: SOD activity assay was based on the method of Beauchamp and Fridovich (1971) that measures SOD ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). POD activity was assayed as described by Ngo and Lenhoff (1980) while CAT activity was measured according to Wang (1995). The total non-enzymatic antioxidant potential of

sample was determined using a ferric reducing antioxidant power (FRAP) assay and expressed as FRAP values [μM] (Benzie and Strain 1996). All spectrophotometric analyses were conducted in a *Shimadzu UV-1601* spectrophotometer at room temperature (25 ± 2 °C).

Polyacrylamide gel electrophoresis (PAGE): Equal amount of protein (50 μg) from original extracts were loaded per well on polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, using a Mini Protean III electrophoresis cell (*Bio-Rad*, Hercules, CA, USA) at 4 °C. Anionic PAGE was performed using a 7.5 % (m/v) polyacrylamide resolving gel, topped with 4 % stacking gel as the separation medium, as described by Laemmli (1970), with the exception that SDS was omitted from all buffers.

Staining of POD isoforms on the gels was conducted according to Shimoni (1994). Negative stain of SOD and CAT isoforms was performed as described by Beauchamp and Fridovich (1971), and Woodbury *et al.* (1971), respectively. In order to identify the different type of SOD isoforms, either 3 mM mM KCN (inhibitor of the Cu/ZnSOD) or 5 mM H_2O_2 (inhibitor of the Cu/ZnSOD and FeSOD) were added to the incubation buffer.

Leaf anatomy: Small leaf segments of explants were fixed for 3 h in 5 % glutaraldehyde buffered with 0.025 M Na-phosphate (pH 7.2). Postfixation was followed for 4 h, in 1 % osmium tetroxide, similarly buffered. Tissue dehydration was carried out in an alcohol series followed by infiltration in Spurr's resin. Semithin sections (1 μm thick) of plastic embedded leaves were obtained in a *Reichert Om* U_2 (Vienna, Austria) ultramicrotome, stained with 1 % toluidine blue O in borax and examined with a photomicroscope (*Zeiss III*, Oberkochen, Germany).

Morphometric assessment of the relative volume of the leaf histological components was conducted using a transparent sheet bearing a square lattice of point arrays, 10 mm apart, laid over light micrographs of leaf cross-sections (×800). The point-counting analysis technique was then applied (Steer 1981). Similar sections were used to estimate the thickness of the leaf histological components.

Statistical analyses: Each treatment included at least 30 replications (tubes). Statistical analysis of the data was performed using the program *SPSS-11* (Chicago, USA) and significance was determined at 95 % confidence limits.

Results

Compared to control, mannitol, sorbitol, NaCl and KCl decreased WC; the decrease induced by NaCl was significantly greater than that of mannitol and sorbitol (Table 1). A decrease in Chl content was noted for the salts and mannitol media, but the influence of salts was significantly greater. In addition, both saline media significantly elevated Cl^- content, while NaCl and KCl further increased Na^+ and K^+ contents, respectively (Table 1). NaCl decreased K, Ca, Mg, Mn and Fe contents whereas KCl decreased Ca, Mg, Mn, and Fe contents. The inclusion of mannitol and sorbitol in the MS medium resulted in considerable decreases of Mn and Fe contents.

SOD activity increased in the leaves and the stems of explants exposed to osmotica and salts (Fig. 1*A*). However, NaCl-treated leaves had higher SOD activity than the others. Native PAGE was performed in order to determine whether the increase in SOD activity was due to an increase in the activity of the constitutive isoenzymes or due to the induction of new isoforms. In the leaves and stems of explants one isoform of Cu/Zn-SOD 1 and one of Mn-SOD 2 were observed (Fig. 2*A*). Moreover, an additional SOD isoform (identified as Mn-SOD 1) was visualized in the NaCl-treated leaves (Fig. 2*A*). Fe-SOD isoform was not observed in the native gels.

