
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA VEGETAL)**

**O ALUMÍNIO INTERFERE NA MOVIMENTAÇÃO ESTOMÁTICA DE
LIMOEIRO ‘CRAVO’ EM RESPOSTA À VARIAÇÃO DO DÉFICIT DE
PRESSÃO DE VAPOR**

GISELLE SCHWAB SILVA

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências biológicas (Biologia Vegetal).

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“O que sabemos é uma gota; o que ignoramos é um oceano.”

- Isaac Newton

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O ALUMÍNIO INTERFERE NA MOVIMENTAÇÃO ESTOMÁTICA DE LIMOEIRO ‘CRAVO’ EM RESPOSTA À VARIAÇÃO DO DÉFICIT DE PRESSÃO DE VAPOR?

RESUMO

O primeiro sintoma de toxicidade ao alumínio (Al) é a inibição do crescimento da raiz, que indiretamente as expõe a uma baixa disponibilidade de água, reduzindo, dessa forma, a hidratação do mesofilo e a condutância estomática (g_s). A abertura estomática geralmente aumenta em resposta à baixo déficit de pressão de vapor (DPV) e diminui em elevado DPV. No presente trabalho, plantas de *Citrus limonia* (limoeiro ‘Cravo’) foram cultivadas em solução nutritiva com 1480 μM de Al por 90 dias, para testar a hipótese de que presença de Al impede que g_s responda ao DPV. Como esperado, g_s não respondeu ao aumento no DPV em plantas expostas ao Al. Além disso, o Al também reduziu o conteúdo relativo de água e o potencial de água “midday” (Ψ_{md}) aos 60 e 90 dias após o plantio. A assimilação de CO_2 (A) seguiu o mesmo padrão de resposta observado em g_s , enquanto que as respostas fotoquímicas foram ligeiramente reduzidas em plantas expostas ao Al, indicando que diminuições em A induzidas pelo Al foi dependente de g_s e menos atribuída a baixa performance fotoquímica. Como em condições de seca, longo tempo de exposição ao Al reduz a hidratação foliar comprometendo g_s de responder a atmosfera, consequentemente prejudicando A em plantas de limoeiro ‘Cravo’.

Palavras-chave: Trocas gasosas; Hidratação foliar; efeitos do Al a longa distância; *Citrus limonia*.

ABSTRACT

Aluminum (Al) is toxic for most plants. Aluminum (Al) inhibits root growth, indirectly exposing the roots to low water availability, which decreases leaf hydration and, consequently, the stomatal conductance (g_s). Stomatal aperture generally increases in response to low vapor pressure deficit (VPD) and decreases at high VPD. In this study, *Citrus limonia* ('Rangpur' lime) was cultivated in nutrient solution with 1480 μM Al for 90 days, and we expected that the presence of Al could prevent g_s from responding to VPD. As expected, g_s did not respond to the increase in VPD in plants exposed to Al. Aluminum also reduced the relative water content and midday leaf water potential (Ψ_{md}) at 60 and 90 days after planting. The CO_2 assimilation rate (A) followed the same response pattern exhibited by g_s while photochemical responses were mildly reduced in plants exposed to Al, indicating that the Al-induced decrease in A was dependent on g_s and less ascribed to low photochemical performance. Like in drought conditions, the long-term exposure to Al reduces leaf hydration and compromises g_s responses to the atmosphere, eventually impairing A in 'Rangpur' lime plants.

Keywords: Leaf gas exchange; Leaf hydration; Long-distance Al effects; *Citrus limonia*;

1. INTRODUÇÃO

O Alumínio (Al) é o terceiro elemento mais abundante da crosta terrestre, naturalmente, encontrado no solo nas formas não tóxicas de óxidos e aluminosilicatos (Vitorello et al, 2005). Em solos ácidos ($\text{pH} < 5.0$), o Al é solubilizado na forma Al^{3+} que é potencialmente tóxico para a maioria das plantas (Horst et al., 2010). Pelo fato de solos ácidos ocorrerem em aproximadamente 30 – 45% das terras livres de gelo no mundo, sendo que na América do Sul cerca de 60% dos solos têm pH abaixo de 5,0 (vonUexküll & Mutert, 1995), a toxicidade ao Al é um dos principais fatores limitantes à produtividade vegetal em solos ácidos (Kochian, 1995).

Um dos primeiros sintomas de toxicidade ao Al é a inibição do crescimento da raiz (Horst et al., 2010; Samac & Tesfaye, 2003), que pode ser detectado em poucas horas de exposição ao Al (Koppitke et al., 2008). Acredita-se que essa inibição ocorra devido à ligação do Al com as substâncias pécticas no apoplasto das células da raiz (Koppitke et al., 2015), que diminui a extensibilidade da parede celular (Ma et al., 2004) e, indiretamente, expõe as raízes à uma baixa disponibilidade de água.

