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A novel in vitro tissue culture approach to study salt stress responses in citrus

Almudena Montoliu · María F. López-Climent · Vicent Arbona · Rosa M. Pérez-Clemente · Aurelio Gómez-Cadenas

Abstract In citrus, a major crop throughout the world, growth and yield are seriously affected by salinity. ferent approaches, including agronomical, physiological and molecular methods, have been used to address this problem. In this work, an in vitro experimental system has been developed to study the toxic effect of NaCl on three citrus genotypes, avoiding the ion filter that represents the root system. To carry out the experiments, shoots were obtained from nodal segments of Cleopatra mandarin, Carrizo citrange and citrumelo CPB4475 plants growing in a greenhouse. Shoots were cultured in control or NaCl-supplemented media. After testing several salt concentrations, 60 mM NaCl was selected as saline treatment. Shoots accumulated similar levels of chloride when cultured without roots and exhibited similar leaf damage. No increases in malondialdehyde levels were observed in any genotype (as a measure of oxidative stress). Similar patterns of hormonal signalling (in terms of abscisic acid and salicylic acid contents) were exhibited in the three genotypes, despite their different tolerance under field conditions. All data together indicate that, without root system, all genotypes had the same behaviour under salt stress. The in vitro culture system has been proved as a useful tool to study biochemical processes involved in the response of citrus to salt stress.

Keywords Oxidative stress · Plant hormones · Stress tolerance · Toxic ions

Introduction

Salts in the substrate affect plant growth in two ways: First, the presence of salt reduces the ability of the plant to take up water which leads to reductions in the growth rate. This is referred to as the osmotic effect of salt stress, which starts immediately after the salt concentration around the roots increases over a threshold level. There is a second and slower response due to the accumulation of ions in leaves. This ion-specific phase of plant response to salinity starts when accumulated salt reaches toxic concentrations in the leaves (Gómez-Cadenas et al. 1998). Within many species, documented genetic variation exists in the rate of accumulation of Na^+ and Cl^- in leaves, as well as in the degree to which these ions can be tolerated (Munns and Tester 2008). For most species, Na^+ appears to reach a toxic concentration before Cl^- does. However for some species, as in the case of citrus, Cl^- is considered to be the more toxic ion (Moya et al. 2002, 2003; López-Climent et al. 2008).

The differences found among citrus rootstocks regarding salt tolerance have been related mainly to their ability to exclude chloride (Bañuls et al. 1997; Moya 2003) although the ability to keep a high performance of the photosynthetic system also seems important (López-Climent et al. 2008). Certain citrus genotypes such as Cleopatra mandarin (CM) or Rangpur lime rootstocks can be classified as relatively tolerant due to their ability to restrict chloride ions to roots while others, such as Carrizo citrange (CC) or citrumelo CPB4475 (Cit), have proved to be more sensitive to salinity (López-Climent et al. 2008).

Salinity causes suberization of root tissues (Walker et al. 1984), a decrease in root hydraulic conductivity, an impaired assimilation of mineral nutrients (Ruiz et al. 1997), visual toxicity symptoms (Chapman 1968) and eventually leaf abscission (Gómez-Cadenas et al. 1998, 2002). Furthermore, chloride accumulation in citrus leaves decreases net photosynthetic rate, transpiration and stomatal conductance while activating plant antioxidant machinery (Arbona et al. 2003; Iglesias et al. 2004).

The analysis of endogenous levels of plant hormones such as abscisic acid (ABA) ethylene, and its direct precursor, 1-aminocyclopropane-1-carboxilic acid, revealed a general pattern of hormonal change composed by a two phase response that paralleled the chloride accumulation in salt-stressed plants (Gómez-Cadenas et al. 1998, 2002). Therefore, ABA and ethylene have been involved as modulators of some of the responses of citrus to high salinity (Gómez-Cadenas et al. 1998).

