Chapter 6

Original Article

Electrical signaling, stomatal conductance, ABA and ACC content in avocado trees in response to drought or root hypoxia

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Submitted to Environmental and Experimental Botany

Abstract

Avocado (*Persea americana* Mill.) trees are very sensitive to drought and soil flooding. This study was designed to determine if changes in extra-cellular electrical voltage signals (variation potentials) in avocado trees could be detected in response to short-term (min) or long-term (days) exposure to drought or root hypoxia and if these signals could be related to stomatal conductance (gs) or root and leaf ABA and ACC concentrations as well as leaf ethylene emission and abscission in plants exposed to root hypoxia. Short-term and long-term drought caused an increase in root to leaf electrical potential differences, whereas there was no significant effect of root hypoxia on root to leaf electrical signals. There was no effect of short-term drought or root hypoxia on root or leaf ABA concentrations. Long-term root hypoxia resulted in increased ethylene concentration in the leaves and increased leaf abscission. The results confirm that extra-cellular electrical signals between the roots and the leaves in avocado trees are related to stomatal closure in response to drought stress. However, for avocado trees exposed to root hypoxia, typically encountered in flooded or poorly drained soils, electrical signals do not appear to be the primary root-to-shoot communication mechanism. Also, in avocado trees, ABA does not appear to be involved in rapid signaling for stomatal closure as a result of stress caused by drought or root hypoxia.

Keywords: *Persea americana*, electrical signals, drought, hypoxia, stomatal conductance, variation potential.

Abbreviations: ABA, abscisic acid; gs, stomatal conductance; ACC, 1-aminocyclopropane-1-carboxylic acid; PEG, polyethylene glycol PPF, photosynthetic photon flux; ω, gravimetric soil water content, ΔV_{L-S}, voltage differences between the base of the stem and the leaf petiole, ΔV_{l-b}, voltage differences between leaf zone and base of the trunk.

INTRODUCTION

Avocado (*Persea americana* Mill.) trees are very sensitive to drought and flooding. The relatively low productivity of this species may, in part, be related to the water status of the crop, which at times is under irrigated causing water stress or over irrigated resulting in root asphyxiation. An excess or lack of water during growth limits avocado fruit production and quality, particularly if stress occurs between spring and the beginning of summer (Whiley *et al.*, 1988a; 1988b). For avocado trees, drought causes reductions in biomass production and is linked to fruit disorders and severely limits fruit production when water stress occurs during flowering (Whiley *et al.*, 1988a; Whiley and Schaffer, 1994).

Early physiological plant responses to drought include reductions in xylem turgor, inhibition of leaf expansion, leaf abscission, and stomatal closure which results in reductions of stomatal conductance (gs). Many studies have suggested that abscisic acid (ABA) is the major chemical root-to-shoot stress signal associated with stomatal closure in drought-stressed plants (Davies and Zhang, 1991; Davies *et al.*, 2005). Drought also increases root concentrations of the ethylene precursor,

ACC (1-aminocyclopropane-1-carboxylic acid) (Gómez-Cardenas *et al.*, 1996). In avocado, in response to drought, gs begins to decline when stem water potential (SWP) reaches -0.4 MPa and continues to decline until stomatal closure occurs at SWP of -1.0 to -1.2 MPa (Sterne *et al.*, 1977; Bower, 1978; Scholefield *et al.*, 1980; Whiley *et al.,* 1988a).

Root anoxia or hypoxia often results in increased concentrations of ACC (Bradford and Yang, 1980), ethylene and ABA in leaves (Bradford and Yang, 1980; Kozlowski, 1997). Elevated concentrations of ACC and ABA in leaves of flooded plants can accelerate abscission (Kozlowski, 1997). Additionally, an increase in leaf ABA concentration has been implicated in causing stomatal closure in flooded plants (Else *et al.*, 1995; Kozlowski, 1997). Low soil oxygen content can result in root tissue damage, inhibition of vegetative and reproductive growth, anatomical and morphological changes (i.e., development of hypertrophied stem lenticels, development of adventitious roots or aerenchyma development in stem tissue), premature senescence, and plant mortality (Schaffer *et al.*, 1992; Drew, 1997; Kozlowski, 1997).

Avocado is among the most susceptible of fruit tree species to poor soil aeration (Schaffer *et al.*, 1992; Whiley and Schaffer, 1994; Schaffer and Whiley, 2002). In avocado, root hypoxia or anoxia usually reduces gs, transpiration, net CO_2 assimilation, root and shoot growth, and leaf expansion, and causes moderate to severe stem and leaf wilting, leaf abscission, and root necrosis (Ploetz and Schaffer, 1989; Schaffer and Ploetz, 1989; Schaffer *et al.*, 1992; Schaffer, 1998; Schaffer and Whiley, 2002).

The presence of fast conducting electrical signals transmitted between different plant organs has been identified in several plant species. These signals can either be in the form of action potentials (Fromm, 1991; Fromm and Spanswick, 1993; Fromm *et al.*, 1997; Fromm, 2006) or variation potentials (Stahlberg and Cosgrove, 1992; 1994, Davies, 2004; 2006; Stahlberg *et al.*, 2006). Both action potentials and variation potentials transmit information about local stimuli to distant cells, promoting its physiological response (Brenner *et al.*, 2006). An action potential elicits an "all or nothing" response when the stimulus reaches a critical threshold, whereas a variation potential varies in amplitude and range depending on the intensity of the stimulus (Dziubinska *et al.*, 2003; Fromm and Lautner, 2007; Davies and Stankovic, 2007). It has been postulated that electrical signals could be a communication mechanism between roots and shoots when plants are water stressed (Fromm and Fei, 1998; Gil *et al.*, 2007). For example, stimulation of roots of *Salix viminalis* by the application of nutrients, hormones or changes in pH caused changes in the electrical potential difference between the roots and leaves. These changes were followed by a modification of leaf respiration and photosynthetic rates within three minutes after treatments were applied, indicating that changes in the electrical signals may reflect or be a direct mechanism of communication between roots and the leaves (Fromm and Eschrich, 1993). Similarly, osmotic stress suddenly applied to maize (*Zea mays*) roots generated an electrical potential difference between the roots and the leaves and a concomitant decrease in gs (Fromm and Fei, 1998). It has been shown in fava bean (*Vicia fava minor*) that thermal stimulation (scorching) of leaves results in the generation of electrical signals transmitted to distant, non-stimulated leaves which enhance

ethylene emission from those non-stimulated distant leaves (Dziubinska *et al.*, 2003). In response to drought in maize (*Zea mays*), an electrical signal (action potential) was transmitted from the root to the shoot via the phloem (Fromm and Fei, 1998; Grams *et al.*, 2007). This signal initiated stomatal closure and resultant decreases in gs and net CO_2 assimilation. A root to leaf electrical signal (variation potential) conducted through the phloem was measured and correlated with gs in avocado trees subjected to soil drying and rewatering (Gil *et al.*, 2007). The electrical potential differences between the base of the stem and the lower third of the canopy in avocado trees suggested that voltage changes play a role in root to shoot communication triggered by changes in soil water content.

The mechanisms for reductions of gs in avocado trees as a result of drought or soil hypoxia have not been elucidated. It is possible that changes in root to shoot electrical voltage differentials in response to drought or soil hypoxia may directly trigger stomatal closure which results in reduced gs or may indirectly affect stomatal closure by triggering changes in leaf ABA concentrations. Also, factors that stimulate leaf abscission in avocado trees in response to root hypoxia have not been elucidated, but increased ethylene concentrations presumably plays a role and concentrations of ethylene or its precursor, ACC, may be affected by root-to-shoot electrical signals generated in response to root hypoxia.

The main objective of this study was to test the hypothesis that electrical voltage signals (variation potentials) in avocado trees could be detected in response not only to short-term (min) and long-term (days) drought but to root hypoxia as well. An additional objective was to determine if these electrical signals are related to gs,

root and leaf ABA and ACC concentrations, and for plants subjected to hypoxia, to leaf ethylene emission and abscission.

MATERIALS AND METHODS

Three experiments were conducted to determine the effects of drought or root hypoxia on electrical voltage differences between the root and shoots, gs, leaf and root ABA and ACC concentrations, and ethylene emission from leaves of avocado trees. Experiment 1 was designed to study the effects of very short-term (75-90 min) reductions in water or oxygen availability to roots on changes in root to shoot electrical voltage differences, gs, and leaf and root ACC and ABA concentrations. Experiment 2 tested the effects of 3 days of drought on root to shoot electrical voltage differences and gs. Experiment 3 was designed to study the effects of 14 days of root hypoxia on root to shoot electrical voltage differences, gs, ethylene emission from leaves and leaf abscission.

