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Aluminum-induced decrease in CO_2 assimilation in 'Rangpur' lime is associated with low stomatal conductance rather than low photochemical performances

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ABSTRACT

Aluminum (Al) is toxic to most plants. Although inhibition of root elongation can occur even under 10 μM Al, above 1000 μM damage to photochemical performances has been reported, reducing the CO_2 assimilation rate (*A*). However, Al is retained in the roots of most plants, including *Citrus limonia* ('Rangpur' lime) with no direct explanation for the low *A*. In this study, 'Rangpur' lime plants were cultivated hydroponically in a nutrient solution with 1480 μM Al, and we expected to determine the time range within which Al-induced decrease in *A* starts. To our surprise, low stomatal conductance (*gs*) rather than low photochemical performances was evident 45 days after exposing the roots to Al. Aluminum was mostly retained in the roots and histochemically observed in the stele, suggesting reduction in water uptake/transport in the xylem that became fibrous. We also found a 35% reduction in the midday leaf water potential in plants exposed to Al, indicating association between the fibrous xylem vessels and low *gs*, which could explain the Al-induced decrease in *A* in 'Rangpur' lime plants.

1. Introduction

Aluminum (Al) is the most abundant element in the Earth's crust and, in the soil, it naturally occurs as Al_3SiO_4; however, in acidic soils (pH < 5.0) it is hydrolysed to Al^{3+}, which is toxic to most plant species (Foy, 1988; Horst et al., 2010). Acidic soils occupy approximately 30–45% of the world's ice-free land (von Uexküll and Mutert, 1995), therefore, Al is a factor limiting plant growth (Horst et al., 2010) and crop yields (Foy, 1988; Vitorello et al., 2005) throughout the world.

Inhibition of root elongation is the first and most conspicuous symptom observed in plants exposed to Al (Horst et al., 2010; Sun et al., 2010). Low root growth can be detected within hours at Al concentrations as low as 10 μM, causing ruptures on the rhizodermis (Kopittke et al., 2008; Blamey et al., 2011). Aluminum is retained in the roots of most Al-sensitive species (Vitorello et al., 2005), including 'Rangpur' lime (*Citrus limonia*) (Santos et al., 1999), 'Sour Pummelo' (*Citrus grandis*) and sweet orange (*Citrus sinensis*) (Yang et al., 2011). In the roots, the Al seems to bind to the cell walls (Rangel et al., 2009), where it can be histochemically evidenced with chrome azurol S (Wehr et al., 2010). Besides the local, rapid and direct effect of Al on root elongation, it also exerts long-distance effects on the shoots, such as reduced shoot growth (Jiang et al., 2009).

Another long-distance indirect effect of Al on shoots is an Al-induced decrease in CO_2 assimilation rate (*A*) observed in many species, including *Citrus* spp. (Pereira et al., 2000; Chen et al., 2005a,b; Jiang et al., 2008, 2009; Yang et al., 2011), coffee plants (*Coffea arabica*) (Konrad et al., 2005), maize (*Zea mays*) (Lidon et al., 1999), and rye (*Secale cereale*) (Silva et al., 2012). In 'Cleopatra' tangerine (*Citrus reshni*) (Chen et al., 2005a,b) and in 'Sour Pummelo' (Jiang et al., 2008; 2009), Al presence in the root environment is associated with low photochemical performance in leaves, as a result of low electron transport rate (ETR) of photosystem II (PSII),

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and attenuated photochemical quenching (qP), reducing the energy and electron transporters for maintaining carboxylation reactions. This low photochemical performance is also reported for coffee plants exposed to Al (Konrad et al., 2005). Another reason for Al-induced decrease in *A* could involve a reduced nitrogen (N) uptake by leaves, since N is a constituent of many photosynthetic enzymes. In maize, increasing Al to up to 2960 μM in the root environment decreased shoot N concentration (Lidon et al., 1999), but in 'Rangpur' lime, leaf N concentration was unchanged when the roots were exposed to up to 1110 μM Al (Santos et al., 1999). Given the relationship between N and photosynthesis (Kumar et al., 2004), it is noteworthy that the rate of carboxylation reactions are not decreased by Al in 'Cleopatra' tangerine (Chen et al., 2005b), coffee plants (Konrad et al., 2005) or rye (Silva et al., 2012). On the other hand, most of these studies show 30–80% decrease in stomatal conductance (*gs*) when the roots are exposed to Al, reiterating a multi-factor effect of Al on *A*.

Citrus is cultivated in humid and sub-humid areas of temperate, tropical and subtropical regions of the world, mostly on acidic soils that are usually rich in Al. However, there are different species and cultivars of *Citrus*, and specific combinations of scions/rootstocks for each region may determine fruit yields (Carr, 2012). The effects of Al on *A* are rarely studied in the 'Rangpur' lime rootstock, which is largely used in subtropical areas of the Americas due to its high drought resistance (Ribeiro and Machado, 2007; Magalhães Filho et al., 2008) and, therefore, this rootstock is useful in rain-fed *Citrus* plantations.

