

Abscisic Acid Reduces Leaf Abscission and Increases Salt Tolerance in Citrus Plants

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ABSTRACT

This paper describes the physiological effects of abscisic acid (ABA) and 100 mM NaCl on citrus plants. Water potential, leaf abscission, ethylene production, photosynthetic rate, stomatal conductance, and chloride accumulation in roots and leaves were measured in plants of Salustiana scion [*Citrus sinensis* (L) Osbeck] grafted onto Carrizo citrange (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf) rootstock. Plants under salt stress accumulated high amounts of chloride, increased ethylene production, and induced leaf abscission. Stomatal conductance and photosynthetic rates rapidly dropped after salinization. The addition of 10 μM ABA to the nutrient

solution 10 days before the exposure to salt stress reduced ethylene release and leaf abscission. These effects were probably due to a decrease in the accumulation of toxic Cl⁻ ions in leaves. In non-salinized plants, ABA reduced stomatal conductance and CO₂ assimilation, whereas in salinized plants the treatment slightly increased these two parameters. The results suggest a protective role for ABA in citrus under salinity.

Key words: Abscisic acid; Citrus; Leaf abscission; Ethylene; Photosynthetic rate; Salt stress

INTRODUCTION

In citrus, a major crop throughout the world, growth and yield are seriously affected by salinity. Although substantial differences in salt tolerance within species scions and rootstocks have been described (see Storey and Walker 1999, for a recent review), the genus *Citrus* and closely related species can be classified as salt sensitive (Chapman 1968).

A high concentration of salts in the root zone substantially decreases leaf water potential. However, turgor potential in leaves of citrus trees grown under salt stress remains at levels similar to those in non-salinized plants. The osmotic adjustment is achieved by accumulation of Na⁺ and Cl⁻ ions and compatible solutes (Bañuls and Primo-Millo 1992; Gómez-Cadenas and others 1998; Lloyd and others 1987). This effective mechanism to avoid water stress has negative consequences in the long term, since citrus plants are chloride-sensitive (Bañuls and Primo-Millo 1995; Bañuls and others 1997; Romero-Aranda and others 1998; Walker and others 1982) and the progressive accumulation of these

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ions in plant tissues leads to well-documented injurious effects (Gómez-Cadenas and others 1998; Romero-Aranda and others 1998).

Salinity affects a wide range of physiological parameters in plants. In citrus, canopy growth decreases whereas the root system appears to sustain less damage (Lloyd and Howie 1989; Syvertsen and others 1988). Increases in leaf succulence in salt-stressed citrus leaves have also been observed (Cerdá and others 1977; Romero-Aranda and others 1998; Zekri 1991). Photosynthesis and stomatal conductance rapidly decrease in the presence of high concentrations of salt in the root zone (Bañuls and others 1997; Bañuls and Primo-Millo 1992; Gómez-Cadenas and others 1998). Root hydraulic conductivity is also impaired by increased levels of NaCl (Zekri and Parsons 1989). When chloride ion concentration in leaves reaches deleterious levels for cell metabolism, citrus plants prematurely shed their intoxicated leaves (Bañuls and Primo-Millo 1995; Gómez-Cadenas and others 1998). Depending on the intensity, this defoliation can even result in the death of the plant.

Plant physiological responses to stress are modulated by hormones such as abscisic acid (ABA), ethylene (Gómez-Cadenas and others 1998), and jasmonic acid (Xu and others 1994). ABA is known to play an important role in the efficiency of water use in plants under environmental stresses. This hormone modulates stomatal opening and therefore transpiration. Citrus plants rapidly respond to a water deficit or salinity by increasing endogenous ABA levels (Gómez-Cadenas and others 1996, 1998). It has also been shown that ethylene modulates leaf abscission in citrus plants under water (Gómez-Cadenas and others 1996; Tudela and Primo-Millo 1992) or salt stress (Gómez-Cadenas and others 1998). In salinized citrus leaves, the increase in chloride levels has been correlated with the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) and its subsequent oxidation to ethylene (Gómez-Cadenas and others 1998).

To alleviate the deleterious effects of salinity in plants, treatments with several plant growth regulators have been studied. Pre-treatments with ABA effectively increased salt tolerance of several species such as tobacco (LaRosa and others 1987), barley (Popova and others 1995), and jack pine (Rajasekaran and Blake 1999). Similarly, treatments with paclobutrazol (an inhibitor of gibberellin biosynthesis) reduced the negative effects of salt stress in peach (AbouELKhasahb and others 1997).

