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Foliar sprays with ABA promote growth of *Ilex paraguariensis* by alleviating diurnal water stress

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Abstract

Stomatal closure, relative water content (RWC) and vegetative growth were monitored in *Ilex paraguariensis* plants grown under well-watered conditions with a photosynthetic photon flux density (PPFD) varying from 100% to 1.5%, and sprayed weekly with either distilled water (control) or 1.89 mM abscisic acid (ABA). ABA treatments caused stomatal closure, ranging from 62% to 73%. These treatments also increased RWC in the early evening from 82% to 92% and 88% to 94% in mature and immature leaves, respectively. Such alleviation of the water stress was correlated with increases in leaf area, leaf dry weight (DW), shoot length and shoot DW. On day 35 from the beginning of the experiment, the increases in DW of both leaves and shoots were 1.5-fold at the 1.5% PPFD and 3-fold (for leaves) and 4.5-fold (for shoots) under 100% PPFD. In water-sprayed control plants grown under 1.5% PPFD shoot length also increased significantly, although these shoots contained more ABA (assessed by capillary gas chromatography–mass spectrometry) than those of plants grown under 100% PPFD. These results show that ABA sprayed on to leaves promotes growth in *I. paraguariensis* plants by alleviating diurnal water stress.

Abbreviations: ABA – abscisic acid; GC–MS – capillary gas chromatography–mass spectrometry; GC–SIM – capillary gas chromatography–selected ion monitoring; DW – dry weight; FW – fresh weight; HPLC – high-pressure liquid chromatography; PPFD – photosynthetic photon flux density; RH – relative humidity; RWC – relative water content; Sat W – fresh weight after saturation in distilled water

Introduction

From an economical point of view, *Ilex paraguariensis* St. Hil. (yerba mate) is a very important tree in South America. It is spontaneous as well as being extensively cultivated in north-eastern Argentina, south-eastern Brazil, and Paraguay (Giberti 1995). The leaves and young shoots are industrialised and hence used to prepare a tea-like infusion which is much appreciated for its peculiar flavour and stimulating properties; such characteristics are mainly due to the high content of caffeine and theobromine (Filip et al. 1998). The natural habitat of the plant is the forest, under the shadows of *Araucaria angustifolia* trees, where conditions of low photosynthetic photon flux density

(PPFD) and high humidity predominate. The introduction of yerba mate as an extensive culture is quite recent. Therefore, few physiological studies have been done with this species in relation to this new environment, particularly regarding the increase in sunlight irradiance and the resulting water demand by evapotranspiration (Sansberro 2000).

It has been shown that diurnal water stress is a condition normally found in most of the species growing in temperate climates during the noon and afternoon hours, even though the soil water status may be at its standard field capacity. This temporary stress might then affect the growth rate (Granier and Tardieu 1999). In fact, mild water deficits that cause reduction of the plant turgor (equivalent to a reduction of 10–15% in the plant water content) result in large changes in growth and metabolism. Nonetheless, they rarely cause plant death unless these conditions persist for a long time (Mullet and Whitsitt 1996). A number of classical studies have shown that expansion growth can be stopped or greatly inhibited by water deficit while transpiration continues unabated (Acevedo et al. 1971, 1979). Leaf tissues are especially sensitive to reduction in water status (Hsiao et al. 1970).

There is strong evidence from a number of different studies that abscisic acid (ABA) plays an important role in the regulation of stomatal behaviour in plants under drought (Mansfield et al. 1990). The main concept is that ABA originating from the roots modulates water loss and promotes stress avoidance (Tardieu 1996; Zhang and Outlaw jr. 2001). However, it has been suggested recently that an additional root signal may be necessary to modulate the stomatal response to ABA (Borel et al. 2001). In any case, it is clear that the stomatal response to ABA can also be enhanced if the leaf tissue is experiencing a water deficit (Tardieu and Davies 1992).

Considering the antecedents, the hypothesis is that *I. paraguariensis* is not well adapted to a high evaporative demand, so that the water losses generate diurnal stress that affects the vegetative growth. Therefore, the aim of this work was to investigate the effect of ABA sprays on the leaf water status and the subsequent vegetative mass production of *I. paraguariensis* plants grown under different PPFD.

