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Running title: Calcium and antioxidant enzymes in salt treated plants

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The effect of calcium on the antioxidant enzymes from salt-treated loquat and anger plants

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ABSTRACT

Salt treatment (50 mM NaCl) reduced plant growth of loquat (*Eribotria japonica* Lindl.) (by to 40%) but not of anger (*Cydonia oblonga* Mill.). Salt stress induced a strong leaf Na^+ accumulation in both species. However, the increase observed in leaf Cl^- level was higher in loquat (13-fold) than in anger plants (3.8-fold). Ca^{2+} addition (25 mM) significantly reduced Na^+ and Cl^- concentrations in both salt-treated species. In anger leaves, calcium addition to the nutrient media did not change the leaf calcium contents in salt-treated or untreated plants, this value being lower in salt-treated plants. However, in loquat plants, an increase in leaf Ca^{2+} was observed for the calcium addition. Surprisingly, an increase in Ca^{2+} concentration was also observed in salt-treated loquat plants. In general, anger plants had higher constitutive antioxidant enzyme levels in both control and salt-treated plants. Salt stress did not change antioxidant enzyme levels in loquat plants. A similar effect was observed in anger plants, but in this case an induction of MDHAR activity was observed (2-fold).

In both species, salinity produced an oxidative stress, indicated by an increase in lipid peroxidation, this value being much higher in loquat (83%) than in anger (40%) plants. In salt-treated plants, Ca^{2+} addition produced some protection to the membranes, because the increases observed in TBARS were not significant.

On the other hand, in control plants, Ca^{2+} treatments increased GR and decreased catalase activity for anger, but increased MDHAR, DHAR, GR and SOD in loquat plants. In salt-treated plants, Ca^{2+} additions decreased CAT and APX for anger and raised DHAR, GR and SOD for loquat. However, the mechanism by which Ca^{2+} could regulate antioxidant enzymes remains to be determined.

These results suggest that anger plants have a higher capacity to scavenge AOS, both under control and salinity conditions. Accordingly, and related to the smaller Cl^- increase observed, anger plants are more salt tolerant, at least partly, due to the higher antioxidant enzyme levels observed.

Keywords:antioxidant enzymes, Calcium, *Cydonia oblonga* Mill., *Eribotria japonica* Lindl, salt stress, oxidative stress.

Abbreviattions: AOS, activated oxygen species; ASC-GSH cycle, ascorbate-glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR,; glutathione reductase; MDHAR, monodehydroascorbate reductase; SOD superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Introduction

Salinity is one of the major limiting environmental factors in crop production. Under salt stress, plants have to cope with water stress imposed by the low external water potential and with ion toxicity due to accumulation inside the plants (Bohnert and Jensen 1996). In addition to its known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress, all of which contribute to its deleterious effects (Gueta-Dahan *et al*. 1997; Hernández *et al*. 2001; Rios-Gonzalez *et al*. 2002).

The effects of various environmental stresses in plants are known to be mediated, at least partially, by an enhanced generation of activated oxygen species (AOS) such as

superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) (Alscher *et al*. 1997; Shalata and Tal 1998; Hernández *et al*. 2001; Vitória *et al*. 2001; Able *et al*. 2003). Plants contain a complex antioxidant system to detoxify AOS that includes carotenoids, ascorbate, glutathione, tocopherols and anthocyanin pigments (Neill *et al*. 2002; Del Río *et al*. 2002), and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases, and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle; Foyer and Halliwell 1976): ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2). The components of this antioxidant defence system can be found in different subcellular compartments (Jiménez *et al*. 1997).

Salinity impairs the uptake of Ca^{2+} ions by plants, either due to ionic interactions and reduction of their activity in the soil solution and therefore their availability to the plant (Cramer *et al*. 1986; Suárez and Grieve 1988) or possibly by displacing it from the cell membranes or in some way affecting membrane function (Lynch *et al*. 1987; Läuchli 1990). It is known that the addition of Ca^{2+} ameliorates the adverse effects of salinity on plants (Cramer *et al*. 1986; Martínez-Ballesta *et al*., 2000). However it has been reported that Ca^{2+} addition has no effects on the salt tolerance of some lettuce genotypes (Cramer and Spurr 1986), and in blueberry plants treated with high NaCl levels, high Ca^{2+} concentrations are even harmful (Wright *et al*. 1995).

Several studies have shown that Ca^{2+} is involved in the regulation of plant responses to various environmental stresses, including heat (Jiang and Huang 2001), salt, and drought stress (Knight *et al*. 1997; Liu and Zhu 1998).

Loquat plants (*Eribotria japonica* Lindl.) are widely grown in the Marina Baja region (Alicante, Spain), where the climatic conditions are suitable for their growth. Loquat plants are normally grafted on *franco* stock, which exhibits a great development and longevity (Vidal 1989). However, loquat plants grafted on *Anger* stock (*Cydonia oblonga* Mill.)

produce trees of short height, showing little tendency for vertical development, these being important characteristics which permit low production costs (Burló-Carbonell *et al*. 1997).

In this work, we studied the effect of salt stress and calcium treatments on the antioxidant systems in leaves of loquat and anger plants, used as rootstock for loquat plants under field contitions. For this,l we analysed plant growth, Na^+, Cl^-, and Ca^{2+} levels as well as CAT, SOD and the ASC-GSH cycle enzymes and the extent of lipid peroxidation.

Material and Methods

Plant material and treatments

Loquat (40 plants) and anger (40 plants) plants were grown for two years in 12-L plastic containers filled with siliceous sand. Groups of 10 uniform plants of each rootstock were used for each treatment. The plants were irrigated generously three times per week to ensure the flow through the pots, and to avoid excessive accumulation of salt in the growing medium. The nutrient solution had the following composition: 3 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM H_3PO_4, 2 mM $MgSO_4$, 5 mM NaCl, 9.25 μM H_3BO_3, 35.8 μM Fe-EDDHA, 1.8 μM $MnSO_4$, 1.35 μM $ZnSO_4$, 0.79 μM $CuSO_4$ and 0.21 μM $(NH_4)_6MO_7O_{24}$, pH 5.8. After six months of acclimation, treatments were begun by adding NaCl and calcium acetate until the required concentrations were reached (NaCl mM + Ca^{2+} mM): 5 + 5 (control plants); 5+25 (control/high calcium plants); 50+5 (salt-treated plants), and 50+25 (NaCl/highCa^{2+} plants), respectively. The control water used in our study is that of the best quality usually used by farmers, and it contains about 5 mM NaCl and 5 mM calcium salts. We think that this reflects better the real situations that occurrs in our region under field conditions. Ca^{2+} addition as calcium acetate has been used successfully by Bañuls *et al.* (1991). In order to avoid an osmotic shock, NaCl and calcium acetate were added gradually over 5 days.