O efeito deletério do Al nas raízes ocasiona efeitos indiretos, levando a reduções no crescimento da parte aérea. Dentre esses efeitos, o Al reduz a assimilação de CO_2 (A) em plantas de tangerineira ‘Cleopatra’ (*Citrus reshni* Hort. Ex Tanaka) (Chen et al., 2005), em cafeeiro (*Coffea arabica*) (Konrad et al., 2005) e em *Citrus grandis* (Jiang et al., 2008, 2009). Esses autores atribuem as reduções em A à baixa performance fotoquímica, apesar de reduções na condutância estomática (g_s) também serem observadas nesses estudos. Por outro lado, em plantas de limoeiro ‘Cravo’ (*Citrus limonia*) expostas ao Al, a redução pronunciada de g_s parece ser mais importante do que variações no desempenho fotoquímico para explicar reduções em A (Banhos et al., 2016). Esses autores também evidenciaram a presença de vasos de xilema fibrosos no estelo, sugerindo uma dificuldade intrínseca da raiz em absorver e transportar água para o mesofilo, causando, dessa forma, diminuições em g_s .

A condutância estomática responde à alterações que ocorrem no ambiente, tais como mudanças na concentração de CO_2 , luminosidade, temperatura do ar, umidade relativa, disponibilidade de água, etc. (Farquhar and Sharkey, 1982; Chaves et al., 2016). A disponibilidade de água no solo afeta a hidratação do mesofilo, de maneira que baixo potencial da água no solo (Ψ_{solo}), pode ocasionar diminuição na hidratação do mesofilo e, conseqüentemente, reduzir g_s (Schulze, 1986; Chaves et al., 2002). Além disso, a água chega ao mesofilo proveniente das raízes para substituir a água perdida para a atmosfera que,

dependendo da temperatura do ar e da umidade, pode ter diferentes déficits de pressão de vapor (DPV). O DPV também é um fator que exerce influência em g_s para uma grande variedade de espécies (Lange et al., 1971; Habermann et al., 2003; McAdam and Brodribb, 2015), levando à diminuição em g_s quando as folhas são expostas ao ar seco (DPV elevado) e aumentando g_s quando as folhas são expostas a uma atmosfera com maior umidade relativa do ar (baixo DPV). Embora os mecanismos por trás dessas respostas não sejam ainda bem estabelecidos, acredita-se que os estômatos se fechem passivamente devido a uma baixa concentração de água no mesofilo, possivelmente induzido pela demanda evaporativa (Lange et al., 1971; Mott and Peak, 2013).

Plantas de *Citrus* são cultivadas em regiões temperadas, tropicais e subtropicais do mundo, crescendo principalmente em solos ácidos ricos em Al. O limoeiro ‘Cravo’ (*Citrus limonia*) é um importante porta-enxerto muito utilizado na citricultura brasileira e paulista não irrigada (Santos et al., 1999; Pereira et al., 2000). Isso porque possui sistema radicular profundo, o que garante resistência à seca (Magalhães Filho et al., 2008). Portanto, a presença de Al no solo pode causar um grande impacto nas relações hídricas dessas plantas, com implicações em suas taxas de trocas gasosas e em seu crescimento. Dessa maneira, no presente trabalho, investigamos a sensibilidade dos estômatos de plantas de limoeiro ‘Cravo’ cultivadas em solução nutritiva com 1480 μM Al à mudança no DPV. Para isso, modificamos o DPV de 1.5 kPa para 2.5 kPa na câmara de medidas de um medidor de trocas gasosas, para testar a hipótese de que a presença de Al na solução nutritiva impede g_s de responder ao DPV.

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Capítulo único:

Aluminum prevents stomatal conductance from responding to vapor pressure deficit in

***Citrus limonia*¹**

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Abstract

Stomatal aperture generally increases in response to low vapor pressure deficit (VPD) and decreases at high VPD. Aluminum (Al) inhibits root growth, indirectly exposing the roots to low water availability, which decreases leaf hydration and, consequently, the stomatal conductance (g_s). In this study, *Citrus limonia* ('Rangpur' lime) was cultivated in nutrient solution with 1480 μM Al for 90 days, and we expected that the presence of Al could prevent g_s from responding to VPD. As expected, g_s did not respond to the increase in VPD in plants exposed to Al. Aluminum also reduced the relative water content and midday leaf water potential (Ψ_{md}) at 60 and 90 days after planting. The CO_2 assimilation rate (A) followed the same response pattern exhibited by g_s while photochemical responses were mildly reduced in plants exposed to Al, indicating that the Al-induced decrease in A was dependent on g_s and less ascribed to low photochemical performance. Like in drought conditions, the long-term exposure to Al reduces leaf hydration and compromises g_s responses to the atmosphere, eventually impairing A in 'Rangpur' lime plants.

Keywords: Indirect Al effect; Leaf gas exchange; Leaf hydration; 'Rangpur' lime;

Introduction

Stomatal aperture allows leaf CO₂ assimilation while restricting water vapor loss to the atmosphere through transpiration. In well-hydrated plants, stomatal conductance (g_s) exerts control over transpiration rate (E) maintaining leaf at optimal temperature for photosynthesis and also preventing heat damage under excessive irradiance (Schulze, 1986; Chaves et al., 2016). Then, a well-hydrated mesophyll is critical for providing turgor pressure for stomatal movement and fully control of E .