Studies of the effects of Al and its movement in the plant with the objective of determining the time range within which Al induce a decrease in *A* are rare for *Citrus*, a plant that drives its photosynthetic responses according to factors that shift every season (Ribeiro et al., 2012). Therefore, using rootstocks in hydroponic system with a nutrient solution containing more than 1000 μM Al, as performed by Chen et al. (2005a,b) and Jiang et al. (2008, 2009), we measured *A*, ΦPSII, ETR and qP in 'Rangpur' lime plants growing in 1480 μM Al, over a 45-day experiment. We predicted that Al-induced decrease in *A* is triggered by an early reduction in photochemical performance. Following this prediction, we sought to detect the time range within which the low photochemical performance occurs in 'Rangpur' lime plants. In addition, histochemical analyses of roots and leaves were performed and provided evidence to support the functional analyses.

2. Material and methods

2.1. Plant material and experimental conditions

We used three-month-old and 13 ± 0.6 cm-high 'Rangpur' lime (*Citrus limonia*) plants for studying the effects of Al and its movement in the plant within a 45-day period. The plants were maintained in hydroponic system and grew directly on an aerated nutrient solution (Furlani and Furlani, 1988) inside opaque plastic boxes (50 cm in length × 30 cm in width × 15 cm in height; 20 L).

The nutrient solution shows a chemical composition based on Clark's solution (Clark, 1975) that has been used to test Al resistance in *Citrus* rootstocks (Santos et al., 2000). It contained the following macronutrients (in mM): NO_3^- 0.96; NH_4^+ 0.41; P, 0.013; K, 0.86; Ca, 1.43; Mg, 0.33; S, 0.22; and micronutrients (in μM): Cl, 214.1; Fe (EDTA), 23.3; B, 8.33; Mn, 2.91; Zn, 0.76; Cu, 0.32; Mo, 0.31. Besides macro and micronutrients, this solution contained 0 and 1480 μM (40 mg/L) Al provided through $AlK(SO_4)_2\ 12H_2O$. This Al concentration was used because most studies have usually observed Al-induced decrease in photosynthetic parameters only when evaluating non-grafted plants under more than 1000 μM Al (Chen et al., 2005a,b; Konrad et al., 2005; Jiang et al., 2008, 2009; Silva et al., 2012). The pH of the solution was maintained at 4.0 ± 0.1 in order to keep the Al as soluble as possible. In addition, the nominal chemical composition of this solution was tested on Geochem-EZ software (Shaff et al., 2010), and it resulted in more than 85% free Al^{3+} available. Solution pH was monitored daily and replaced every ten days. Besides testing the solution on Geochem-EZ software, we noted that nominal 1480 μM Al supply resulted in 1100 ± 5.3 μM Al, which was measured colorimetrically (Sarruge and Haag, 1974). Expanded polystyrene (Isopor®) 50 × 30 cm plates (2-cm thick), with five holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around

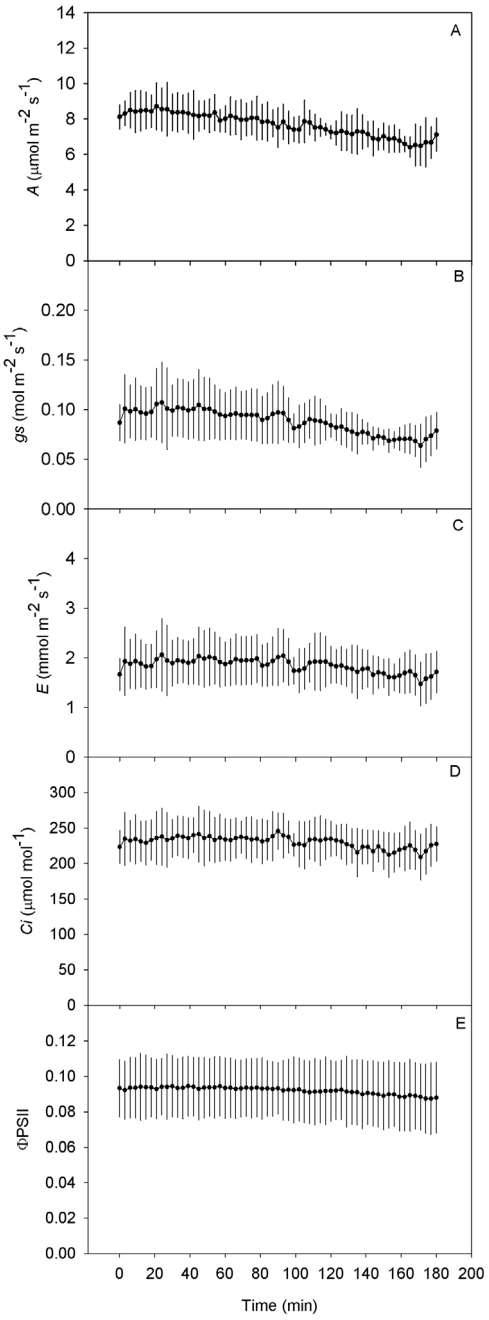

Fig. 1. Leaf gas exchange rates (A, B, C, and D) and effective quantum yield of photosystem II (E) of leaves of 'Rangpur' lime plants measured for three hours after being transferred to a nutrient solution containing 1480 μM Al. Absence of letters indicates lack of significant difference (RM Anova; $p > 0.05$) between mean values ($n = 5$ plants) and values previously obtained. Bars are s.d.