Experiment 1: responses to short-term drought and root hypoxia

Plant material. Six–month-old seedling 'Mexicola' avocado (*Persea americana* Mill.) trees obtained from a commercial nursery were used in this study. Plants were grown in a medium composed of 100% compost and irrigated and fertilized according to standard nursery practices. Plants were removed from the potting medium and roots were washed with tap water and placed in a hydroponic medium of Hoagland's solution (Hoagland and Arnon, 1950) in a 30-L plastic container. Air was continuously pumped into the container via an air pump (Elite model 802, Hagen Manufacturing, Canada) with 2 exit tubes at a flow rate of 3 L min^{-1} and an

injection pressure of 24 KPa. At the time that trees were placed into the hydroponic medium, plants ranged in height from 20 to 35 cm with 10 to 16 leaves per plant.

Experimental design. Plants were divided into three treatments: 1) control plants, 2) plants exposed to simulated drought stress (drought), and 3) plants with roots exposed to low oxygen pressure (pO_2) (root hypoxia). For the control treatment, five plants (replications) were each placed in a 2-L plastic container of Hoagland's solution. Electrical voltage signals (variation potentials) were measured for about 90 minutes under stable environmental conditions in a laboratory to determine voltage differences between the base of the stem and the leaf petiole ($\Delta V_{L\text{-}S}$) in the absence of environmental alterations (control or baseline condition). For the simulated drought stress treatment, five plants were each placed in a 2-L plastic container of Hoagland's solution. After 15 min of baseline electrical potential measurements, polyethylene glycol (PEG) 6000 (15%, 15ºC) was added to the Hoagland's solution to block water availability to the roots and simulate water stress (Fromm and Fei, 1998; Olsovska and Brestic, 2001). According the equation established by Michel and Kaufmann (1973), the PEG 6000 solution at 15% and 15ºC presented an osmotic potential (Ψ_s) of -0.35 MPa. Electrical potentials were then measured for an additional 75 min. For the root hypoxia treatment, five plants were each placed in a 2-L plastic container of Hoagland's solution. After 5 min of baseline recording, 100% gaseous N_2 was bubbled through the Hoagland's solution to displace the O_2 and create a hypoxic condition in the root zone. For all treatments, the electrical potential difference between roots and leaves was measured for at least 75 min.

Air temperature during the experiment was between 20 and 23 ºC and leaf temperature ranged from 22 to 25ºC. The photosynthetic photon flux (PPF) measured with a quantum sensor (QSS-01 light meter, Lehle Seeds, Round Rock, Texas, USA) directly above the adaxial leaf surface was about 90 μmol photons m^{-2} s^{-1}, which is above the light compensation point of this species which is 30 μmol photons m^{-2} s^{-1} (Schaffer and Whiley, 2002).

Electric voltage signals. Voltage differences between the base of the stem and the leaf petiole (ΔV_{L-S}) were measured and recorded. Surface contact electrodes were placed on the stem, 5 cm above the solution surface, and in the petiole of a leaf located in the upper half of the plant canopy (Fromm and Fei, 1998; Gil *et al.*, 2007). The electrodes consisted of a thick cotton thread absorbed with KCl 0.1 M dipped in a 2.0 mL Eppendorf tube containing KCl 0.1 M. The Ag/AgCl electrodes (0.4 mm in diameter) were immersed in the Eppendorf tubes and were connected to an amplifier with an input impedance of 10^{11} ohm and DC-1 kHz bandwidth (M-707 Microprobe System; World Precision Instruments, Sarasota, Florida, USA). The signal was digitized with an analog-digital board (Digidata 1200A, Axon Instruments, Sunnyvale, California, USA) for later analysis. Prior to measuring the variation potential in plants, the electrodes were placed in KCl 0.1 M and balanced to 0 mV to compensate the junction potential. To record ΔV_{L-S}, the electrode located on the leaf petiole acted as the recording electrode while the electrode located on the stem served as the reference. A schematic diagram of the electrical potential measurement system is shown in Fig. 1A.

Oxygen content in the root zone. Oxygen pressure (pO_2) was continuously measured with a needle oxygen electrode (Diamond General, Michigan, USA) connected to a Chemical Microsensor Pro 1221 (Diamond Electro Tech, Inc, Michigan, USA).

Stomatal conductance. Stomatal conductance (gs) was measured with a steady state porometer (Li-Cor 1600, Lincoln, Nebraska, USA) as described by Prive and Janes (2003) and Raviv *et al.* (2001). Stomatal conductance was measured on the same leaf on which the electrode was placed before and after the voltage was recorded for each treatment.

ABA and ACC analyses. Immediately after each treatment period, a sample of mature leaf tissue and live root tissues from each plant were frozen in liquid nitrogen (-192ºC). For ABA analysis, samples of about 1 g of leaf and 1 g of root tissue were weighed and homogenized in 99.8% methanol in semi-darkness to avoid ABA photo-oxidation. The homogenized solution was placed in Eppendorf tubes, incubated for 1 h at 4 ºC and then centrifuged for 7 min at 3000 g at 4 ºC as described by Peña-Cortés *et al.* (1989). The ABA concentration was determined separately for leaf and root samples by an ABA indirect ELISA assay using an ABA Phytodetek kit (AGDIA Inc., Elkhart, Indiana, USA).

For ACC measurement about 5 g of leaf and 5 g of root tissue were collected from each replication and leaf and root tissues were homogenized separately in 0.9% TCA. The homogenate was centrifuged for 20 min at 27,000 g at 4 ºC. The ACC concentration in roots and leaves was determined through the conversion of ACC

to ethylene by a reaction with NaOCl using the method described by Lizada and Yang (1979) modified by Hoffman and Yang (1980). Samples and standards were stored and measured from sealed tubes with rubber serum caps that were hermetically sealed. The concentration of ethylene liberated during the reaction was determined by injecting a 1 ml headspace sample in a Shimadzu gas chromatograph equipped with a flame ionization detector (FID) and alumina column.

Experiment 2: Responses to 3 days of drought

Plant material. Two-year-old 'Hass' avocado trees grafted on clonal Duke 7 rootstock were obtained from a commercial nursery. The plants were grown in a medium composed of soil, compost and sand (1/3 of each component) in 7-L polyethylene container bags and irrigated and fertilized according to standard nursery practices. The plants ranged in height from 1.1 to 1.5 m with 20 to 43 leaves per plant.

Experimental design. Plants were divided into two treatments: 1) control plants and 2) drought treatment, with 5 single-plant replications per treatment. Five plants in each treatment were randomly placed in a Faraday cage (3.5 m x 1.25 m x 3.0 m) to prevent external electromagnetic signals from interfering with the electrical measurements. The Faraday cage was used because the experiment was performed in an environmentally-controlled greenhouse. Plants in the control treatment were kept in polyethylene bags containing the roots and potting medium, and irrigated at 2-day intervals to keep the soil water content near field capacity.

For plants subjected to the drought treatment, the polyethylene container bags (containing the roots and potting medium) were kept open and had no irrigation throughout the entire experimental period. The electric voltage differences in both treatments were measured at 2-min intervals for 3 days to determine voltage differences between the base of the trunk and the canopy ($\Delta V_{l\text{-}b}$).

Air temperature during the experiment was between 23 and 25 ºC. The PPF measured with a quantum sensor (QSS-01 light meter, Lehle Seeds, Round Rock, Texas, USA) at the top of the canopy ranged from 0 μmol photons m^{-2} s^{-1} (night) to 430 μmol photons m^{-2} s^{-1} (noon).

Electrical voltage signals. Microelectrodes were constructed according to the technique described by Sawyer *et al.* (1995). Briefly, microelectrodes were made from hypodermic stainless steel syringe needles (0.4 mm diameter) containing Ag/AgCl electrodes (0.1 mm in diameter) immersed in 3.5 M KCl; the needle was sealed with heat-fused polyethylene coated insulation at both ends. The electrodes were inserted in the base of the trunk and in the stem within the leaf canopy of each plant. To record $\Delta V_{l\text{-}b}$, one electrode was located on the leaf zone, while the other electrode was located on the base of the trunk. One electrode was placed in the trunk 14 cm above the soil surface and the second electrode was placed in the stem within the upper half of the canopy height (44 cm above the first microelectrode), with both microelectrodes reaching the xylem tissue. A third electrode was placed in the soil to act as a ground electrode. Microelectrodes were connected to a Keithley 20 channel Differential Multiplexer model 7700 (Keithley

Instruments, Inc., Cleveland, Ohio, USA). The multiplexer was operated using a Keithley Multimeter/Data Acquisition System model 2701 (Keithley Instruments, Inc., Ethernet Multimeter/Data Acquisition System, Cleveland, Ohio, USA) with high input resistance (>10^9 Ω), a DC-60 Hz bandwidth, voltage recording from 100 nV to 1000 V and AC/DC converter. The signal was analyzed with ExceLINX-1A software, an add-on utility provided by Microsoft© Excel. A schematic diagram of the electrical potential measurement set-up is shown in Fig. 1B.