The plants were grown in a greenhouse with partially-controlled conditions. Temperature was maintained between 13 and 32ºC and relative humidity was always kept

over 55%. The plants were treated for four months, and at the end of this period, the plants were removed from their pots and the fresh and the dry weight were determined.

Mineral content

Ten plants from each rootstock were harvested at the end of the experimental period. The leaves of each plant were washed with distilled water, dried at 65^{o}C, ground, and stored at room temperature for inorganic solute analyses. Cl^- contents were analysed in the aqueous extracts by potentiometric titration with $AgNO_3$ (0.01N) in an automatic Metrohm 702 SM Titrino. Na^+ contents were determined in a HNO_3:$HClO_4$ (2:1,v/v) digestion extract by flame photometry (Jenway Ltd. PFP7) and Ca^{2+} by atomic absorption spectrophotometry (Unicam Solaar 969).

Leaf enzyme extraction

All operations were performed at 4ºC. Leaves (2 g) were homogenized with a mortar and pestle in 4 mL of ice-cold 50 mM Tris-HCl buffer (pH 6.5), containing 0.1 mM EDTA 8 mM cysteine, 2% (w/v) PVPP, 0.1 mM PMSF, and 0.2% (v/v) Triton X-100. For APX activity, 20 mM sodium ascorbate was added. The homogenate was centrifuged at 14000 g for 20 min and the supernatant fraction was filtered through Sephadex G-25 NAP columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with the same buffer used for the homogenization, with or without 5 mM sodium ascorbate.

Enzymatic determinations

APX, DHAR, MDHAR, and GR activities were assayed according to previously published protocols, as described by Hernández *et al*. (2001). Enzyme activities were corrected for non-enzymatic rates and for interfering oxidations (Jiménez *et al*. 1997). APX was measured in the presence and absence of the specific inhibitor p-chloromercuryphenyl sulfonic acid (pCMPS) (0.5 mM). pCMPS-sensitive ascorbate peroxidase activity was considered as being due to class I ascorbate peroxidase (EC 1.11.1.11).

Catalase and total SOD activity were assayed as described in Hernández *et al*. (2001)

Assays

The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS). The leaf samples were immediately frozen in liquid nitrogen. Leaf material (200 mg) was homogenized in 2 mL of 0.1% TCA solution. The homogenate was centrifuged at 15000 g for 10 min and 0.5 ml of the supernatant obtained was added to 1.5 mL 0.5% TBA in 20% TCA. The mixture was incubated at 90º C in a water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice-water bath. Then, the samples were centrifuged at 10000 g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted (Cakmak and Horst 1991). The amount of TBARS (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1} (Cakmak and Horst 1991).

Protein was estimated according to Bradford (1976).

Statistical analysis

Comparisons among means were made using the Least Significant Difference (LSD) test, calculated at $P<0.05$. Statistical procedures were carried out with the software package SPSS 10.0 for Windows.

Results

Growth inhibition of 40% was observed in loquat plants submitted to 50 mM NaCl, whereas no significant effects could be observed in salt-treated anger plants (Fig. 1). The addition of 25 mM calcium to the nutrient media did not improve the growth of salt-treated loquat plants under our experimental conditions.

The leaf Na^+ concentration was increased dramatically in both salt-treated plants species (nearly 5-fold) (Fig. 2*a*). However, the increase observed in leaf Cl^- levels was higher

in loquat (13-fold) than in anger plants (3.8-fold) (Fig. 2*b*). Ca^{2+} addition (25 mM) significantly reduced Na^{+} and Cl^{-} concentrations in both salt-treated plant species.

In relation to Ca^{2+} levels, a different behavior was produced. In anger leaves, the addition of calcium acetate to the nutrient media did not change the leaf Ca^{2+} contents in salt-treated or untreated plants, values being lower in salt treated-plants (Fig. 2*c*). However, in loquat plants, an increase in leaf Ca^{2+} was observed after the addition of calcium to the nutrient media (Fig. 2*c*). Surprisingly, an increase in Ca^{2+} concentration was also observed in salt-treated loquat plants (Fig. 2*c*).

In general, anger plants had higher constitutive antioxidant enzyme levels in both control and salt-treated plants. Under control conditions, the CAT level, was 3.7-fold higher in anger than in loquat plants (Fig. 3*a*). A differential behavior was observed in Ca^{2+}-treated plants depending on the plant species. Calcium produced a significant decrease in CAT activity from anger plants, whereas in loquat a 65% increase was observed. However, no changes were observed under saline conditions, in either anger or loquat plants (Fig. 3a).

The constitutive APX levels were nearly 7-fold higher in anger than in loquat plants (Fig. *3b*). Unlike CAT, APX levels were unchanged by 25 mM Ca^{2+} addition. Salt stress did not change APX activity, neither in loquat nor in anger plants, although, in this case, APX levels in anger were 3.7-fold higher than in loquat plants. On the other hand, under salt stress conditions, Ca^{2+} addition produced a decrease in APX activity from anger plants, but under these conditions activity values were 3.3-fold higher in anger than in loquat plants (Fig. 3*b*).

In the absence of salt, calcium treatment increased MDHAR only in loquat plants (Fig. 4*a*). Salt stress raised this activity only in anger plants (2-fold), whereas no changes were observed with Ca^{2+} treatments under saline conditions, neither in Anger nor in loquat plants (Fig. 4*a*).

No changes were observed in DHAR activity in anger plants due to salt and/or Ca^{2+} additions. However, Ca^{2+} treatment (25 mM) raised DHAR in loquat plants, both in the presence and absence of salt (Fig. 4*b*).

The constitutive levels of GR activity were 5.3-fold higher in anger than in loquat plants. In both species, Ca^{2+} increased GR activity, although this increase was higher in loquat (3.7-fold) (Fig. 5*a*). Under salt stress conditions GR increased only in loquat, although in these conditions, the activity levels were 2-fold higher in anger plants (Fig. 5*a*). GR activity was unchanged by Ca^{2+} addition in salt-treated anger plants. However, Ca^{2+} raised this activity in loquat plants, both in the absence and presence of salt, but the activity levels were lower than those exhibited by anger plants.