It has been shown that the root system plays a key role in controlling water and chloride uptake (Moya et al. 2002). An adaptative improvement of the salt-tolerant genotype CM can be inferred from the linear correlation between chloride and water usage (Moya et al. 2003). It appears that CM has a more restrictive mechanism than CC for chloride influx at the root level, being highly efficient in limiting chloride uptake to the aerial part (Moya et al. 2002). Since differences are not only restricted to the aerial part or the root system, it is very difficult to study, under conditions, other putative

moss, perlite and vermiculite (80:10:10) as a substrate. Plants were watered when needed with a 0.5 L of a half-strength Hoagland solution (Bañuls et al. 1997). Three months after germination, salt stress was applied by increasing NaCl concentration in the watering solution to 90 mM. Percentages of salt affected plants, chloride, and malondialdehyde (MDA) contents were recorded at 10, 20 and 30 days of culture.

In a second set of in vitro experiments, greenhouse-grown plants of the same citrus rootstocks were used as a source of plant material. Stem pieces (15 cm long) were stripped of their leaves, disinfected by immersion for 10 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween wetting agent, and rinsed three times with sterile water. Node stem segments (1 cm long) were cultured in Petri dishes with basal medium (BM), containing the inorganic salts of Murashige and Skoog (1962), 100 mg/l i-inositol, 1 mg/l pyridoxine-HCl, 0.2 mg/l thiamine-HCl, 1 mg/l nicotinic acid and 30 g/l sucrose. The pH was set at 5.7 $\pm$ 0.1 with 0.1 N NaOH before autoclaving. The medium was solidified by the addition of agar (Pronadisa, Madrid, Spain).

Shoots recovered from nodal stem segments were excised from the explant and cultured into 150 $\times$ 20 mm tubes on multiplication medium (MM) to promote the development of axillary buds. MM consisted of BM medium supplemented with 0.4 mg/l 6-benzylaminopurine. During the growth
formed from buds located at leaf axils. When these shoots

collected after 2, 5, 10 and 20 days of the imposition of salt stress and MDA, ABA and salicylic acid (SA) contents measured.

To assess whether the growth regulators used in the culture media had some effect on the results obtained, a new experiment was carried out using the following culture media: BM as control and the same media supplemented with 60 mM NaCl for the salt treatment, MT and MT2. After 20 days of treatment, percentage of plants affected by salt was recorded and plant material collected for chloride and MDA analyses.

In all cases, plant material was cultivated in culture rooms at 24°C with a 16-h photoperiod. Leaves or shoots were collected, rinsed with distilled water to eliminate any residue and frozen in liquid nitrogen. Plant material was kept at −80°C until further analyses.

Visible symptoms of leaf damage

The presence of yellowish spots at the leaf tip that progressively led to severe burning injuries was considered to be a good visible estimate of chloride-induced damage to leaves. The number of damaged leaves was regularly recorded during the experimental period and expressed as a percentage of the total number of leaves. Plants or shoots showing a percentage of damaged leaves equal to or over 50% were considered salt "affected".

Chloride content

Chloride content was measured by automatic titration as described in López-Climent et al. (2008). Samples were oven-dried for 72 h at 70°C. After desiccation, samples were minced and incubated overnight in a 0.1 N HNO3 (PA grade, Panreac, Barcelona, Spain) and 10% glacial acetic acid (Baker grade, JT Baker, Barcelona, Spain) solution. After filtering, 0.5 ml of the solution was used for determination in a chloridometer (Model 626, Sherwood Scientific Ltd., Cambridge, UK).

Malondialdehyde concentration

Malondialdehyde concentration was measured following the procedure described in Hodges et al. (1999). Plant material was homogenized in 5 ml of 80% cold ethanol (Panreac, Barcelona, Spain) using a tissue homogenizer (Ultra-Turrax; IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 4°C to pellet debris and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid (TCA) (Panreac, Barcelona, Spain) or a mixture of 20% TCA and 0.5% thiobarbituric acid (Sigma-Aldrich, Madrid, Spain). Both mixtures were allowed to react in a water bath at 90°C for 1 h. After this time, samples were cooled down in an ice bath and centrifuged. Absorbance at 440, 534 and 600 nm was read in the supernatant against a blank. The MDA concentration in the extracts was calculated as in Arbona et al. (2008).