In this paper, the effect of ABA treatments on citrus responses to salinity was studied by using grafted citrus plants (Salustiana scion on Carrizo

citrange rootstock) grown under salt stress. The study includes measurements of water potential, chloride accumulation, photosynthetic rate, stomatal conductance, ethylene production, and leaf abscission. The data obtained suggest that ABA can play a role in increasing salt tolerance of citrus plants.

MATERIALS AND METHODS

Plant Material

Two-year-old plants of Salustiana cultivar [*Citrus sinensis* (L) Osbeck] grafted on Carrizo citrange (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf) rootstock were used in the experiments. Plants were obtained from a commercial nursery and transplanted into 2-l plastic containers filled with inert sand. Plants were then cultivated in a greenhouse under the following conditions: day temperature, 24–28°C; night temperature, 18–20°C; photoperiod, 16:8 (L:D), and 60–95% relative humidity. Each plant was watered twice a week with 500 ml of a half-strength modified Hoagland solution (Bañuls and others 1997). Only intermediate leaves and young roots were used for measurements. Abscission was expressed as the percentage of leaves that were shed with a gentle touch.

Chemical and Salt Treatments

Abscisic acid (Sigma-Aldrich, Madrid, Spain) was added to the watering solution to achieve a final concentration of 10 µM. Treatment with the phytohormone was initiated 10 days before the beginning of salinization and maintained during the whole experiment. Salt stress was imposed by adding NaCl to the modified Hoagland solution to reach a concentration of 100 mM.

Leaf Water Potential

Leaf water potential was determined by using a pressure chamber (model 3000, Soilmoisture Equipment, Santa Barbara, CA) as described in Gómez-Cadenas and others (1996).

Photosynthesis and Stomatal Conductance Measurements

Net photosynthetic rate and stomatal conductance were measured by a LI-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) using a 250 ml cuvette. Determinations were performed at

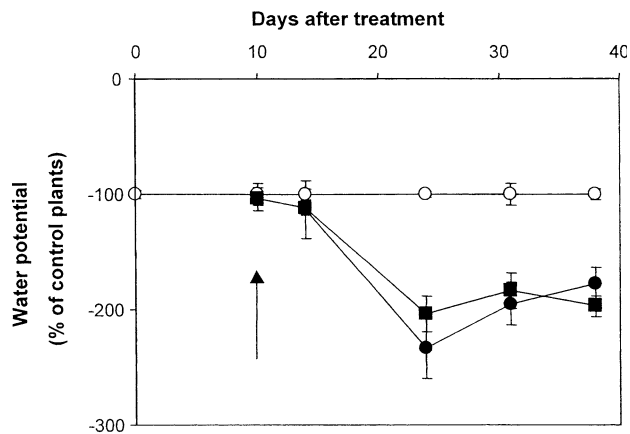


Figure 1. Effect of salt stress on leaf water potential. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 5 independent measurements. The arrow indicates onset of salinization.

9 a.m. During the measurements, radiation within the cuvette was supplemented with a 150 W lamp (Philips EFR A1/232) after cooling with external fans. All measurements were performed at a photosynthetic photon flux (PPF, 400–700 nm) of 900–1000 μ mol/m² s, which exceeds saturating PPF for citrus.

Chloride Analysis

Total chloride content in leaf and root tissue was determined by AgNO₃ titration (Chapman and Pratt 1961). Chloride ions were extracted from previously calcined leaf and root tissue in basic medium. Briefly, to basify the medium, 0.25 g of CaO (PRS grade, Panreac, Barcelona, Spain) and a few drops of water were added to 1 g of lyophilized tissue in porcelain capsules. All samples were calcined in a muffle oven for 90 min at 550°C. Ashes were re-suspended in 15 ml of hot (70°C) ultrapure water. This suspension was filtered through a Whatman #1 filter paper *in vacuo*. The filtered residue was cleaned 5 times with 10 ml of hot ultrapure water and the pH was then adjusted to a value between 6.0 and 7.0 with diluted acetic acid. The titration was performed with 0.05 N AgNO₃ using 5% KCrO₂ as an indicator.

