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Hydraulic and Chemical Responses of Citrus Seedlings to Drought and Osmotic Stress

M. Ángeles Forner-Giner · Juan Rodríguez-Gamir · Eduardo Primo-Millo · Domingo J. Iglesias

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Abstract In this work we investigated the function of abscisic acid (ABA) as a long-distance chemical signal communicating water shortage from the root to the shoot in citrus plants. Experiments indicated that stomatal conductance, transpiration rates, and leaf water potential decline progressively with drought. ABA content in roots, leaves, and xylem sap was also increased by the drought stress treatment three- to sevenfold. The addition of norflurazon, an inhibitor of ABA biosynthesis, significantly decreased the intensity of the responses and reduced ABA content in roots and xylem fluid, but not in leaves. Polyethylene glycol (PEG)-induced osmotic stress caused similar effects and, in general, was counteracted only by norflurazon at the lowest concentration (10%). Partial defoliation was able to diminish only leaf ABA content (22.5%) at the highest PEG concentration (30%), probably through a reduction of the active sites of biosynthesis. At least under moderate drought (3–6 days without irrigation), mechanisms other than leaf ABA concentration were required to explain stomatal closure in response to limited soil water supply. Measurements of xylem sap pH revealed a progressive alkalinization through the drought condition (6.4 vs. 7.1), that was not counteracted with the addition of norflurazon. Moreover, in vitro treatment of detached leaves with buffers iso-osmotically adjusted at pH 7.1 significantly decreased stomatal conductance (more than 30%) as much as 70% when supplemented with ABA. Taken together, our results suggest that increased pH generated in drought-stressed roots is transmitted by the xylem sap to the leaves, triggering reductions in shoot water loss. The parallel rise in ABA concentration may act synergistically with pH alkalinization in xylem sap, with an initial response generated from the roots and further promotion by the stressed leaves.

Keywords Abscisic acid · Leaf water potential · Norflurazon · Polyethylene glycol · pH · Stomatal conductance · Root hydraulic conductance · Transpiration · Water stress

M. Á. Forner-Giner · J. Rodríguez-Gamir · E. Primo-Millo · D. J. Iglesias (✉)
Departamento de Citricultura, Instituto Valenciano de Investigaciones Agrarias, Oficial Box, E-46113 Moncada, Valencia, Spain
e-mail: iglesias_dom@gva.es

Introduction

It is widely accepted that the root acts as a "sensor" of soil dryness, generating a chemical signal that is translocated to the shoots (Davies and others 1990). Soil drying results in an increased synthesis of abscisic acid (ABA) in the roots, which is transported to the shoots through the xylem by the transpiration stream, thus inhibiting stomatal opening and leaf growth (Zeevaart and Boyer 1984; Zhang and Davies 1987, 1989a, 1990; Zhang and others 1987). This is supported by the enhanced concentration of ABA in the xylem sap from plants exposed either to dry soil (Zhang and Davies 1989a, 1990) or to a polyethylene glycol (PEG) osmoticum (Hoad 1975; Neales and others 1989; Davies and Zhang 1991; Neales and McLeod 1991; Comstock 2002). Such responses can be induced rapidly in some plants, because stomatal closure may occur before any detectable change in the total water or turgor potential (Gollan and others 1986; Gowing and others 1990; Zhang and Davies 1990; Trejo and Davies 1991). Also, the increase in ABA concentration in the xylem sap of drought-stressed plants may be very rapid (Hoad 1975).

In drought-stressed leaves the ABA concentration increases as turgor decreases (Zhang and Davies 1989b). Thus, these organs should be a potential source of ABA that could contribute to the increased transport through the xylem flow. In this sense, some reports suggest that the ABA derived from the stressed leaves is transported downward to the roots via phloem and then released to the transpiration stream (Hoad 1973, 1978; Wolf and others 1990).

Although ABA transported in the xylem from root to shoot and perceived at the guard cell appears to be the main factor in the regulation of stomatal closure in water-stressed leaves (Davies and Zhang 1991; Wilkinson 2004; Christmann and others 2005, 2006; Israelsson and others 2006), there is also considerable evidence that plants are able to respond directly to root-to-shoot hydraulic signals caused by soil drying (Cochard and others 2002; Boodribb and Holbrook 2003; Christmann and others 2007). Such signals could be involved in stomatal responses to decreased leaf water potential (Comstock and Mencuccini 1998; Saliendra and others 1995) or to the drop of hydraulic conductance during drought (Sperry and others 2002). In this context, it has been reported that in many species, root hydraulic conductance decreases as water availability in the soil is reduced (Cruz and others 1992; North and Nobel 1996; Lo Gullo and others 1998). Moreover, this reduction due to soil drying can be reversed when soil is rewetted (North and Nobel 2000; Martre and others 2001), and further regulatory processes appear to involve aquaporin expression (North and others 2004).

Changes in xylem sap or apoplastic pH (Wilkinson 1999; Bahrun and others 2002; Sharp and Davies 2009) or even electrical signals (Grams and others 2007) may be strongly involved in the regulation of stomatal dynamics under water stress. Although not completely understood, the combination of some of those signals has been recently proposed to play a main role in the responses to drought (Lovisolo and others 2002).

In citrus, numerous studies have reported the consequences of drought at both leaf and root levels (Syvertsen and others 1988; Bryla and others 1997; Pérez-Pérez and others 2007; Poggi and others 2007). Differences in drought tolerance related to morphology and physiology (Savé and others 1995; Garcia-Sanchez and others 2007) have also been described and, more recently, wide alterations of gene expression reported as a consequence of drought (Gimeno and others 2009). However, there is a lack of knowledge about the relative contributions of chemical and hydraulic signaling to the final responses.

In this work we examine the possibility that soil water deficit generates other signals which, in addition to ABA signaling, modulate stomatal closure. Experiments were conducted to test the effect of norflurazon (NF)-induced inhibition of ABA synthesis in roots (Sandermann and Böger 1989) on stomatal conductance, water relations, root hydraulic conductance, and ABA content in citrus plants under drought and osmotic stress conditions. An in vitro approach is assayed to manipulate sap pH and ABA content in detached leaves to study their interactions and the way in which they complement each other in modulating stomatal closure. Finally, the relative contributions of roots and leaves to ABA concentration in xylem sap with respect to stress intensity were analyzed.

Material and Methods

Plant Material and Growth Conditions

Ten-month-old seedlings of a common citrus rootstock Citrange carrizo [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] were used in this experiment. Plants were cultured under glasshouse conditions with supplementary light (50 μmol m^{-2} s^{-1}, 400–700 nm) to extend the photoperiod to 16 h. The greenhouse was equipped with an evaporative cooling pad combined with a fogging system. Temperature and humidity were continuously monitored throughout the study period. Temperatures ranged between 16 and 18°C at night and between 24 and 28°C during the day, and relative humidity was maintained at approximately 80%.