Stomatal conductance. Stomatal conductance was measured in 3 mature leaves per tree as described for Experiment 1. Stomatal conductance was measured during the morning (10:00 to 11:00 h) on the first and last days of the experiment.

Soil moisture content. A sample of soil was collected from each container at the beginning and end of the experiment and gravimetric soil water content (ω) was determined with the formula:

ω = ((wet soil weight - dry soil weight) / dry soil weight)*100

Experiment 3: Responses to 14 days of root hypoxia

Plant material. Two-year-old 'Hass' avocado trees grafted on clonal Duke 7 rootstock obtained from the same commercial nursery in the same potting mix and irrigated and fertilized the same as plants in Experiment 2 were used in this experiment. The plants ranged in height from 1.2 to 1.6 m with 23 - 40 leaves per plant.

Experimental design. Plants were divided into two treatments: 1) control and 2) root hypoxia treatment. For each treatment, five single-plant replications were randomly placed in the same Faraday cage used for experiment 2. For plants in the control treatment, each polyethylene bag (containing the roots and potting medium) was placed in a larger plastic container with drainage holes that allowed rapid drainage from the potting medium. Plants were irrigated at 2-day intervals to maintain soil water content near field capacity. For the root hypoxia treatment, each original polyethylene bag (containing the roots and potting medium) was placed in a larger plastic container (with no drainage holes) and filled with tap water to the surface of the soil. For plants in both treatments, electrical voltage signals were measured at 1-min intervals for 14 days to determine stem voltage differences between the leaf zone and the base of the trunk ($\Delta V_{l\text{-}b}$).

Electric voltage signals. Electrical voltages were measured as described for Experiment 2. Air temperature during the measurement period was between 23 and 25 ºC. The PPF measured with a quantum sensor (QSS-01 light meter, Lehle Seeds, Round Rock, Texas, USA) at the top of the canopy ranged from 0 μmol photons m^{-2} s^{-1} (night) to 430 μmol photons m^{-2} s^{-1} (noon).

Stomatal conductance. Stomatal conductance was measured as described for Experiment 2. However, in this experiment gs was also measured 7 days after the beginning of the treatment period.

Oxygen diffusion rate (ODR). The ODR was measured at 3 or 4-day intervals during the treatment period at a soil depth of 10 cm with a platinum electrode (Oxygen Diffusion Meter, Eijkelkamp, Netherlands) as described by Letey and Stolzy (1964).

Leaf ethylene concentration and abscission. Three leaves per tree were enclosed in a 5.5 x 8.5 x 0.5 cm Plexiglass box that was hermetically sealed with hot silicone and plastic tape. A sample of air in each Plexiglass box was extracted with a 1-ml syringe after inserting the syringe needle through a rubber septa. The concentration of ethylene gas in the extracted air sample was analyzed with a gas chromatograph (GC-8000 series, Fison Instruments, Rodano, Milan, Italy) equipped with FID Detector and a Boraplot Q 0.32 mm x 10 m column (Chrompack capillary column, Varian Inc., Walnut Creek, California, USA).

The number of abscised leaves was determined and recorded weekly for plants in each treatment.

Data analyses

Data were expressed as means. For Experiment 1, the effects of treatment on the maximum ΔV_{L-S} – initial ΔV_{L-S} (ΔV_{L-S} Maximum Difference), Absolute value of maximum ΔV_{L-S} – initial ΔV_{L-S} (Abs ΔV_{L-S} Maximum Difference) and the change in stomatal conductance from the beginning to the end of the treatment period (Δgs) were analyzed by a one-way ANOVA and Tukey's Studentized Range Test for post-hoc comparison of means. Also, the ΔV_{L-S} differences, both raw and absolute

values measured continuously after treatments were imposed, were analyzed by repeated measures two-way ANOVA and mean ΔV_{L-S} differences at specific points in time were analyzed using Tukey´s Studentized Range Test. The ABA and ACC concentrations were analyzed by linear regression analysis and one-way ANOVA, respectively. Differences in means were analyzed using Tukey´s Studentized Range Test.

For Experiments 2 and 3, final ΔV_{l-b} – initial ΔV_{l-b} (ΔV_{l-b} difference), Absolute value of final ΔV_{l-b} – initial ΔV_{l-b} (Abs ΔV_{l-b} difference), and Δgs were analyzed by a one-way ANOVA and Bonferroni's Test was used for post-hoc comparison of means. The relationships between ΔV_{l-b} difference and Δgs, and Abs ΔV_{l-b} difference and Δgs were analyzed by robust linear regression analysis which detects data outliers and provides stable regression analysis in the presence of data outliers (Rouseeuw and Leroy, 2003). For the third experiment, ODR, leaf ethylene concentration, the number of abscised leaves and gs values over time were analyzed by repeated measures two-way ANOVA and mean ΔV_{l-b} differences at specific points in time were analyzed by Bonferonni's Test.

Statistical analyses in all three experiments were performed with the SAS statistical software package (SAS Institute, Cary, North Carolina, USA).

RESULTS

Experiment 1: Responses to short-term drought and root hypoxia

Electrical voltage signals. The ΔV_{L-S} difference in plants in the hypoxia treatment was not significantly different from that of plants in the control treatment (data not shown). However the Abs ΔV_{L-S} difference in plants in the drought treatment was significantly higher than that of the control or hypoxia treatments ($P \leq 0.05$; Table 1). Time series analysis indicated that there was a significant difference in ΔV_{L-S} ($P \leq 0.05$) between the drought and hypoxia treatments 10 minutes after treatments were initiated and there were no statistically significant differences among treatments after that time. Although not statistically significant, ΔV_{L-S} tended to be higher for the drought treatment than the other treatments from 20 to 75 min after treatments were initiated (Fig. 2A). The difference in ΔV_{L-S} between the drought treatment and the other treatments was greatest at 75 min, but the difference was not statistically significant at that time due to high between-plant variability within treatments. The Abs ΔV_{L-S} difference tended to be greater for plants in the drought treatment than for plants in the other treatments from 15 to 75 minutes after treatments were initiated. However, those differences were only statistically significant ($P \leq 0.05$) after 60 minutes and 75 minutes, presumably due to large between-plant variability within each treatment (Fig. 2B). The ΔV_{L-S} values for individual replications in each treatment are shown in Fig. 3 (A, B and C).

Oxygen content in the root zone. For plants in the root hypoxia treatment, the oxygen content in the root zone rapidly decreased 5 min after treatments were

imposed, whereas it remained relatively constant for plants in the control treatment (Fig. 4).

Stomatal conductance. The Δgs of plants in the drought treatment was significantly greater (more negative) than that of plants in the control treatment ($P \leq 0.05$; Table 1).

ABA and ACC concentrations. There were no statistically significant differences in leaf ABA or ACC concentrations or root ABA concentrations among treatments (data not shown). Root ACC concentrations were significantly higher ($P \leq 0.05$) for plants in the drought treatment than for plants in the hypoxia or control treatments (Table 1).

Experiment 2: Responses to 3 days of drought

Soil moisture content (ω). The control and drought treatments had similar ω at the beginning of the treatment period. The ω at the beginning of the treatment period was 22.5 and 20.3% for the control and drought treatments, respectively. By the last day of the experiment, ω decreased considerably to 1.5% in the drought treatment, whereas ω in the control treatment ω was 16.1% at the end of the experiment (data not shown).

Electrical voltage signals. The ΔV_{l-b} for individual plants (replications) in the control and drought treatments are shown in Fig. 5A and B. For plants in the drought treatment, the ΔV_{l-b} tended to decrease from the beginning to the end of the treatment period, whereas for plants in the control treatment, there was a slight

increase in $\Delta V_{l\text{-}b}$ from the beginning to the end of the treatment period. There was a significant difference ($P \leq 0.05$) in voltage changes ($\Delta V_{l\text{-}b}$ Difference) between trees in the drought and trees in the control treatment (Table 2). However, there was no significant difference ($P > 0.05$) in the Abs $\Delta V_{l\text{-}b}$ difference between the control and drought treatment (Table 2).

Stomatal conductance. The difference in gs between the first and the last days of the treatment period (Δgs) was significantly higher for plants in the drought treatment than in the control (Table 2). There was a positive linear correlation (R^2 = 0.32) between Δgs and raw $\Delta V_{l\text{-}b}$ difference; however there was a negligible correlation (R^2 = 0.06) between the Δgs and Abs $\Delta V_{l\text{-}b}$ difference (Fig. 6).

Experiment 3: Responses to 14 days of root hypoxia

Electrical voltage signals. There was little variation in $\Delta V_{l\text{-}b}$ measured during 14 days for plants in both the control and hypoxia treatments. However there appeared to be a daily cyclical fluctuation in the electrical signal, whereby the electrical potential drastically changed at noon (exhibiting a minimum or maximum value), then changed again at the beginning of the night and remained constant during the night and part of the next morning (Fig. 7). These daily fluctuations were more pronounced for plants in the hypoxia treatment than for plants in the control treatment.