Table 1. Effect of the different culture media (all corresponded to -1.0 MPa) on mineral, WC and Chl contents in the MM 106 explants. The mean values (n = 3) in a column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Culture medium	K [mg g^{-1}(d.m.)]	Ca	Mg	Cl	Na [μg g^{-1}(d.m.)]	Mn	Fe	WC [%]	Chl [mg g^{-1}(d.m.)]
Control	18.3 b	8.0 a	1.9 a	1.1 b	2.0 bc	100 a	115 a	94.43 a	4.58 a
Mannitol	17.6 b	7.7 a	1.9 a	1.0 b	2.2 b	78 b	86 b	90.40 bc	3.74 b
Sorbitol	17.8 b	7.9 a	1.7 ab	1.2 b	2.3 b	72 bc	79 bc	91.50 b	4.03 ab
NaCl	15.2 c	6.1 b	1.5 bc	3.5 a	3.1 a	67 c	66 c	88.30 c	3.01 c
KCl	22.8 a	6.3 b	1.4 c	3.3 a	1.9 c	68 c	77 bc	89.10 c	3.11 c

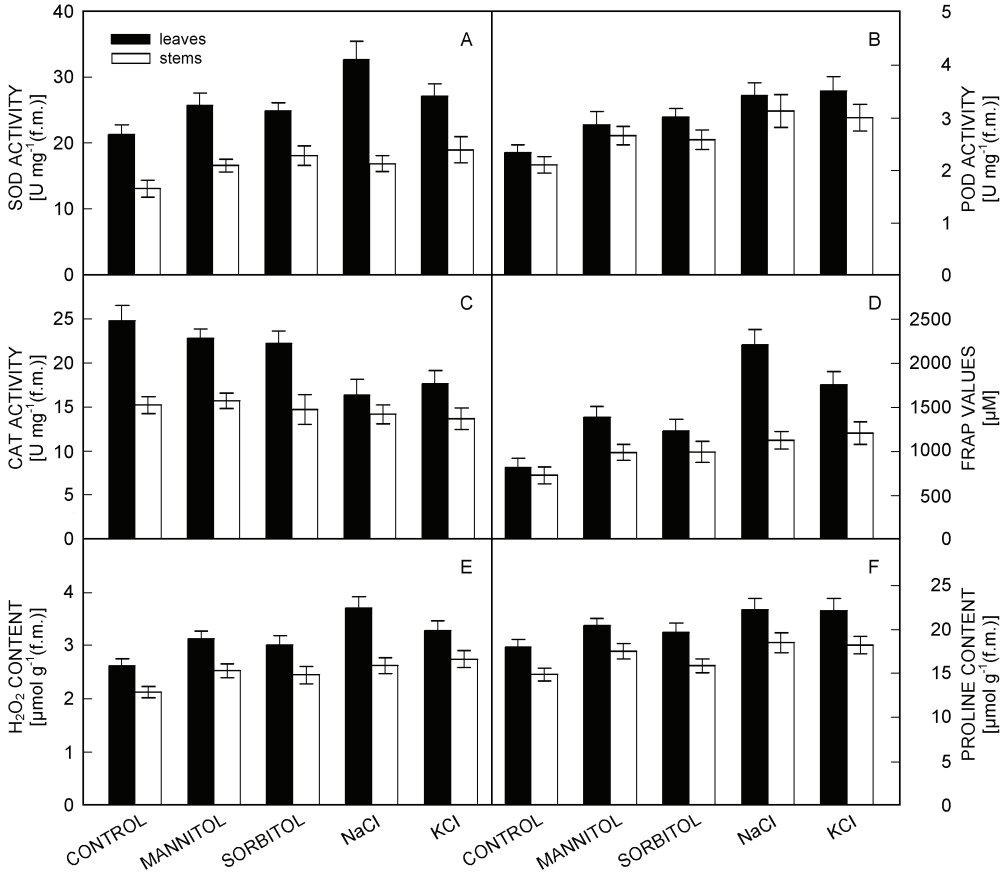

Fig. 1. Effect of different culture media (all corresponded to -1.0 MPa) on SOD (*A*), POD (*B*), CAT (*C*) and non enzymatic antioxidant (FRAP values) (*D*) activities as well on H_2O_2 (*E*) and proline (*F*) content in the leaves and stems of MM 106 explants. *Vertical bars* indicate SE for three samples each one from three different extracts.