Plants were grown individually in 1-L pots filled with coarse sand. All plants were irrigated every 2 days, until the beginning of the experiment, with the following nutrient solution: 3 mM $Ca(NO_3)_2$, 3 mM KNO_3, 2 mM $MgSO_4$, 2.3 mM H_3PO_4, 17.9 μM Fe-EDDHA, and trace elements as prescribed by Hoagland and Arnon (1950). Nutrient solution pH was adjusted to 6.0 with 1 M KOH or 1 M H_2SO_4. Approximately 0.2 L of solution per pot was used in each watering event. Excess solution was drained out of the pot, thereby avoiding any salt accumulation.

Plants growing as a single shoot were selected for uniformity of size at the beginning of the treatments. In all experiments plants were randomized over the experimental area and analyzed individually. A row of plants, not included in the experiment, was placed around the perimeter as a border row.

Experimental Design

Four different experiments were performed as follows.

Experiment 1: Effect of Water Stress upon Hydraulic Signaling and ABA Content

The purpose of this experiment was to study the differential contribution of hydraulic and chemical signals (represented

by ABA content) in water-stressed citrus plants. Seedlings treated with or without NF were maintained under drought stress conditions to test the influence of the inhibition of root ABA synthesis on leaf stomatal conductance (g_s), transpiration (E), relative water content (RWC), root hydraulic conductance (K_r), and abscisic acid (ABA) concentrations in roots, leaves, and xylem fluid. Changes in pH of sap extracted from roots were also monitored. The three treatments were (1) fully watered plants (control) with the nutrient solution alone, (2) drought-stressed plants by withholding irrigation, and (3) drought-stressed plants by withholding irrigation after application of 1 mM NF to the nutrient solution at the beginning of the experiment. Treatments were applied for 9 days and determinations done at 0, 3, 6, and 9 days. Six plants per treatment were used for each sampling date to measure g_s, E, total water (Ψ_W) and osmotic (Ψ_Π) potentials, RWC, K_r, and xylem sap pH (described below). Gas exchange, water relationships, K_r, and sap pH were determined in four independent groups of plants cultured under similar conditions. Three plants were used for analysis of ABA in leaves, roots and xylem sap.

Experiment 2: Responses of Citrus Plants to PEG-induced Osmotic Stress

The objective of this experiment was to determine the responses of citrus plants to PEG-induced osmotic stress, looking for other signals distinct to ABA through the inhibition of its biosynthesis by NF. The six treatments were

A. Nutrient solution alone
B. Nutrient solution added with 1 mM NF
C. Nutrient solution added with 10% PEG
D. Nutrient solution added with 10% PEG + 1 mM NF
E. Nutrient solution added with 30% PEG
F. Nutrient solution added with 30% PEG + 1 mM NF

Plants were irrigated with these solutions at the beginning of the experiment and were maintained for 48 h before determinations. Six plants per treatment were used to measure g_s, E, Ψ_W andΨ_Π, RWC, and K_r. Gas exchange, water relationships, and K_r were determined in independent groups of plants cultured under similar conditions. Three plants were used for ABA analysis in leaves and roots.

Experiment 3: Effect of Defoliation upon ABA Changes Promoted by PEG-Induced Osmotic Stress

In this experiment the effects of PEG and defoliation treatments on the ABA concentration in xylem sap from the upper part of the shoot were studied to elucidate the contribution of leaves to the ABA transported by the transpiration stream. The six treatments were

A. Nutrient solution alone applied to intact plants (controls)
B. Nutrient solution alone applied to 66% defoliated plants
C. Nutrient solution added with 10% PEG applied to intact plants
D. Nutrient solution added with 10% PEG applied to 66% defoliated plants
E. Nutrient solution added with 30% PEG applied to intact plants
F. Nutrient solution added with 30% PEG applied to 66% defoliated plants

Plants of treatments B, D, and F were defoliated by removing approximately two thirds of the leaves from the lower part of the stem and leaving leaves on the top 15–20 cm of the shoot. Defoliation was performed before PEG treatments. Forty-eight hours after the treatments stems were cut just above the defoliated part and at approximately the same height in intact control plants. Sap was extracted from the excised part of the shoot (upper part) and ABA concentration in the xylem fluid determined. Six plants per treatment were used; samples from three of them were collected for analysis.

Experiment 4: Effect of pH and ABA upon Stomatal Conductance of Detached Leaves

The 4 treatments were

A. Potassium phosphate buffer, pH 6.4
B. Potassium phosphate buffer, pH 6.4 added with 0.2 ppm ABA [(±)-abscisic acid (Sigma-Aldrich, Madrid, Spain)]
C. Potassium phosphate buffer, pH 7.1
D. Potassium phosphate buffer, pH 7.1 added with 0.2 ppm ABA

Six uniform, fully expanded mature leaves from six different plants with 40–50 leaves were used per treatment at each sampling time to measure g_s. Determinations were performed every hour for up to 6 h.

Gas Exchange Parameters

g_s and E were determined using at least two uniform, fully expanded mature leaves from the midstem zone of each of the six plants considered per treatment. The average value of the two leaves was considered as representative of each individual plant.

Measurements were taken outdoors between 10:00 and 11:30 a.m. on sunny days, under stable environmental conditions. Photosynthetically active radiation (PAR) at the leaf surface was adjusted to a photon flux density of

1000 μmol m^{-n} s^{-s}, which exceeds the saturating value for citrus (Iglesias and others 2002). A closed gas exchange CIRAS-2 (PP-systems, Hitchin, UK) was used to make the measurements. Leaf laminae were fully enclosed within a PLC 6(U) universal leaf autocuvette in a closed circuit mode and kept at 25 ± 0.5°C, with a leaf-to-air vapor deficit of 1.7 Pa. The air-flow rate through the cuvette was 0.5–1.5 L min^{-m}. In general, ten consecutive measurements were taken at 3-s intervals. Similar conditions were employed to estimate gas exchange parameters in detached leaves (see below). When needed, immediately after measurements, leaves were tightly wrapped in aluminum foil, frozen by immersion in liquid nitrogen, and stored at −20°C for further analyses.

Root Hydraulic Conductance

k_r was determined using the high-pressure flow meter (HPFM) method using the Dynamax flow meter (Dynamax, Inc., Houston, TX, USA). Measurements were performed under conditions similar to gas exchange parameters following Tyree and others (1995). The stem was cut 5 cm above the soil surface, the stump connected to the HPFM with a water-tight seal, and the root conductance measured by a few transient (1-min) measurements. Water flow into root (F) and applied pressure (P) were registered every 3 s while increasing the applied pressure at a constant rate of 3–7 kPa s^{-1}. k_r was calculated as the slope of the plot of F versus P:

$$k_r = dF/dP$$

where dF/dP was computed from the regression line. All conductance values were normalized (made root-specific) by dividing k_r by the total root weight to yield K_r. Intact root systems from six plants per treatment were considered for determinations.

Leaf Water Relations

All leaf tissue evaluations were performed using two uniform fully expanded mature leaves from the midstem zone of each of the six plants per treatment. The average value of the measurements on the two leaves was considered as representative of each individual plant.

