

Effect of salt stress on the superoxide dismutase activity in leaves of *Citrus limonum* in different rootstock-scion combinations

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Abstract

The effect of salinity on leaf superoxide dismutase (SOD) activity of lemon trees of different rootstock-scion combinations was studied. In leaves from *Citrus limonum* cv. Verna scions on *Citrus macrophylla* and *C. reticulata* rootstocks, salinity treatment clearly caused a significant depression in both Fe-SOD and Mn-SOD activities and an increase in Cu,Zn-SOD activity. However, in leaves from *Citrus limonum* on *Citrus aurantium* rootstock, the reduction observed in the activity values of Fe-SODs and Mn-SODs was not statistically significant. Salt stress also produced a decrease in the content of soluble proteins and chlorophylls. However, this drop was greater in *C. limonum* leaves on *C. macrophylla* than for other combinations.

Additional key words: chlorophyll, *Citrus aurantium*, *Citrus limonum*, *Citrus macrophylla*, *Citrus reticulata*, mineral elements, proteins.

Introduction

In south-eastern Spain (Murcia), irrigation water, usually containing chloride concentrations higher than 10 mM, is used to irrigate a variety of horticultural crops, including *Citrus*, which are generally reported as salt-sensitive plants (Cerdá *et al.* 1990). Salt tolerance of *Citrus* plants varies depending on the rootstock-scion combination and water and soil quality (Cerdá *et al.* 1990).

Plants exposed to salt stress undergo changes in their metabolism in order to cope with the changes taking place in their environment. Salt stress, in addition to its known components of osmotic stress and ion toxicity, is also manifested as an oxidative stress, all of which contribute to its deleterious effect (Hernández *et al.* 1993a, 1995, 2000, Gómez *et al.* 1999). Superoxide dismutases (SOD; E.C. 1.15.1.1) are a group of metalloenzymes that catalyse the disproportionation of

$O_2^{\cdot -}$ radicals to molecular oxygen and H_2O_2 , and play an important role in protecting cells against superoxide-derived oxidative damage (Halliwell and Gutteridge, 1989).

Citrus rootstocks differ widely in their ability to exclude sodium and/or chloride from scion foliage (Walker and Douglas 1983). While the influence of salinity and *Citrus* rootstock-scion combinations in the leaf gas exchange, water relation, ion content and concentration of compatible solutes is reasonably well documented, at present there is no information about the effect of salinity and rootstock combination on the isozyme pattern of SODs. In addition, changes in some stress parameters such as ion content, protein concentration and leaf chlorophyll level were also studied.

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Abbreviations: AOS - activated oxygen species; Cu,Zn-SOD - copper, zinc-containing superoxide dismutase; Fe-SOD, iron-containing superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; $O_2^{\cdot -}$ - superoxide radicals.

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Materials and methods

Plants and treatments: Uniform seedling of sour orange (*Citrus aurantium* L.), Cleopatra mandarine (*Citrus reticulata* B.) and *Citrus macrophylla* rootstock were transplanted from a nursery to the experimental site. Two years later they were grafted with Verna lemon (*Citrus limonum* R. cv. Verna) scion as described previously by Cerdá *et al.* (1990).

Two NaCl concentration (12 and 28 mM), applied through the irrigation water, were used over a five year period. Tap water, having 6 mM NaCl, was considered as control. Each experimental plot had 4 trees of each rootstock planted 6 × 5 m apart with no guard trees.

Preparation of extracts and enzyme assays: All operations were performed at 4 °C. Samples of leaves (8 - 10 months old) were blended and total SOD activity was determined as described by Almansa *et al.* (1989). SOD isozymes (Cu,Zn-SOD, Mn-SOD and Fe-SOD) were separated by isoelectric focusing and identified

Results and discussion

Citrus reticulata and *C. macrophylla* rootstock have been considered as salt sensitive and *C. aurantium* rootstock as salt tolerant (Cerdá *et al.* 1990).

We have previously described that leaves from *C. limonum* cv. Verna contain nine SOD isozymes: 4 Cu,Zn-SOD, 3 Fe-SOD and 2 Mn-SOD (Sevilla *et al.* 1984, Almansa *et al.* 1989). Under normal growth conditions Cu,Zn-SOD and Fe-SOD are the most abundant SOD isozymes.