Abscisic acid and salicylic acid analyses

Plant hormones were analyzed by HPLC coupled to tandem mass spectrometry as described in Durgbanshi et al. (2005) and Arbona and Gómez-Cadenas (2008). Briefly, frozen citrus shoots were ground to a fine powder with a pre-chilled mortar and a pestle and then 0.5 g of powdered tissue was extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany). Before extraction, samples were spiked with 100 ng of [$^2H_6$]-ABA, and 100 ng of [$^2H_4$]-SA. After extraction and centrifugation, the pH of the supernatant was adjusted to 3.0 and partitioned twice against di-ethyl-ether (Panreac, Barcelona, Spain). The organic layers were combined and evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was thereafter resuspended in a water:methanol (9:1) solution, filtered, and injected into a HPLC system (Alliance 2695, Waters Corp., Milford, USA). Hormones were then separated in a reversed-phase Kromasil 100 C18 column (100 × 2.1 mm 5-µm particle size) using methanol and ultrapure water both supplemented with glacial acetic acid to a concentration of 0.05%. The mass spectrometer, a triple quadrupole (Quattro LC, Micromass Ltd., Manchester, UK), was operated in negative ionization electrospray mode and plant hormones were detected according to their specific transitions using a multiresidue mass spectrometric method (Durgbanshi et al. 2005).

Statistical analyses

Data mean comparisons and regression analyses were performed with STATGRAPHICS PLUS v.5.1. (Statistical Graphics Corporation, Herndon, VA) software. One-way ANOVA and comparisons between means were made following the LSD test at $P < 0.05$.

Results

Effect of salt stress on intact plants of different citrus genotypes

In a first experiment, 3-month-old intact seedlings of the three citrus genotypes CC, Cit and CM, were watered with an increased concentration of NaCl to study the effect of salt stress on young plant material (Table 1). Leaf damage was obvious from the first day of measurement in plants of

Table 1 Damage, chloride concentration and malondialdehyde (MDA) content in leaves of intact plants of three citrus genotypes subjected to salt stress

		Time of treatment (days)					
		10		20		30	
		Control	90 mM NaCl	Control	90 mM NaCl	Control	90 mM NaCl
Leaf damage (% of affected plants)	Carrizo citrange	0.00 ± 0.00	6.15 ± 0.04*	0.00 ± 0.00	12.50 ± 0.80*	0.00 ± 0.00	48.42 ± 1.91*
	Citrumelo CPB 4475	0.00 ± 0.00	12.02 ± 1.01*	0.00 ± 0.00	21.12 ± 3.29*	0.00 ± 0.00	61.08 ± 1.54*
	Cleopatra mandarin	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.42*	0.00 ± 0.00	11.64 ± 0.29*
Cl^- (mg/g fresh tissue)	Carrizo citrange	4.14 ± 0.84	6.32 ± 0.39*	3.87 ± 0.65	14.47 ± 2.09*	5.11 ± 0.56	32.19 ± 0.98*
	Citrumelo CPB 4475	2.36 ± 0.64	6.96 ± 0.80*	2.14 ± 0.58	18.24 ± 1.57*	2.94 ± 0.20	35.12 ± 2.67*
	Cleopatra mandarin	1.94 ± 0.20	2.53 ± 0.49	1.47 ± 0.11	4.5 ± 0.95*	1.53 ± 0.01	12.61 ± 0.19*
MDA (nmol/g fresh tissue)	Carrizo citrange	29.57 ± 2.15	30.00 ± 1.58	27.41 ± 1.21	42.01 ± 1.29*	27.78 ± 2.52	37.56 ± 2.00*
	Citrumelo CPB 4475	25.65 ± 1.92	32.09 ± 2.19*	23.99 ± 2.08	51.19 ± 2.84*	26.39 ± 1.90	48.52 ± 3.25*
	Cleopatra mandarin	25.30 ± 1.81	24.22 ± 0.33	21.33 ± 0.63	23.76 ± 0.88	22.43 ± 1.32	22.03 ± 0.50

* Symbols followed with an asterisk denote statistical significance at $P < 0.05$. Data in insets are normalized mean values ± relative SE

Cit. In this genotype, after 30 days of stress, affected plants were 61% of the total. Plants of CC also showed evident damage due to the increased concentration of NaCl from day 10, being 50% of the plants affected by the stress at day 30. Contrastingly, the percentage of CM plants affected by salt stress was only 11% over a 30-day period. Leaf Cl^- concentration mimicked damage and leaves of Cit and CC plants accumulated the highest concentration of the toxic ion whereas in leaves of CM, chloride content was much lower throughout the experimental period. Basal levels of Cl^- were lower in CM than in the rest of genotypes. Leaf MDA concentration (an indirect marker of salt-stress induced oxidative damage) increased in the sensitive genotypes (Cit and CC) until a certain extent and then remained constant. In contrast, leaf MDA content in salt-stressed plants of CM was similar to that in control plants throughout the experimental period.