Ψ_W of leaves was measured at daybreak (6:30–7:30 a.m.) with a Scholander-type pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA) (Scholander and others 1965). After thawing, nonturgid Ψ_Π was registered in expressed cell sap collected from a syringe at 25 ± 1°C and placed in an osmometer (Digital Osmometer, Wescor, Logan, UT, USA). Turgor potential (Ψ_P) was calculated as the difference between Ψ_W and Ψ_Π.

Also at daybreak, two other similar leaves were detached and leaf fresh weight (FW) determined. Leaf petioles were placed in a beaker of water overnight in the dark so that the leaves would become fully hydrated. Leaves were blotted dry with paper towels, reweighed to obtain turgid weight (TW), and dried at 80°C for 24 h to obtain the dry weight (DW). RWC of the leaves was calculated as $RWC = (FW - DW) \times (TW - DW)^{-1} \times 100$ according to Morgan (1984).

Extraction and Quantification of Xylem Fluid

Xylem fluid was extracted from roots using a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA). Six plants per treatment were cut 5 cm above the first roots, and whole bare roots were placed into the pressure chamber cylinder with the cut end out of the cylinder. A similar procedure was used to obtain sap from the upper part of the stem when considered. The shoot of each of six plants per treatment was cut at 15–20 cm below the terminal meristem and the entire shoot tip with about 8–12 intact leaves was sealed in the pressure chamber, leaving only the cut end protruding. In both cases, a plastic tube was connected to the cut end to collect xylem fluid with a micropipette and a constant pressure of 2 MPa was applied for 5 min (Tudela and Primo-Millo 1992).

ABA Analysis

The procedure for ABA quantification was performed as previously described (Agusti and others 2007). In brief, samples were extracted with 80% ethanol and further purification was obtained with C_{18} Sep-Pak cartridges and reverse-phase high-pressure liquid chromatography (HPLC). The fraction of HPLC containing ABA was methylated and analyzed using a Varian Star 3400 CX gas chromatograph coupled to a Varian Saturn mass spectrometer (MS). The samples (1–2 μl) were injected in splitless mode, the He inlet pressure was 85 kPa, and the injector, interface, and MS source temperatures were 250, 250, and 200°C, respectively. ABA was quantified based on the use of internal standards (Gaskin and MacMillan 1991). Base peaks of standard and deuterated [$^2H_6$] ABA (190 and 194 m/z, respectively) were monitored for ABA identification and quantification. Three plants per treatment were used for analysis.

Measurement of Xylem Sap pH

After extraction of xylem fluid (see above for details), pH was directly measured with a microelectrode Metrohm model 6.02.34.100 (Metrohm AG, Switzerland) interfaced with a pH-meter Crison model Basic 20 (Crison Instruments

SA, Spain). Six different plants per treatment were used for determinations.

Assays on Detached Leaves

Fully expanded intact natural leaves (including petioles) were detached from the midstem zone of plants and immediately placed in 100-ml vessels containing 20 ml of potassium phosphate buffer (10 mol m^{-3} KH_2PO_4/K_2HPO_4). Buffers were adjusted to pH values of either 6.4 or 7.1 by altering the ratio of the two salts such that different treatments were iso-osmotic. In some treatments ABA (0.2 ppm) was added to both buffer solutions. Six leaves were kept in each vessel, with the cut ends of the petioles immersed in the pH solutions to a depth of approximately 2.0 cm. Vessels were randomized within a controlled-environment chamber (Sanyo MCR-350 H, Sanyo Electric Biochemical Co., Japan) under continuous light (200 μmol m^{-2} s^{-1} photon flux density) at 28°C and 80% RH. Gas exchange parameters were measured in leaves after 6 h as described above.

Statistical Analyses

Parameters were tested by analyses of variance (ANOVA) and comparisons of means determined through the least significant differences (LSD) method at 95% confidence level. Statistical analyses were performed with Statgraphics Plus ver. 5.1 (Statistical Graphics, Englewood Cliffs, NJ, USA).

Results

Experiment 1: Effect of Water Stress upon Hydraulic Signaling and ABA Content

The values of g_s decreased progressively throughout the drought treatment (Fig. 1A), reaching minimum values on day 9. Seedlings without norflurazon (−NF) underwent a marked reduction in g_s by day 3, whereas NF-treated (+NF) plants maintained higher levels from day 3 to day 6 but showed similar levels to −NF seedlings by day 9. Transpiration rates showed analogous changes (Fig. 1B).

Leaf Ψ_W, Ψ_Π, and Ψ_P all progressively decreased similarly in drought-stressed plants (Fig. 2). As drought stress progressed, reductions in leaf Ψ_W caused by drought stress were not completely compensated for with reductions in leaf Ψ_Π, and therefore leaf turgor potential (Ψ_P) decreased in all stressed seedlings. After 9 days, Ψ_P in water-stressed plants treated or not with NF decreased more than 70% with respect to control values. Leaf RWC

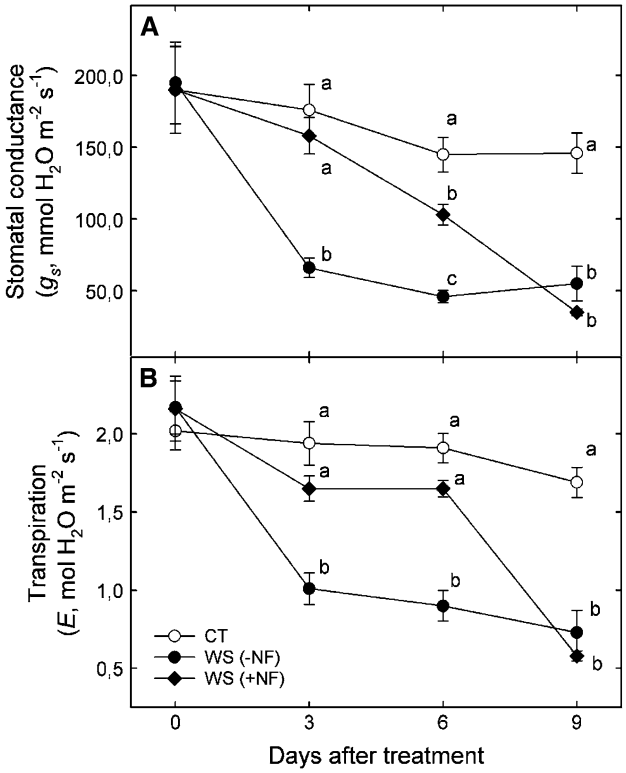

Fig. 1 Stomatal conductance (g_s) and transpiration (E) of Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(-NF)] with 1 mM norflurazon. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

of droughted plants decreased progressively to 50% (Fig. 3), similar to patterns in Ψ_P.

ABA concentration in roots from drought-stressed seedlings increased progressively from the beginning of the experiment, reaching the highest values at day 9 (Fig. 4A). This increase was lower in +NF (2.3-fold) than in −NF seedlings (6.5-fold) relative to well-watered controls. In addition, water stress increased leaf ABA concentration, which reached values 7-fold above controls after 9 days without irrigation (Fig. 4B). However, no significant differences were found among stressed plants treated with or without NF. Drought stress also raised the ABA transported by xylem sap, as −NF seedlings had higher ABA content than +NF seedlings (Fig. 4C).