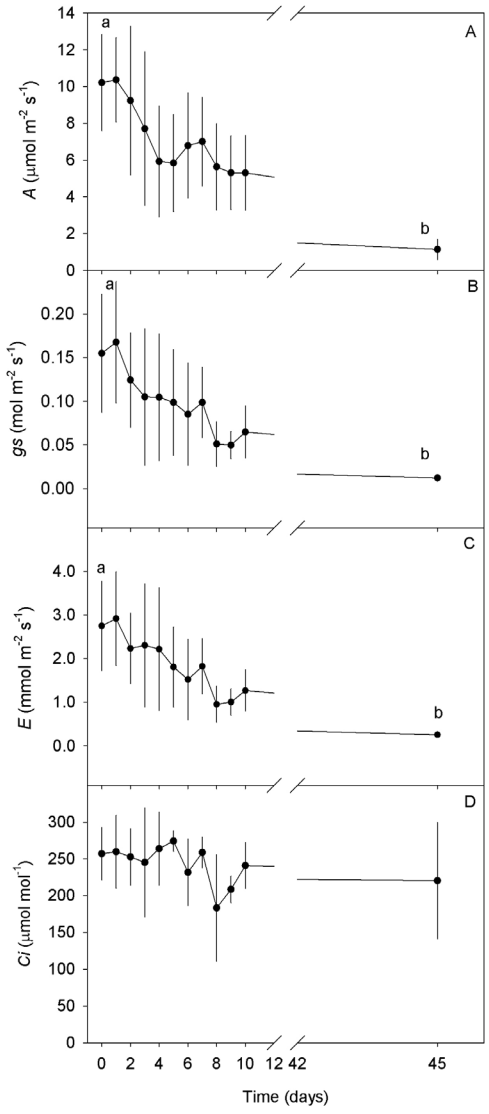

Fig. 2. Leaf gas exchange rates of 'Rangpur' lime plants measured for 45 days after being transferred to a nutrient solution containing 1480 μM Al. Absence of letters indicates lack of significant differences (RM Anova; $p > 0.05$), and distinct letters indicate significant differences (RM Anova; $p < 0.05$) between mean values ($n = 5$ plants) and values previously obtained. Bars are s.d.

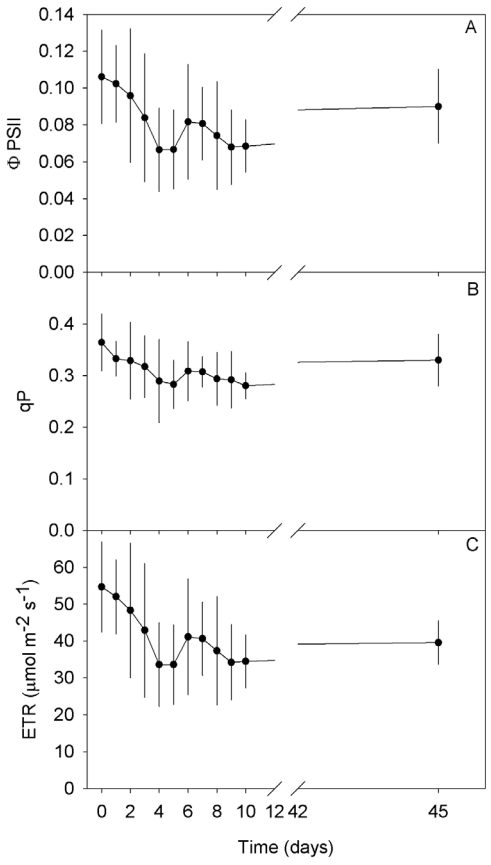

Fig. 3. Photochemical parameters in leaves of 'Rangpur' lime plants, measured for 45 days after being transferred to a nutrient solution containing 1480 μM Al. Absence of letters indicates lack of significant differences (RM Anova; $p > 0.05$) between mean values ($n = 5$ plants) and values previously obtained. Bars are s.d.

the plant collar. The boxes were kept on benches, maintained inside a greenhouse, under semi-controlled conditions (air temperature $28.5 \pm 0.7\,^{\circ}C$; relative humidity $63.3 \pm 1.3\%$; 753.4 ± 176.1 μmol photons $m^{-2}\,s^{-1}$; approximately 14 h of natural photoperiod).

2.2. Experimental design

We measured leaf gas exchange and fluorescence of chlorophyll *a* in light-adapted leaves, after transferring 'Rangpur' lime plants from the nutrient solution with no Al to the solution containing 1480 μM Al. After the transference, these parameters were assessed for three hours (every three minutes), every day for 10 days after the transference (DAT), and at 45 DAT when leaf water potential (Ψw) was measured. At 115 DAT (to ensure a long-term Al stress for plant organs and growth) the plant size, biomass, Al concentration in each organ, and anatomical and histochemical analysis of leaves and roots were performed in the plants maintained in the nutrient solution with 0 and 1480 μM Al.

2.3. Photosynthetic parameters

CO_2 assimilation (***A***) and transpiration (***E***) rates, stomatal conductance (***gs***), and intercellular CO_2 (***Ci***) were measured with an open portable gas exchange system (LI-6400xt, LI-COR, Lincoln, NE, USA). The CO_2 concentration entering the leaf cuvette averaged 390 μmol mol^{-1}, as provided by the 6400-01CO_2 mixer (LI-COR). Measurements were taken between 9:00 and 11:00 h (Feistler and Habermann, 2012) on cloudless days, under the natural fluctuation of air temperature and vapor pressure deficit (VPD) inside the greenhouse. The photosynthetic photon flux density (PPFD) was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to provide 1200 μmol photons $m^{-2}\,s^{-1}$ in the leaf cuvette, as this value saturates *A* for *Citrus* (Habermann et al., 2003).