Ethylene Production

Ethylene was measured in intermediate leaves by gas chromatography. Leaves were separated from the plant by making a cut in the base of the petiole

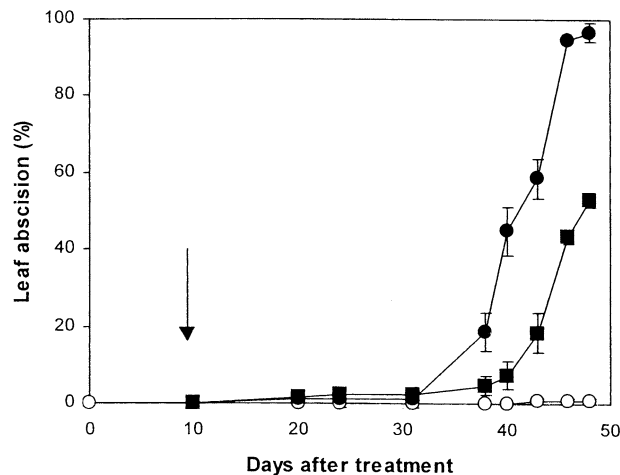


Figure 2. Effect of salt stress on leaf abscission. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 5 independent measurements. The arrow indicates the onset of salinization.

using a razor blade. Leaves were then enclosed individually in 12-ml tubes with the petiole submerged in 100 μ l of water placed in the bottom of the tube. After 30 minutes, the tube was aerated and then sealed with a silicone cap. Following a 4 h incubation period, 1 ml of the enclosed atmosphere was injected into a gas chromatograph (Agilent 4890D, Agilent technologies, Inc. Wilmington, DE, USA) equipped with an activated alumina column and a flame ionization detector. The instrument was previously calibrated with known amounts of an ethylene standard.

RESULTS

As a first approach to evaluate the effect of salinity on citrus plants, leaf water potential was measured (Figure 1). Non-salinized plants showed leaf water potential values that oscillated between -8.42 and -12.50 bar throughout the period studied (data not shown). To facilitate the interpretation of data, values of control plants were always taken as 100%, and leaf water potential of the salinized plants were normalized to these values. The addition of ABA to the watering solution did not modify the water potential before salinization (Figure 1, day 10). After salinization, both ABA-treated and non-treated plants showed parallel reductions in leaf water potential. The lowest levels were detected

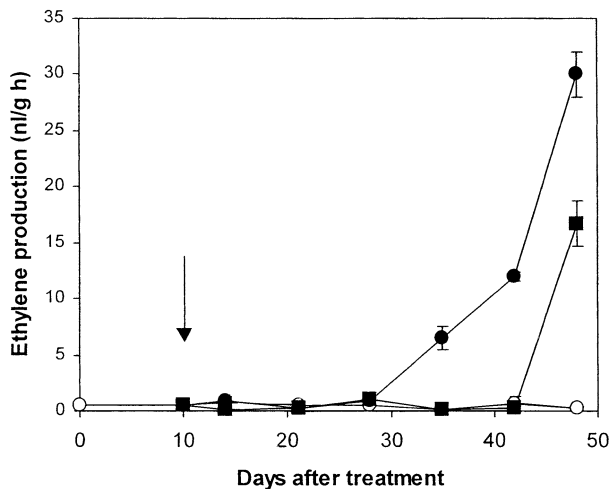


Figure 3. Effect of salt stress on ethylene production in leaves. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of at least 5 independent measurements. The arrow indicates the onset of salinization.

after 14 days of salt treatment (-18.7 Bar, which was 2.3-fold lower than control values at day 24). Thereafter, leaf water potential in salt-stressed plants remained low but it did not decrease further with regard to control plants (Figure 1). These data indicate that ABA did not modify leaf water potential and that all plants were subjected to the same stress conditions independent of the hormone treatment.

Leaves in non-salinized plants did not abscise during the period studied (Figure 2). However, addition of 100 mM NaCl to the watering solutions resulted in a massive drop of leaves. Thus, after 20 days of salt treatment (day 30), leaf abscission was obvious and in a short period of time (18 days), plants lost most of their leaves. Treatment with 10 μ M ABA resulted in an important reduction of the salt-induced abscission (56% in ABA-treated versus 98% in non-treated plants, 38 days after the beginning of salinization).