Using a pressure of 2 MPa for 5 min, between 150 and 180 μl of fluid was obtained from control plants. After 3 days of drought, no effect upon extracted volume was observed in stressed plants with or without NF. However, at day 6 this volume was reduced in stressed plants to less than 30%, and at the end of the experiment (day 9), it was too low to be measured.

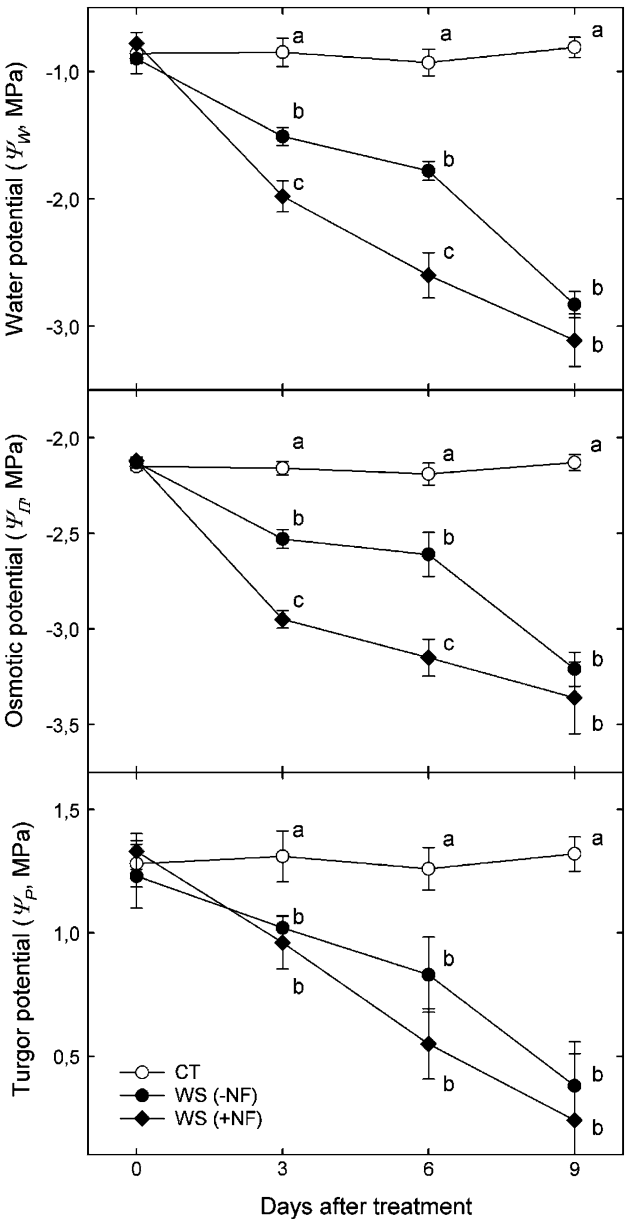

Fig. 2 Leaf water (Ψ_W), osmotic (Ψ_Π), and turgor (Ψ_P) potential from Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(−NF)] with 1 mM norflurazon. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

In drought-stressed plants with or without NF, K_r values remained similar to control plants until day 6. Significant reductions (higher than 50%) were registered only by day 9 regardless of NF treatment (Fig. 5).

Sap extracted from roots of drought-stressed plants was significantly more alkaline by day 2 than that from well-irrigated control plants regardless of NF treatment (Fig. 6).

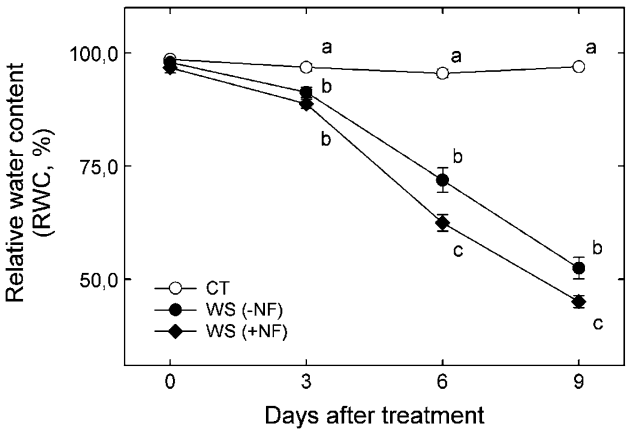

Fig. 3 Relative water content (RWC) of Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(−NF)] with 1 mM norflurazon. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

Well-watered control plants presented similar values of g_s, E, Ψ_W, Ψ_Π, Ψ_P, RWC, ABA content, K_r, and sap pH through the course of the experiment. There were no significant differences between control plants and fully watered NF-treated plants (data not shown).

Experiment 2: Responses of Citrus Plants to PEG-Induced Osmotic Stress

Irrigation of plants with PEG solutions caused a reduced stomatal conductance, especially at high external PEG concentration (Fig. 7A). The addition of NF to PEG solutions significantly reduced the effect on g_s at 10% PEG but not that of the already lower g_s at 30% PEG. Treatment effects on E were similar to those on g_s (Fig. 7B). External PEG decreased Ψ_W in leaves especially with 30% PEG but regardless of NF treatment (Fig. 8). The reduction in leaf Ψ_W caused by the 10% PEG solution was compensated for by a reduction in Ψ_Π, thus maintaining Ψ_P at the same level as that in control plants. However, in plants treated with 30% PEG, the reduction in Ψ_Π was not enough to offset the decrease in Ψ_W, so leaf turgor reached values below 34% of those observed in control plants. Leaf RWC responses to treatments were similar to Ψ_P (Fig. 9).

PEG-induced osmotic stress significantly increased the ABA concentration in roots and leaves similarly (Fig. 10). The increases in root ABA with respect to control plants ranged from 3.2- to 5.5-fold in roots supplemented with 10 and 30% PEG, respectively. In plants treated with 10% PEG, addition of NF decreased root ABA content by 48% in relation to 10% PEG, whereas no NF effect was detected in plants treated with 30% PEG. On the other hand, leaf ABA content from plants treated with 10 and 30% PEG