Stomatal conductance varies continuously according to changes in the environment, such as atmospheric CO₂ concentration, sunlight, air temperature, air relative humidity, wind, etc (Farquhar and Sharkey, 1982; Chaves et al., 2016). Soil water content directly affects leaf hydration, so that low soil water potential (Ψ_{soil}), under drought conditions for example, may reflect in low leaf hydration and, consequently, low g_s (Schulze, 1986; Chaves et al., 2002). In addition, water reaches the mesophyll coming from the roots to replace water lost to the atmosphere that depending on air temperature and humidity may have different vapor pressure deficits (VPD). VPD also influences g_s , so that stomatal aperture decreases when exposed to drier air (high VPD) and increases at high air humidity (low VPD) for a wide range of species (Lange et al., 1971; Habermann et al., 2003; McAdam and Brodrribb, 2015). Although the mechanisms for these responses are not well known, it is believed that stomata may passively close due to a low water content in the mesophyll possibly induced by the evaporative demand (Lange et al., 1971; Mott and Peak, 2013); another hypothesis involves the abscisic acid (ABA) (Xie et al., 2006; Bauer et al., 2013), a plant hormone known to cause stomatal closure (Kriedemann et al., 1972; Merilo et al., 2015). Therefore, any factor affecting the leaf hydration may also influence g_s , which in turn may stop responding to the VPD.

Among environmental factors, drought is directly associated with low g_s (Farquhar and Sharkey, 1982; Chaves et al., 2002), and drought may induce root growth so as to reach

water in deep soil layers (Sharp et al., 2004; Yang et al., 2013). In contrast to drought, aluminum decreases root growth and development (Horst et al., 2010; Yang et al., 2013), indirectly exposing the roots to low water availability. Aluminum is the third most abundant element in the Earth's crust and in acidic soils (pH < 5.0), which composes 30-45% of the world's ice-free land (von Uexküll and Mutert, 1995) it is hydrolysed to its toxic form (Al³⁺). Mostly retained in the roots (Vitorello et al., 2005; Horst et al., 2010), Al binds to the pectic net in the apoplast of root cells (Kopittke et al., 2015), primarily stunting the root growth. Despite causing direct effects on the root system, Al also causes 40% lower CO₂ assimilation rate (*A*) in 'Cleopatra' tangerine (*Citrus reshni* Hort. ex. Tanaka) (Chen et al., 2005), 50% lower *A* in coffee plants (*Coffea arabica*) (Konrad et al., 2005), and 60-70% lower *A* in *Citrus grandis* (Jiang et al., 2008, 2009). These studies attribute the Al-induced decrease in *A* to low photochemical performance rather than low *g_s*. However, in these same studies *g_s* was also significantly reduced in the presence of Al. In addition, for 'Rangpur' lime (*Citrus limonia*), Al-induced decrease in *A* is better explained by the low *g_s* rather than low photochemical responses (Banhos et al., 2016). These authors have also evidenced fibrous xylem vessels in the stele associated with low water uptake/transport causing low *g_s* and leaf hydration. Al-induced decrease in water transport was also evidenced in barley (Ahmed et al., 2016). Then, as plants exposed to Al develop still unknown mechanisms for impairments to water uptake, Al is associated with low leaf hydration and it could prevent *g_s* from responding to VPD.

Here we chose 'Rangpur' lime plants to test the hypothesis that the presence of Al in nutrient solution prevents *g_s* from responding to VPD. 'Rangpur' lime is an important rootstock largely used in rain-fed *Citrus* plantations in subtropical areas of the Americas due to its high drought resistance (Magalhaes Filho et al., 2008); therefore Al could cause a great impact on its water relations and implications to leaf gas exchange and plant growth. To test this hypothesis, we measured leaf gas exchange rates, including *g_s*, and photochemical

parameters measured at 1.5 and 2.5 kPa of VPD, as well as leaf relative water content, predawn and midday leaf water potential and plant biomass in a 90-day study exposing plants to 0 and 1480 μM Al.

Material and Methods

Plant material and experimental conditions

We used three-month-old and 15 ± 0.5 cm-high ‘Rangpur’ lime plants (*Citrus limonia*) that were obtained from a commercial *Citrus* nursery (Sanicitrus, Araras, SP, Brazil). After rinsing the roots of these plants under tap water, the plants were transferred to a hydroponic system (aerated solution) and grew directly on a nutrient solution inside opaque plastic boxes (50 cm in length \times 30 cm in width \times 15 cm in height; 20 L) for 90 days.