The pattern of ethylene production in leaves mimicked that of leaf abscission. Leaves on control plants always produced small amounts of ethylene ranging between 0.30 and 0.68 nl/g h (Figure 3). In salinized plants, leaf ethylene production remained low until day 28. However, after this date leaves continuously increased the endogenous production of the plant hormone and on day 48, showed accumulation rates of 30 nl/g h, which was 100 times higher than the values in non-salinized plants

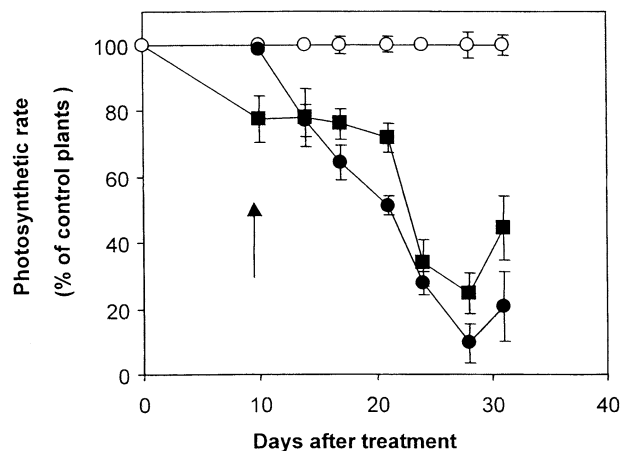


Figure 4. Effect of salt stress on photosynthetic rate. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 30 independent determinations. The arrow indicates the onset of salinization.

(Figure 3). Abscisic acid treatment suppressed ethylene production until 32 days after the onset of salt stress. Thereafter, leaf ethylene production in ABA-treated plants increased but levels on day 48 were still 55% lower than those of non-treated plants.

To show the effect of salinity on photosynthetic rates, the same normalization method as above was used. Non-salinized plants showed photosynthetic rates that varied between 5.7 and 7.9 μ mol/m² s throughout the study period (data not shown). High concentrations of NaCl continuously inhibited CO₂ assimilation in citrus plants until day 18 after the onset of salinization (Figure 4) when the photosynthetic rate in salt-stressed plants was 90.5% lower than in non-salinized plants. In ABA-treated plants, the photosynthetic rate decreased by 20% (Figure 4, day 10) but remained constant for the next 12 days. Thus, after 10 days of salinization (Figure 4, day 20), ABA-treated plants showed CO₂ assimilation values 20% higher than non-treated plants. From that point, photosynthesis in ABA-treated plants substantially decreased although it still remained slightly higher than in non-treated plants (Figure 4).

The response of stomata to salt stress is shown in Figure 5, where data are presented in relative terms compared to control plants. Non-salinized plants showed variations in this parameter ranging between 250 and 100 mmol/m² s in these experiments (data not shown). Salinity strongly and rapidly reduced the stomatal aperture. Thus, 7 days after the

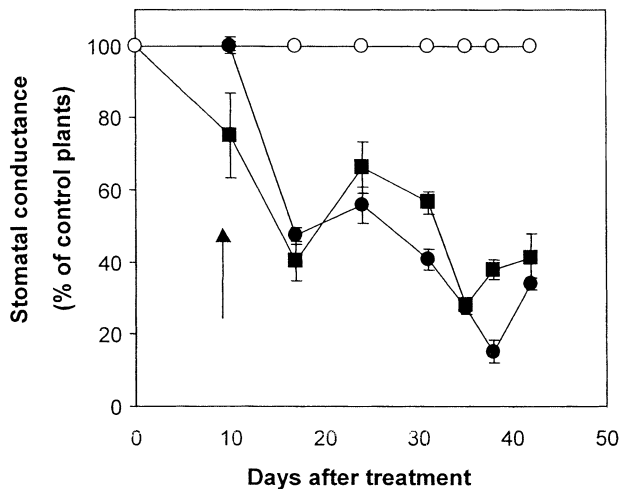


Figure 5. Effect of salt stress on stomatal conductance. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 30 independent determinations. The arrow indicates the onset of salinization.

beginning of salt treatment, stomatal conductance in salt-stressed plants was 53% lower than in non-salinized plants (Figure 5, day 17). After a relative stabilization period, values further decreased to reach 85% reduction compared with control plants. Abscisic acid treatment decreased stomatal conductance in non-salinized plants by 25% (Figure 5, day 10). After NaCl exposure, treated and non-treated plants showed similar changes, although the values measured were slightly higher in the ABA-treated plants.