Constitutive SOD activity levels were 2-fold higher in anger than in loquat (Fig. 5*b*). In anger leaves, SOD activity was not changed by Ca^{2+} and/or NaCl addition. However, in loquat leaves, 25 mM Ca^{2+} increased it nearly 4-fold and 2.6-fold in the absence and presence of NaCl, respectively. In loquat plants treated only with NaCl, no significant change in SOD was observed. However, in NaCl/high Ca^{2+} loquat plants the SOD activity was 40% higher than that showed by Anger plants (Fig. 5*b*).

In both species, salt stress produced an oxidative stress, indicated by the increase in lipid peroxidation (measured as TBARS), indicating damage to membranes due to salt stress, this value being much higher in loquat (83%) than in Anger (40%), in relation to control plants (Fig. 6). In salt-treated plants, Ca^{2+} addition produced some protection to the membranes, because the increases observed in TBARS were not significant.

Discussion

Under saline conditions, plant growth is generally reduced, although it is known that the degree of growth inhibition by salinity may differ between species and between cultivars

within a species (Hernández *et al*. 1995; Shannon and Grieve 1999), and even between scion-rootstock combinations (Nieves *et al*. 1991).

The results for nutrient uptake suggest that anger leaves had a higher capacity to compartment toxic ions in vacuoles, or that the problem was osmotic rather than toxic, or both at the same time. Ca^{2+} addition (25 mM) produced a lower absorption and/or transport of Na^{+} and Cl^{-} to the leaf in both species. A similar effect has been observed in other plant species (Maas and Grieve 1987; Bañuls *et al*. 1991). It could be that an anionic competition between Cl^{-} and CH_3COO^{-} occurred. However, it has been reported that the Cl^{-} decrease is due to a Ca^{2+} effect and not a CH_3COO^{-} effect, and that calcium probably also plays a regulatory role in membrane permeability to Cl^{-} (Bañuls *et al*. 1991). However, the Ca^{2+} addition did not overcome the adverse effects of NaCl on loquat growth. This effect could be attributed to the following possible reasons: 1.- The fact that our control nutrient solution had a sufficient Ca^{2+} concentration such that the maximum beneficial effects had been manifested already, and a higher Ca^{2+} addition, although it decreased the leaf Na^{+} levels, was not able to improve plant growth. 2.- The effect on plant growth could be attributed to an osmotic stress, in agreement with other authors (Reid and Smith 2000). However, Bañuls *et al*. (1991) reported that Ca^{2+} addition also neutralized the decrease in leaf water and osmotic potentials induced by salinity. 3.- The plant genotype, since Ca^{2+} addition improved plant growth in tomato, cucumber, melon and *Citrus* (Bañuls *et al*. 1991; Al-Harbi, 1995; Martínez-Ballesta *et al*. 2000), but no beneficial effects on growth have been described in several lettuce and wheat genotypes (Cramer and Spurr 1986; Weimberg 1988), in agreement with our results for loquat plants.

The decrease in leaf Ca^{2+} levels in salt-treated anger plants could be explained by a decrease in Ca^{2+} activity in the nutrient solution (Cramer *et al*. 1986; Suárez and Grieve 1988), and possibly by its displacement from the cell membranes or by an effect on membrane function (Lynch *et al*. 1987; Läuchli 1990). However, the increase in leaf Ca^{2+} observed in salt-treated

loquat plants is more difficult to explain, but this result has been corroborated recently in other experiments obtained in loquat plants grafted on the same franco stock (unpublished results).

In the present work, we used four-month treated plants because in the short-term no obvious physiological changes were observed. After two months of the treatments no changes in plant growth were observed. On the other hand, we carried out a longer-term experiment to know which plant was more salt-tolerant. Once this was established, we wanted to study the effect of salt stress on the antioxidant enzymes levels in both plant species. The antioxidant enzyme responses and AOS generation for long-term salt treatment have been measured also in pea, *Citrus* and *Lycopersicon pennellii* plants (Hernández *et al*. 1993, 1995, 2000, 2001, Almansa *et al*. 2002; Mittova *et al*. 2003).

Contradictory results were obtained by different authors in relation to the effect of salt on the activity and protein levels of the various antioxidant enzymes. Some authors have attributed salt tolerance to higher constitutive levels of some antioxidant enzymes (Gueta-Dahan *et al*. 1997; Shalata and Tal 1998; Comba *et al*. 1998; Tsugane *et al*. 1999). However, others authors have found that, rather than the constitutive levels, the coordinated up-regulation of the activities of antioxidative enzymes seems to be one of the mechanisms involved in the salt-tolerance response (Hernández *et al*. 1993, 1995, 2000, 2001; Olmos *et al*. 1994; Piqueras *et al*. 1996; Gómez *et al*. 1999; Mittova *et al*. 2003). Moreover, in this activity response, the importance of the cellular compartment in which the specific antioxidative enzyme(s) is (are) located has been described (Hernández *et al*. 1993, 1995, 2001; Bueno *et al*. 1998; Gómez *et al*. 1999; Mittova *et al*. 2003).

Lycopersicon pennellii L. plants, tolerant to 100 mM NaCl, had SOD, APX, and DHAR activity levels inherently higher than *L. esculentum* L. plants, a salt-sensitive species (Shalata and Tal 1998). Under salinity, APX, CAT, and SOD activity levels were also higher in *L. pennellii* than in *L. esculentum.* These results suggest that the wild, salt-tolerant *L. pennellii* plants are better protected against AOS, inherently and under salt stress conditions,

than the relatively sensitive *L. esculentum* plants. In *pst1* Arabidopsis mutant plants, tolerance to salt stress was correlated with higher SOD and APX activity levels (Tsugane *et al*. 1999). Our results agree with those described by other authors, who suggested that salt tolerance is due, at least partially, to higher constitutive antioxidant enzyme activities (Gueta-Dahan *et al*. 1997; Shalata and Tal. 1998; Tsugane *et al*. 1999). In agreement with these authors, one of the reasons why anger plants are more salt-tolerant than loquat plants could be their higher antioxidant enzyme levels, both under control and saline conditions, suggesting that these plants have a higher capacity to scavenge AOS, both in control and salt stress conditions, a situation in which the AOS production is stimulated, increasing the risk of oxidative damage (Hernández *et al*. 1993, 1995, 2001).

Lipid peroxidation is the symptom most easily ascribed to oxidative damage and it is often used as an indicator of oxidative damage (Hernández and Almansa 2002; Hernández *et al*. 1995, 2001, 2002; Gómez *et al*. 1999). In the present work, the lower TBARS increases observed in salt-treated anger plants could be due, at least partly, to the higher antioxidant enzyme levels found under these conditions.