The fluorescence of chlorophyll *a* was measured with a portable modulated fluorometer (6400-40 LCF, LI-COR, Lincoln, NE, USA), which was integrated into the LI-6400xt gas exchange system. The effective quantum yield of PSII (ΦPSII) was calculated as $(F_m' - F_s)/F_m'$, where F_m' and Fs are the maximum and the steady-state fluorescence in light-adapted leaves, respectively. Apparent electron transport rate (ETR = ΦPSII PPFD 0.5 0.85) was calculated, using 0.5 as the fraction of excitation energy distributed to PSII, and 0.85 as the fractional leaf light absorbance; the proportion of open PSII reaction centers (qP) was measured as $(F_m' - F_s)/(F_m' - F_o')$ (Bolhàr-Nordenkampf and Öquist, 1993), where F_o' is the minimal fluorescence in light-adapted leaves, measured after exposing the leaf to a far red light pulse before the actinic light goes off.

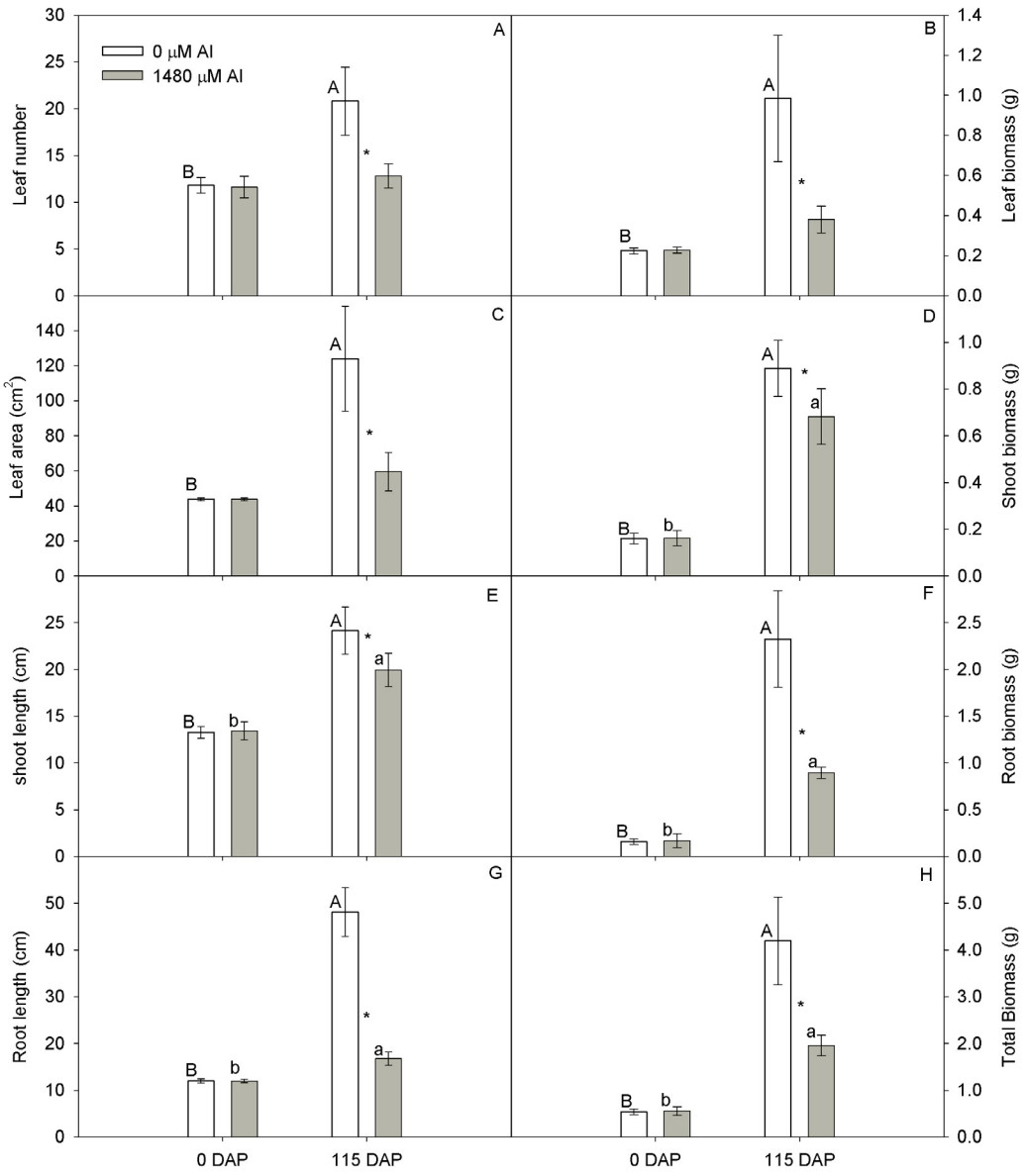

Fig. 4. Growth and biomass of organs of 'Rangpur' lime plants cultivated for 115 days in nutrient solutions containing 0 and 1480 μM Al. Asterisks indicate significant differences by the Tukey test ($\alpha = 0.05$) between Al treatments. For the control plants, distinct uppercase letters indicate significant differences ($p < 0.05$) between 0 and 115 DAP; for plants exposed to Al, distinct lowercase letters indicate significant differences ($p < 0.05$) between 0 and 115 DAP. Columns are mean values (n = 5 plants), and bars are s.d. DAP = days after planting.