Table 2. Effect of the different culture media on the thickness and the relative volume of leaf components in the MM 106 explants. The mean (n = 5) values in a column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Culture medium	Thickness [μm]					Relative volume [%]		
	upper epidermis	palisade parenchyma	spongy parenchyma	lower epidermis	lamina thickness	epidermal cells	mesophyll cells	intercellular spaces
Control	15.0 b	20.0 a	58.2 d	11.2 b	104.4 d	25.0 a	50.5 a	24.5 b
Mannitol	15.0 b	17.8 b	72.8 c	10.5 b	115.3 c	21.2 ab	50.1 a	28.7 b
Sorbitol	14.7 b	20.2 a	56.9 d	11.4 b	103.2 d	25.9 a	50.2 a	23.9 b
NaCl	20.0 a	20.5 a	110.6 a	12.9 a	165.9 a	19.2 b	42.4 b	38.4 a
KCl	16.3 ab	18.4 b	91.0 b	12.8 a	138.5 b	22.9 ab	48.8 ab	28.3 b

In comparison to control, POD activity of leaves and stems of explants exposed to osmotica and salt was increased (Fig. 1*B*). Native-PAGE of leaf extracts revealed four POD isoforms (POD 1, POD 2, POD 4, POD 5) in all media (Fig. 2*B*), whereas an additional POD isoform (POD 3) was visualized in the salinity-treated leaves. In the stems of explants three POD isoforms (POD 1, POD 2, POD 4) were detected in all media while the POD 5 isoform was expressed only in the stems of explants exposed to salinity. CAT activity, unlike SOD and POD, exhibited a significant decrease in the salt-treated leaves as compared with control (Fig. 1*C*) whereas in the stem extracts no significant change of CAT activity was recorded. One CAT isoform (CAT 1)

was detected in the leaves and stems in the all treatments (Fig. 2*C*). In addition, both osmotic and salinity media increased significantly the non-enzymatic antioxidant activity (FRAP values) in the leaves and stems of explants (Fig. 1*D*). However, the increases in FRAP values of the leaves caused by salts were apparently greater than that of osmotica, whereas the effect of NaCl exceeded that of KCl.

In both the leaves and stems of explants exposed to osmotic and salinity media the H_2O_2 content was increased compared with control (Fig. 1*E*). H_2O_2 was more accumulated in the NaCl-treated leaves, as compared to mannitol, sorbitol and KCl-treated ones. Furthermore, the addition of mannitol, NaCl and KCl in the MS medium resulted in a significant increase of proline content in the leaves and stems of explants compared to control whereas sorbitol had no effect on the proline content (Fig. 1*F*).

Leaves of the apple explants exhibited the typical anatomy for the dicots (Fig. 3). The upper epidermis had larger cells than the lower one (Table 2) but it was devoid of stomata, which, however, were numerous on the lower epidermis. Palisade parenchyma ordinarily consisted of one layer of short cells, not so elongated as in the typical dicot leaf. The major portion of the mesophyll was composed of spongy parenchyma cells and large intercellular spaces (Fig. 3). Cross-sections of differently treated explant leaves disclosed apparent variations in thickness (Table 2). Mannitol and sorbitol-treated leaves were significantly thinner than the salt-treated ones, while all explants, except the sorbitol-treated, had thicker leaves than the control. NaCl-treated leaves had the thickest lamina due to an extensive increase of the cell size of epidermal and spongy parenchyma cells (Table 2, Fig. 3). In addition, the relative volume of the intercellular spaces was obviously higher in the NaCl-treated leaves (Table 2).