Most studies on the effect of salt stress on the activity of antioxidant enzymes have been carried out in herbaceous plants or in cell cultures. However, the studies carried out in woody plants are more scarce. In salt-sensitive *Quercus robur* L. plants, salt stress produced an increase in SOD activity, whereas no changes could be observed in APX and GR activities (Sehmer *et al*. 1995). These authors suggested that an increase in SOD and the lack of APX induction might have increased H_2O_2 levels. So, the deleterious effects observed in salt-stressed *Q. robur* plants could be due not only to ion toxicity but also to an increase in the levels of H_2O_2 and derivative species in whole cells (Sehmer *et al*. 1995).

By using different rootstock-scion combinations in lemon trees, a correlation has been observed between a higher constitutive SOD level and a higher salt tolerance in *Citrus limonum* on sour orange, in relation to the other combinations (Almansa *et al*. 2002).

Calcium has been found to be involved in the regulation of various responses of plants to environmental stresses (Knight *et al*. 1997; Liu and Zhu 1998; Jiang and Huang 2001). Calcium may be involved in plant tolerance of heat stress by regulating antioxidant metabolism (Gong *et al*. 1998; Jiang and Huang 2001). External Ca^{2+} also increased drought resistance in *Vigna catjang* L., soybean, and cotton (Mukherjee and Choudhuri 1985; Yang *et al*. 1993), and increased salt tolerance in bean (Cachorro *et al*. 1993).

The effect of Ca^{2+} on the activity of antioxidant enzymes seems to depend on the plant species. In heat-stressed tall fescue (*Festuca arandinacea* L.) and Kentucky blue grass (*Poa pratensis* L.), Ca^{2+} treatment increased CAT, GR, and APX activities and reduced lipid peroxidation (Jiang and Huang 2001). Calcium also enhanced ABA-induced thermotolerance in maize seedlings, increasing the SOD and APX activities and lowering the heat stress-induced lipid peroxidation (Gong *et al*. 1998). In contrast, pre-treatment of maize seedlings with the Ca^{2+} chelator EGTA (plus ABA), which lowered calcium content and weakened the ABA-induced thermotolerance, also lowered the ABA-induced SOD and APX activities (Gong et al. 1998). However, in tobacco plants, Price *et al*. (1994) suggested that $[Ca^{2+}]_{cyt}$ mediated the inhibition of SOD by H_2O_2. However, it is not known how $[Ca^{2+}]_{cyt}$ mediates this inhibition and whether it does so directly or indirectly, but either phosphorylation by calcium-dependent protein kinases or the action of specific calcium-dependent proteases are possibilities (Price *et al*. 1994). However, there is no information about the effect of Ca^{2+} on antioxidant enzymes under saline conditions. One may consider transient changes in cytosolic calcium as a common mediator affected by oxidative stress (Price *et al*. 1994) and salinity (Lynch *et al*. 1987).

Some studies have suggested that calcium loading in root cells induces a dramatic increase in $O_2^{\cdot -}$ release during wound stress (Minibayeba *et al*. 1998). Other authors reported that production of AOS is stimulated by Ca^{2+} and calmodulin in purified plasma membrane from wheat roots and transgenic tobacco expressing a foreign calmodulin gene (Qiu *et al*.

1995; Harding *et al*. 1997). However, excessive Ca^{2+} released into the cytosol and a sustained high cytosolic Ca^{2+} concentration might be cytotoxic (Hepler and Wayne 1985; Biyasheva *et al*. 1993) and, probably, this toxic effect could be due, at least partly, to the increased AOS generation. Under saline conditions, calcium-treated loquat plants had higher SOD levels than anger plants. Probably, the increase in SOD activity, observed in loquat plants under these conditions, could have been mediated by the higher $O_2^{\cdot -}$ production, that could have been induced by excessive Ca^{2+}. In this sense, it should be pointed out that loquat plants accumulated much more Ca^{2+} in leaves than did anger plants. It should be borne in mind that SOD activity is an important H_2O_2 source and that loquat plants had lower CAT and APX levels (H_2O_2-scavenging enzymes). This suggests that loquat plants treated with NaCl plus Ca^{2+} could generate more H_2O_2, and that they have a lower capacity to eliminate it than anger plants. This was reflected in higher TBARS levels and a growth reduction in loquat plants. In others work, a positive correlation between salts accumulation and oxidative stress has been observed (Hernández et al., 1993, 1995, 2001, Sehmer et al. 1995). Sjölin and Mϕller (1991) have demostrated that salts stimulated electron transport in submitochondrial particles (SMPs) from potato through electrostatic charge screening, and this could be the reason for the enhanced production of $O_2^{\cdot -}$ observed in pea leaf SMPs from salt-treated plants (Hernández et al., 1993). This higher $O_2^{\cdot -}$ production was correlated with an increase in mitochondrial lipid peroxidation (Hernádez et al., 1993). In chloroplasts from salt-treated pea plants, an increase in H_2O_2 and in lipid peroxidation has been described (Hernández et al., 1995). In both cases, the increase in $O_2^{\cdot -}$ and H_2O_2 was correlated with an increase in the Na^+ and Cl^- contents in leaves. In the present work, a correlation between lower Cl^- levels and lower lipid peroxidation levels was observed in anger plants, and this was accompanied by a higher levels of antioxidant enzymes

The mechanism by which Ca^{2+} could regulate antioxidant enzymes remains to be determined. It has been described that Ca^{2+} acts by regulating NAD kinase and Δ^1-pyrroline-

5-carboxylate synthetase (Delumeau *et al*. 2000; Knight *et al*. 1992). In plants, calcium-dependent protein kinases, calmodulin and calcineurin-B-like proteins (AtCBLs) are also obvious candidates for primary decoders of $[Ca^{2+}]_{cyt}$ oscillations (Evans *et al*., 2001). Probably, changes in these proteins could be involved in the regulation of antioxidant enzymes mediated by Ca, directly or indirectly, involving either phosphorylation by calcium-dependent protein kinases or the action of specific calcium-dependent proteases, as suggested for the H_2O_2-inhibition of SOD mediated by Ca in tobacco plants (Price et al. 1994). However, these proteins are regulated by μM increases in cytosolic calcium concentrations (Evans *et al*., 2001), and, unfortunately, the method used for Ca^{2+} determination only provides total leaf content and thus cannot inform us about Ca^{2+} distribution in different cell compartments.