The nutrient solution shows a chemical composition based on the solution proposed by [Clark \(1975\)](#), and it has been already used to test Al resistance in ‘Rangpur’ lime plants ([Banhos et al., 2016](#)). It consisted of 1372.8 μM $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$, 507 μM NH_4NO_3 , 224.4 μM KCl, 227.2 μM K_2SO_4 , 218.6 μM KNO_3 , 483.2 μM $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 12.9 μM KH_2PO_4 , 26.01 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 23.8 μM NaEDTA, 3.5 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 9.9 μM H_3BO_3 , 0.9 μM $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.2 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 μM $\text{NaMoO}_2 \cdot 2 \text{H}_2\text{O}$. In a previous study ([Banhos et al., 2016](#)), we observed that 1480 μM Al causes Al-induced decrease in gas exchange rates in ‘Rangpur’ lime plants after 45 days. Then, the solution contained 0 and 1480 μM Al provided through 1479.5 μM $\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$. The pH of the solution was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible, and the solution was totally replaced every 15 days. The nominal chemical composition of this solution was also tested on Geochem-EZ software ([Shaff et al., 2010](#)), resulting in more than 85% free Al^{3+} available. Samples of the nutrient solution were also collected ($n = 3$) every 15 days and sent to the routine plant nutrition lab at University of São Paulo (USP, Esalq, Piracicaba), where Al was measured in

the solutions using the colorimetric method (Sarruge and Haag, 1974), and nominal 1480 μM Al resulted in $1064 \pm 26 \mu\text{M}$ Al.

Expanded polystyrene (Isopor®) 50×30 cm plates (2-cm thick), with six equidistant 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 the plant collar. The boxes were kept on benches, maintained inside a greenhouse, under semi-controlled conditions ($891.7 \pm 167.4 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; approximately 14 h of natural photoperiod). The mean air temperature and vapor pressure deficit (VPD) were measured between 9:00 and 11:30 h (to follow the same daily time course used for measuring leaf gas exchange and chlorophyll fluorescence), using an aspirated psychrometer (ModelSK-RHG, Sato, Tokyo, Japan), and these values were $24.2 \pm 0.7^\circ\text{C}$ and 1.64 ± 0.39 kPa, respectively.

Experimental design

In plants cultivated in nutrient solution with 0 and 1480 μM Al, we measured leaf gas exchange and chlorophyll fluorescence under 1.5 and 2.5 kPa of VPD inside the leaf cuvette of a gas exchange system, at 1, 7, 15, 30, 45, 60 and 90 days after planting (DAP). On these same evaluation dates, we used leaves to measure the relative water content (RWC) and water potential at predawn (Ψ_{pd}) and midday (Ψ_{md}). At the beginning (0 DAP) and the end of the study (90 DAP), we measured the number of leaves, leaf area, shoot length, root length, biomass of root, shoot, leaves, and total biomass, as well as Al contents in roots, shoots and leaves. In addition, at 90 DAP, A/C_i curves were assessed in leaves of plants exposed and not exposed to Al measured at 1.5 and 2.5 kPa in order to estimate the apparent carboxylation efficiency.

Photosynthetic parameters

CO₂ assimilation (*A*), transpiration (*E*) rates, stomatal conductance (*g_s*), and intercellular CO₂ (*C_i*) were measured with an open portable gas exchange system (LI-6400xt, LI-COR, Lincoln, NE, USA). The water use efficiency (WUE; *A/E*) and intrinsic water use efficiency (IWUE; *A/g_s*) were also calculated. The CO₂ concentration entering the leaf cuvette (LCF chamber; 2 cm², LI-COR) averaged 400 μmol mol⁻¹, as provided by the 6400-01 CO₂ mixer (LI-COR). Measurements were taken between 9:00 and 11:30 h (Banhos et al., 2016) on cloudless days. The photosynthetic photon flux density (PPFD) was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to provide 1500 μmol photons m⁻² s⁻¹ in the leaf cuvette, as this value saturates *A* for *Citrus limonia* as observed in *A/C_i* curves (see below), and as also performed by Banhos et al. (2016).

The incoming air (500 μmol s⁻¹) to the LI-6400xt was provided by a dew point generator (LI-610, LI-COR), which had a known temperature and 100% relative humidity. The air temperature in the leaf cuvette was the same of that in the external ambient (greenhouse), and with these values and based on psychometric equations it was possible to calculate and set the air temperature produced by the dew point generator, so that 1.5 and 2.5 kPa of VPD was reached inside the leaf cuvette, according to Habermann et al. (2003).

Chlorophyll *a* fluorescence was measured with a portable modulated fluorometer (6400-40 LCF, LI-COR), which was integrated into the LI-6400xt gas exchange system. The saturating light pulse was approximately 7000 μmol m⁻² s⁻¹ during 0.7 s. The light fraction used for photosystem II (PSII) in photochemistry [**P** = ((*F'_v* - *F_s*) / *F'_m*)], heat dissipation in the antenna [**D** = 1 - (*F'_v* / *F'_m*)] and heat dissipation in reaction centers [**E** = (1 - *qP*) (*F'_v* / *F'_m*)] was calculated according to Demming-Adams (1996). For these calculations, *F'_v* is the variable fluorescence between the maximal (*F'_m*) and minimal (*F'_o*) fluorescence from light-adapted leaf and *F_s* is the steady state fluorescence from light-adapted leaf. The

photochemical quenching (qP) was calculated as $[qP = (F'_m - F_s)/(F'_m - F'_o)]$, according to [Baker \(2008\)](#). The effective quantum yield of PSII $[\Phi_{PSII} = (F'_m - F_s)/F'_m]$, and the apparent electron transport rate $[ETR = \Phi_{PSII} \times PPFD \times 0.5 \times 0.84]$ were also calculated.