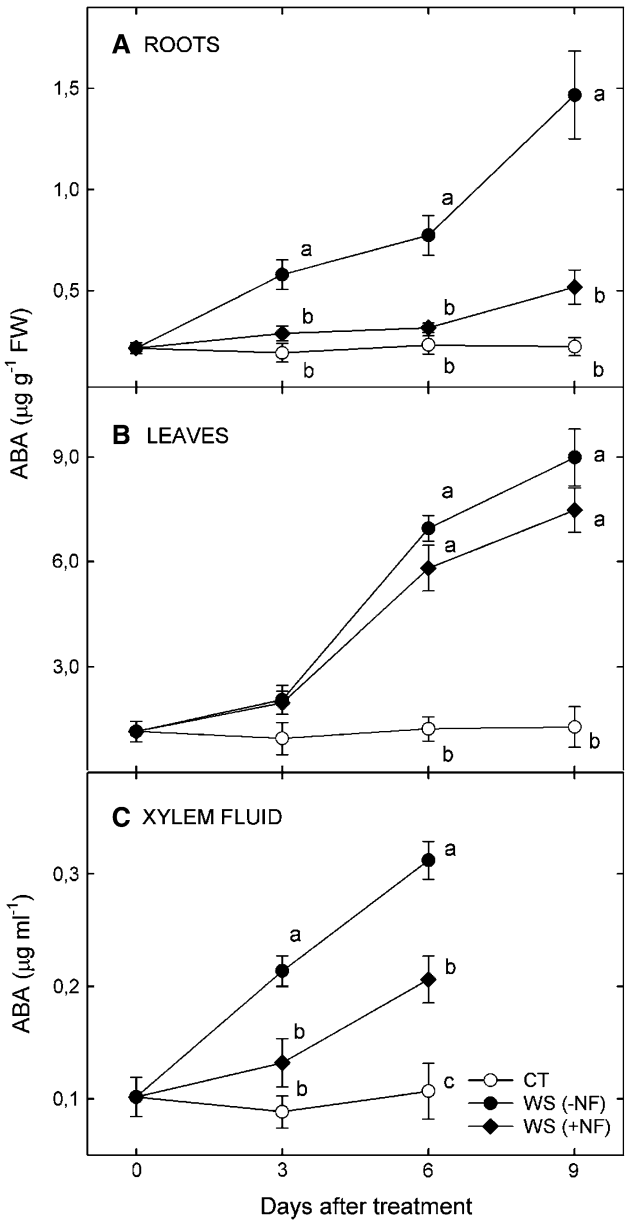

Fig. 4 Abscisic acid (ABA) concentration in roots (**A**), leaves (**B**), and xylem fluid (**C**) from Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(−NF)] with 1 mM norflurazon. Data are mean values of independent extractions performed from $n = 3$ different plants; the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

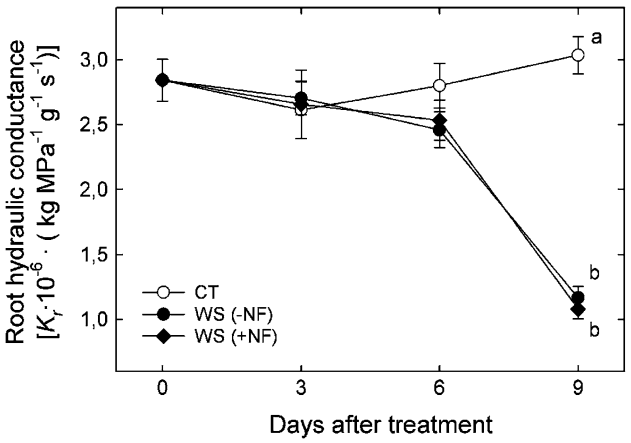

Fig. 5 Root hydraulic conductance (K_r) measured from Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(−NF)] with 1 mM norflurazon. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

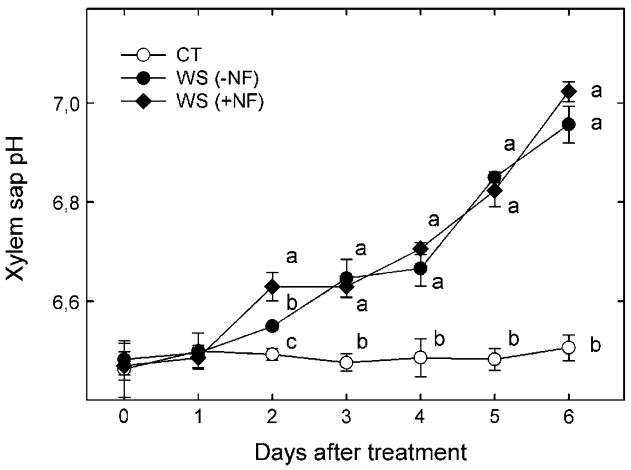

Fig. 6 pH of xylem sap extracted from Citrange carrizo seedlings during water stress treatment. Plants were either nonstressed (CT) or water-stressed, treated [WS(+NF)] or not [WS(−NF)] with 1 mM norflurazon. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$) for the same sampling date

was 4.7- and 8.3-fold higher than in control plants, respectively. In this case, NF significantly decreased (more than 40%) values found in leaves from 10% PEG-treated plants. However, no effects of NF on ABA content were found in those leaves from plants treated with 30% PEG.

There was no significant effect of PEG or NF on K_r (Fig. 11).

Experiment 3: Effect of Defoliation upon ABA Changes Promoted by PEG-Induced Osmotic Stress

Only treatments with 30% PEG significantly increased the ABA concentration in xylem sap from shoot tips (Table 1). In addition, defoliation decreased by more than 20% the ABA concentration in sap from plants treated with 30% PEG. This treatment reduced the volume of extracted xylem sap from shoot tips by about 40%, whereas defoliation showed no significant effect. Interestingly, the total

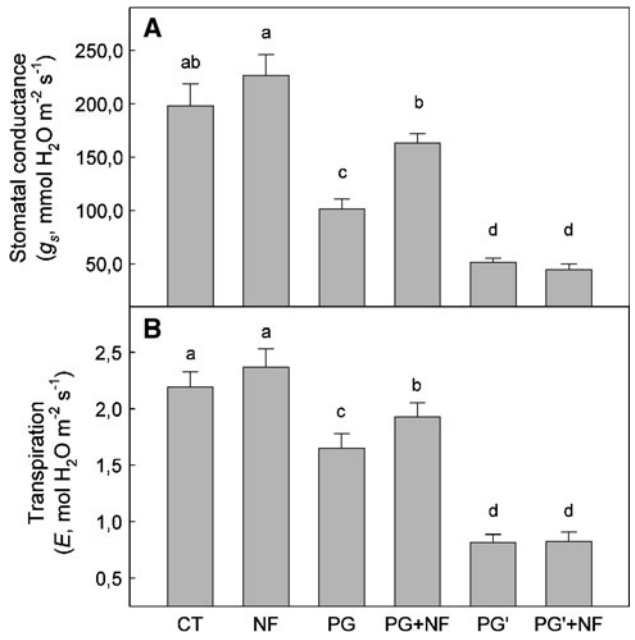

Fig. 7 Stomatal conductance (g_s) and transpiration (E) of Citrange carrizo seedlings during PEG-induced osmotic stress combined or not with 1 mM norflurazon (NF). Plants were nonstressed (CT) or stressed with 10% (PG) or 30% PEG (PG′). Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

amount of ABA in the extracted xylem sap volume was unaffected by defoliation in any case.

Experiment 4: Effect of pH and ABA upon Stomatal Conductance of Detached Leaves

In this experiment, the g_s response of detached leaves maintained in buffer solutions with 0.2 ppm ABA or without ABA was determined. The pH values of buffers used in this experiment were equivalent to those measured in xylem sap extracted from well-watered and drought-stressed plants (6.4 and 7.1, from Experiment 1, Fig. 6).