Total SOD activity shows a decrease in salt-treated *C. limonum* trees, but it was only significant in trees on *C. reticulata* rootstock treated with 28 mM NaCl (Fig. 1). In *C. limonum* trees on *Citrus macrophylla* and *C. reticulata* combinations, salinity treatment clearly determined a significant depression in both Fe-SOD and Mn-SOD activities and an increase in Cu,Zn-SOD activity (Fig. 1). However, in leaves from *C. limonum* on *C. aurantium* rootstock treated with 12 mM NaCl only Cu,Zn-SOD activity decreased. It is important to note that leaves from *C. limonum* trees on *C. aurantium* rootstock had a higher SOD isozymes constitutive activity than the other combinations (Fig. 1). In pea plants, the induction of antioxidant defences, among other factors, seems to be important in the tolerance mechanisms of peas to long term salt treatment (Hernández *et al.* 1993a, 1995, 2000). However in *C. limonum* trees, it seems that high constitutive SOD levels could be important in the protection against salt stress.

Mn-SOD has been localized in mitochondria and peroxisomes (del Río *et al.* 1989). An increased production of O₂⁻ has been described in mitochondria

according to Almansa *et al.* (1989). The isozyme activity was quantified by recording the transmittance of the gels in a Shimadzu CS-9000 densitometer (Kyoto, Japan).

Ion analysis: Leaf samples were oven-dried at 65 °C and ground to a fine powder. Nutrient concentrations of intact leaves were determined by atomic absorption spectrometry, except for chloride contents, which were measured by a potentiometric method (Hernández *et al.* 1993b).

Chlorophylls were extracted with N-N'dimethylformamide and estimated by the methods of Inskeep and Bloom (1985). Proteins were determined by the Bradford (1976) procedure.

Statistics: Each experiment was replicated four times with four different batches of leaves. The average values were calculated and the data included in the tables were analysed according to Duncan's Multiple Range Test.

from salt-treated pea cultivars (Hernández *et al.* 1993a), whereas an induction in mitochondrial Mn-SOD was only found in the NaCl-tolerant pea cultivar and not in the sensitive one, in which Mn-SOD even decreased (Hernández *et al.* 1993a). In this sense, in *C. limonum* leaves on *C. reticulata* and *C. macrophylla* rootstocks treated with 12 and 28 mM NaCl the Mn-SOD activity decreases (Fig. 1), indicating that an oxidative stress mechanism mediated by O₂⁻ could be involved at mitochondrial and peroxisomal levels.

In plants, Cu,Zn-SODs and Fe-SODs are present mainly in chloroplasts (Bowler *et al.* 1992). Chloroplasts are important AOS generators like O₂⁻ and H₂O₂ under salt-stress (Hernández *et al.* 1995). In *C. limonum* leaves on *C. macrophylla* and *C. reticulata* combinations, the decrease on Fe-SOD activity was compensated with an increase in the Cu,Zn-SOD activity to cope with the O₂⁻ radicals produced during saline conditions. However, this increase would not be sufficient to avoid oxidative stress because Cu,Zn-SOD are sensitive to H₂O₂. However, in *C. limonum* leaves on *C. aurantium*, Cu,Zn-SOD activity decreased slightly. This decrease may be compensated by maintaining Fe-SOD activity. It has been described that H₂O₂ completely deactivates Cu,Zn-SOD, whereas Fe-SOD is deactivated to a limit of 90 % (Kanematsu and Asada 1994) and that this residual H₂O₂-resistant activity may play an important role during stress conditions.

Salt stress produced a decrease in soluble proteins from leaf extracts especially with the highest salt level used (Table 1). However, this drop was stronger in *C. limonum* leaves on *C. macrophylla* reaching a 40 %

decrease in relation to control trees (Table 1).

Total chlorophyll reduction at 28 mM NaCl in relation to the control treatment was 28, 23, and 43 % for trees on *C. reticulata*, *C. aurantium*, and *C. macrophylla* rootstock combinations, respectively (Table 1). This response has been generally found in NaCl-sensitive

plants subjected to salt stress (Hernández *et al.* 1993a). The reduction in chlorophyll contents, and subsequently on photosynthesis activity, could be responsible for the depression of growth and yield shown in all combinations, especially in trees on *C. macrophylla* (Cerdá *et al.* 1990).