Adjustment of NaCl concentration in the in vitro system

Shoots of the CC, Cit and CM cultivated in vitro were subjected to different saline treatments (30, 60 and 90 mM NaCl) and the pattern of Cl^- concentration followed over a 30 day period (Fig. 1). Chloride concentration in control shoots showed similar basal values among the three genotypes throughout the experimental period. After salinisation, chloride in shoots progressively increased in all genotypes and for all treatments, being the highest levels found in the most severe salt treatment. Although the studied citrus genotypes exhibited slightly different accumulation patterns (chloride accumulation was faster in CC and Cit than in CM), all tended to similar maximum values (Fig. 1). For the subsequent experiments, 60 mM NaCl was set as the salt stress treatment because this intermediate concentration did not promote a high mortality (as that observed in shoots treated with 90 mM NaCl), but allowed an important and fast Cl^- accumulation (higher than in the 30 mM treatment) useful for further measurements.

Effect of salt stress on in vitro cultured shoots of the different citrus genotypes

It is well known that, under field conditions, salinity causes yellowing, bronzing, or browning of leaves and premature foliage drop. In our experimental system, damage caused by exposure of citrus shoots to 60 mM NaCl was evidenced by the apparition of characteristic leaf symptoms (Fig. 2). It was observed that leaf injury become more severe as the period of salt treatment progressed. After 10 days, light yellowing was observed in all genotypes. Leaf chlorosis increased after 20 days in all the cases and, at the end of the treatment (30 days), browning was evident in leaf tissues of all genotypes.

To quantify the occurrence of toxicity due to chloride ions, we considered as affected shoots those showing necrosis in at least 50% of their leaves. Figure 3 represents the percentage of CC, CM and Cit shoots affected by salinity (60 mM) after 0, 10, 20 and 30 days of treatment. Leaf damage increased very fast in Cit (60% of affected plants vs. 20% in CM and 17% in CC at day 10). After 20 days of salt treatment, 75 and 66% of CC and CM shoots were damaged respectively whereas almost all Cit shoots showed significant leaf damage. In all cases, and despite the slight different rates of leaf damage occurrence, shoots were in very bad conditions after 30 days of salt treatment (Figs. 2 and 3).

When chloride concentration was determined in shoots of CC, CM and Cit after 10, 20 and 30 days of treatment, no differences were found among the three citrus genotypes (Table 2). The accumulation of Cl^- ions took place