Prior to the imposition of pH and ABA treatments, leaves had a mean g_s of 108.0 ± 5.8 mmol H_2O m^{-2} s^{-1} (Fig. 12). Treatment with buffer adjusted to pH 7.1 significantly reduced g_s of detached leaves compared to those immersed in buffer adjusted to pH 6.4. The decreases in g_s were observed after 2 h of the treatments (data not shown), and after 6 h, mean values had decreased to approximately 68% of leaves at pH 6.4. ABA (0.2 ppm) added to buffer solutions caused a reduction in g_s of 32 and 53% on detached leaves exposed for 6 h to buffers adjusted to pH 6.4 and 7.1, respectively. The greatest reduction in g_s occurred at pH 7.1 with ABA (0.2 ppm).

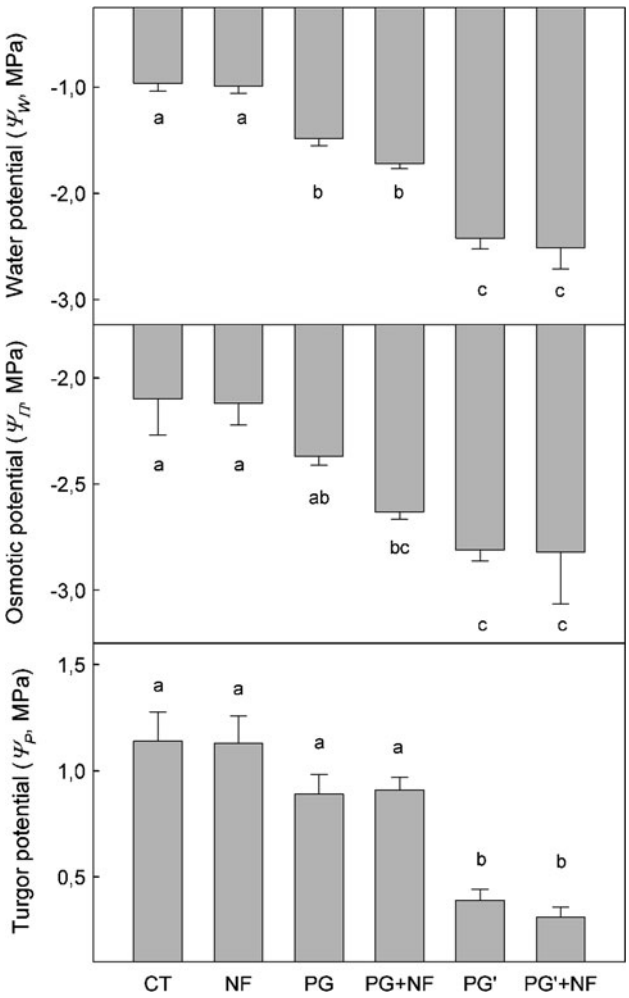

Fig. 8 Leaf water (Ψ_W), osmotic (Ψ_Π), and turgor (Ψ_P) potential from Citrange carrizo seedlings during PEG-induced osmotic stress combined or not with 1 mM norflurazon (NF). Plants were nonstressed (CT) or stressed with 10% (PG) or 30% PEG (PG′). Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

Discussion

Withholding water from the pots reduced stomatal conductance, and under moderate drought stress (3-6 days without irrigation), this effect was diminished in plants treated with NF. Moreover, high transpiration rates under water-stress conditions were associated with lower leaf water potentials, as evident in plants treated with NF. Because of osmotic adjustment, no significant differences of the effect +NF and −NF on leaf turgor or RWC of water-stressed leaves were observed.

Drought increased ABA concentration in roots, contributing to the increase in the ABA concentration in xylem sap and likely to the rise of ABA concentration in leaves. Treatment with NF decreased ABA concentration in roots

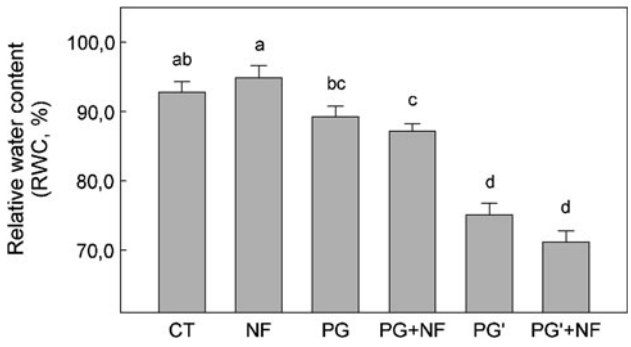

Fig. 9 Relative water content (RWC) of Citrange carrizo seedlings during PEG-induced osmotic stress combined or not with 1 mM norflurazon (NF). Plants were nonstressed (CT) or stressed with 10% (PG) or 30% PEG (PG′). Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

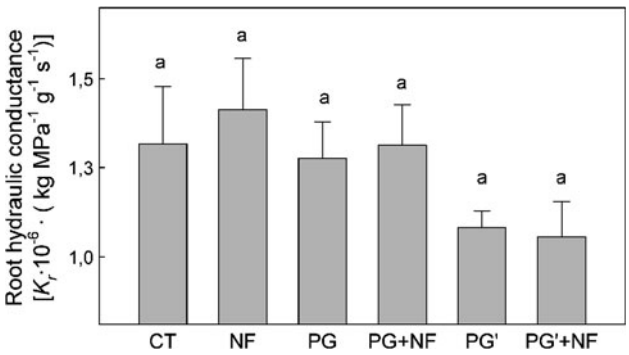

Fig. 11 Root hydraulic conductance (K_r) measured from Citrange carrizo seedlings during PEG-induced osmotic stress combined or not with 1 mM norflurazon (NF). Plants were nonstressed (CT) or stressed with 10% (PG) or 30% PEG (PG′). Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

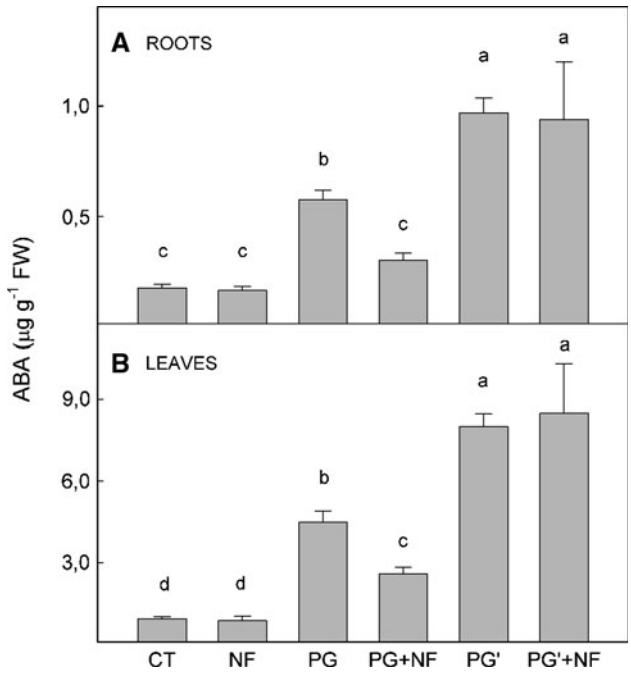

Fig. 10 Abscisic acid (ABA) concentration in roots (**A**) and leaves (**B**) from Citrange carrizo seedlings during PEG-induced osmotic stress combined or not with 1 mM norflurazon (NF). Plants were nonstressed (CT) or stressed with 10% (PG) or 30% PEG (PG′). Data are mean values of independent extractions performed from $n = 3$ different plants; the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