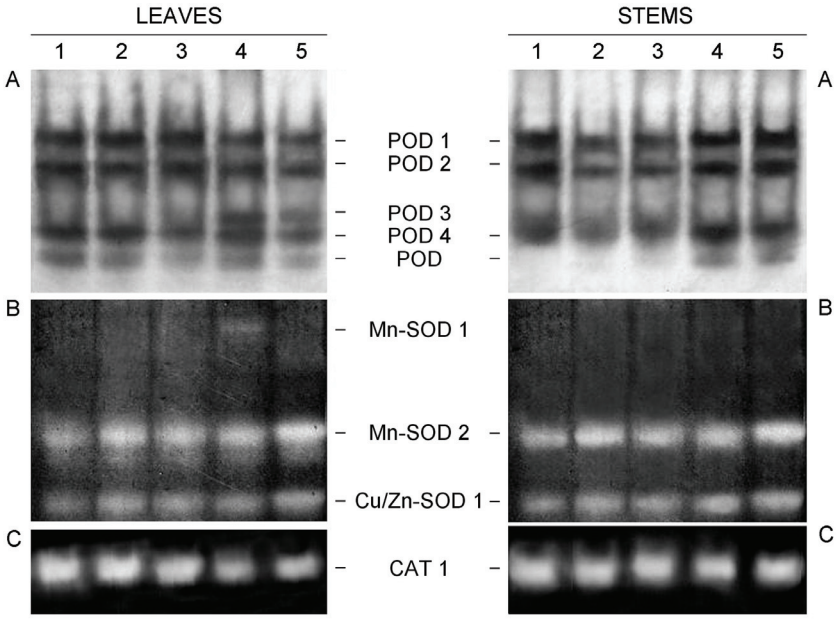

Fig. 2. Native-PAGE and activity staining for SOD (*A*), POD (*B*) and CAT (*C*) activities in the leaves and stems of MM 106 explants in response to different culture media. *Lane 1* - control; *lane 2* - mannitol; *lane 3* - sorbitol; *lane 4* - NaCl; *lane 5* - KCl-treated samples.

Discussion

Under our experimental conditions, both osmotica and especially salts reduced WC, which at cellular level is translated into changes in f.m. and d.m. depicting growth inhibition. Apart from the WC, saline treatments caused severe decreases in Chl content, whereas the impact of polyols was less severe. Bahaji *et al.* (2002) also verified this effect of salinity on Chl content. Although WC is a very sensitive parameter of plant development, Chl formation is impaired at slightly more severe stresses (Hsiao 1973). Judging from the changes in WC and Chl content, salinity is probably a more severe stress than the pure osmotic and thus Chl decrease can be attributed to the other parameters of salinity and not to osmotic stress included.

At the nutrients level, the inclusion of osmotica in the MS medium did not seem to cause severe nutrient imbalance. One possible explanation could be the ability of mannitol and sorbitol to enter the cell wall space

resulting in osmotic adjustment as well the milder stress conditions (Xiong and Zhu 2002). On the contrary, salinized explants displayed Na, K and Cl accumulation, depending on the salt added, as well as decreases in Ca, Mg, Mn, Zn, Fe concentrations and K in the case of NaCl. Grattan and Grieve (1998) have also reported previously Mg, Mn, Zn and Fe decreases in response to salinity. According to Blumwald *et al.* (2000) the decrease of K concentration due to NaCl may be attributed to high external Na concentration. Since the two ions have similar hydrated ionic radii, transport proteins find difficult to discriminate them, making easy the Na entry to the cell through low-affinity and high affinity K carriers, excluding K uptake (Maathuis and Amtmann 1999). Furthermore, translocation of Na to the leaves leads to a displacement of the apoplastic Ca causing depolarization of membrane systems (Nakamura *et al.* 1992). Subsequently, the ability of membranes to selectively absorb some ions is impaired and ion imbalance is inevitable.