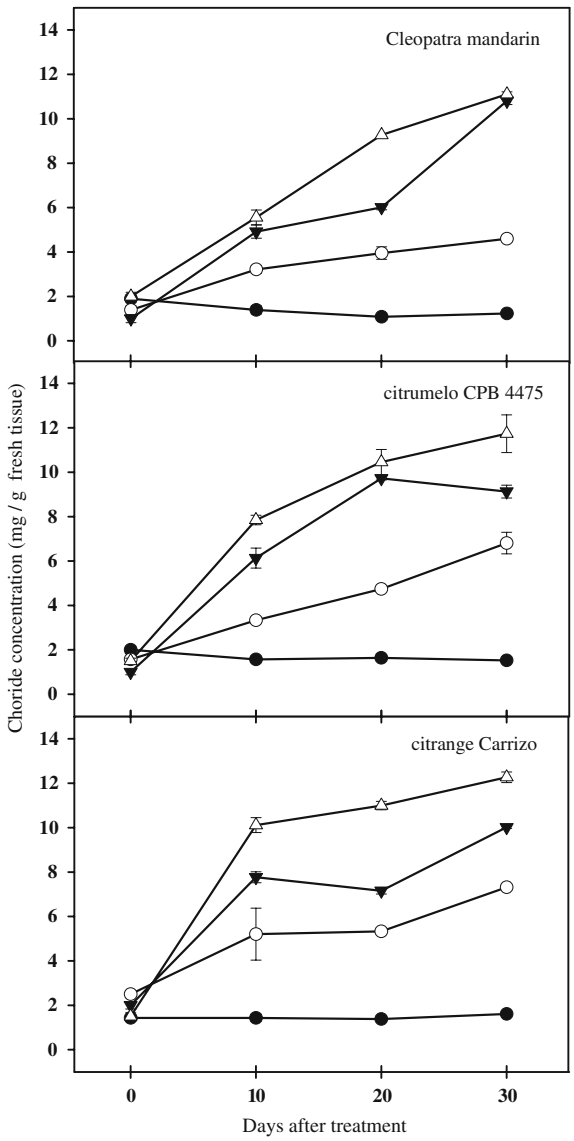

Fig. 1 Shoot chloride concentration in three citrus genotypes subjected to different concentrations of NaCl. In control (*filled circle*), 30 (*open circle*), 60 (*filled inverted triangle*), and 90 (*open triangle*) mM NaCl supplemented medium. Each point corresponds to the average ± standard error of four independent determinations

progressively throughout the experimental period. After 10 days of treatment, values threefold above controls were recorded in salinized shoots (ranging from 6.80 to 8.89 mg/g in control shoots vs. 20.85–27.00 mg/g in salinized ones). At the end of the experiment, Cl^- concentration in salinized shoots achieved values even higher (ranging from 6.55 to 13.20 mg/g in control shoots vs. 33.73–39.08 mg/g in salinized ones).

As an indicator of oxidative damage, MDA content was measured in shoots of citrus genotypes. No significant differences were found between salt-stressed and control shoots of Cit and CM (Table 2). However in CC, 60 mM NaCl treatment induced reductions in MDA content at day 10 and 30 and a slightly significant increase at day 20 (1.2-fold above controls), suggesting a poor correlation between MDA content and leaf damage.

Salt treatment did not induce significant ABA accumulation in shoots of any genotype, regardless the extent of saline treatment (Table 2). ABA contents in control shoots were higher than in stressed ones in all genotypes throughout the experimental period except for a transient increase in salt-treated Cit shoots after 20 days. Elevated SA levels were observed in shoots of all studied genotypes after 10 days of treatment (Table 2), although it was no significantly different in the case of CC. This could evidence an early signaling of SA as a consequence of salt stress.

Early effects of salt stress on in vitro shoots of Carrizo citrange

To investigate the early effects of salt stress, chloride concentration, MDA, ABA and SA contents were measured on in vitro cultured CC shoots 2, 5 and 10 days after the imposition of saline conditions (Table 3). Chloride content remained almost invariable throughout the experimental period in control shoots. On the contrary, chloride concentration in salinized shoots progressively increased with time. Two days after the onset of the treatment, Cl^- concentration in salinized shoots was 1.5 times higher than that found in control ones, and achieved levels 4.6 times higher than in control shoots at day 10.

No differences in MDA levels were found between control and salinized shoots after short saline treatment periods. However a slight increased of MDA took place at day 10; in comparison with the values obtained at day 2, in both, control (27.3 vs. 16.48 nmol/g fresh tissue) and salinized-shoots (29.74 vs. 20.79 nmol/g fresh tissue).

Abscisic acid content in control shoots was similar to that measured in salinized ones; only after 5 days of saline treatment the differences between them were statistically significant. As observed before, ABA content in control shoots was much higher than in stressed ones (33.20 vs. 9.6 ng/g fresh tissue).

No differences were found in the content of SA between control and salinized shoots after 2 and 5 days. After 10 days of stress, a slight although no significant increase in this hormone was recorded (Table 3).

Effect of plant growth regulators

To elucidate whether the addition of plant growth regulators to the culture medium had some effect on the studied parameters, changes in foliar damage, chloride and MDA contents were measured after 20 days in CC shoots cultured in medium with or without plant growth regulators

Fig. 2 Effect of salt stress in different citrus genotypes. Yellowing, browning and necrosis in leaves of Cleopatra mandarin (**a**), Citrumelo CPB 4475 (**b**) and citrange Carrizo (**c**) affected by salinity. In each picture and from left to right: control leaves and after 10, 20 and 30 days of saline treatment. **d** Necrotic shoot of Cleopatra mandarin after 30 days saline treatment (*right*) and control (*left*)

and with or without treatment with 60 mM NaCl (Table 4). Shoots grown on media, supplemented or not with plant growth regulators, showed the same behaviour. Healthy leaves (without evident damage) were observed in shoots cultured in both media (with or without plant growth regulators, Table 4). This suggests that no additional hormones are required to maintain shoots in vitro for 20 days. Shoots growing in salt-stress conditions showed foliar damage (approximately a 70% of affected shoots) without significant differences due to the presence of plant growth regulators in the medium.