The presence of antioxidant isozymes in different subcellular compartments has been described (Foyer and Halliwell 1976; Jiménez *et al*. 1997; Gómez *et al*. 1999; Hernández *et al*. 2000; Mittova *et al*. 2003), although the presence of the ASC-GSH cycle enzymes in apoplast is doubtful (Hernández *et al*. 2001). The results obtained by several authors show that the activity of antioxidant enzymes and their transcript levels varied considerably in response to NaCl stress (Bueno *et al*. 1998; Savouré *et al*. 1999; Hernández *et al*. 2000). On the other hand, it has been suggested that the different response of SOD isozymes induced by NaCl stress could be related to the subcellular compartment where these isozymes are present (Bueno *et al*. 1998; Hernández *et al*. 2000). It has been suggested that the enhanced tolerance to NaCl requires induction of organelle-specific antioxidant enzymes (Hernández *et al*. 1993, 1995, 2000; Gómez *et al*.1999; Mittova *et al*, 2003). Enzymes functioning in elimination of AOS in the cytoplasm were induced by salt stress in salt-tolerant *Citrus* and in pea plants (Gueta-Dahan *et al*. 1997; Hernández *et al*. 2000), as well as in chloroplast and mitochondria from salt-tolerant pea and *Lycopersicom pennellii* plants (Hernández *et al*. 1993, 1995; Gómez *et al*. 1999; Mittova *et al.* 2003).

In the present work, results for antioxidant enzymes must be considered with caution, because analyses have been restricted to total specific activity, and changes observed could be due to changes in the levels of particular isoforms. It has been suggested that changes in the levels of particular isoforms of such enzymes, rather than changes in the level of total activity, may be more important (Stevens *et al*. 1997; Hernández *et al*. 2000). In *Citrus* plants, salt induction of cytosolic APX, which is not exhibited in total APX activity, indicated that although its contribution to the total activity is minor, its localization might be of great importance (Gueta-Dahan *et al*. 1997). Such importance for the cytosolic compartment in the plant's response to salt stress has been suggested also for pea (Hernández *et al*. 2000).

In conclusion, these results suggest that anger plants have a higher capacity to scavenge AOS, both under control and saline conditions. Accordingly, and related to the smaller leaf increase in Cl^- observed, anger plants are more salt-tolerant, at least partly due to the higher antioxidant enzyme levels observed. On the other hand, in loquat plants althought Ca^{2+} addition did not improve plant growth, it decreased the Cl^- and Na^+ absorption and/or transport to the leaves.

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Legend to Figures

Fig. 1.- Effect of NaCl and Ca additions (expressed as mM) to the nutrient media on leaf growth of anger and loquat plants. Data are the means ± SE of at least ten different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

Fig. 2.- Effect of NaCl and Ca additions (expressed as mM)to the nutrient media on Na^+ (*a*), Cl^- (*b*) and Ca^{2+} (*c*) concentrations in anger and loquat leaves. Data are the means ± SE of at least ten different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

Fig 3.- Effect of NaCl and Ca additions (expressed as mM) to the nutrient media on catalase (*a*) and APX (*b*) activities of anger and loquat leaves. Data are the means ± SE of at least four different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

Fig. 4.- Effect of NaCl and Ca additions (expressed as mM) to the nutrient media on MDHAR (*a*) and DHAR (*b*) activities of anger and loquat leaves. Data are the means ± SE of at least four different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

Fig. 5.- Effect of NaCl and Ca additions (expressed as mM) to the nutrient media on GR (*a*) and SOD (*b*) activities of anger and loquat leaves. Data are the means ± SE of at least four different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

Fig. 6.- Effect of NaCl and Ca additions (expressed as mM) to the nutrient media on lipid peroxidation (given as TBARS) in anger and loquat leaves. Data are the means ± SE of at least four different plant samples. Different letters indicate significant differences ($p< 0.05$) according to Duncan's Multiple Range Test.

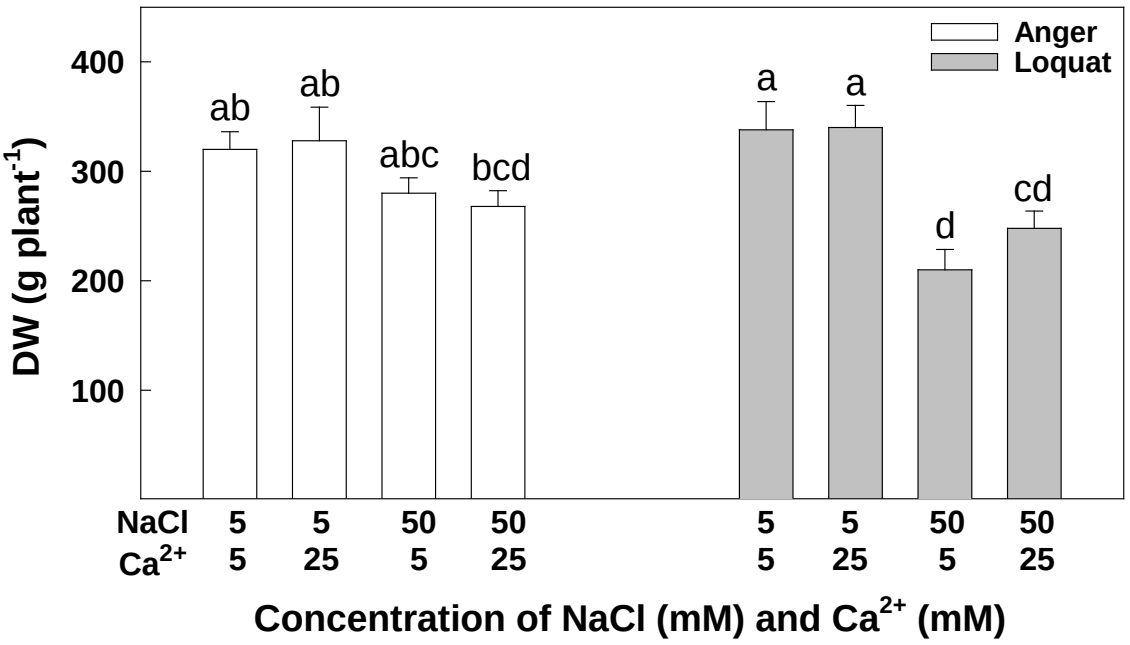
Anger
Loquat
DW (g plant-1)
400
300
200
100
ab
ab
abc
bcd
a
a
d
cd
NaCl
Ca2+
5
5
5
25
50
5
50
25
5
5
5
25
50
5
50
25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig 1