A/Ci curves were performed under 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 25°C, at 1.5 and 2.5 kPa in the leaf cuvette. The CO₂ concentrations inside the leaf chamber (reference chamber) were set in the following sequence to compose the *A/Ci* curves: 400, 300, 200, 100, 50, 25, 400, 600, 800, 1000 and 1200 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air, according to [Kissmann et al \(2014\)](#).

Leaf water potential and relative water content

Leaf water potential was measured using four leaves per replicate (plant) at predawn (Ψ_{pd}) and midday (Ψ_{md} ; under maximum VPD), and values in MPa were obtained by the pressure chamber method ([Turner, 1981](#)), using a DIK-7000 (Daiki Rika Kogyo, Tokyo, Japan) chamber.

The relative leaf water content (RWC) was assessed using six leaf discs per replicate (plant). It was calculated as $RWC (\%) = [(FW - DW)/(TW - DW)] \times 100$, where FW is the fresh weight; TW is the turgid weight after rehydrating samples for 24 h in 100 mL deionized water; and DW is the dry weight after oven-drying the discs at 60°C for 48 h, according to [Habermann et al. \(2011\)](#).

Biometric parameters

After separating the plant into leaves (plus petioles), shoots and roots, the number of leaves was counted and the shoot and root lengths were measured with a ruler (cm). The leaf area per plant was measured with an area meter (LI-3100C, LI-COR). Leaf, shoot and root samples were oven-dried at 60°C until constant mass, and biomass (g) was measured using a scale.

Aluminum concentration in plant organs

Dried samples of leaves, shoots and roots were sent to a routine Plant Nutrition laboratory at University of São Paulo (Esalq, USP, Piracicaba) where they were ground and digested in a solution of sulfuric:nitric:perchloric acids (1:10:2, v/v/v). After digestion, Al concentrations were determined by the atomic absorption spectrophotometer method (Sarruge and Haag 1974) and expressed as mg Al per kg dry mass.

Data analysis

The study was conducted with six plants per treatment. A two-way analysis of variance (two-way ANOVA) was conducted using the VPD factor (1.5 and 2.5 kPa) and the Al factor (0 and 1480 μM Al) in order to test A , E , g_s , C_i , WUE, IWUE, **P**, **D**, **E**, ΦPSII , qP and ETR separately, for each evaluation date and the estimation of carboxylation efficiency at 90 DAP (angular coefficient of A/C_i curves). A one-way ANOVA was performed between plants exposed to 0 and 1480 μM Al to test differences in the number of leaves, leaf area, shoot length, root length, biomass of root, shoot, leaves, and total biomass as well as Al concentration in plant organs at 0 and 90 DAP. The Tukey test ($P < 0.05$) was used to conduct post hoc comparisons to determine the least significant difference between mean results. In addition, a Student t-test ($\alpha = 0.05$) was used to test differences in RWC, Ψ_{pd} , Ψ_{md} at every evaluation date.

Results

Leaf gas exchange rates, mainly A , g_s and E were reduced ($P < 0.05$) in plants exposed to Al (Fig. 1). In plants not exposed to Al, as expected, A and g_s decreased under 2.5 kPa when compared with 1.5 kPa (Fig. 1A, C). The transpiration rate (E), however, was higher at

2.5 kPa in relation to 1.5 kPa (Fig. 1G), as well as C_i values that were higher at 2.5 kPa at 7, 15 and 30 DAP (Fig. 1E). In plants exposed to Al, g_s and A did not respond to the increase in VPD from 7 DAP until the end of the study (Fig. 1B, D). Transpiration rate and C_i , however, were higher at 2.5 kPa at 7, 15, 30 and 45 DAP (Fig. 1F, H).

At 90 DAP, A_{max} and the angular coefficient of A/C_i curves were higher in plants not exposed to Al in relation to those exposed to Al ($P = 0.004$) (Table 1; Fig. 2). Only when measured at 1.5 kPa was the angular coefficient of A/C_i curves higher in plants not exposed to Al in relation to those exposed to Al (Table 1). In addition, as C_i is related to the apparent carboxylation efficiency, it is important to note that, regardless of the VPD, this parameter was higher ($P < 0.05$) in plants exposed to Al in relation to those not exposed to Al, at 90 DAP (Fig. 1E, F).

Compared to plants not exposed to Al, regardless of the VPD, WUE was lower in plants exposed to Al at 15 ($P < 0.001$) 60 ($P = 0.030$) and 90 ($P = 0.016$) DAP (Fig. 3A, B), and IWUE was also reduced in plants exposed to Al at 7 ($P = 0.018$), 15 ($P = 0.007$) and 90 ($P < 0.001$) DAP (Fig. 3C, D). Considering that E responded to VPD in both Al treatments (Fig. 1G, H), WUE followed almost the same response pattern of leaf transpiration (Fig. 3A, B), while IWUE did not respond to VPD on most days for both Al treatments (Fig. 3C, D).