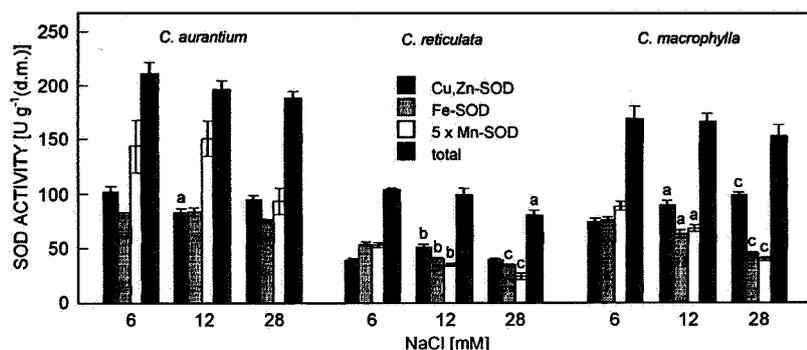


Fig. 1. Effect of salt stress on the SOD activities in leaves from *Citrus limonum* cv. Verna on different rootstocks (a, b, c - differences from control values were significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to Duncan's Multiple Range Test).

Table 1. Protein and chlorophyll contents [$\text{mg g}^{-1}(\text{d.m.})$] in leaves from *Citrus limonum* cv. Verna in relation to rootstocks and salinity in the irrigation water (a - differences from control values significant at $P < 0.05$, according to Duncan's Multiple Range Test)

Rootstock	NaCl [mM]	Protein	Chlorophyll a	Chlorophyll b
<i>C. aurantium</i>	6	2.35 ± 0.02	0.390 ± 0.014	0.142 ± 0.008
	12	3.05 ± 0.01	0.371 ± 0.012	0.128 ± 0.006
	28	$1.96 \pm 0.01a$	$0.317 \pm 0.004a$	0.092 ± 0.006
<i>C. reticulata</i>	6	1.69 ± 0.03	0.384 ± 0.014	0.114 ± 0.010
	12	1.75 ± 0.01	0.359 ± 0.018	0.113 ± 0.060
	28	1.66 ± 0.04	$0.264 \pm 0.012a$	0.093 ± 0.004
<i>C. macrophylla</i>	6	4.38 ± 0.07	0.478 ± 0.022	0.146 ± 0.004
	12	$3.38 \pm 0.08a$	$0.394 \pm 0.016a$	$0.126 \pm 0.002a$
	28	$2.62 \pm 0.02a$	$0.259 \pm 0.040a$	$0.094 \pm 0.016a$

Table 2. Mineral element content [$\text{mg g}^{-1}(\text{d.m.})$] in leaves from *Citrus limonum* cv. Verna in relation to rootstocks and NaCl concentration [mM] in the irrigation water (a, b, c - differences from control values were significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to Duncan's Multiple Range Test).

Rootstock	NaCl	Cl	Ca	K	Na	Fe	Mn	Cu	Zn
<i>C. aurantium</i>	6	15.8 ± 0.8	83.0 ± 4.5	271.5 ± 21.3	8.1 ± 0.4	97.7 ± 8.9	29.9 ± 4.5	8.4 ± 0.7	21.6 ± 4.9
	12	14.3 ± 1.2	76.2 ± 4.0	262.5 ± 18.8	6.8 ± 0.3	104.5 ± 8.7	40.9 ± 8.1	9.7 ± 0.6	29.5 ± 2.1
	28	$33.2 \pm 2.8b$	92.1 ± 5.2	245.5 ± 19.7	7.3 ± 0.3	110.1 ± 7.6	$66.7 \pm 9.3a$	11.1 ± 1.1	$44.2 \pm 4.8a$
<i>C. reticulata</i>	6	13.3 ± 1.0	78.5 ± 3.7	251.5 ± 17.6	6.9 ± 0.5	118.0 ± 10.6	52.9 ± 6.1	7.1 ± 0.6	29.5 ± 3.3
	12	13.1 ± 0.9	79.9 ± 4.2	250.0 ± 16.5	7.1 ± 0.2	117.5 ± 9.8	56.5 ± 2.0	8.7 ± 0.7	33.5 ± 2.8
	28	17.5 ± 0.9	89.5 ± 6.0	249.0 ± 18.1	7.9 ± 0.6	110.0 ± 8.8	$77.2 \pm 4.5a$	7.8 ± 0.5	38.3 ± 4.7
<i>C. macrophylla</i>	6	12.0 ± 0.8	59.8 ± 3.0	256.0 ± 17.8	7.3 ± 0.3	104.0 ± 7.6	55.4 ± 5.1	7.0 ± 0.5	35.9 ± 2.5
	12	$132.3 \pm 10.2c$	68.9 ± 5.1	285.0 ± 19.8	7.9 ± 0.2	98.3 ± 5.4	36.0 ± 3.1	7.8 ± 0.6	29.8 ± 2.0
	28	$327.4 \pm 25.7c$	70.6 ± 4.0	310.0 ± 35.5	10.2 ± 0.9	94.3 ± 8.8	$75.5 \pm 6.2a$	7.6 ± 0.7	$50.1 \pm 4.7a$