Similar increases in Cl^- concentration were observed in shoots growing in both salinized media (4.71 mg/g in media supplemented with plant growth regulators vs. 5.23 mg/g in media without them). In the same way, no significant variations in MDA content were observed between plants cultured with additional plant growth regulators or without them. In this case, as observed before, salt treatment did not modify MDA levels.

Discussion

The literature extensively describes how h extens

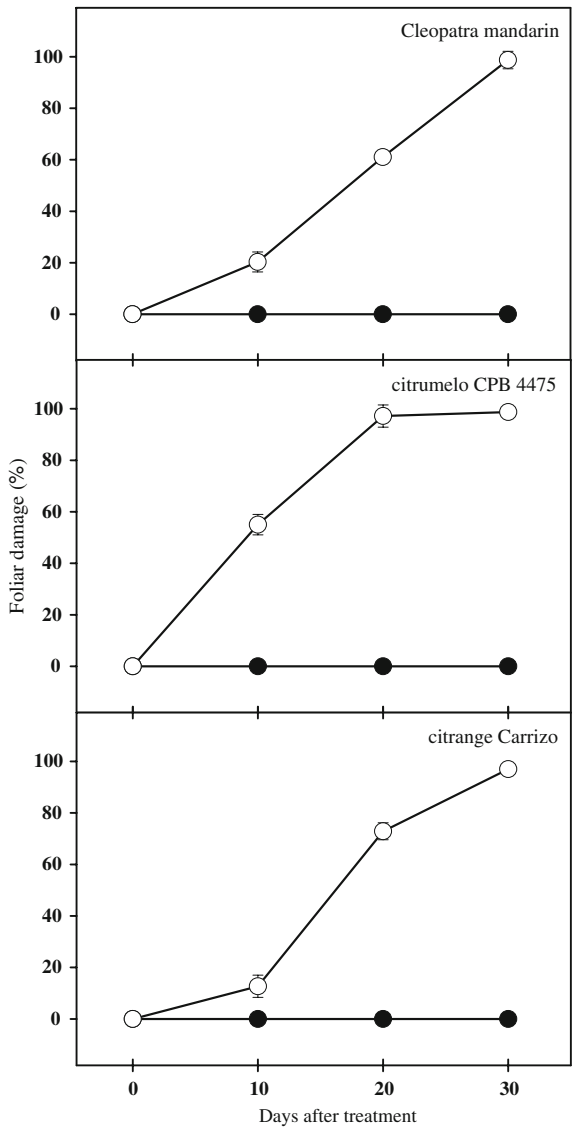

Fig. 3 Percentage of citrus shoots affected by salinity. Shoots were grown in control (*filled cirlce*) or 60 mM NaCl supplemented (*open circle*) medium. Each point corresponds to the average ± standard error of 18 independent determinations

both salt-tolerant and salt-sensitive genotypes accumulating similar amounts of chloride, which is really complicated under field conditions. Therefore, the method proposed appears to be a good tool for studying biochemical processes involved in the response of citrus to salt stress. Singh et al. (2004) cultured calli from different citrus rootstocks differing in their tolerance to salt stress. In this study, a good correlation between salt tolerance and ion accumulation was found. The differences found between both systems must be due to the totally different type of tissue cultured. Furthermore, while the system used by Singh et al. (2004) could be used as a practical tool to evaluate salt tolerance of citrus germoplasm (due to the similar behaviour of calli and intact plants), our system is oriented to elucidate common and specific response to ion toxicity among genotypes, avoiding the root filter. Salt stress tolerance has been correlated with an improved oxidative stress response in several crops (Shalata et al. 2001; Perl-Treves and Perl 2002). Increased MDA levels in leaves of intact plants of CC and Cit plants were also observed under salt treatment (Table 1). However, no significant differences were found between salt-stressed and control shoots of Cit and CM (Table 2) when cultured in vitro without root system. In the case of CC, a slight decrease in MDA content was found in some points. From these results, we can conclude that there is no correlation between foliar damage and oxidative stress: characteristic leaf symptoms caused by salinity (yellowing, browning, etc) were observed in all studied genotypes while no consistent MDA accumulation was detected.