The fraction of absorbed light utilized in photochemistry (**P**) was lower in plants exposed to Al in relation to those not exposed to Al, at 15, 30, 60 and 90 DAP ($P < 0.001$). The heat dissipation in the antenna (**D**) increased in plants exposed to Al when compared to plants not exposed to Al, at 15 ($P = 0.019$), 30 ($P < 0.001$), 45 ($P < 0.001$) and 60 DAP ($P < 0.001$) (Fig. 4). In plants not exposed to Al, **P** was similar (except at 60 DAP) between 1.5 and 2.5 kPa ($P = 0.833, 0.222, 0.147, 0.281, 0.251$ and 0.419 at 1, 7, 15, 30, 45, and 90, respectively) while **D** increased with the amplification of VPD ($P = 0.008, 0.038, 0.007, 0.022, 0.042, < 0.001$ and 0.009 at 1, 7, 15, 30, 45, 60 and 90, respectively) (Fig. 4A, C).

However, in plants exposed to Al, **P** was reduced at 2.5 kPa in relation to 1.5 kPa but only at 1 ($P = 0.008$), 7 ($P = 0.041$) and 60 DAP ($P = 0.012$), and **D** increased under the highest VPD at 1 ($P < 0.001$) and 7 DAP ($P = 0.034$) (Fig. 4B, D).

The values of Φ PSII and ETR were lower in plants exposed to Al when compared to plants not exposed to Al, at 7, 15, 30, 60 and 90 DAP, while the presence of Al reduced qP only at 15 and 30 DAP. In both Al treatments, Φ PSII and ETR decreased at 2.5 kPa only at 1 and 60 DAP, and qP was similar between both VPD throughout the study (Supplementary material; Fig. S1).

In relation to plants exposed to Al, the RWC of plants not exposed to Al was 5% higher at 15 DAP, 15% higher at 30 and 45 DAP, and 20% higher at 60 and 90 DAP (Fig. 5). Predawn leaf water potential (Ψ_{pd}) was the same between Al treatments throughout the study (Fig. 6A). Midday leaf water potential (Ψ_{md}) of plants not exposed to Al was significantly higher than that of plants exposed to Al at 60 and 90 DAP, although opposite results occurred at 7 and 45 DAP (Fig. 6B).

The number of leaves, leaf area, shoot length, root length, biomass of root, shoot, leaves, and total biomass were higher in plants not exposed to Al in relation to those exposed to Al at 90 DAP (Fig. 7). In plants exposed to Al, shoot length was the only parameter that did not increase from 0 to 90 DAP (Fig. 7E).

At 90 DAP, Al accumulation was higher in the leaf (Fig. 8A), shoot (Fig. 8B), root (Fig. 8C), and in the whole plant (Fig. 8D) when cultivated in the solution containing 1480 μ M Al in relation to plants not exposed to Al. Notwithstanding, the roots of plants exposed to Al accumulated 10 times more Al than those of plants not exposed to Al at 90 DAP (Fig. 8C).

Discussion

In the present study, we confirmed that leaf hydration of plants exposed to Al is considerably reduced, and it led to a lack of response of g_s to the increase in VPD (Fig. 1C,D), which corroborates our hypothesis. Most studies on the effect of VPD on stomatal conductance (g_s) of unstressed plants have shown that g_s decreases with the increase in VPD (Monteith 1995; Habermann et al., 2003; Peak and Mott 2011; Aliniaiefard and Meeteren, 2013), which occurred in plants not exposed to Al and submitted to 2.5 kPa (Fig. 1C). Leaves are believed to possess a feedback on leaf water relations in order to stabilize leaf transpiration (E) (Sinclair and Allen, 1982), and an increase in VPD usually results in an increase in E with an adjustment in g_s in order to match water flux from roots into the leaf (Sinclair et al., 2017). In plants not exposed to Al, the increase in VPD led to an increase in E throughout the study (Fig. 1G), following the model proposed by most studies (Peak and Mott 2011; Sinclair et al., 2017). In plants exposed to Al, E was higher under 2.5 kPa when compared to 1.5 kPa, but only until 45 DAP, being unresponsive to the increase in VPD at 60 and 90 DAP (Fig. 1H). This matches the RWC and Ψ_{md} responses that were significantly reduced at 60 and 90 DAP. This indicates that water supply to the mesophyll was considerably compromised from 60 DAP.

Studies using 'Rangpur' lime plants (Pereira et al., 2000) and coffee plants (Konrad et al., 2005) reported lower values of WUE when these plants were exposed to 400 and 1480 μM Al, respectively. In the present study, IWUE followed the g_s response pattern, either for plants exposed or not exposed to Al (Fig. 3C, D). The WUE, however, was higher at 1.5 kPa than 2.5 kPa in plants not exposed to Al throughout the study (Fig. 3B). In plants exposed to Al, WUE became unresponsive to VPD at 60 and 90 DAP (Fig. 3B), as also occurred with E . This reinforces that in the presence of Al for more than 45 days the water supply to the mesophyll is significantly affected. Evidence of low leaf hydration in plants exposed to Al

was already observed, as Ψ_{md} of ‘Rangpur’ lime exposed to 1480 μM Al was 35% lower when compared to plants not exposed to Al (Banhos et al., 2016).