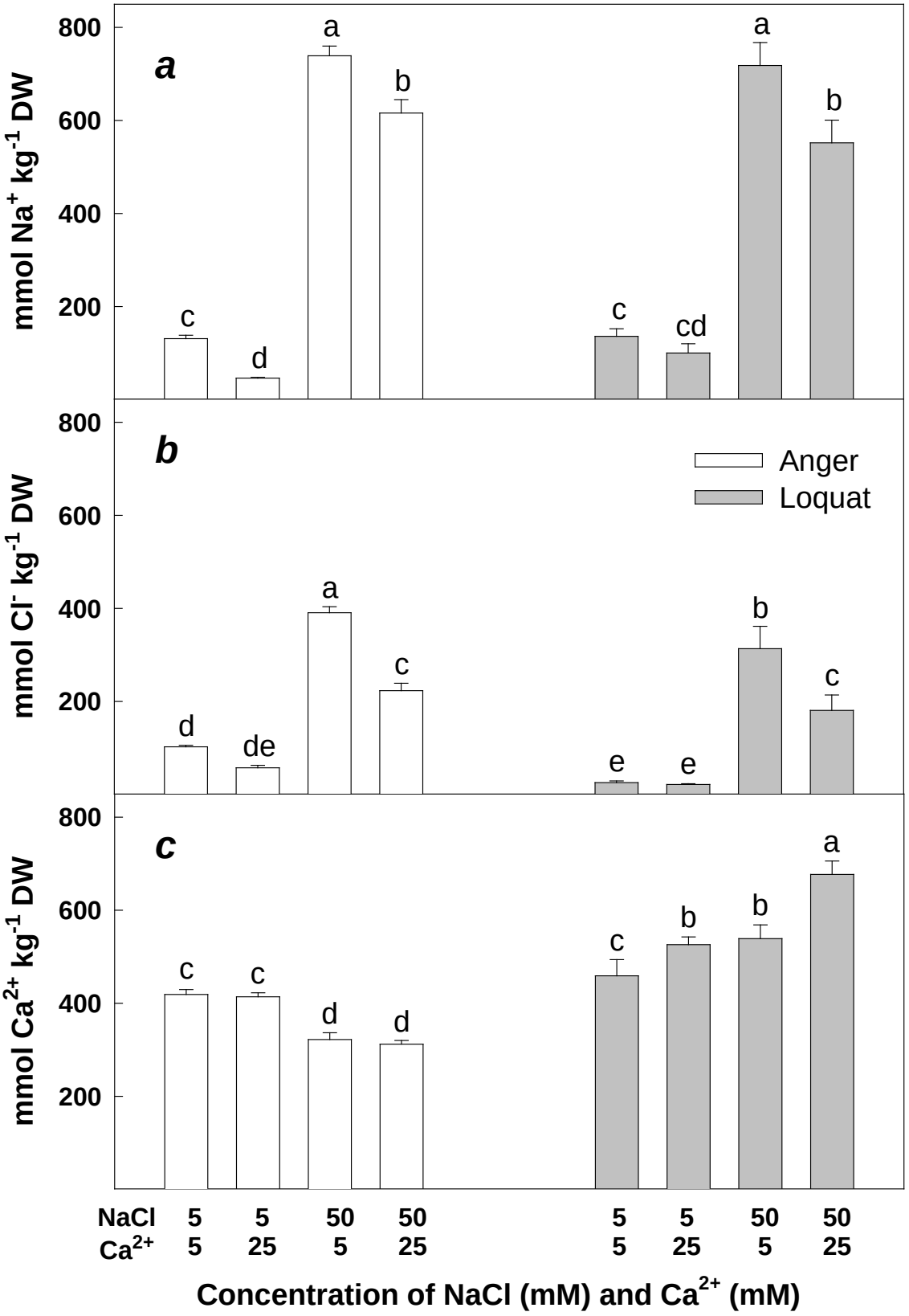
a
b
c
Anger
Loquat
mmol Na+ kg-1 DW
mmol Cl- kg-1 DW
mmol Ca2+ kg-1 DW
800
600
400
200
NaCl 5 5 50 50 5 5 50 50
Ca2+ 5 25 5 25 5 25 5 25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig 2

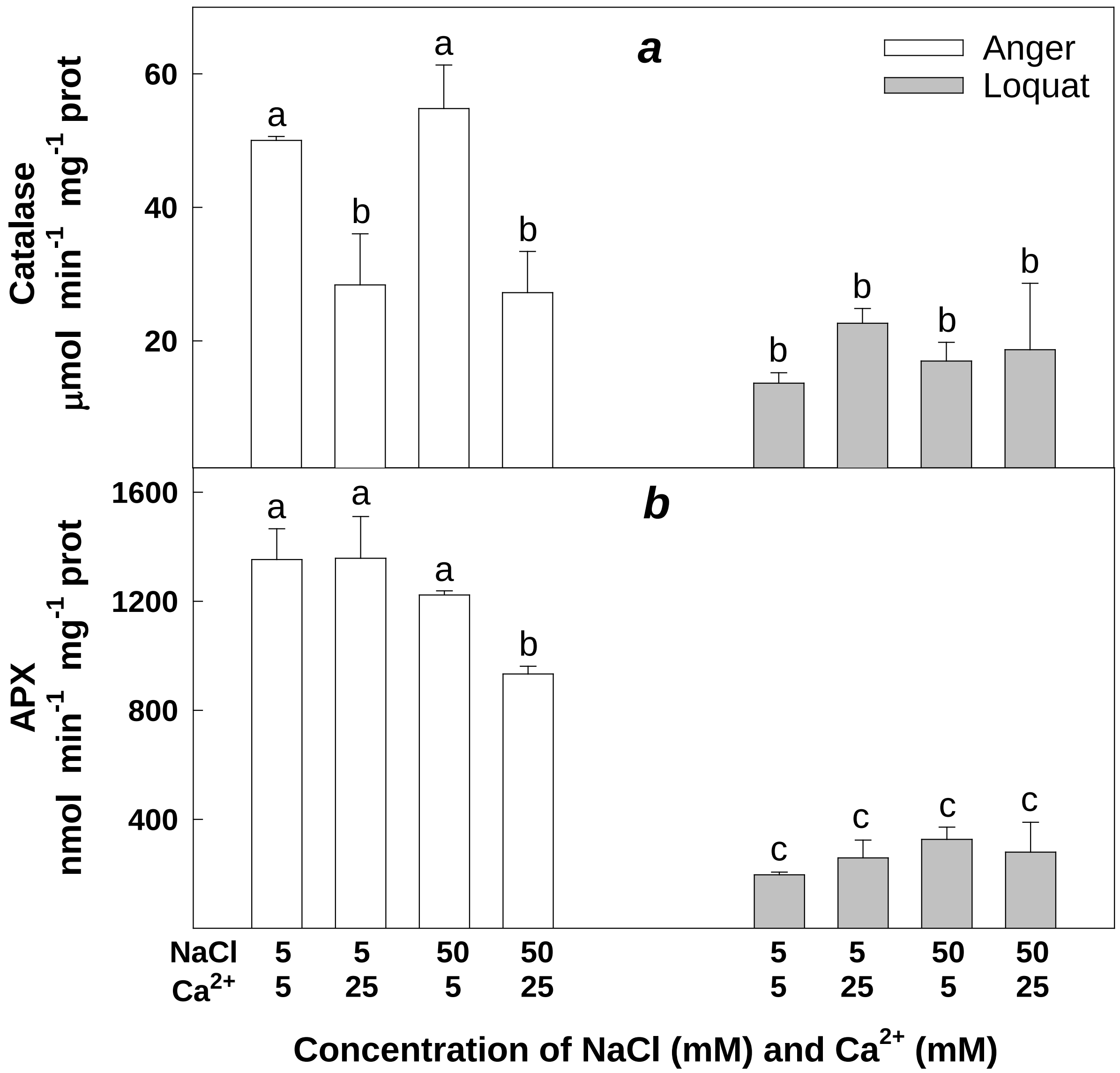
a
Anger
Loquat
Catalase
µmol min-1 mg-1 prot
60
40
20
b
APX
nmol min-1 mg-1 prot
1600
1200
800
400
NaCl 5 5 50 50 5 5 50 50
Ca2+ 5 25 5 25 5 25 5 25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig. 3