Material and methods

The commercially available clone "Garruchos 18" of *I. paraguariensis* St. Hil. was used. It was kindly provided by "Establecimiento Las Marías S.A.", Gobernador Virasoro, Corrientes, Argentina. Two-year-old plantlets obtained by stem cuttings from 22-year-old plants were grown in 4 l pots filled with a mixture of lateritic (Alfisols) red soils and peat (3 : 1). They were pruned at the beginning of the experiment in order to obtain plants of a similar size. These plants were placed under field conditions at Instituto de Botánica del Nordeste, Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Argentina. The environmental conditions registered throughout the 35-day-experiment were as follows: average temperature was 26.8 °C with a minimum of 21.2 °C and a maximum of 32.7 °C; average RH was 65% with a minimum and a maximum of 42% and 89% respectively. Only running tap water was added to the pots in order to keep the soil moisture at field capacity.

Treatments consisted of all combinations of five (100%, 50%, 20%, 6% and 1.5%) PPFD (corresponding to the average values measured at different levels along the canopy of a commercial plantation) and two concentrations of ABA (0 and 1.89 mM). The different sunlight irradiances were achieved by covering the roof, eastern and western sides of iron-framed boxes with different layers of shading nets. The PPFD levels were assessed with a Li-Cor 191SA (Li-Cor Inc, Lincoln, NE) using a quantum sensor. Each box contained 12 randomly distributed pots including 6 control plants (0 mM) and 6 (1.89 mM) ABA-treated plants. The light treatments (boxes) were repeated three times.

The day after pruning (day 1), the leaves were submitted to a single high-pressure foliar spray of distilled water (control) or ± cis, trans-ABA (99% purity, Sigma-Aldrich, St. Louis, MO, USA) 1.89 mM until incipient runoff (approximately 10 ml of aqueous solution per plant). Both solutions included Triton® 0.1% as surfactant and a minimum amount of ethanol used to dissolve the ABA. Sprays were repeated five times on days 7, 14, 21 and 28.

Stomatal apertures were measured on mature leaves at 8, 14 and 19 h during five days starting on day 21, when leaf expansion and shoot lengthening of the new sprouts were taking place.

Thus, the results were a measurement average covering days 21 to 26. Two leaves per treatment were chosen and five microscope fields (0.25×0.25 mm) were counted on each leaf. A layer of acrylic (synthetic nail amend) was brushed on to the abaxial side of the leaf, allowed to dry for a few seconds, then carefully extracted and mounted for microscope (Olympus BX 50) observation. Stomatal aperture was measured by using Bio Scan (OPTIMAS, Washington, USA) software.

At the same time that stomata measurements were done, the relative water content (RWC) was calculated from three mature and three new immature leaves per treatment as: $RWC = [FW - DW/SatW - DW] \times 100$; where FW, fresh weight, DW, dry weight and SatW, fresh weight after saturation in distilled water.

On day 35, leaf area, shoot length, FW and DW of shoots and leaves were measured separately.

On day 35, ABA was quantified in new shoots of control plants grown under two extreme light intensities (100% and 1.5% PPFD), following the procedure used by Volmaro et al. (1998) with modifications. The plant material was frozen in liquid nitrogen and dehydrated. One gram DW of shoot was extracted for 16 h at 4 °C with methanol : water : acetic acid (80 : 19 : 1, v/v) plus 200 mgl^{-1} of BHT (Fluka) as antioxidant. Then, 200 ng of [$^2H_6$] ABA (a gift from J.D. Cohen, University of Minnesota, USA) was added as internal standard. One hour later, after allowing isotope equilibration, the sample was filtered, re-extracted with the same solvent mixture mentioned above, and methanol was evaporated under low pressure. The remaining aqueous fraction was filtered and centrifuged for 15 min at 8000 rpm. The supernatant was adjusted to pH 3.0 and partitioned four times with ethyl acetate saturated with aqueous acetic acid 1%. The pooled ethyl acetate fractions were then submitted to reverse phase HPLC (C_{18} μ-Bondapack, 300×3.9 mm, Water Associates, USA) eluted with a gradient of methanol : water (1% acetic acid) from 10% to 73% of methanol at 2 ml min^{-1}. The fraction to be co-chromatographied with authentic ABA was transformed to its methyl derivative with fresh diazomethane and analysed by capillary gas chromatography-selected ion monitoring (GC–SIM) on a Hewlett-Packard A 5890 GC (HP-5 column, 25 m long, i.d. 0.22 mm, film thickness 0.25 μm) connected with a direct interface to a HP B 5970 MSD. The gas carrier was He at 1 ml min^{-1}. The amount of ABA was assessed comparing the area of m/z 194/166 and 190/162 for deuterons and endogenous ABA, respectively. Measurements were done by triplication of three different extractions.