Chloride accumulation was also studied in roots and leaves (Figures 6 and 7). Cl^- concentration in the leaves of both groups of salt-stressed plants (that is, ABA-treated and non-treated) continuously increased throughout the 32 days of salinization (Figure 6). However, the rate of accumulation varied between the non-treated and ABA-treated plants, with the latter being substantially lower. As a result, on day 42, leaves of ABA-treated plants contained 30% less of the toxic ions than those in non-treated plants. In roots (Figure 7), the pattern of Cl^- accumulation differed from leaves. An important increase was observed during the first 12 days after salinization and then levels remained constant. Abscisic acid-treated plants showed levels of chloride in roots slightly lower than those of non-treated plants during the experimental period. This reduction was more obvious after 33 days of salinization when levels of Cl^- in roots of ABA-treated plants were 20% lower (Figure 7).

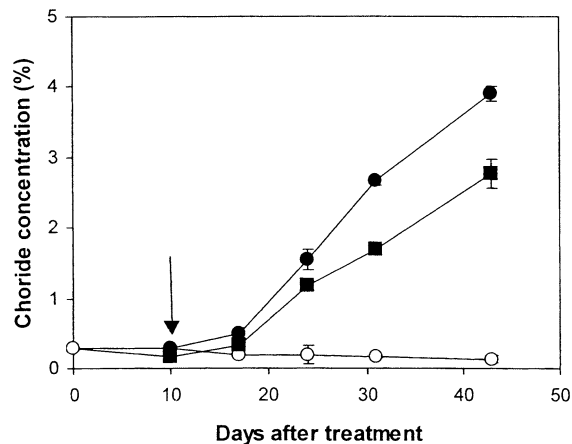


Figure 6. Effect of salt stress on chloride concentration in leaves. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 3 independent determinations. The arrow indicates the onset of salinization.

DISCUSSION

It has been previously shown that one of the primary responses of Carrizo citrange rootstock to severe salt conditions (200 mM NaCl) is the massive abscission of leaves (Gómez-Cadenas and others 1998). As with many other physiological and anatomical disturbances produced by salinity in citrus (Bañuls and others 1997; Romero-Aranda and others 1998; Walker and Douglas 1983), leaf drop appears to be correlated with Cl^- accumulations in leaves (Bañuls and Primo-Millo 1995; Gómez-Cadenas and others 1998). Furthermore, ethylene is recognized as the hormone that triggers leaf and fruit abscission in response to adverse growth conditions, such as salinity, drought or nutritional deficiencies (Gómez-Cadenas and others 1996, 1998, 2000; Tudela and Primo-Millo 1992). In the present work, the massive leaf abscission observed in salinized plants (Figure 2) was preceded by increases in leaf ethylene production (Figure 3), and was also coincident with a progressive accumulation of chloride in the leaves (Figure 6). Thus, data presented here agree with those conclusions and extend the findings to grafted citrus plants.

Treatment with 10 μ M ABA effectively reduced abscission and ethylene production in leaves of salt-stressed plants (Figures 2, 3). Considering that chloride accumulation is responsible for the ethylene increase and the subsequent leaf abscission, the observed reduction in leaf Cl^- concentrations in

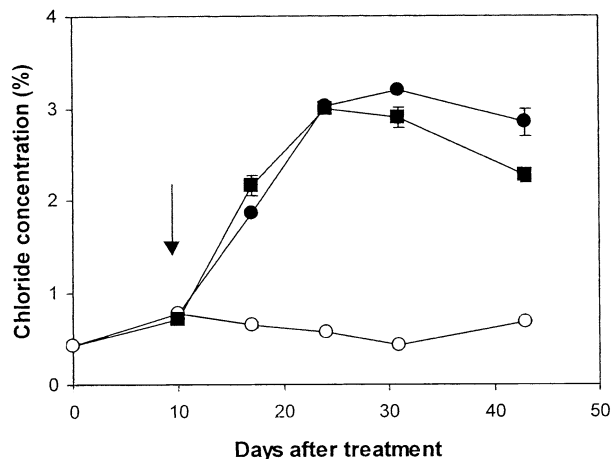


Figure 7. Effect of salt stress on chloride concentration in roots. Control plants (○); non-ABA treated, salinized plants (●); ABA-treated, salinized plants (■). ABA (10 μ M) was added to the watering solution 10 days prior to the onset of the salt stress (100 mM NaCl). Data are means \pm SE of 3 independent determinations. The arrow indicates the onset of salinization.