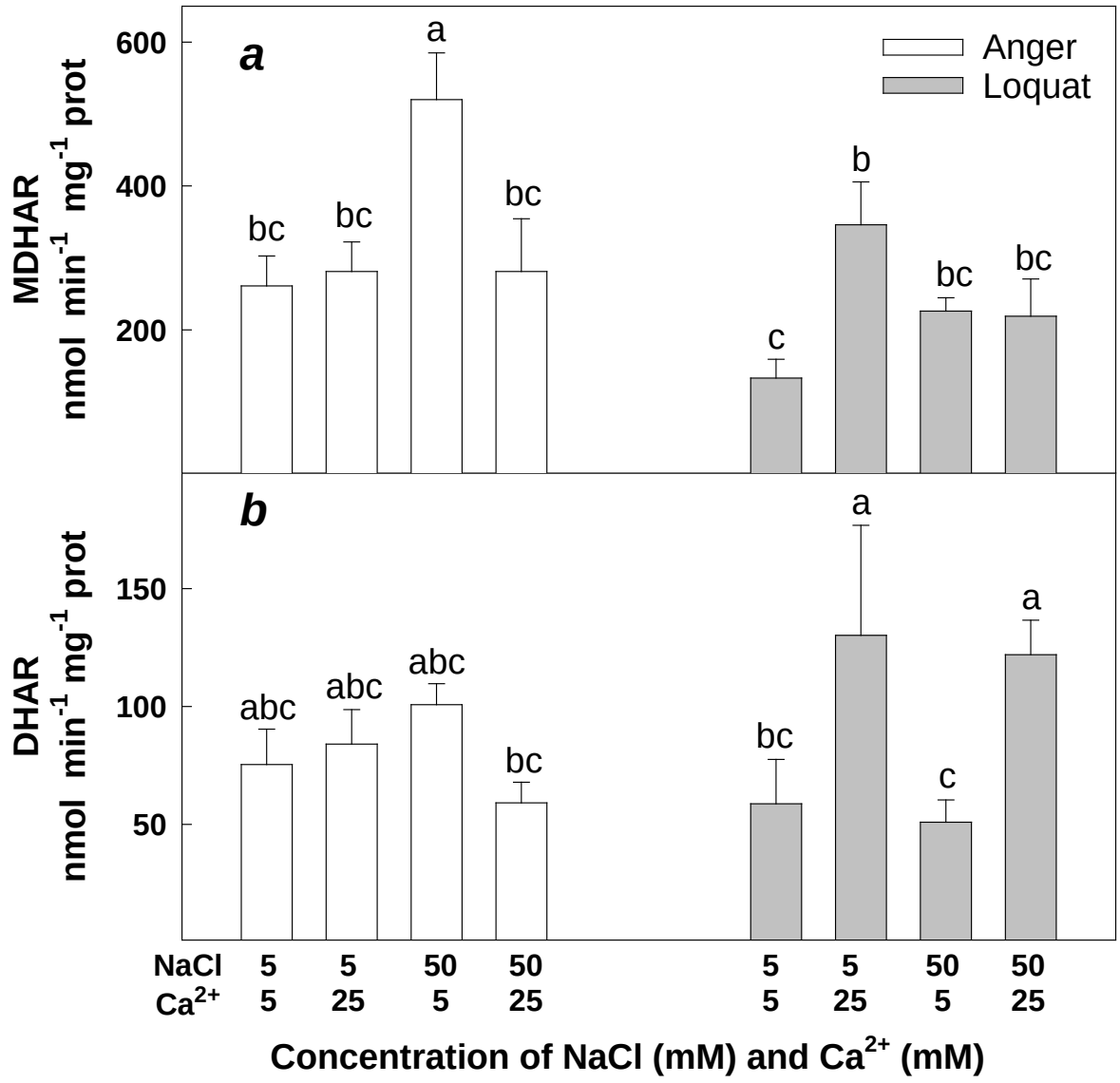
a
b
Anger
Loquat
MDHAR
nmol min-1 mg-1 prot
DHAR
nmol min-1 mg-1 prot
600
400
200
150
100
50
bc
bc
a
bc
c
b
bc
bc
abc
abc
abc
bc
bc
a
c
a
NaCl
Ca2+
5 5
5 25
50 5
50 25
5 5
5 25
50 5
50 25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig. 4