Statistical analysis of data was performed with ANOVA analysis (Graph Pad Software, San Diego, CA, USA) and the Tukey and Bonferoni tests ($p < 0.05$) were used in order to compare differences among treatments.

Results

The number of stomata by unit of leaf area in *I. paraguariensis* varies from 430 to 480 per mm^2. The stomata are localised at the upper (abaxial) side of the leaves. The average number as well as the stomata size did not change under the different treatments (e.g., different PPFD levels and ABA treatments) during the experiment, even in the youngest leaves (results not shown).

The proportion of closed stomata in mature leaves from control plants submitted to 50% PPFD varied from a minimum of 18.6% (±4.5) at 8 h up to a maximum of 32.6% (±5.1) at midday (14 h). When ABA was applied, stomatal closure was promoted throughout the day as compared with untreated control. Figure 1(A–D) shows the physiological parameters assessed as average values of measurements performed at 8, 14 and 19 h during 5 days starting on day 21 (e.g. when leaf expansion and shoot lengthening of the new sprouts were taking place) from mature leaves of plants grown under 50% PPFD. Stomatal closure (Figure 1A) and the consequent reductions in stomatal pore area (Figure 1B) were achieved with ABA (ranging from 62% to 73%). This control of stomatal behaviour was reflected as an increase in RWC, especially in the afternoon and early evening (Figure 1C and D). In effect, RWC was increased in the early evening from 82% to 92% and 88% to 94% in mature and immature leaves, respectively.

The stress alleviation described (Figure 1C and D) was then correlated with increases in total leaf area, leaf DW, shoot length, and shoot DW (Figure 2A–D). On day 35 of the experiment, the increases in DW of leaves and shoots (i.e., the harvest material in commercial plantations) were of