The CO_2 assimilation rate (A) followed the same response pattern exhibited by g_s . In plants not exposed to Al, A decreased in response to the increase in VPD, which has been already noted for unstressed plants (Habermann et al., 2003; Chaves et al., 2016). In plants exposed to Al, A was the same at 1.5 and 2.5 kPa (Fig. 1A,B). These results indicate that A was dependent on g_s in plants exposed (and not exposed) to Al. In studies using *Citrus* (Chen et al., 2005; Jiang et al., 2008, 2009) and coffee (Konrad et al., 2005) plants exposed to more than 1000 μM Al, low A and g_s values are also reported. However, these studies attribute the Al-induced decrease in A to the low photochemical performance rather than the low g_s . In plants exposed to Al, D was higher and P was lower when compared to those not exposed to Al, corroborating the above-mentioned studies, but D was the same between VPD, and ΦPSII and ETR showed differences between VPD only at 1 and 60 DAP while qP was the same between VPD throughout the study (Supplementary material; Fig S1). Therefore, these results suggest that low g_s and photochemical performance occur concomitantly in plants exposed to Al (being these parameters associated with the Al disturbance), or that low g_s could lead to low photochemical responses in these plants, since Al-induced decrease in g_s was more consistent than the Al-induced decrease in photochemical responses. Plants of *C. limonia* grown in 1480 μM Al do not show reduced photochemical responses until 45 days, while g_s is affected by Al within the same period (Banhos et al., 2016). Thus, it could also be a matter of time of Al exposure and/or a species-dependent response. For instance, studies attributing the Al-induced decrease in A to the low photochemical performance were conducted submitting ‘Cleopatra’ tangerine (*Citrus reshni* Hort. Ex. Tanaka) to Al, for 60 days (Chen et al., 2005), *Citrus grandis* for 150 days (Jiang et al., 2008), *C. grandis* for 126 days (Jiang et al., 2009), and coffee plants (*Coffea arabica*) for 97 days (Konrad et al., 2005).

At 90 DAP, C_i was higher in plants exposed to Al regardless of the VPD, suggesting an impairment of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme. In fact, the estimation of apparent carboxylation efficiency was higher in plants not exposed to Al in relation to those exposed to Al. Rubisco activity was also lower in rye exposed to 1110 and 1850 μM Al in relation to plants not exposed to Al (Silva et al., 2012), and in coffee plants A/C_i values were reduced in plants exposed to 1480 μM Al for 97 days (Konrad et al., 2005). However, in the present study, the reduction of the angular coefficient of A/C_i curve in plants exposed to Al did not occur at 2.5 kPa, indicating that stomatal responses may be, somehow, more important than biochemical responses, at 90 DAP. Notwithstanding, A/C_i curves were measured only at 90 DAP and accurate carboxylation efficiency measurements performed over the time of Al exposure merits further investigation in similar studies.

The present study reiterates that Al reduces leaf hydration in 'Rangpur' lime plants. As a novelty, we show that this effect leads to a lack of response of g_s to the increase in VPD. The possible causes of the Al-induced decrease in leaf hydration could be related to the impairment of water uptake by roots, where Al is mostly retained. For instance, 65% of the absorbed Al is immobilized in the roots of *Citrus grandis* and *C. sinensis* when exposed to 1200 μM Al (Yang et al., 2011) and 80% in the roots of *Citrus limonia* exposed to 1110 μM Al (Santos et al., 1999) and 1480 μM Al (Banhos et al., 2016). In the present study, 85% of the absorbed Al was retained in the roots (Fig. 8). Thus, the Al retained in the apoplast of root cells could impair water flux, as evidenced by a marked reduction in flow rate on addition of Al to solution flowing through a calcium pectate membrane in an *in vitro* system (Blamey et al., 1993), although these results are controversial (Horst et al., 2010). In fact, it has been identified that the first lesion of toxic Al is the binding of this metal to the pectic nets on walls of outer cells in the root system, reducing the root growth (Kopittke et al., 2015). Fibrous xylem vessels with structural modifications of the stele and reduced Ψ_{md} were already

reported in 'Rangpur' lime plants exposed to 1480 μM Al, suggesting a lack of functional capacity of the fibrous xylem vessels to transport water (Banhos et al., 2016). Therefore, sap flow rate measurements and gene expression of aquaporins are candidates for future investigations aiming at revealing the cause of reduced water uptake and transport, eventually explaining the low g_s and leaf hydration in plants exposed to Al.

One may still argue that the first and most conspicuous symptom of Al toxicity is the inhibition of the root growth (Koppittke et al., 2008; Horst et al., 2010), and that some studies (Vitorello et al., 2005; Kochian et al., 2015; Rengel et al., 2015) have supported that Al-induced decrease in root growth would lead to reduced water uptake, eventually explaining the Al-induced decrease in g_s . However, compared to plants not exposed to Al, plants exposed to 1480 μM Al showed reduced leaf number (-40%), leaf area (-75%), shoot length (-60%), leaf biomass (-57%) and shoot biomass (-60%) observed at 90 DAP (Fig. 7), therefore compensating for the decrease in their root length (-44%) and root biomass (-65%).