At the end of the experiment, there were no significant differences in the raw or absolute $\Delta V_{l\text{-}b}$ values between treatments ($P > 0.05$). Although the Absolute $\Delta V_{l\text{-}b}$

differences of plants in the hypoxia treatment were higher than those of plants in the control treatment, the differences were not statistically significant (Table 3).

Stomatal conductance. There was a significant difference ($P \leq 0.05$) in Δgs between the hypoxia and control treatments (Table 3). From 7 to 14 days after treatments were initiated, gs of plants in the hypoxia treatment decreased, whereas there was a slight increase in gs of plants in the control treatment. There was a significant difference in gs between plants in the hypoxia and control treatments 14 days after treatments were initiated (Fig. 8D).

Oxygen diffusion rate. On all measurement dates, ODR in the soil was significantly lower ($P \leq 0.05$) in the hypoxia treatment than in the control treatment and the magnitude of this difference was greatest 14 days after treatments were initiated (Fig. 8 A).

Ethylene and number of abscised leaves. Leaf ethylene concentration was higher in plants in the hypoxia treatment than in the control treatment 3 and 7 days after treatments were initiated, but these differences were only statistically significant ($P \leq 0.05$) 3 days after treatments were initiated. For plants in both treatments, there was a sharp decrease in leaf ethylene concentration from 7 to 14 days after treatments were initiated, but the decrease in leaf ethylene concentration was greater for plants in the hypoxia treatment (Fig. 8B).

The accumulated number of abscised leaves during the treatment period was significantly greater ($P \leq 0.05$) for plants in the hypoxia treatment than for plants in the control treatment on the last day (Day 14) of the experiment (Fig. 8C).

DISCUSSION

Short-term (min) exposure to drought stress simulated by the addition of PEG-6000 to the hydroponic medium resulted in a decrease in gs. Similarly, Olsovska and Brestic (2001) reported a sharp decline in gs of juvenile plants when 15% PEG-6000 was added to a hydroponic medium. In the present study, long-term (3 days) drought stress of avocado also resulted in a significant decrease in gs. A significant increase in $\Delta V_{L\text{-}S}$ or $\Delta V_{l\text{-}b}$ in avocado was observed as a result of short-term or long-term drought. The increase in gs was linearly correlated with $\Delta V_{l\text{-}b}$ after 3 days (Fig. 6). Also, in Experiment 1, there was a significant increase in $\Delta V_{L\text{-}S}$ between roots and leaves within minutes after subjecting plants to simulated drought stress, whereas non-stressed control plants did not exhibit a significant increase in $\Delta V_{L\text{-}S}$. Thus, in avocado an extra-cellular electrical signal appears to be involved in root to leaf communication initiating stomatal closure at a very early stage of drought stress. This is consistent with a recent study by Gil *et al.* (2007), who observed a fast conducting signal transported from roots to leaves in the phloem of avocado in response to soil drying that was correlated with decreased stomatal conductance. Similar to responses of avocado trees to short-term simulated drought stress, maize plants subjected to drought stress simulated by the addition of PEG-6000 to a hydroponic medium exhibited a reduction in water potential to -0.5 MPa, which evoked a rapid depolarization of the electrical potential. It was possible to

demonstrate that the response was an effect of osmotic shock and not due to any specific toxic effect of PEG (Fromm and Fei, 1998).

There was no effect of short-term drought on root or leaf ABA concentrations. Increased ABA concentration synthesized in the roots and translocated to the leaves has been proposed as the signal for stomatal closure when plants are drought stressed (Sauter *et al.*, 2001; Hartung *et al.*, 2002; Davies *et al.*, 2005). However in the present study, the lack of significant increases in root or leaf ABA concentrations, coupled with a significant increase in $\Delta V_{L\text{-}S}$ during short-term drought indicates that the root-to-shoot communication mechanism in avocado is most likely via a fast-conducting extra-cellular electrical signal rather than transport of ABA. A recent study with *Arabidopsis thaliana* also suggested that ABA is not the long-distance signal for communicating water deficit from the root to the shoot, and that the shoot response to limited soil water supply is not affected by the capacity to generate ABA in the roots, but first by a hydraulic response in the shoot of *Arabidopsis*, which precedes ABA signaling and stomatal closure (Christmann *et al.*, 2007).

Short-term drought stress also resulted in an increase in ACC concentration in avocado roots. This is consistent with observations of another woody fruit tree species, 'Cleopatra' mandarin, for which drought stress stimulated ACC production as well as the production of ABA in the roots (Gómez-Cardenas *et al.*, 1996). In that study, it was concluded that increased ABA synthesis in response to drought stress increased ACC synthesis in the roots. However, in the present study with avocado, short-term drought stress had no effect on root ABA concentration. Thus,

the increased concentration of ACC in drought stressed avocado roots may have been due to an inhibition of conversion of ACC to ethylene.

In the present study, short-term or long-term hypoxia, due to lack of oxygen in a hydroponic medium or soil flooding resulted in a ΔV_{L-S} and ΔV_{l-b} differences higher than those in non-stressed control plants. However, these differences were not statistically significant. In a previous study of avocado trees, it was observed that adding water to the soil resulted in an increase in ΔV_{L-S}, but this increase was not significantly different from that of control (baseline) plants, with soil moisture kept relatively constant (Gil *et al.*, 2007). Thus, the electrical signals in avocado trees appear to be less affected by addition of water to the soil or lack of soil oxygen than to soil desiccation. However, in the present study there was a significant reduction in gs as a result of short-term or long-term hypoxia, which indicates that the root-to-shoot communication mechanism for reduced gs as a result of root hypoxia in avocado does not appear to be via a change in variation potentials.

There was no significant effect of short-term root hypoxia on root or leaf ABA concentrations in the present study. Increased ABA concentration in leaves has been implicated by several researchers as a biochemical signal for stomatal closure in leaves of woody plants subjected to root hypoxia or anoxia (Schaffer *et al.*, 1992; Kozlowski, 1997). However, the lack of significant differences in leaf or root ABA concentrations between non-stressed control plants and plants subjected to hypoxic conditions indicates that ABA does not appear to be the root-to-leaf communication signal for stomatal closure in avocado trees. Whiley and Schaffer (1994) postulated that stomatal closure in avocado trees exposed to root hypoxia

resulting from soil flooding was due to a build-up of CO_2 in the leaf which has been associated with stomatal closure in plants (Rashke, 1975; Mansfield *et al.*, 1990). This was based on studies where flooding-induced reductions of gs in avocado trees were observed concomitantly with decreases in net CO_2 assimilation and increased intercellular CO_2 concentration in leaves (Schaffer *et al.*, 1992). Although the precise timing and sequence of hypoxia-induced reductions in gs and net CO_2 assimilation have not be elucidated in avocado trees, reductions in net CO_2 assimilation may occur before reductions in gs resulting in an increased intercellular CO_2 concentration. Thus, reduced gs in avocado leaves as a result of root hypoxia may actually be the result of a build-up of intercellular CO_2 resulting from a reduced rate of carbon fixation (Whiley and Schaffer, 1994).

Subjecting avocado roots to hypoxic conditions for several days resulted in increased ethylene emission from leaves and an increase in the number of abscised leaves compared to non-stressed control trees. Leaf ethylene concentrations were determined to be higher in plants in the hypoxia treatment than in the control treatment only 3 and 7 days after treatments were initiated and differences were only statistically significant on day 3. The number of abscised leaves was higher for plants in the hypoxia treatment than plants in the control treatment on days 7 and 14, with a statistically significant difference only on day 14. Presumably, leaf abscission was stimulated by increased ethylene concentration, but the different response times were most likely due to a time lag between increased ethylene concentration and leaf abscission. Leaf abscission is a common response of avocado trees to root hypoxia (Ploetz and Schaffer, 1989;

Schaffer *et al.*, 1992). In many plants, root hypoxia stimulates ethylene production (Jackson, 1985; Kozlowski, 1997). The conversion of ACC to ethylene is dependent upon an adequate oxygen supply (Bradford and Yang, 1980). Thus, in anoxic conditions, the total lack of O_2 in the root zone halts the conversion of ACC to ethylene. However, in hypoxic conditions, when partial pressures of O_2 in the root zone are between 0 and that of air, conversion of ACC to ethylene is actually stimulated (Jackson, 1985). In the present study, exposing avocado roots to short-term hypoxia did not result in increased or decreased root or leaf ACC concentrations compared to non-stressed control plants, although ethylene emission from leaves was greater for plants in the long-term root hypoxia treatment than in the control treatment. Thus, it is likely that in avocado trees, root hypoxia resulted in increased production of ACC in the roots which could not be measured because it was quickly converted to ethylene and translocated to the leaves, thus stimulating leaf abscission.