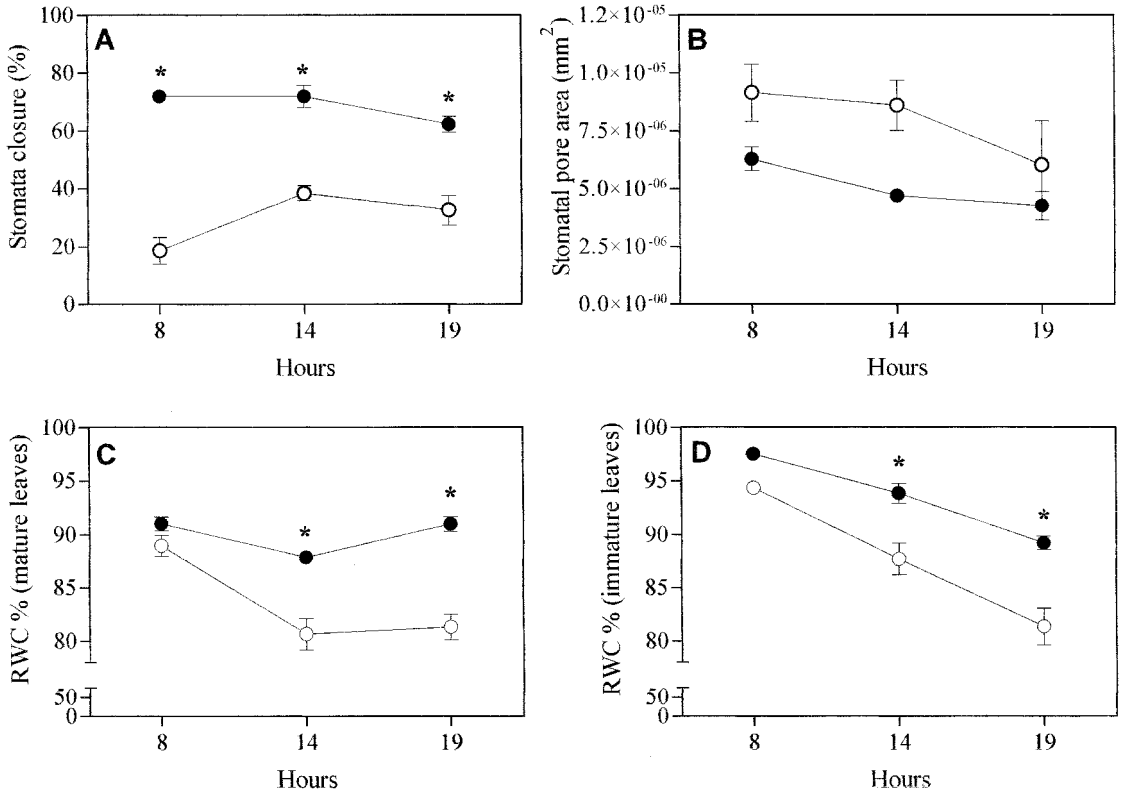

Figure 1. A, stomatal closure (as % of closed ostiols), B, stomatal pore area (in mm^2), C, RWC of mature (pre-existent when the experiment began) leaves and D, RWC of young (new sprouted after the experiment began) leaves. Measurements were done at 8, 14 and 19 h of a 24-h period, in yerba mate plants grown under 50% PPFD. Open circles, control plants; dark circles, ABA-treated plants. Bars indicate standard error of the mean value. *Indicates significant differences ($P < 0.05$, Tukey's multiple comparison test) respect to the control.

1.5-fold for both at 1.5% PPFD and 3-and 4.5-fold, respectively, under full sunlight (100% PPFD). These increases were achieved by enhanced growth of the new tissues (immature leaves plus new shoots).

The ABA content of new shoots of control plants grown under 1.5% and 100% PPFD is shown in Table 1. There was an increase in ABA levels in plants grown under 1.5% PPFD. However there was no correlation with shoot length (Figure 2B). On the contrary, these increased ABA levels did not have any inhibitory effect on shoot growth since the shoots grew longer than those in full sunlight.

Discussion

Our results suggest that exogenous ABA sprayed on to leaves promotes growth in *I. paraguariensis* plants by alleviating diurnal water stress. ABA promoted stomatal closure that was positively correlated with an increase of the RWC in leaves. This situation was reflected in higher leaf expansion implying augmentation of expansion growth; that is, the new leaves and sprouts of the ABA-treated plants grew more than those of untreated control plants. These results are in agreement with the concept that expansion growth is very sensitive to water deficit, especially in leaf tissues (Granier and Tardieu 1999). In fact, the role of ABA in the adaptation of a variety of plant species under extreme conditions of water deficit has been well established (Zeevaart and Creelman 1988). ABA-deficient mutants showed higher damage at leaf level under conditions of water stress, in correlation with their ABA contents (Fambrini et al. 1994). Reciprocally, the ABA-deficient mutants treated with exogenous ABA had higher water