Abscisic acid plays a pivotal role in the adjustment of plants to abiotic stress conditions (Gómez-Cadenas et al. 1998; Christmann et al. 2006); it is the long-distance signal that communicates water stress from the root to the shoot. Evidence for root-derived ABA as a long-distance signal has been obtained from split-root experiments with whole plants in which only one part of the root system experienced water deficit (Dodd et al. 2008). Earlier studies showed that, in many crops, the leaf can accumulate ABA in response to salt stress (Montero et al. 1997; Sibole et al. 1998). In the case of citrus, previous reports indicate that the genotype CC responds to salinity by increasing ABA levels (Gómez-Cadenas et al. 1998). These experiments were performed with whole intact plants and it is possible that such salt treatment in the roots leads to a shoot water deficit that, in turn, triggers ABA accumulation. These results suggested the existence of an osmosensing mechanism and also the organ-specific nature of such a response. This suggestion is also supported by the fact that gene expression in response to salt stress usually is organ- or tissue-specific (Jia et al. 2002). When shoots were cultured in vitro, no accumulations of ABA were observed in any of the studied genotypes, which seem to discard an ABA dependent signaling. These results are similar to those obtained in maize by Jia et al. (2002), who observed that NaCl treatment only induced a small ABA accumulation in leaf tissues, whereas on the contrary, the same treatment of NaCl caused a significant ABA accumulation in root tissues.

The transient increase of SA levels at 10 days of salt treatment could suggest a role for this hormone in the response of citrus to salinity. Although further work should be done to understand this effect, our data suggests a common signal not related to genotype tolerance as observed in all studied genotypes, independently of their tolerance to high salinity.

Table 2 Chloride, malondialdehyde (MDA), abscisic acid (ABA) and salicylic acid (SA) contents in shoots of three citrus genotypes subjected to salt stress (60 mM NaCl)

		Time of treatment (days)					
		10		20		30	
		Control	60 mM NaCl	Control	60 mM NaCl	Control	60 mM NaCl
Cl^- (mg/g fresh tissue)	Carrizo citrange	7.70 ± 0.02	27.00 ± 0.04*	6.06 ± 0.02	30.25 ± 0.04*	13.20 ± 0.02	33.73 ± 0.05*
	Citrumelo CPB 4475	6.80 ± 0.01	27.00 ± 0.09*	7.90 ± 0.01	38.00 ± 0.03*	9.09 ± 0.02	48.34 ± 0.04*
	Cleopatra mandarin	8.89 ± 0.03	20.85 ± 0.10*	8.57 ± 0.08	43.14 ± 0.21*	6.55 ± 0.04	39.08 ± 0.11*
MDA (nmol/g fresh tissue)	Carrizo citrange	37.09 ± 1.46	24.74 ± 1.35*	14.90 ± 0.85	18.42 ± 1.33*	27.31 ± 2.92	16.93 ± 0.70*
	Citrumelo CPB 4475	21.99 ± 1.18	21.55 ± 1.47	22.04 ± 0.50	20.65 ± 0.51	23.75 ± 0.84	20.45 ± 1.55
	Cleopatra mandarin	–	–	21.34 ± 0.96	21.02 ± 0.76	24.83 ± 0.47	25.21 ± 0.74
ABA (ng/g fresh tissue)	Carrizo citrange	37.20 ± 9.35	29.05 ± 4.99	53.55 ± 2.32	23.65 ± 3.84*	91.37 ± 8.31	33.13 ± 3.95*
	Citrumelo CPB 4475	78.75 ± 7.51	52.05 ± 2.74*	40.70 ± 1.85	78.42 ± 14.54*	157.40 ± 7.38	82.39 ± 3.40*
	Cleopatra mandarin	104.50 ± 18.84	90.50 ± 3.16	26.00 ± 0.56	22.00 ± 2.30	24.95 ± 2.30	13.30 ± 2.15*
SA (ng/g fresh tissue)	Carrizo citrange	57.60 ± 11.03	74.90 ± 9.05	79.45 ± 6.05	75.20 ± 2.66	108.15 ± 6.31	57.15 ± 18.54*
	Citrumelo CPB 4475	33.25 ± 5.34	55.35 ± 6.32*	42.60 ± 4.98	54.25 ± 11.49	60.15 ± 5.28	62.20 ± 11.59
	Cleopatra mandarin	67.20 ± 4.88	176.45 ± 5.16*	116.10 ± 7.92	71.35 ± 5.02*	79.60 ± 9.02	62.35 ± 7.56