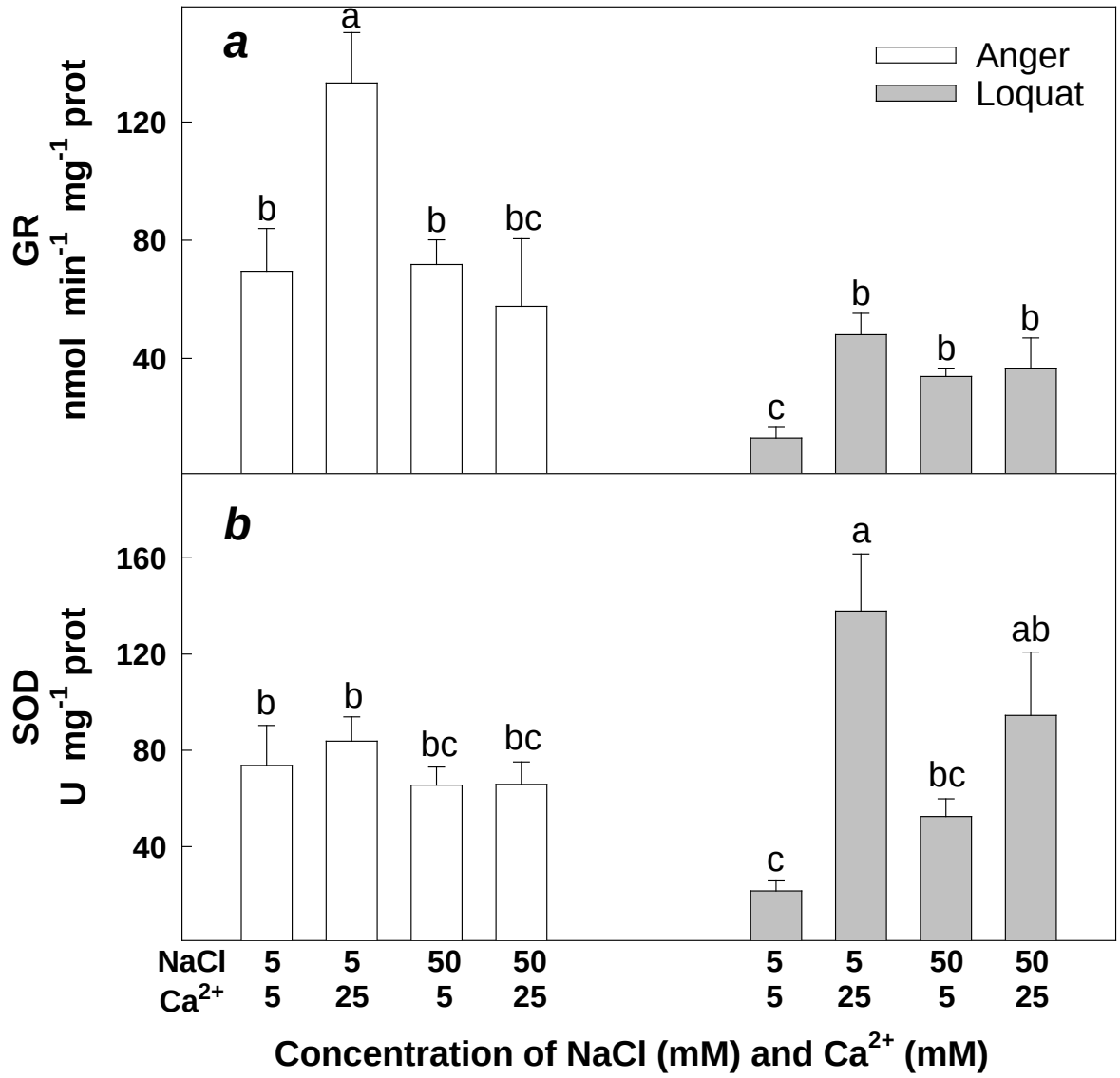
a
Anger
Loquat
GR
nmol min-1 mg-1 prot
120
80
40
b
a
b
bc
c
b
b
b
b
SOD
U mg-1 prot
160
120
80
40
b
b
bc
bc
c
a
bc
ab
NaCl
Ca2+
5 5
5 25
50 5
50 25
5 5
5 25
50 5
50 25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig. 5

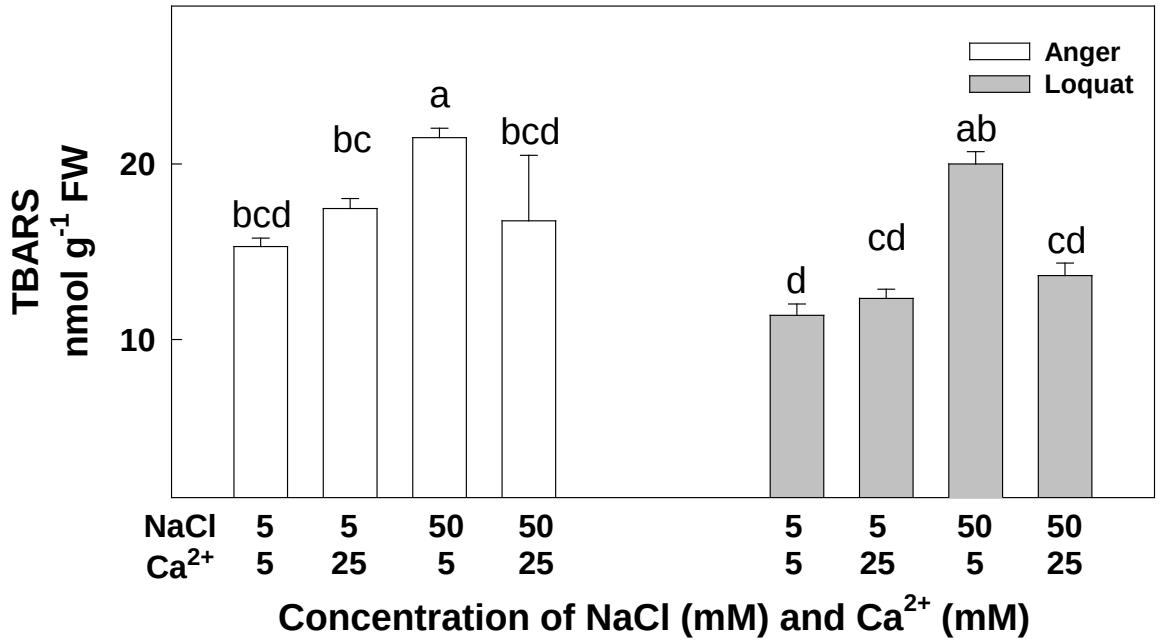
Anger
Loquat
TBARS
nmol g-1 FW
20
10
bcd
bc
a
bcd
d
cd
ab
cd
NaCl
Ca2+
5 5
5 25
50 5
50 25
5 5
5 25
50 5
50 25
Concentration of NaCl (mM) and Ca2+ (mM)

Fig. 6