2.4. Al concentration in plant organs

Leaves, shoots and roots were washed under deionized water and oven-dried at 60 °C to constant dry mass. These samples were, then, ground and digested in a nitric:perchloric acids solution (v:v). The concentration of Al was quantified colorimetrically (Sarruge and Haag, 1974).

2.5. Biometric parameters

The lengths (cm) of stems (from the plant collar to the shoot apex) and roots (from the plant collar to the root tip) were measured with a ruler, and the number of leaves was counted. At 0 and 115 DAT plants were separated into leaves, stems (plus petioles) and roots, which were dried at 60 °C until constant mass. The biomasses (g) of organs and total plant biomass were evaluated, and the leaf area (LA, cm^2) was measured with an area meter (LI-3100C, LI-COR, USA).

2.6. Anatomical analysis

The leaves and root tips were collected and immediately fixed in FAA 50 (37% formaldehyde, glacial acetic acid, 50% ethanol; 1:1:18 v:v:v) and preserved in 70% alcohol (Johansen, 1940). We also used fresh tissues to histochemically test the Al indicator (chrome azurol S – CAS), and the staining patterns were the same as those obtained when the plant material was fixed (FAA 50) and preserved (70% alcohol).

The anatomical study was based on consecutively sliced cross sections of leaf segments (1 cm^2) from leaf midribs containing part of the leaf lamina, and of root tips (approximately 0.5 cm from the root apex). These sections were hand-made with a razor blade. Non-dye treated cuts were immersed in distilled water for 15 min before mounting semi-permanent glass slides. Dye-treated cuts were stained (one or two drops of the staining solution – Kukachka and Miller, 1980) for 45 min (at room temperature) as no staining difference was observed after 20, 30, 40 or 60 min. After the

staining, the cuts were immersed in distilled water for 15 min three times and mounted in semi-permanent glass-slides. All cuts were observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany). The images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) functionally attached to the DMLB.

As Al indicator, we used chrome azurol S (CAS) or 'mordant blue 29′. This indicator is highly specific to detect Al in plant tissues (Bressan et al., 2016) as it strongly complexes Al that is bound tightly to pectate or cell wall material (Wehr et al., 2010). Chrome azurol S (3′′-sulpho-2′′,6′′-dichloro-3,3′-dimethyl-4-hydroxyfuchson-5,5′-dicarboxylic acid), 50% purity (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared dissolving 20 mL of a 41.3 mM CAS solution (25 g/L) into 80 mL of a 760 mM and 4.82 pH sodium acetate solution (Kukachka and Miller, 1980). Therefore, CAS final concentration was 8.3 mM (5 g/L) (pH = 4.76 ± 0.01).

2.7. Leaf water potential (Ψ_w)

Leaf water potential at predawn (Ψ_{pd}) and midday (Ψ_{md}; under maximum VPD) was measured by the pressure chamber method (Turner, 1981), using a DIK-7000 (Daiki Rika Kogyo, Tokyo, Japan) portable chamber.

2.8. Data analysis

The study was conducted with five plants per treatment. A one-way analysis of variance (Anova) was performed between plants exposed to 0 and 1480 μM Al, testing Ψ_{pd} and Ψ_{md} at 45 DAP, as well as the leaf number, LA, shoot and root lengths, leaf, shoot, root and total biomasses and Al concentrations in plant organs at 0 and 115 DAP. In addition, a repeated measures Anova (RM-Anova) was performed to test for differences in the effect of Al on *A*, *gs*, *E*, *Ci*, ΦPSII, ETR and qP over time on the same group of plants transferred from 0 to 1480 μM Al. In this case, we did not compare plants maintained under 0 and 1480 μM Al, because we aimed at studying the dynamics of ecophysiological responses on the same individuals after the transference, and RM-Anova is more appropriate for such purposes, when the group of individuals serves as its own control (Girden 1992).

The Tukey test (α = 0.05) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results of growth, biomass, Al concentrations in the organs and Ψw.

3. Results

Values of *A*, *gs*, *E*, *Ci* and ΦPSII remained unchanged within three hours after 'Rangpur' lime plants were transferred from 0 to 1480 μM Al (Fig. 1). However, these parameters decreased consistently throughout the 45-day period, and significant differences were observed for *A* (Fig. 2A), *gs* (Fig. 2B and *E* (Fig. 2C) at 45 DAT in relation to day 0; *Ci* (Fig. 2D), ΦPSII (Fig. 3A), qP (Fig. 3B) and ETR (Fig. 3C) remained unchanged over this period.

The 'Rangpur' lime plants cultivated in the nutrient solution with 1480 μM Al showed lower leaf number (Fig. 4A), leaf biomass (Fig. 4B) and LA (Fig. 4C) at 115 DAP, in relation to the control plants. Aluminum significantly reduced plant growth from 0 to 115 DAP, with a conspicuous impact on root length (Fig. 4G) and root biomass (Fig. 4F) when compared to the impact on shoot length (Fig. 4E) and shoot biomass (Fig. 4D).