and sap, although no effect was observed in leaves, probably because the reduced ABA levels in sap were compensated for by a higher transpirational flux or by ABA synthesis in stressed leaves (Zhang and Davies 1987, 1989b, 1990; Zhang and others 1987; Nambara and Marion-Poll 2005). ABA is synthesized in larger amounts in drought-stressed roots, moving to leaves through the transpiration stream causing stomatal closure quite independently of any change in leaf water content. In this sense, ABA production in roots seems to provide a sensitive indication to the shoots of reduced water availability in the soil (Zhang and Davies 1989a). Leaf responses might involve additional modulation of water loss through the reduction of stomatal conductance (Zhang and Davies 1990; Israelsson and others 2006).

There was a discrepancy between leaf ABA concentration and stomatal conductance between 3 and 6 days after the drought stress treatments began. Although there were similar concentrations of ABA in leaves from drought-stressed plants regardless of NF treatment, levels of stomatal conductance were higher in NF-treated plants than in non-NF treated plants. Results might indicate that the concentration of ABA in xylem sap rather than total ABA delivery (as estimated by ABA content, Table 1), appeared to be better correlated to stomatal closure as also shown by Jia and Zhang (1999). Because ABA found in leaves is the result of different processes of absorption, compartmentation, accumulation, conjugation, and metabolization by mesophyll and epidermal tissues, it may modify the composition of the xylem stream before reaching the guard cells (Slovic and Hartung 1992a, b). Moreover, it is likely that only the fraction of the xylem-transported ABA moving through the leaf apoplast, which remains available to the guard cell receptors, would be transduced into a closing signal (Hartung and others 1998). However, no correspondence between stomatal conductance and xylem ABA concentration was shown to occur in sunflower plants (Schurr and others 1992). Therefore, under moderate water stress, the supply of ABA to leaves may not be solely responsible for the limitations in stomatal conductance induced by soil drying.

In droughted plants there was a substantial decrease in the turgor and water content of leaves 9 days after the

Table 1 Volume of xylem fluid from defoliated (DF) and non-defoliated (ND) citrange carrizo seedlings ($n = 6$ plants per treatment) osmotically stressed with polyethylene glycol (PEG), and ABA concentration and ABA content in the extracted xylem sap ($n = 3$ plants per treatment)

Treatment	Xylem sap volume (μl)		ABA concentration (ng ml^{-1})		ABA content (ng)	
	ND	DF	ND	DF	ND	DF
CT	55.3a	52.3a	113.0c	102.6c	6.3a	5.4a
PG	49.7a	50.0a	147.5c	152.8c	7.3a	7.6a
PG'	34.0b	34.7b	285.1a	221.3b	9.7a	7.7a

Treatments were controls (CT) and 10% (PG) or 30% PEG (PG′). Data show mean values; the different letters indicate statistically significant differences ($p \leq 0.05$) per parameter

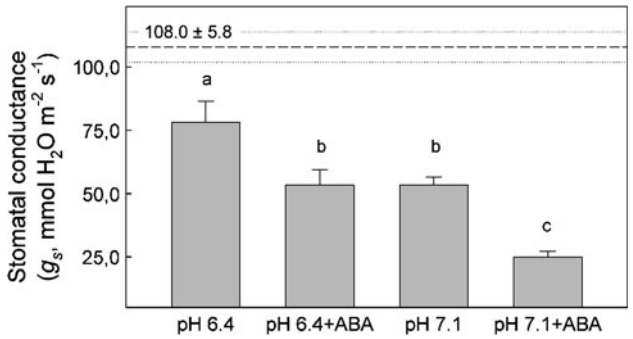

Fig. 12 Stomatal conductance (g_s) of detached leaves from Citrange carrizo immersed for 6 h in iso-osmotic buffers adjusted to pH 6.4 and 7.1 combined or not with abscisic acid (0.2 ppm ABA). The dashed line indicates the mean value (±standard error) measured before treatment. Data are mean values ($n = 6$ plants); the error bars show standard errors and the different letters indicate statistically significant differences ($p \leq 0.05$)

beginning of the experiment. Leaves may produce increasing amounts of ABA as turgor or water content decreases (Zhang and Davies 1989b; Neales and McLeod 1991). This is in agreement with our results where at low levels of soil water potential, a lower Ψ_Π could not sustain turgor and leaves became partially dehydrated. Moreover, this may have resulted in the stimulation of ABA production in leaves which, independent of the NF treatment, appeared to be responsible for stomatal closure.

PEG-induced osmotic stress increased ABA concentration in roots and leaves and decreased stomatal conductance and transpiration rate, especially in plants treated with the highest external PEG concentration (30%). The fact that NF had a significant effect on stomatal conductance only when supplemented with the 10% PEG solution but not with 30% PEG could be explained by the reductions in turgor and water content in leaves from plants treated with 30% PEG. The high PEG concentration may have enhanced ABA biosynthesis in leaves and reduced the dependence of g_s responses on ABA transported from roots, therefore making NF ineffective.

The main difference between the responses of plants to drought (experiment 1) and osmotic stresses (experiment 2) appears to be related to the effect of NF on the leaf ABA concentration in plants under moderate drought or osmotic stress. In the case of drought stress, NF did not affect ABA concentration in leaves, whereas in the case of PEG stress, leaf ABA was reduced by NF. Thus, the correlation between ABA concentration in leaves and stomatal conductance was lower when considering all the data from experiment 1 ($R^2 = 0.72$, df = 11) compared to the data from experiment 2 ($R^2 = 0.96$, df = 5). In unwatered plants, the ABA response of roots to soil drying may have required a sufficiently low soil water potential in a sufficiently large volume of the root zone, which required some time to be reached as the soil gradually dried. However, it is likely that PEG osmotic treatments may have acted more rapidly throughout the whole root system. Therefore, the ABA synthesized in response to osmotic stress appears to represent a stronger signal for stomatal closure than drought stress over 3-6 days. Moreover, the results also suggest that at least under moderate drought, the concept of stomatal control based only on a chemical signal (ABA) is not fully adequate. It has been previously suggested (Tardieu and Davies 1993) that a system based on the integration of hydraulic and chemical signals in the control of stomatal conductance in drying soils is more likely than control based on chemical signals alone.