* Symbols followed with an asterisk denote statistical significance at $P < 0.05$. Data in insets are normalized mean values ± relative SE

Table 3 Chloride, malondialdehyde (MDA), abscisic acid (ABA) and salicylic acid (SA) contents in Carrizo citrange shoots after 2, 5 and 10 days of saline treatment

	Time of treatment (days)					
	2		5		10	
	Control	60 mM NaCl	Control	60 mM NaCl	Control	60 mM NaCl
Cl^- (mg/g fresh tissue)	1.68 ± 0.03	2.73 ± 0.04*	1.72 ± 0.08	5.02 ± 0.09*	1.15 ± 0.04	5.38 ± 0.13*
MDA (nmol/g fresh tissue)	16.48 ± 1.49	20.79 ± 0.99	18.41 ± 2.08	17.31 ± 1.38	27.30 ± 1.35	29.74 ± 1.76
ABA (ng/g fresh tissue)	25.20 ± 2.80	30.07 ± 3.87	33.20 ± 5.91	9.60 ± 1.40*	8.40 ± 1.60	11.33 ± 0.40
SA (ng/g fresh tissue)	34.60 ± 13.00	27.73 ± 6.82	28.93 ± 5.28	37.80 ± 5.24	35.00 ± 3.60	56.73 ± 12.91

* Symbols followed with an asterisk denote statistical significance at $P < 0.05$. Data in insets are normalized mean values ± relative SE

Table 4 Foliar damage, chloride and malondialdehyde (MDA) contents in Carrizo citrange shoots grown in medium supplemented or not with 0.2 mg/l of gibberellic acid and 6-benzylaminopurine. Data were recorded after 20 days of saline treatment

	Treatment			
	+		−	
	Control	60 mM NaCl	Control	60 mM NaCl
Foliar damage (%)	0.00 ± 0.00	72.90 ± 1.10*	0.00 ± 0.00	68.00 ± 0.20*
Cl^- (mg/g fresh tissue)	1.67 ± 0.03	4.71 ± 0.12*	1.63 ± 0.07	5.23 ± 0.12*
MDA (nmol/g fresh tissue)	28.06 ± 1.55	33.24 ± 0.69*	17.51 ± 1.67	18.35 ± 1.98*

* Symbols followed with an asterisk denote statistical significance at $P < 0.05$. Data in insets are normalized mean values ± relative SE

The lack of ABA accumulation within in vitro cultured shoots of citrus genotypes under stress conditions could be due to the fact that the triggering signal for increasing biosynthesis must occur in the roots and the lack of this tissue in our system makes impossible for this early signal to occur. It was also possible that hormone-signalling had taken place before 10 days of saline treatment, when first data of ABA concentration were recorded. To exclude this last possibility and to elucidate the early effects of salt stress on in vitro grown citrus shoots, the pattern of chloride concentration, MDA and hormone concentrations in CC shoots were determined after 2, 5 and 10 days of salt

stress treatment. Chloride accumulation in shoots was again gradual throughout this short experimental period, but no differences were found in MDA, ABA or SA levels between control and salinized shoots after short saline treatment periods, which do not support a putative early ABA- or SA-dependent signalling pathway.

After analyzing all data, we can conclude that when shoots are cultured without a root system, all genotypes accumulate the same levels of chloride and exhibit similar leaf damage as a consequence of the imposition of a salt stress treatment. The lack of an increase in MDA levels in all genotypes, and the common patterns of hormonal signalling, in both short and long periods of study indicate that, under the same salt conditions and with the same level of leaf chloride intoxication, no biochemical differences exist among tolerant and sensitive genotypes. This points to the roots as a key organ not only as a filter of chloride ions but also as a signalling system in citrus.

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