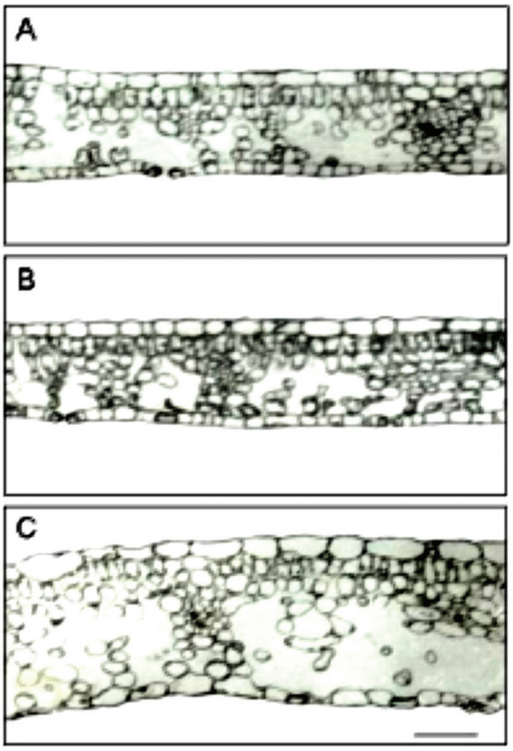

Fig. 3. Light micrographs of leaf cross sections of control (*A*), sorbitol (*B*) and NaCl-treated (*C*) MM 106 explants. The NaCl-treated leaf appeared thicker with more voluminous intercellular spaces. *Bar* represents 50 μm.

Our results illustrated that both osmotica and salts could up-regulate the antioxidant mechanism of MM 106. Indeed, we showed SOD activity to increase under osmotic and saline conditions whereas an additional Mn-SOD isoform was detected in the NaCl-treated leaves. The enhancement of SOD activity by salinity has also been observed by Lee *et al.* (2001). Although SOD functions as the first line of defense against oxidation at the membrane boundaries, its end product is the toxic H_2O_2 (Mittler 2002). In the present study saline and iso-osmotic conditions caused a H_2O_2 accumulation particularly evident for the leaves of explants exposed to NaCl. Therefore, an efficient H_2O_2-scavening system is required to enable rapid removal of H_2O_2 in the MM 106 cells. We showed that POD activity increase due to osmotica and salts whereas additional POD isoforms were detected in the explants exposed to salinity. The enhancement of POD activity by salinity has also been observed in the rice leaves (Lee *et al.* 2001) and callus cultures of *Suaeda nudiflora* (Cherian and Reddy 2003). Lee *et al*. (2001) pointed out that the induction of specific POD isoforms by salinity occurs under CAT deactivation. Our results confirmed the inability of CAT to cope with salinity since CAT activity depressed in the saline-treated leaves. Furthermore osmotic and salt media stimulated the non-enzymatic antioxidants in the leaves and stems of explants, however, this increase was more pronounced in the NaCl-treated leaves. From the overall results obtained by the antioxidant study, it is clear that NaCl conditions led to a strong oxidative stress in respect to the other iso-osmotic media tested.

One of the most challenging and controversial issues arose from this study concerns POD activation in the polyol-treated explants without the subsequent detection of additional isoforms, contrary to the case of salts. Recent studies have demonstrated that mannitol and sorbitol act also as compatible osmoprotectants detoxifying ROS (Xiong and Zhu 2002). Hence, due to the scavenging properties of mannitol and sorbitol and the enzymatic antioxidant mechanism involving both POD and CAT less ROS were present in the cell, so there was no need for the explants to mobilize the expression of additional POD isoforms. For these reasons, we suggest that the increased POD activity of osmotic treated explants could be probably owing to the activating effect of mannitol and sorbitol on the transcriptional level of already existing POD isoforms.

Data on proline accumulation pattern indicated that mannitol and salt treatments triggered an accumulation of proline in the leaves and stems of explants. Our results are in agreement with those were reported by Dash and Panda (2001) for salt-treated seeds of *Phaseolus mungo*. Of particular interest in the reported investigation is the finding that proline content was not increased in the leaves and stems of MM 106 exposed to sorbitol as reported previously in rice by Al-Khayri and Al-Bahrany (2002). According to Wang and Stutte (1992) sorbitol was the main sugar in the cell sap and accounted for up to 50 % of total osmotic adjustment in apple trees under water stress. This observation suggests that sorbitol plays a major role in active osmotic adjustment in apple. Hence, it is reasonable to assume that a high proportion of exogenous sorbitol may be absorbed by the MM 106

explants, and therefore, its osmotic adjustment ability increases. This fact might probably explain why the content of Chl and proline was not significantly affected in the explants exposed to sorbitol.