Variation potentials, which were measured in this study, are directly related to the strength of the stimulus unlike action potentials, which respond to a critical stimulus threshold (Dziubinska *et al.*, 2003; Fromm and Lautner, 2007; Davies and Stankovic, 2007). In the first experiment, expressing the electrical potential as absolute values (without taking into account the sign of the value) provided a better indication of the stimulus strength and a clearer statistical separation of treatment means than expressing the data as raw values (Fig. 2, Table 1). Thus, analyzing ΔV_{L-S} differences as absolute values may be a better approach for evaluating plant responses to changes in variation potentials as affected by some variables such as

root hypoxia. On the other hand, linear regression analysis between raw ΔV_{L-S} or Abs ΔV_{L-S} differences and Δgs as the result of short-term drought in Experiment 1 showed low R^2 values (R^2= 0.1 and R^2= 0.22, for raw and absolute values, respectively; data not shown). However, when plants were exposed to a longer drought period, there was a much stronger linear correlation between raw ΔV_{l-b} differences and Δgs than between Abs ΔV_{l-b} differences and ∆gs (Fig. 6), indicating that raw values are more closely related to stomatal response than absolute values.

Day-to-night fluctuations in electrical potentials observed in avocado trees (Figs. 5 and 7) appeared to correspond to the time of day (morning, afternoon or night) and were therefore presumably affected by diurnal changes in ambient light and temperature rather than plant stress factors. After relating electrical variations in trees to meteorological (precipitation) and atmospheric electricity conditions, Koppán *et al.* (2000) and Gibert *et al.* (2006) concluded that daily changes in stem voltages cannot only be interpreted as electrokinetic effects associated with sap flow, but also are affected by other mechanisms of charge exchange in xylem elements. In avocado trees, rapid voltage changes were observed from morning to noon and from night to morning, indicating that these diurnal voltage changes may be associated with changes in sap flow.

In conclusion, the results of this study confirm that extra-cellular electrical signal between the roots and leaves in avocado trees are related to stomatal closure in response to drought stress. However, for trees exposed to root hypoxia, typically

encountered in flooded or poorly drained soils, electrical signals do not appear to be the primary root-to-shoot communication mechanism. Also, in avocado trees, ABA does not appear to be involved in signaling stomatal closure as a result of stress caused by drought or root hypoxia.

Acknowledgments

We thank Dr. Julio Alcayaga from Facultad de Ciencias, Universidad de Chile, for access to part of the equipment for electrical potential measurements, Dr. Bruno Defilippi and Dr. Reynaldo Campos from INIA La Platina for access to equipment and assistance with ACC analysis, Dr. Eduardo Oyanedel from the Pontificia Universidad Católica de Valparaíso, Facultad de Agronomía, for access to equipment for laboratory ABA determination, Mr. Francisco Rivera, Dr. Claudio López and Ms. Paulina Naranjo from the Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Mrs. Paulina Arias at the Laboratory of Neurobiology of the Pontificia Universidad Católica de Chile, and Mrs. Marisol Pérez from INIA La Platina for their assistance with the experimental set-up and laboratory measurements. We also thank Mr. Oscar Godoy from Fundo las Chacras (Calera, Chile) for providing seedling ‘Mexicola’ avocado plants. This work is part of a Ph.D. thesis in Agricultural Science (P.M. Gil) at the Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile.

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Table 1. Experiment 1. Effects of root hypoxia and drought on the absolute maximum voltage difference between the leaf petiole and the base of the stem (Abs ΔV_{L-S}), the change in stomatal conductance from the beginning to the end of the treatment period (Δgs) and root ACC concentration.

Treatment	Abs ΔV_{L-S} Maximum Difference (mV)	Δgs (cm s^{-1})	ACC (nmol g^{-1})
Control	5.2 ± 1.03 b	-0.013 ± 0.01 a	0.059 ± 0.02 b
Hypoxia	6.0 ± 1.05 b	-0.046 ± 0.02 ab	0.060 ± 0.01 b
Drought	20.8 ± 5.50 a	-0.069 ± 0.01 b	0.121 ± 0.01 a

Values are means value ± SE (n = 5). Different letters within columns indicate significant differences (P ≤ 0.05) among treatments (Control, Hypoxia and Drought) (one-way ANOVA and Tukey´s Studentized Range Test).

Table 2. Experiment 2. Effect of drought on maximum voltage difference between the leaf zone and the base of the trunk ($\Delta V_{l\text{-}b}$ Maximum Difference), absolute value of maximum voltage difference between the leaf zone and the base of the trunk (Abs $\Delta V_{l\text{-}b}$ Maximum Difference) and the change in stomatal conductance (Δgs) from the beginning to the end of the treatment period (Δgs).

Treatment	$\Delta V_{l\text{-}b}$ Maximum Difference (mV)	Abs $\Delta V_{l\text{-}b}$ Maximum Difference (mV)	Δgs (cm s^{-1})
Control	32.6 ± 6.5	32.6 ± 6.5	0.079 ± 0.027
Drought	-11.6 ± 13.5	26.4 ± 6.4	-0.013 ± 0.005
Significance	*	NS	*

Values are means ± SE (n = 5). An asterisk indicates significant difference ($P \leq 0.05$) and NS indicates no significant difference between treatments (one-way ANOVA and Bonferroni's Test).

Table 3. Experiment 3. Effect of root hypoxia treatment on maximum voltage difference between the leaf zone and the base of the trunk ($\Delta V_{l\text{-}b}$ Maximum Difference), absolute value of maximum voltage difference between the leaf zone and the base of the trunk (Abs $\Delta V_{l\text{-}b}$ Maximum Difference (mV)) and the change in stomatal conductance from the beginning to the end of the treatment period (Δgs).

Treatment	$\Delta V_{l\text{-}b}$ Maximum Difference (mV)	Abs $\Delta V_{l\text{-}b}$ Maximum Difference (mV)	Δgs (cm s^{-1})
Control	26.5 ± 16.8	37.5 ± 10.2	0.09 ± 0.017
Hypoxia	4.6 ± 121.9	178.9 ± 82.8	-0.02 ± 0.013
Significance	NS	NS	*

Values are means ± SE (n = 5). An asterisk indicates significant difference ($P \leq 0.05$) and NS indicates no significant difference between treatments (one-way ANOVA and Bonferroni's Test).

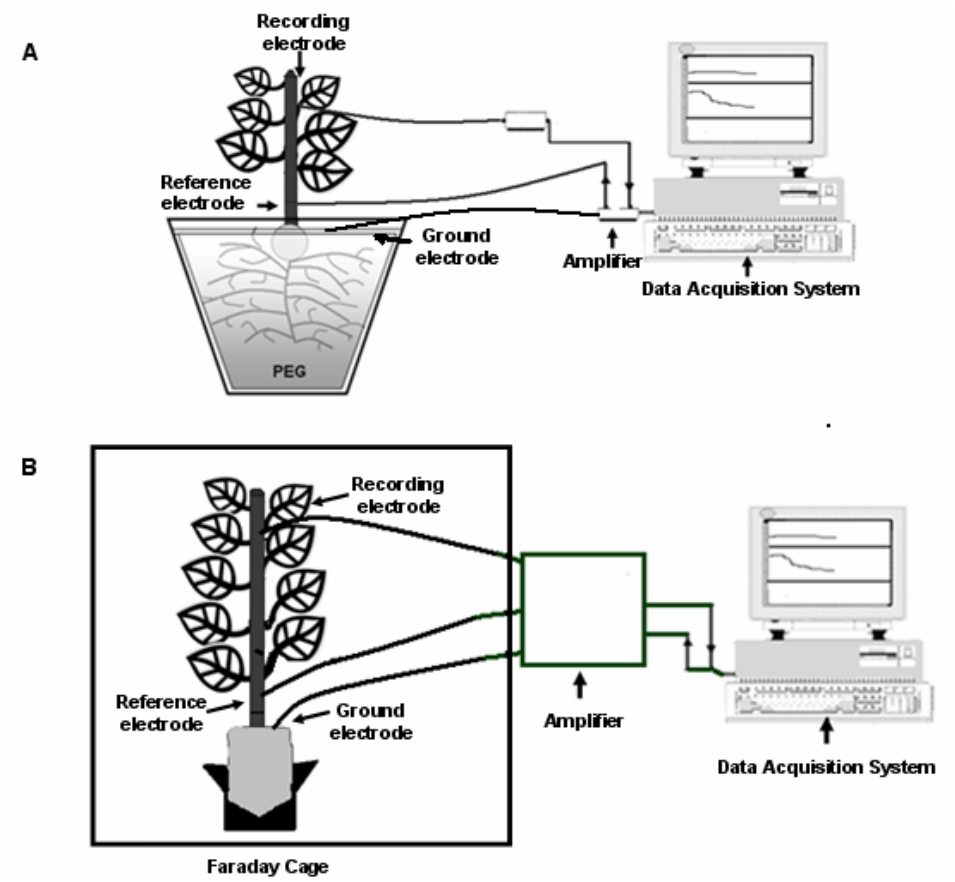

Figure 1. A: Experiment 1: Schematic diagram of the digital acquisition system for recording extra-cellular voltage difference between the leaf petiole and the base of the stem ($\Delta V_{L\text{-}S}$) in 'Mexicola' avocado trees. B: Experiment 2 and 3: Schematic diagram of the digital acquisition system for recording voltage differences between the base of the trunk and the leaf zone ($\Delta V_{l\text{-}b}$) in 'Hass' avocado trees.