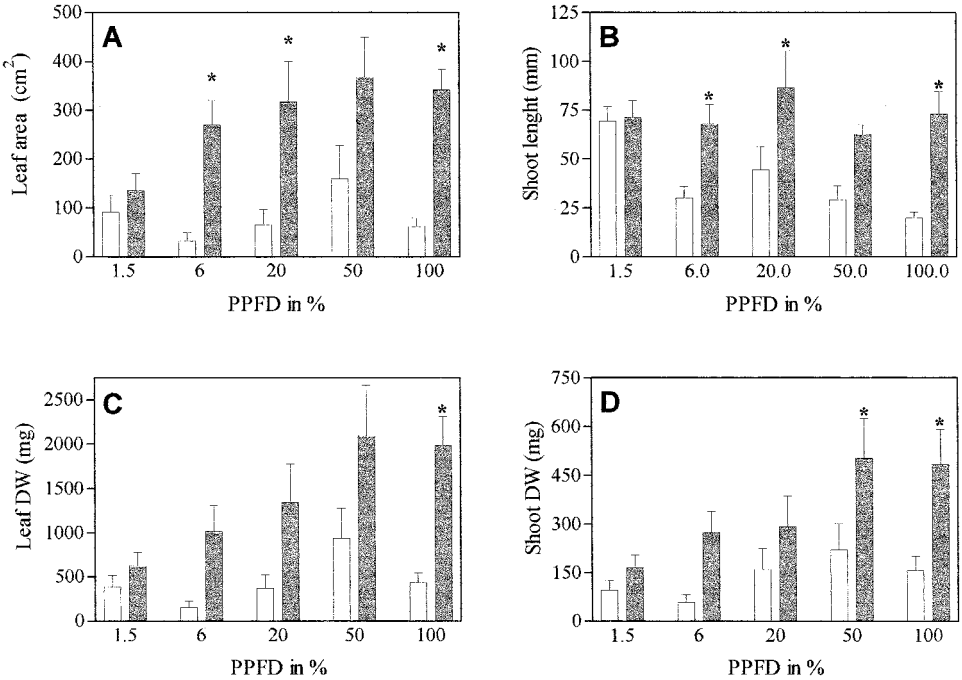

Figure 2. A, leaf area (cm^2), B, shoot length (mm), C, leaf DW (mg) and D, shoot DW (mg) of yerba mate plants grown under field conditions and different PPFD levels. Open bars, control plants; dark bars, ABA-treated plants. * Indicates significant differences ($P < 0.05$, the Bonferroni's test) respect to the control.

Table 1. ABA content (ng g^{-1} d was measured by GC-MS with deutero internal standards) of control plants grown in pots under field conditions and two different PPFD levels.

PPFD (%)	ABA (ng g^{-1} DW)
1.5	149.4 ± 12.6
100	86.6 ± 21.2

potential and greater turgor, indicating less damage.

Surprisingly, stress alleviation also had the consequence of promoting shoot length. It has been claimed that one of the functions of ABA is to inhibit shoot elongation (Hoffmann-Benning and Kende 1992), possibly through disruption of microtubule arrangement (Sakiyama and Shibaoka 1990). While the results presented here do not rule out such an effect in certain situations when cell turgor is maximal, it seems that the major role for ABA *in planta* is related to water economy and not to growth inhibition. In fact, shoot elongation under field conditions for different species is mainly affected by gibberellin levels (Foster and Morgan 1995; Clúa et al. 1996) including woody angiosperms (Junttila 1991) and even yerba mate (Sansberro et al. 2002). Although ABA may increase water use efficiency, it has been emphasized that its role in stress defense mechanisms is related to growth restraint as a way of moderating the effect of stressful situations (Rademacher et al. 1989; Creelman et al. 1990). Nevertheless, ABA has been reported to promote mesocotyl growth in etiolated seedlings of a dwarf mutant of foxtail millet (Chen and Zhou 1998), and accumulation of ABA is also required for maize primary root elongation (Sharp et al. 1994). It has also been demonstrated that ABA-deficient mutants of tomato show less growth than the wild type even under adequate water supply, indicating that "specific" levels of ABA may be required for normal growth (Sharp et al. 2000). All these ABA-promoting effects seem to occur through limiting ethylene production (Spollen et al. 2000; Sharp and LeNoble 2002). According to the results presented here, the stress alleviation produced by exogenously applied ABA masked the possible shoot growth-inhibiting effect of the hormone. On the

contrary, ABA had a promoting effect on shoot length of yerba mate plants.

We conclude that ABA, applied weekly to leaf surfaces of *I. paraguariensis* under well-watered soil and high evaporative demand conditions promoted stomatal closure, especially during hours of high water loss by transpiration, so diminishing the water stress in leaves. Consequently, ABA might have promoted higher turgor thus inducing increased growth in terms of volume (as noticed by higher leaf expansion) as well as shoot elongation. In turn, the augmented growth could introduce and increased demand for photosyntates, resulting in subsequent accumulation of dry matter due to a higher photosynthetic activity and/or improved translocation of photosynthetic products.

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