Besides the increases in the antioxidant capacity, explants under salinity and osmotic stress, take on anatomical adjustments in order to save water while salinity imposes further adaptations so as to avoid the toxic effects of salts. To differentiate the anatomical responses related to osmotic stress, and Na and Cl accumulation, we examined the anatomy of osmotic and saline treated leaves. In this respect, the greater thickness appeared in the saline-treated leaves can be attributed to high Na and Cl concentrations. The fact that the NaCl-treated leaves were significantly thicker with more voluminous intercellular spaces than the leaves of the KCl treated explants lead us to assume that the further effects of NaCl are due to the high Na concentration since both saline media caused equal accumulation of Cl in the explants tissues. Apart from leaf thickness, which has also been reported to increase due to salinity in *Citrus* seedlings (Romero-Aranda 1998), the larger size of epidermal and bulliform cells has been further mentioned in *Oryza sativa* salt-stressed leaves (Bahaji *et al.* 2002). Increases in cellular size are usually associated with succulence (Romero-Aranda 1998), which is a typical morphological response to salinity in dicot species (Shannon *et al.* 1994). The relative volume of the intercellular spaces was also found to be significantly higher in NaCl-treated leaves. Similarly, Sam *et al*. (2003) found that salinity increased the volume of the palisade intercellular spaces in leaves of two tomato cultivars. In *Citrus*, the spongy parenchyma of salt-treated leaves was thicker, consisted of enlarged cells, however, the volume of the spongy parenchyma intercellular spaces was reduced in relation to the controls (Romero-Aranda 1998).

Recently, Bahaji *et al*. (2002) supported that most of the saline-related responses in rice seedlings were due to osmotic stress and not due to the ionic component of salinity. The picture that emerges from our study suggests that the presence of osmatica and salts in the MS medium caused several common and distinct responses in the MM 106 explants. However, our results indicate that NaCl induced the most considerable responses as compared with KCl and especially with mannitol and sorbitol. Since the saline media had the same osmolarity with mannitol and sorbitol and the most striking impact on nutrient level concerned Na and Cl toxic concentrations, the differences appeared between the salts and polyols treated explants could be ascribed in the first place to deleterious effects of Na and Cl. As Cl concentration did not vary considerably between the NaCl and KCl treated explants, we conclude that that Na accumulation was the first candidate for these specific responses.

References

Al-Khayri, J.M., Al-Bahrany, A.M.: Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmotic stress in rice. - Biol. Plant. **45**: 609-611, 2002.

Bahaji, A., Mateu, I., Sanz, A., Cornejo, M.J.: Common and distinctive responses of rice seedlings to saline- and osmotically-generated stress. - Plant Growth Regul. **38**: 83-94, 2002.

Bates, L.S., Waldren, S.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Beauchamp, B., Fridovich, I.: Superoxide dismutase: improved assays and applicable to acrylamide gels. - Anal. Biochem. **44**: 276-287, 1971.

Benzie, I.F.F., Strain, J.J.: The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. - Anal. Biochem. **239**: 70-76, 1996.

Blumwald, E., Aharon, G.S., Apse, M.P.: Sodium transport in plant cells. - Biochim. biophys. Acta **1465**: 140-151, 2000.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Cherian, S., Reddy, M.P.: Evaluation of NaCl tolerance in the callus cultures of *Suaeda nudiflora* Moq. - Biol. Plant. **46**: 193-198, 2003.

Dash, M., Panda, S.K.: Salt stress induced changes in growth and enzyme activities in germinating *Phaseolus mungo* seeds. - Biol. Plant. **44**: 587-589, 2001.