the ABA-treated plants (Figure 6) could account for the inhibition of ethylene production and leaf drop. The mechanisms involved in Cl^- uptake and root to shoot transport remain unknown (Storey and Walker 1999) although previous reports indicate that chloride uptake under salinization is primarily driven by passive forces, mainly water absorption (Moya and others 1999; Storey 1995; Storey and Walker 1999; Walker 1986). Hence, the data suggest that the initial reduction in stomatal conductance induced by ABA (Figure 5) might be responsible for the reduction of chloride accumulation observed in leaves. This reduction implies that the toxic threshold for Cl^- in leaves was not reached as quickly as in non-treated plants, and therefore, abscission was reduced in the ABA-treated plants. Other modifications induced by ABA such as a decrease in metabolism would also be consistent with attenuated leaf abscission.

Several studies have found a correlation between the reduction in CO_2 assimilation and high Cl^- and Na^+ levels in leaves (Bañuls and Primo-Millo 1992; Garcia-Legaz and others 1993; Walker and others 1982), whereas others have concluded that neither Cl^- nor Na^+ were directly responsible for the reduction in CO_2 assimilation (Bañuls and Primo-Millo 1995; Bañuls and others 1997). Data in Figure 4 show that the decrease in photosynthesis in salinized non-treated plants was linear and coincident with the increase in leaf Cl^- (Figure 6). On the other hand, ABA treatment seems to modify the pattern of CO_2 assimilation in response to salinity. Follow-

ing an initial reduction in the photosynthetic rate (Figure 4, day 10), CO_2 assimilation remained slightly higher in ABA-treated than in non-treated plants. Although data are not conclusive, we suggest that ABA could play a protective role in the photosynthetic machinery as reported in other species under drought or high salinity conditions (Popova and others 1995; Rajasekaran and Blake 1999).

It has been previously demonstrated that ABA and ethylene interact to modulate abscission in citrus plants under stress (Gómez-Cadenas and others 1996, 2000). ABA appears to act as a mediator between the perception of the stress and the accumulation of ACC in fruits and roots, but not in leaves. For example, under water stress, the root ABA-ACC interaction and the following ACC translocation to the leaves, is the mechanism responsible for leaf abscission (Gómez-Cadenas and others 1996; Tudela and Primo-Millo 1992). Conversely, elevated endogenous ABA in leaves under water stress (Gómez-Cadenas and others 1996) or direct foliar ABA application (Goren 1993) did not stimulate either ACC accumulation or promote leaf abscission. In contrast to water deficit, the amounts of ABA and ACC generated in roots and transported to leaves in salinized citrus plants were very low, probably because of the rapid osmotic readjustment (Gómez-Cadenas and others 1998). Therefore, the ACC build-up observed in salinized leaves is likely induced by toxic Cl^- accumulation *in situ* without ABA involvement. Thus, under salt stress, the pathway that leads to leaf abscission appears to be independent of ABA. The experiments reported in the present work agree with this hypothesis and clearly indicate that the addition of 10 μ M ABA to the watering solution resulted in a decrease in salt-induced leaf abscission.

Besides the involvement of ABA in regulating Cl^- accumulation, leaf abscission and CO_2 assimilation, other protective roles have been suggested for the phytohormone in different plant species under stress. These include reduction in membrane damage (Rajasekaran and Blake 1999), improvement in mineral nutrition (Gadallah 1996), up- and down-regulation of gene expression (Gómez-Cadenas and others 1999), and up-regulation of antioxidant enzyme activity (Bellaire and others 2000). Although these parameters are not the subject of this study, we presume that the beneficial effects of ABA treatment on citrus plants under stress could also be partly due to these aspects.

In conclusion, ABA regularly added to the watering solution reduces the damaging effects that a high NaCl concentration causes in citrus plants. The salt-induced defoliation was delayed by ABA

treatment probably through mechanisms that slow down plant metabolism and chloride uptake and accumulation in leaves, that is, initial reduction of stomatal aperture and transpiration. The decline in photosynthesis associated with salt stress was also more moderate in ABA-treated plants. Therefore, ABA appears to improve tolerance to salinity in citrus.

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