At 115 DAP, Al concentration was significantly higher in the leaf (Fig. 5A), shoot (Fig. 5B), root (Fig. 5C), and in the whole plant (Fig. 5D) when cultivated at 1480 μM Al in comparison to the control plants. After 115 days at 1480 μM Al, tissue Al concentration

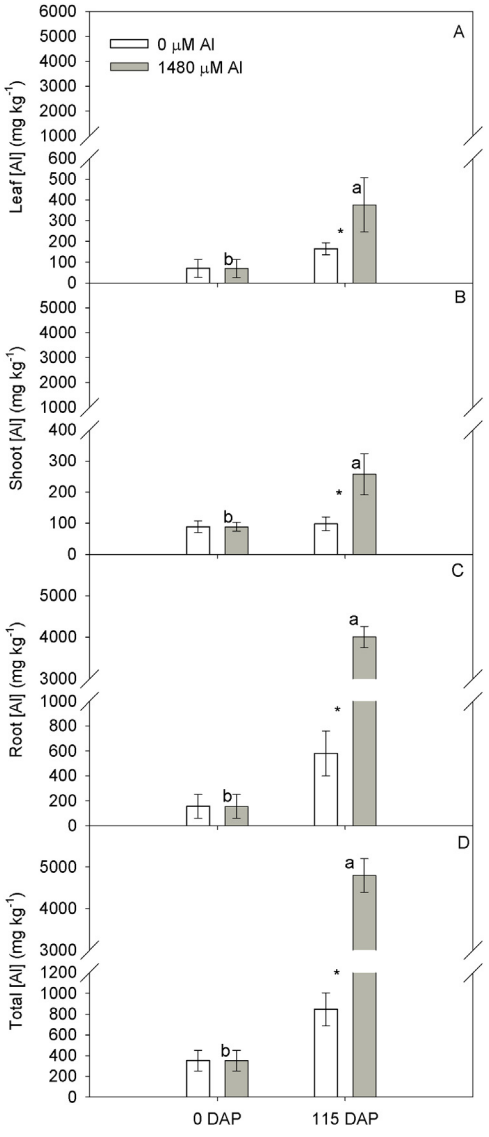

Fig. 5. Mean values (n = 5 plants) of Al concentration in leaves (A), shoots (B), roots (C), and in the whole plant (D) of 'Rangpur' lime rootstocks cultivated for 115 days in nutrient solutions containing 0 and 1480 μM Al. Asterisks represent significant differences by the Tukey test (α = 0.05) between Al treatments. For each treatment, absence of letters indicates lack of significant differences (p > 0.05) and distinct letters indicate significant differences (p < 0.05) between 0 and 115 DAP. Bars are s.d. DAP = days after planting.

was 10 times higher in the roots (Fig. 5C) in relation to the shoots (Fig. 5B) and leaves (Fig. 5A).

Chrome azurol S did not indicate the presence of Al in the roots or leaves of 'Rangpur' lime plants under 0 μM Al (Fig. 6B, C). However, this dye indicated the presence of Al in the roots of plants exposed to 1480 μM Al (Fig. 6E), but not in their leaves (Fig. 6F). In these plants, Al was noted in the root vascular cylinder (Fig. 6H) and, compared to other tissues, Al was noted more intensely in the cytoplasm of the phloem and inside the lumen of xylem vessels (Fig. 6H, I). However, no positive reactions were observed on the cell wall of xylem vessels (Fig. 6H, I). The roots of plants exposed to Al also showed structural modifications to the pericycle and phloem, as well as fibrous xylem vessels (Fig. 6I), which did not occur in the roots of plants not exposed to Al (Fig. 6G).

Predawn leaf water potential was the same for plants at 0 and 1480 μM Al, whereas Ψ_{md} was 35% lower in plants exposed to Al when compared to the control plants (Fig. 7).