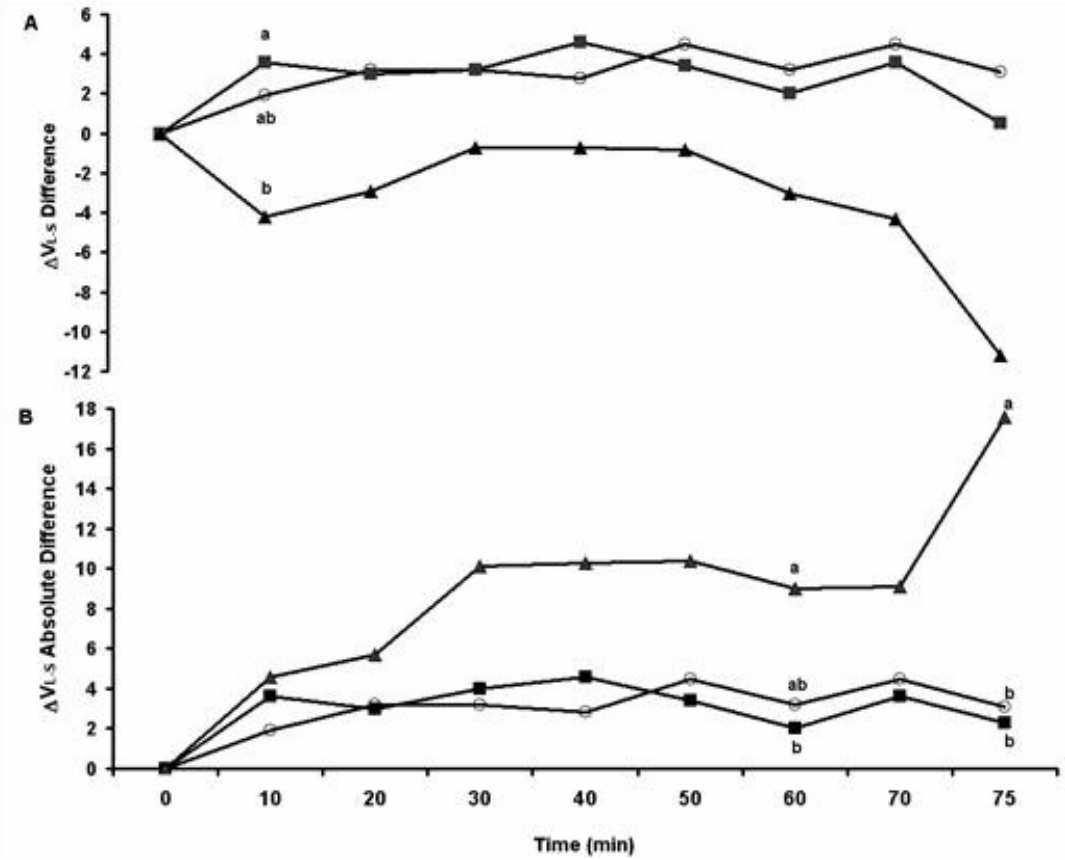

Figure 2. Experiment 1. A: Voltage difference (ΔV_{L-S}) (Raw value) at 10 min intervals from treatments impose. B: Voltage difference (ΔV_{L-S}) (Absolute value) at 10 min intervals from treatments impose. —▲— Drought, —■— Root hypoxia, —○— Control. Different letters (a, b) indicate significant differences (A: $P \leq 0.05$, B: $P \leq 0.1$) among treatments (one-way ANOVA and Tukey´s studentized range test).

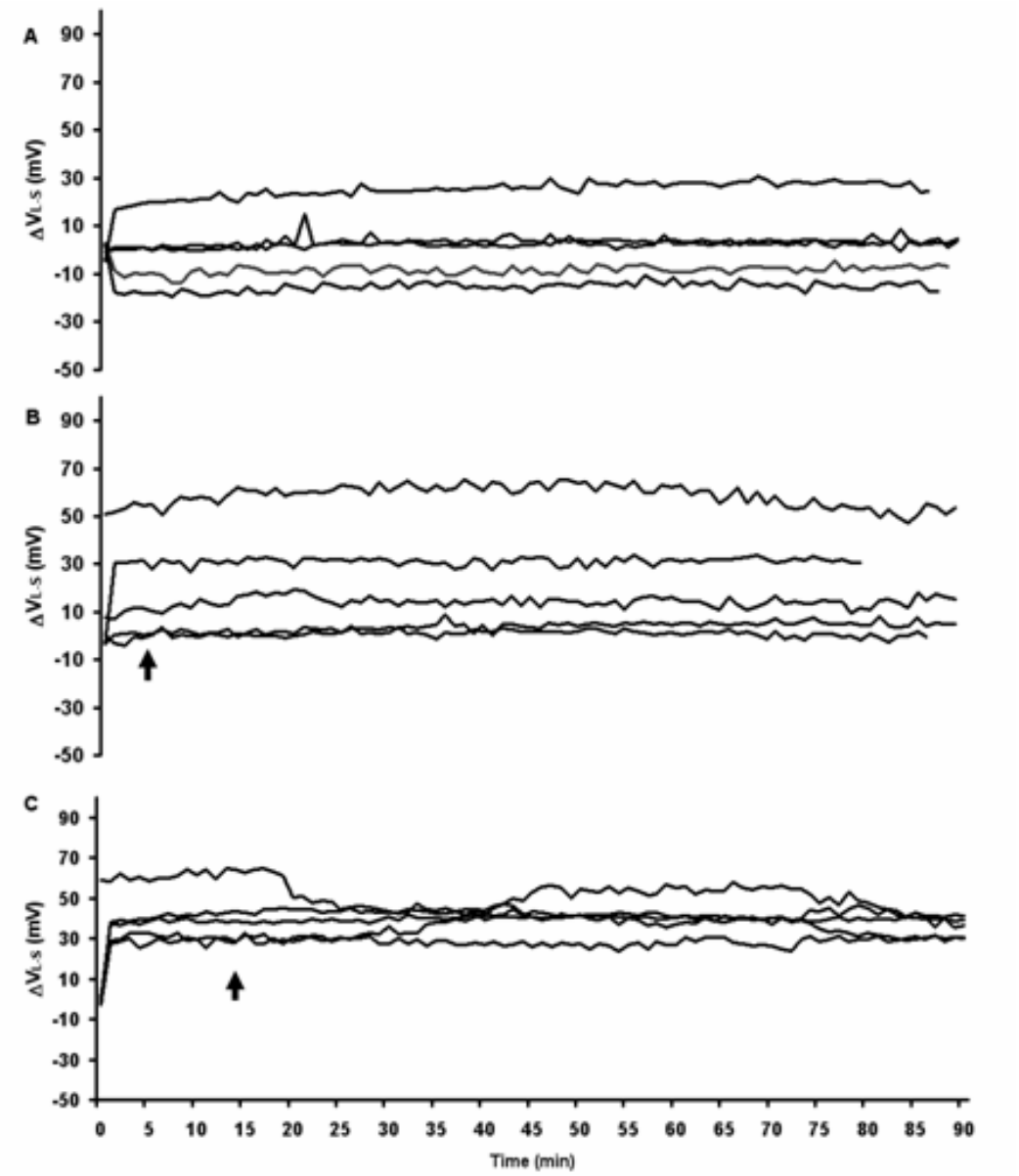

Figure 3. Experiment 1. A: Voltage difference between the leaf petiole and the base of the stem (ΔV_{L-S}) of 5 control plants for 90 min. B: ΔV_{L-S} of 5 plants in the root hypoxia treatment from 5 to 90 minutes. C: ΔV_{L-S} of 5 plants in the drought treatment from 15 to 90 minutes. Data were collected at 2 Hz and plotted at 1 min intervals. The arrows indicate the beginning of each treatment.