Such an integrated system might be summarized as follows:

a. An initial decrease of water potential may be responsible for the drought-induced reduction in stomatal conductance (Comstock and Mencuccini 1998; Bunce 2006). Several studies propose (Tardieu and Davies 1992, 1993; Tardieu and others 1993) a role for leaf water potential in the control of stomatal conductance via a modification of the stomatal sensitivity to ABA. However, this is not the case here since in NF-treated citrus plants, there was no reduction in stomatal conductance relative to control plants during the first 3 days after the water supply was withheld, while a significant reduction in leaf water potential occurred.

b. Hydraulic conductance is altered as a consequence of water deficit (Cruz and others 1992; North and Nobel 1996; Lo Gullo and others 1998) or changes in environmental conditions (Matzner and Comstock 2001) and might be dynamically adjusted through aquaporin expression to achieve control of leaf water potential (Comstock 2002). Then, stomatal conductance is adjusted in response to leaf water potential. Measurements of root hydraulic conductance in drought-stressed citrus plants showed that this parameter was reduced by approximately 30% only after 9 days without irrigation, but not under low to moderate stress conditions after 3-6 days or PEG treatments. Therefore, the possibility that stomatal behavior was affected by changes in root hydraulic conductance appears to be not fully consistent with our data.

c. Soil water stress elicits a hydraulic response in the shoot, which precedes ABA signaling (Christmann and others 2007). The differences found in stomatal conductance between water-stressed citrus plants treated with or without NF during the first 6 days of drought did not support this possibility.

d. Drought-induced increases in the pH of the xylem sap flowing from the roots might act as a signal whereby the information is conveyed to the aerial parts of the plant. Although not universal in higher plant species (Thomas and Eamus 2002; Sharp and Davies 2009), alkalinization of xylem sap has been described to occur in numerous plants growing in drying soil (Hartung and Radin 1989; Gollan and others 1992; Wilkinson and Davies 1997, 2002; Wilkinson and others 1998; Bacon and others 1998, Bahrun and others 2002). In fact, alterations in sap pH in response to water stress has been suggested to be one of the first signals to leaves, leading to stomatal closure, even with low reductions in soil water potential (Bahrun and others 2002; Sobeih and others 2004). Moreover, it has been demonstrated that, in some cases, supplying leaves or roots with buffers adjusted to pH similar to that of the xylem of water-stressed plants can lead to stomatal closure (Dodd and others 2003; Kamaluddin and Zwiazek 2004; Wilkinson and others 2007; Wilkinson and Davies 2008; Sharp and Davies 2009). In this context, increased xylem pH appears to act as a drought signal (Netting 2000) via an ABA-dependent mechanism (Bacon and others 1998; Wilkinson and Davis 1997, 2008; Wilkinson and others 1998). The OH^- source of the increased pH has been reported to be a combination of physiological responses, changes at the membrane level (that is, in H +/ATPase activity or symport dynamics), and even alterations in the xylem charge because of modified ion uptake (Netting 2000; Sharp and Davies 2009). Wilkinson (1999) proposed that this alkalinization of xylem sap pH could cause stomatal closure by different mechanisms: (1) increasing leaf ABA concentration through a higher ABA biosynthesis or lower degradation or conjugation processes (Jia and Zhang 1997; Nambara and Marion-Poll 2005); (2) increasing guard cell sensitivity to ABA (Gollan and others 1992; Schurr and others 1992; Jia and Davies 2007); (3) changing the distribution of ABA among leaf compartments to increase local concentration near the guard cells (Kaiser and Hartung 1981; Zhang and Outlaw 2001a, b), altering ion fluxes at the membrane level, and finally affecting stomatal aperture (Hedrich and others 2001; Felle and Hanstein 2002).

Similar increases in xylem sap pH as shown in citrus roots during drought stress (Fig. 6) have been described in other species (Wilkinson 2004). Moreover, buffers adjusted to pH 7.1 supplied to detached citrus leaves reduced g_s more than 30% compared to those adjusted to pH 6.4 (Fig. 12). The pH values of buffers used in this experiment were equivalent to those measured in sap extracted from well-watered (pH 6.4) and drought-stressed (pH 7.1) plants. Similarly, the addition of ABA at physiological ranges to both buffer solutions resulted in a decrease in g_s. Although statistically significant in both cases, this effect was more than 1.5-fold higher at pH 7.1 than at pH 6.4.

Comparable results in tomato and *Forsythia* plants treated with buffered foliar alkaline sprays have been recently published (Wilkinson and Davies 2008). Also, it has been reported that alkaline solutions decreased g_s when supplied to whole plants of paper birch (Kamaluddin and Zwiazek 2004), detached leaves of pepper (Dodd and others 2003), or detached shoots of maize (Wilkinson and others 2007).

From our results, two facts appear evident: first, citrus plants increase xylem sap pH in response to soil drying; second, artificial buffers at pH 7.1, supplied through the petiole of detached leaves, are able to substantially reduce g_s, even in the absence of exogenous ABA. This seems to indicate that alkalinization of xylem sap by itself constitutes a root signal that can control stomatal conductance of plants in response to soil drying. The data presented also suggest an interaction between pH and ABA content in xylem sap, because the effect of ABA added to buffered solutions on stomatal closure was much stronger at the higher pH value. In addition, the behavior of NF-treated plants may support this interaction. Drought-stressed citrus plants either treated or not with NF had similar increases in pH of sap extracted from roots (Fig. 6). However, the values of g_s were quite different among both groups of plants, indicating the additional effect of the ABA concentration in xylem sap (Fig. 1).

Our results are consistent with the possibility that pH changes generated in drought-stressed roots are transmitted by the xylem sap to the leaf apoplast (Jia and Davies 2007), acting as a signal that triggers the mechanisms that enable the shoot to reduce water loss through stomatal closure. In particular, apoplastic pH can thereby determine the amount of ABA that finally reaches the guard cells (Slovic and Hartung 1992a, b; Wilkinson and Davies 2002; Wilkinson 2004). This effect may explain the observed interactions between pH and ABA.

In summary, ABA has been considered an essential regulating factor in stomatal closure under drought stress. The role of both roots and leaves as potential sources for the increase in ABA in the xylem sap in response to drought has been described (Neales and McLeod 1991). These authors reported that after a PEG treatment to the roots, the initial increase in the ABA content of sap may be due to synthesis in the roots, whereas thereafter the ABA derived from the leaves may constitute a major contribution to the concentration found in the xylem sap. According to this, the results from experiment 3 showed that defoliated plants, which retained only the younger terminal leaves, had significantly higher levels of ABA in xylem sap after 30% PEG treatment compared to untreated controls. The presence of basal leaves increased the ABA concentration in the sap, but only in plants treated with the highest PEG concentration (30%) where leaf turgor was lowered. Zhang and Davies (1989b) reported that in sunflower plants growing in soil that gradually dried, the drought stimulus initially generated an ABA root response, but then when leaf water relations were affected, a subsequent response of the leaves was initiated. Furthermore, our results suggest a complementary role of increased xylem sap pH and changes in ABA concentration in the signaling process modulating stomatal closure in droughted citrus seedlings, and also support the proposal of Zhang and Davies (1989b) that indicates that the rise in ABA concentration in the xylem follows a sequential response. The initial increase is generated by the roots and the subsequent one, at least partially, is promoted by the stressed leaves. Both responses appear to be required to reach increasing levels of plant protection against stress.

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