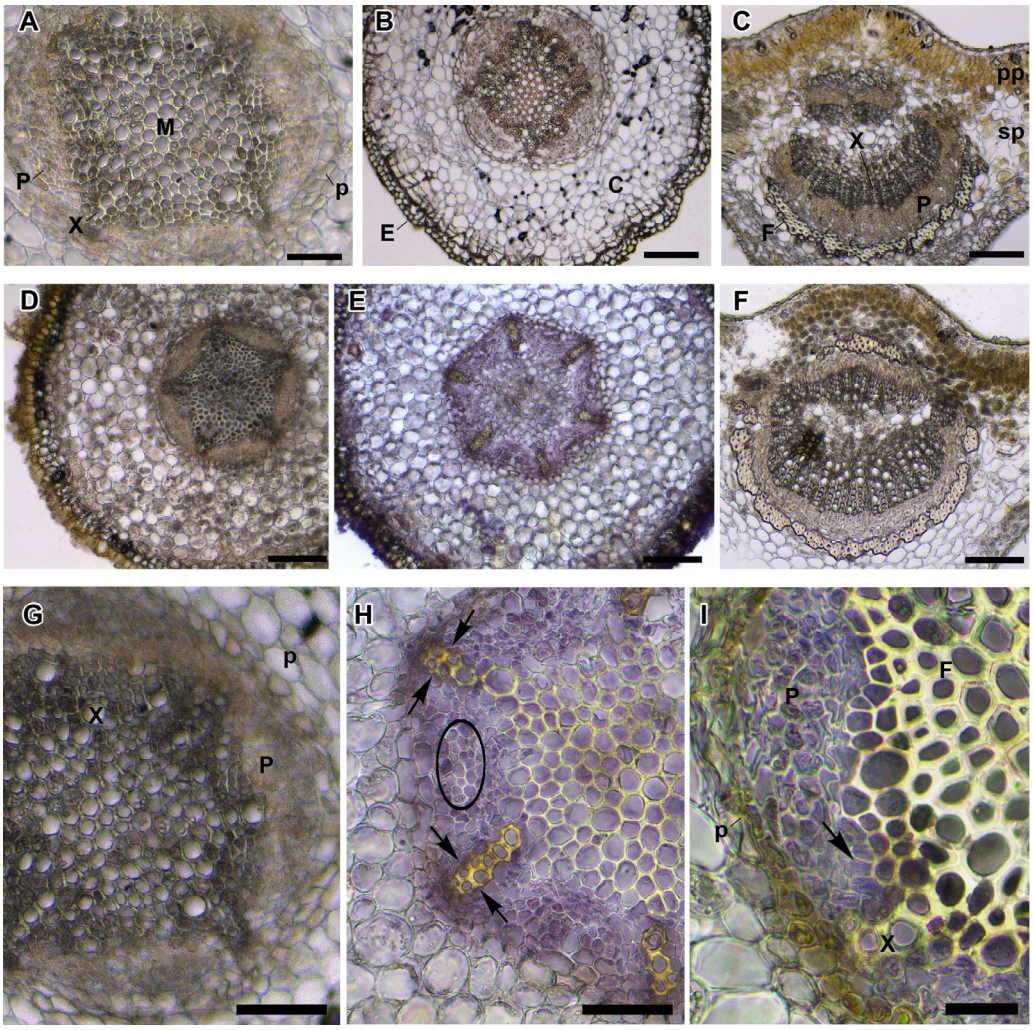

Fig. 6. Anatomical analyses of roots (A, B, D, E, G, H, and I) and leaf midribs (C and F) of 'Rangpur' lime plants. A, B, C and G: cultivated in nutrient solutions containing 0 μM Al; D, E, F, H and I: cultivated in nutrient solutions containing 1480 μM Al; A and D: non-stained; B, C, E, F, G, H and I: stained with chrome azurol S. C = cortex; E = epidermis; F = fibers; M = medulla; p = pericycle; P = phloem; pp = palisade parenchyma; sp = spongy parenchyma; X = xylem. Double-opposite arrows: fibrous xylem; Simple arrows: positive reaction of cellular contents. Scale bars (μm): A = 50; B-F = 100; G-H = 50; I = 20.

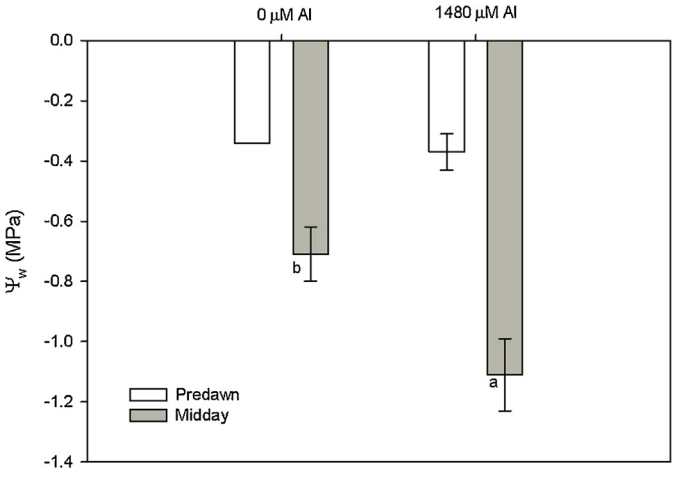

Fig. 7. Mean values (n = 5 plants) of leaf water potentials of 'Rangpur' lime plants cultivated for 45 days in nutrient solutions containing 0 and 1480 μM Al. For each time of day (predawn or midday), absence of letters indicates lack of significant differences by the Tukey test ($p > 0.05$), and distinct letters indicates significant differences ($p < 0.05$) between 0 and 1480 μM Al. Bars are s.d.

4. Discussion

When we transferred 'Rangpur' lime plants from a nutrient solution with 0 to 1480 μM Al, we expected to determine the time range within which Al-induced decrease in photochemical performances would start influencing *A*. Nonetheless, Al did not affect photochemical performances throughout the study. Stomatal conductance, however, consistently decreased throughout the study and was significantly low after 45 days. We observed 90% lower *gs* at 45 DAT in relation to the first day that the plants were transferred to 1480 μM Al (Fig. 2B). This suggests that *gs* reduction may be more important than low photochemical performances for explaining the Al-induced decrease in *A* in 'Rangpur' lime plants. Aluminum-induced decrease in *gs* by 30% in 'Cleopatra' tangerine (Chen et al., 2005b), 40% in 'Sour Pummelo' (Jiang et al., 2008), 65% in coffee plants (Konrad et al., 2005), and 80% in rye (Silva et al., 2012) has already been reported.