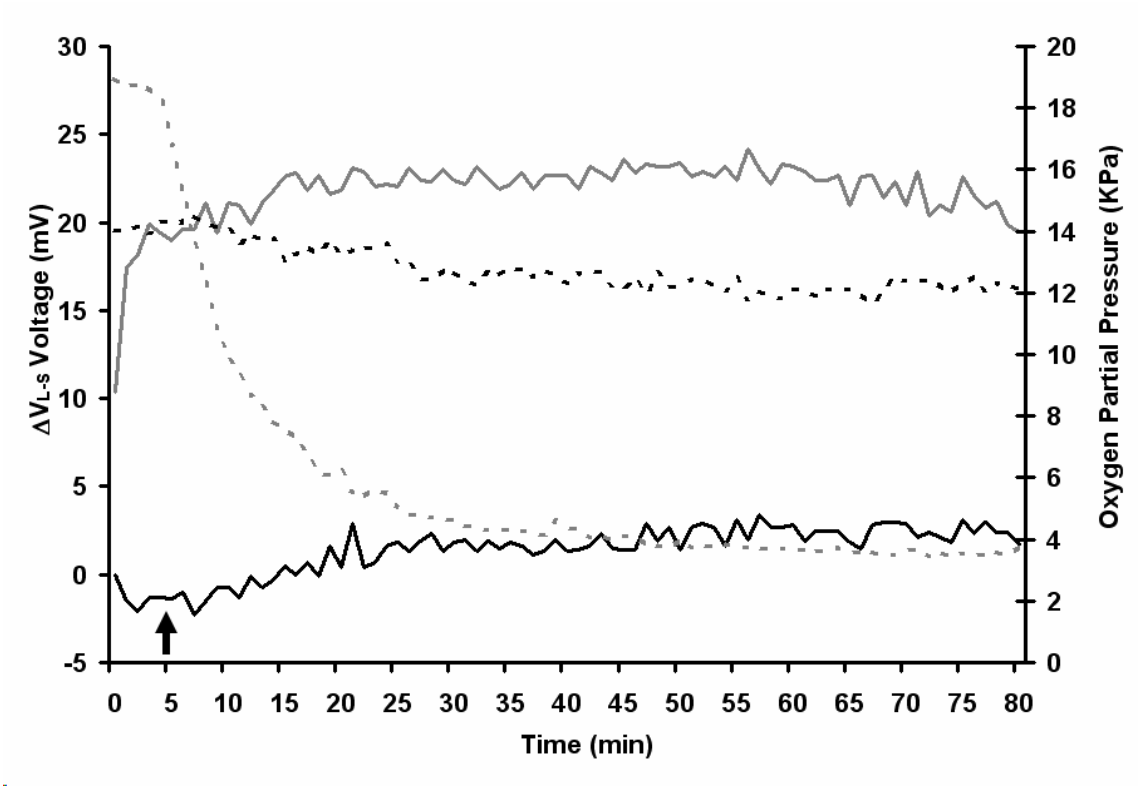

Figure 4. Experiment 1. Average (n=5) voltage (ΔV_{L-S}) and oxygen partial pressure (KPa) in the control and root hypoxia treatments. ——— Control treatment voltage, ——— root hypoxia treatment voltage, - - - control treatment oxygen partial pressure, - - - root hypoxia treatment oxygen partial pressure. Arrows indicate the beginning of the treatment.

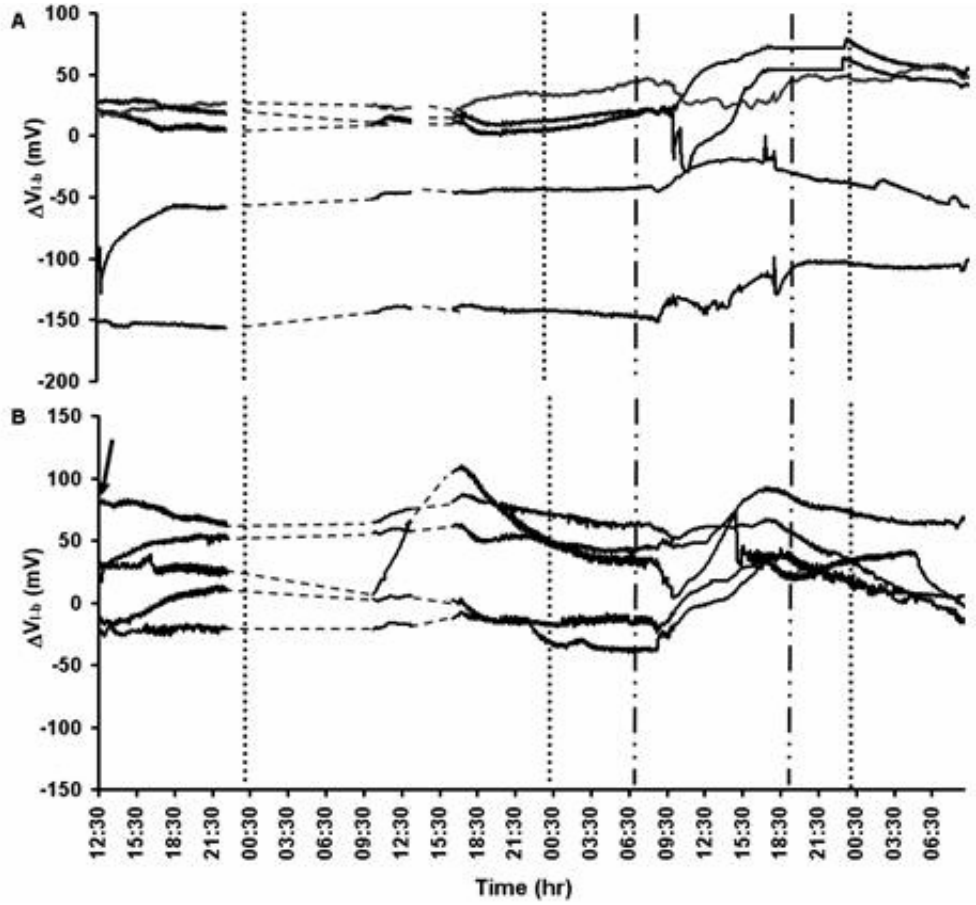

Figure 5. Experiment 2. A: Voltage difference between the base of the trunk and the canopy of the tree ($\Delta V_{l\text{-}b}$) in 5 plants in the control treatment and B: 5 plants in the drought treatment for 68.5 h. Dashed lines represented missing data during the experiment. Vertical dotted lines indicate a new day (12:00 PM); vertical dashed-dotted lines indicate measurements during night.

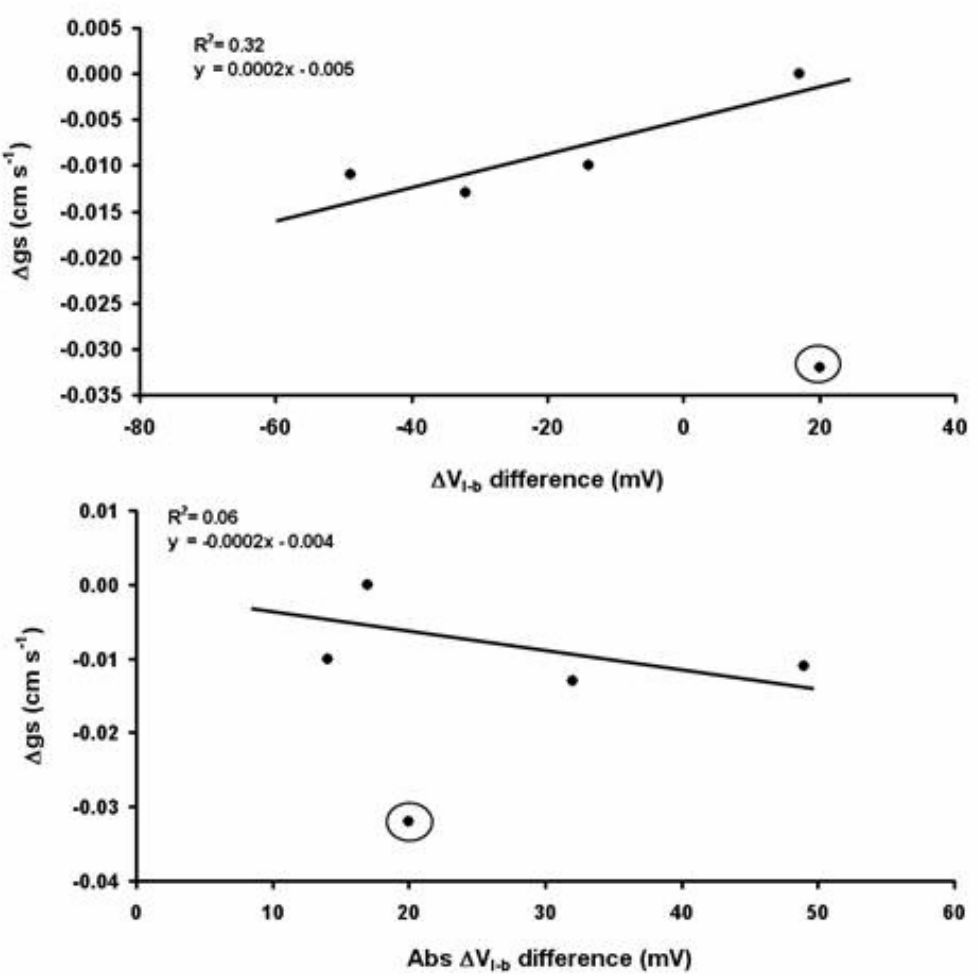

Figure 6. Experiment 2. A: Robust linear regression between $\Delta V_{l\text{-}b}$ Maximum Difference (**(**$\Delta V_{l\text{-}b}$ Difference) and the change in stomatal conductance from the beginning to end of the treatment period (Δgs) of plants in the drought treatment. B: Robust linear regression between absolute value of $\Delta V_{l\text{-}b}$ Maximum Difference (Abs $\Delta V_{l\text{-}b}$ Difference) and Δgs of plants in the drought treatment. The circle around a symbol indicates that the data point was detected as an outlier by robust regression analysis.