Chloride concentration in leaves from trees on *C. macrophylla* for the highest salinity treatment was about 10 and 19 times greater than on *C. aurantium* and

C. reticulata rootstock, respectively (Table 2). These leaf Cl contents are considered to be toxic for *Citrus* plants. Leaf abscission and burn symptoms were clearly evident

in the higher saline treatment. Sodium contents in *C. limonum* leaves from the three different rootstock were scarcely altered by salt treatment and were not proportional to the Na^+ concentration in the irrigation water (Table 2). These results disagree with a recent study carried out using different scion-rootstock combinations showing a strong increase in Na^+ and Cl^- contents in treated trees (García-Lidón *et al.* 1998). However, that research used higher NaCl concentrations (50 mM NaCl) than that used under our experimental conditions (up to 28 mM NaCl). Potassium concentration in leaves was also similar for all rootstocks. The maintenance of K^+ contents has also been shown in other rootstock-scion combinations (García-Lidón *et al.* 1998). It has been reported that the decrease in K^+ levels may be due to a direct effect of Na^+ , which displaces K^+ , or to a loss of K^+ from the root tissues (Walker and Douglas 1983, García-Lidón *et al.* 1998). The Ca^{2+} contents slightly increased in salt-treated plants, but were lower in *C. limonum* leaves on *C. macrophylla* compared to the other rootstocks (Table 2). This content of Ca^{2+} is considered low for *Citrus* plants (García-Lidón *et al.* 1998). It has been reported that the translocation of Na^+ to the leaves leads to a displacement of the apoplastic Ca^{2+} (Zid and Grignon 1985). However, in our work no important changes in Na^+ were observed, which could explain the lack of variation in K^+ and Ca^{2+} concentrations. The concentrations of the remaining nutrients did not vary greatly with the treatment or rootstock, and are considered to be in the optimum range for these plants (García-Lidón *et al.* 1998). Only a slight increase in Mn and Zn was observed in leaves from salt-treated trees (Table 2). Iron content did not change

significantly in any combination. In *C. limonum* leaves on *C. macrophylla* and *C. reticulata*, the Fe-SOD activity decreased significantly but, not the Fe content that could be present as free iron and could catalyze the generation of hydroxyl radicals (OH^\cdot) (Halliwell and Gutteridge 1989). Leaf analysis does not supply information on the physiological activity of a particular trace element but only indicates its chemical content in the leaf (del Río 1983), and Fe-SOD activity has been proposed as a biological marker for Fe content in *Citrus* plants (Sevilla *et al.* 1984). It has been reported that the susceptibility of the cells to oxidative stress is dramatically influenced by the availability of free intracellular iron (Halliwell and Gutteridge 1989). Results obtained showed the usefulness of the metalloenzyme system SOD as a tool for studies of micronutrient imbalances in *Citrus* plants, in agreement with results obtained for peas and soybean plants (del Río *et al.* 1983).

From these results, it is clear that rootstock is important in relation to salt tolerance of lemon trees as previously reported by Cerdá *et al.* (1990).

The strong decrease in protein and chlorophyll contents induced by NaCl in *C. limonum* leaves on *C. macrophylla* rootstock, in relation to the other combinations, could be related with the highest sensitivity to NaCl in irrigation water in this combination. On the other hand, the NaCl produced a strong decrease in Fe-SOD and Mn-SOD activity, in *C. limonum* on *C. macrophylla* and *C. reticulata* rootstocks. This suggests that an increased AOS production could take place in those combinations under a salt stress situation, as has been reported in other plant species and cells (Hernández *et al.* 1993a, 1995, Gómez *et al.* 1999).

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