Since *A* decreased steadily through a 45-day Al treatment (Fig. 2A), and *Ci* stayed unchanged throughout this period (Fig. 2D), one may argue that non-stomatal factors could *also* be involved in the Al-induced decrease in *A* because unchanged *Ci* might imply reduced carboxylation rates. However, we found a large correspondence between *A* (Fig. 2A), *gs* (Fig. 2B) and *E* (Fig. 2C), indicating

that *A* was under diffusive control, *i.e.*, it was dependent on *gs*. In addition, in 'Cleopatra' tangerine (Chen et al., 2005b) and rye (Silva et al., 2012) the Al-induced decrease in *A* is unaccompanied by reduced carboxylation rates, weakening the non-stomatal factor hypothesis. Low mesophyll hydration must be considered as one of the mechanisms that might explain this association between low *A* and *gs* in plants exposed to Al. Samac and Tesfaye (2003) and Vitorello et al. (2005) defend that Al induces the stunting of the primary root and inhibition of lateral root formation, which would lead to reduced water uptake. In the present study, however, the plants exposed to Al showed reduced leaf number (Fig. 4A) and low leaf area (Fig. 4C) at 115 DAP; their leaf biomass did not increase (from 0 to 115 DAP) as it did in the control plants (Fig. 4B), therefore compensating for the decrease in root length (Fig. 4G) and root biomass (Fig. 4F) caused by Al. In addition, plants exposed to Al exhibited lower Ψ_{md} in relation to the control plants (Fig. 7), indicating a greater difficulty for these plants to hydrate their mesophyll during the day. These Ψ_{md} values represent a mild but important water deficit when compared to Ψ_{md} of sweet orange grafted on 'Rangpur' lime plants grown in the field during the dry season under subtropical conditions (−2.0 to −2.5 MPa; Ribeiro and Machado, 2007; Habermann and Rodrigues, 2009), or after 12 days of drought under semi-controlled conditions (−1.6 MPa; Magalhães Filho et al., 2008), or to Ψ_{md} values of sweet orange plants grafted on 'Rough' lemon (*Citrus jambhiri*) exhibiting leaf wilting after 10 days under drought (−2.0 MPa; Vu and Yelenosky, 1988). Therefore, the reduced hydration in mesophyll evidenced by the low Ψ_{md} (Fig. 7) could be associated with a lack of functional capacity of the fibrous xylem vessels (Fig. 6H, I) and, consequently, explain the low *gs* (and *A*) found in 'Rangpur' lime plants exposed to Al.

Our results also show that Al caused an indirect/long-distance effect on *A* because Al was mostly retained in the roots (Fig. 5C), and was *not* anatomically evidenced in the leaves (Fig. 6F). Aluminum immobilization in the roots has already been reported for most crop species (Vitorello et al., 2005), including 'Rangpur' lime (Santos et al., 1999), 'Sour Pummelo', and sweet orange (Yang et al., 2011) plants. Our results demonstrate that Al is associated with structural modifications to the pericycle and phloem; modifications to the xylem may also be noted as this tissue became fibrous in the presence of Al (Fig. 6H, I) when compared to plants not exposed to Al (Fig. 6G). This illustrates how a long-distance effect of Al may have an influence on *gs* and *A* in the leaves. CAS reacts positively with Al bound to pectin from the cell wall (Wehr et al., 2010), and it evidenced the presence of Al on the phloem cell walls (Fig. 6H, I), where pectins are known to be abundant. It also reacted positively with the phloem contents of roots exposed to Al (Fig. 6I). This corroborates the Al localization in phloem cells of *leaves* of Al-accumulating plants (Haridasan et al., 1986; Tolrà et al., 2011). We did not note positive reaction between CAS and xylem cell *walls* (where lignin is more important than pectin), but we observed thick xylem cell wall, characterizing them as fibrous vessels (Fig. 6H) when compared to those from plants not exposed to Al (Fig. 6G). In cowpea, the first 2–3 mm of the primary root is completely recovered from Al stress after 24 h, except for the already damaged cell roots (Blamey et al., 2011). That is, once Al is bound (to the primary cell wall, rich in pectins) during the process of cell development, it is unlikely to be released (Rangel et al., 2009). Therefore, it is possible that the xylem of 'Rangpur' lime plants became fibrous during cell development in response to the presence of Al, and this might have affected water uptake/transport through these vessels, as indirectly evidenced by the low *gs* (Fig. 2B) and Ψ_{md} (Fig. 7).

One could argue that CAS staining the lumen of the xylem vessels of plants exposed to Al (Fig. 6H, I) could be due to an insufficient washing after staining, since this dye is dependent on pectin-bound Al (Wehr et al., 2010). It is uncertain that insufficient washing has occurred because cuts were immersed in distilled water for 15 min three times. On the other hand, we have no further evidence, such as X-ray spectra using scanning electron microscope (Bressan et al., 2016), that Al was *somehow* present in the lumen of these vessels. However, this does not affect the main purpose of our study. We show that low mesophyll hydration is more important than a photochemical limitation. This limited hydration suggests reduction in water uptake/transport in the roots. Despite showing an indirect evidence of such reduction (low *gs* and Ψw), it is assumed that Al-induced callose deposition in the symplast inhibits not only the cell-to-cell flow through plasmodesmata but also the apoplastic bypass flow in root cortical cell walls (Horst et al., 2010). Therefore, further investigation of root water flow and xylem thickening over time of Al exposure merits attention.

In this study, we observed structural modifications to the pericycle and cambial cells, as well as fibrous xylem vessels, suggesting that Al "invaded" the stele, resulting in reduction in water uptake/transport in the roots, as indirectly evidenced by the low *gs* and Ψw after a 45-day Al treatment. Therefore, in 'Rangpur' lime plants, the Al-induced decrease in *A* seems to be an indirect (long-distance) effect of Al that is mostly retained in the roots, with limited involvement of photochemical parameters.

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