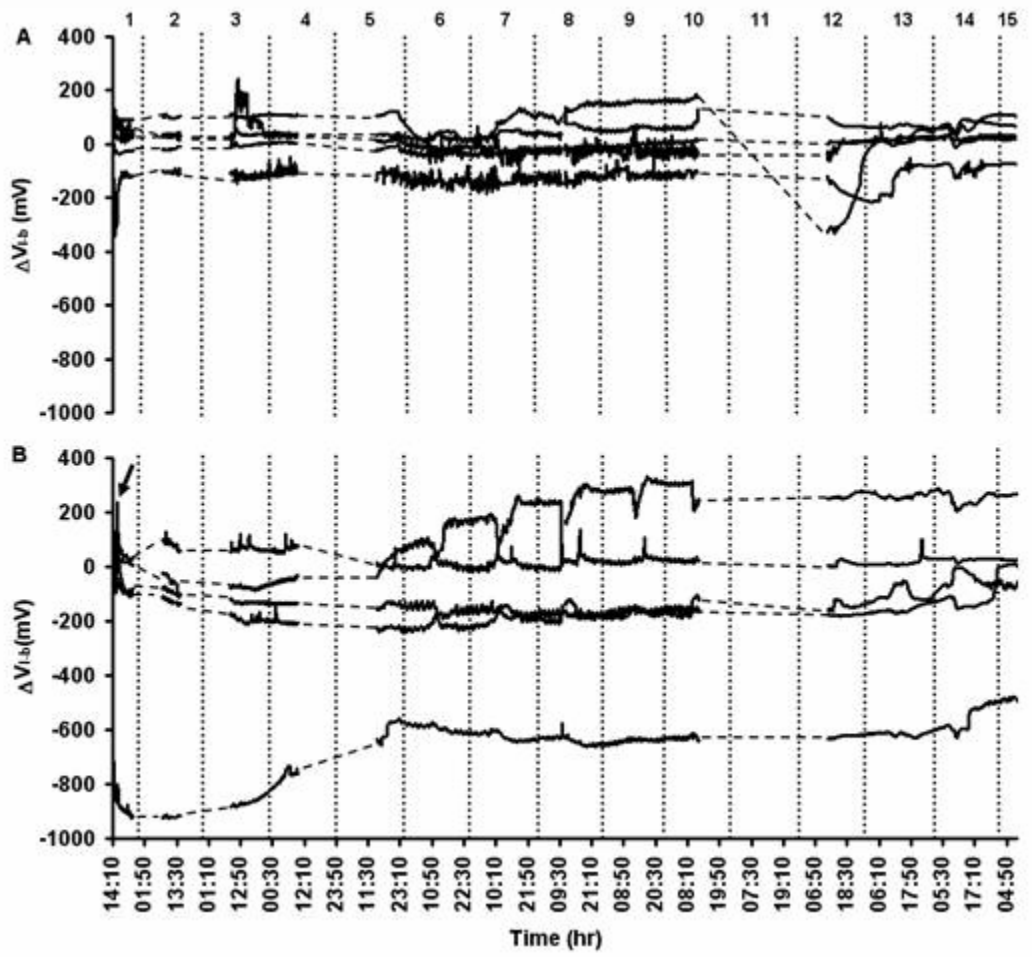

Figure 7. Experiment 3. A: $\Delta V_{l\text{-}b}$ in 5 control plants and B: $\Delta V_{l\text{-}b}$ in 5 plants in the root hypoxia treatment for 14 days. Dashed lines indicate missing data during the experiment. The arrow indicates the beginning of treatment period.

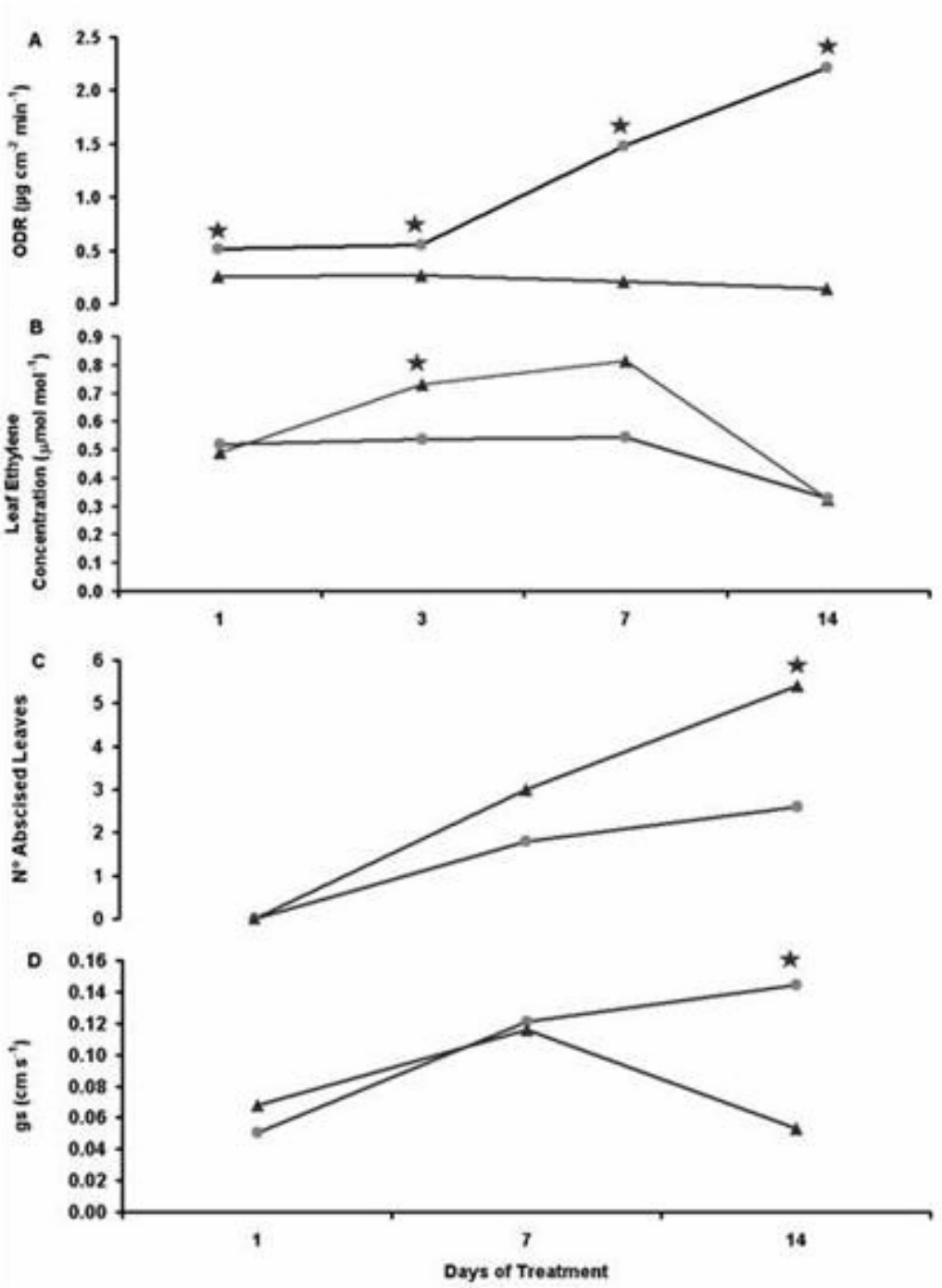

Figure 8. Experiment 3. A: Soil oxygen diffusion rate (ODR) during the experimental period. B: Leaf ethylene concentration (□mol mol^{-1}), C: the number of abscised leaves, and, D: Stomatal conductance (gs) of plants in the root hypoxia and control treatments measured at 3-day intervals during the experimental period. Asterisks indicate a significant difference between treatments according to a Bonferonni's Test after a 2-way ANOVA ($P \leq 0.05$) at each time. ●— Control, —▲— Root hypoxia treatment.