Grattan, S.R., Grieve, C.M.: Salinity-mineral nutrient relations in horticultural crops. - Scientia Hort. **78**: 127-157, 1998.

Hsiao. T.C.: Plant responses to water stress. - Annu. Rev. Plant Physiol. **24**: 519-570, 1973.

Kuo, J.M., Yeh, D.B., Pan, B.S.: Rapid photometric assay evaluating antioxidative activity in edible plant material. - J. Agr. Food Chem. **47**: 3206-3209, 1999.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lee, D.H., Kim, T.S., Lee, C.B.: The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). - J. Plant Physiol. **158**: 737-745, 2001.

Maathuis, F.J.M., Amtmann, A.: K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. - Ann Bot. **84**: 123-133, 1999.

Mittler, R.: Oxidative stress, antioxidants and stress tolerance. - Trends Plant Sci. **7**: 405-410, 2002.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue culture. - Physiol. Plant. **15**: 473-495, 1962.

Nakamura, Y., Kasamo, K., Shimosato, N., Sakata, M., Ohta, E.: Stimulation of the extrusion of protons and H^+-ATPase activities with the decline in pyrophosphatase activity of the tonoplast in intact mung bean roots under high-NaCl stress and its relation to external levels of Ca^{2+} ions. - Plant Cell

Physiol. **33**: 139-149, 1992.

Ngo, T.T., Lenhoff, H.M.: A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. - Anal. Biochem. **105**: 389-397, 1980.

Panda, S.K., Upadhyay, R.K.: Salt stress injury induces oxidative alterations and antioxidative defence in the roots of *Lemna minor*. - Biol. Plant. **48**: 249-253, 2004.

Pazdzioch-Czochra, M., Widenska, A.: Spectrofluorimetric determination of hydrogen peroxide scavenging activity. - Anal. chim. Acta **452**: 177-184, 2002.

Romero-Aranda, R., Moya, J.L., Tadeo, F.R., Legaz, F., Primo-Millo, E., Talon, M.: Physiological and anatomical disturbances induced by chloride salts in sensitive and tolerant citrus: beneficial and detrimental effects of cations. - Plant Cell Environ. **21**: 1243-1253, 1998.

Sam, O., Ramirez, C., Coronado, M.J., Testillano, P.S., Risueno, M.C.: Changes in tomato leaves induced by NaCl stress: leaf organization and cell ultrastructure. - Biol. Plant. **47**: 361-366, 2003.

Shannon, M.C., Grieve, C.M., Francois, L.E.: Whole-plant response to salinity. - In: Wilkinson, R.E. (ed.): Plant-Environment Interactions. Pp. 199-204. Marcel Dekker, New York 1994.

Shimoni, M.: A method for activity staining of peroxidase and β-1, 3-glucanase isozymes in polyacrylamide electrophoresis gels. - Anal. Biochem. **220***:* 36-38, 1994.

Steer, M.W.: Understanding Cell Structure. - Cambridge University Press, Cambridge 1981.

Wang, C.Y.: Effect of temperature preconditioning on catalase, peroxidase and superoxide dismutase in chilled zucchini squash. - Postharvest Biol. Technol. **5**: 67-76, 1995.

Wang, Z., Stutte, G.W.: The role of carbohydrates in active osmotic adjustment in apple under water stress. - J. amer. Soc. hort. Sci. **117**: 816-823, 1992.

Wintermans, J.F., Mots, A.: Spectrophotometric characteristics of chlorophylls *a* and *b* and pheophytins in ethanol. - Biochim. biophys. Acta. **109**: 448-453, 1965.

Woodbury, W., Spencer, A.K., Stahmann, M.A.: An improve procedure using ferricyanide for detecting catalase isozymes. - Anal. Biochem. **44**: 301-305, 1971.

Xiong, L., Zhu, J.K.: Molecular and genetic aspects of plant responses to osmotic stress. - Plant Cell Environ. **25**: 131-139, 2002.

Zhu, J.K.: Plant salt tolerance. - Trends Plant Sci. **6**: 66-71, 2001.