In the present study, we confirm that leaf hydration of plants exposed to Al is considerably reduced, and it leads to a lack of response of g_s to the increase in VPD. The reduced photochemical responses observed in plants exposed to Al were not as consistent as those related to stomatal control. In addition, the apparent carboxylation efficiency was reduced in plants exposed to Al, although under the highest VPD such biochemical reduction did not occur, reinforcing the importance of diffusive responses, which merits further investigation in plants exposed to Al.

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Table:

Table 1. Mean values ($n = 6$) \pm s.e. of the angular coefficient obtained from A/C_i curves to estimate the carboxylation efficiency of ‘Rangpur’ lime plants at 90 days after planting in nutrient solution containing 0 and 1480 μM Al, measured at 1.5 and 2.5 kPa of vapor pressure deficit.

Treatments		Estimation of carboxylation efficiency
0 μM Al	1.5 kPa	0.0425 ± 0.0061 a
	2.5 kPa	0.0316 ± 0.0038 ab
1480 μM Al	1.5 kPa	0.0282 ± 0.0014 b
	2.5 kPa	0.0198 ± 0.0010 b

Different letters indicate significant differences by the Tukey test ($P < 0.05$) between treatments

Figure captions:

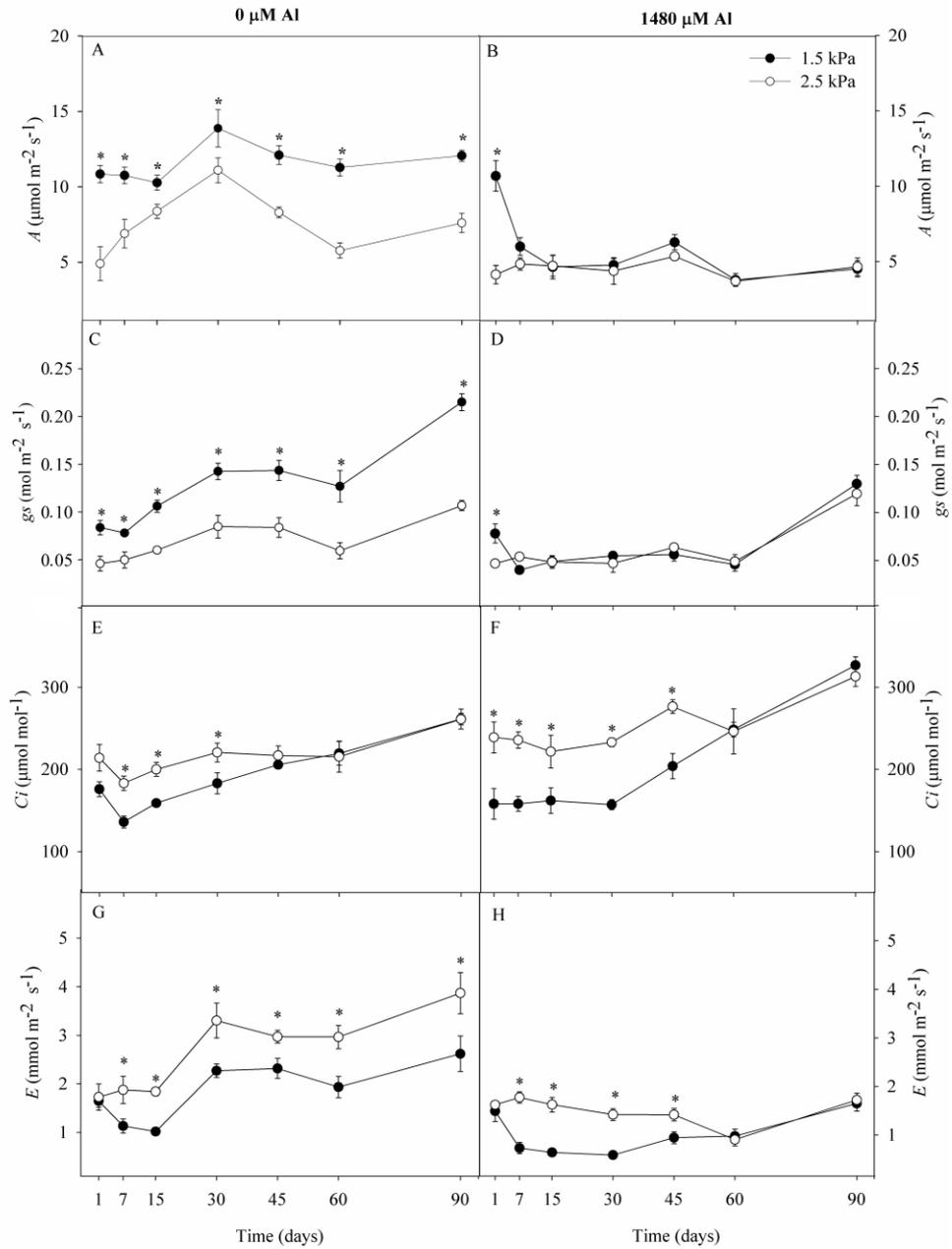


Fig 1. Leaf gas exchange of ‘Rangpur’ lime plants, cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al, measured at 1.5 and 2.5 kPa of VPD. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between mean values ($n = 6$ plants) measured at 1.5 and 2.5 kPa of VPD. Bars are s.e.

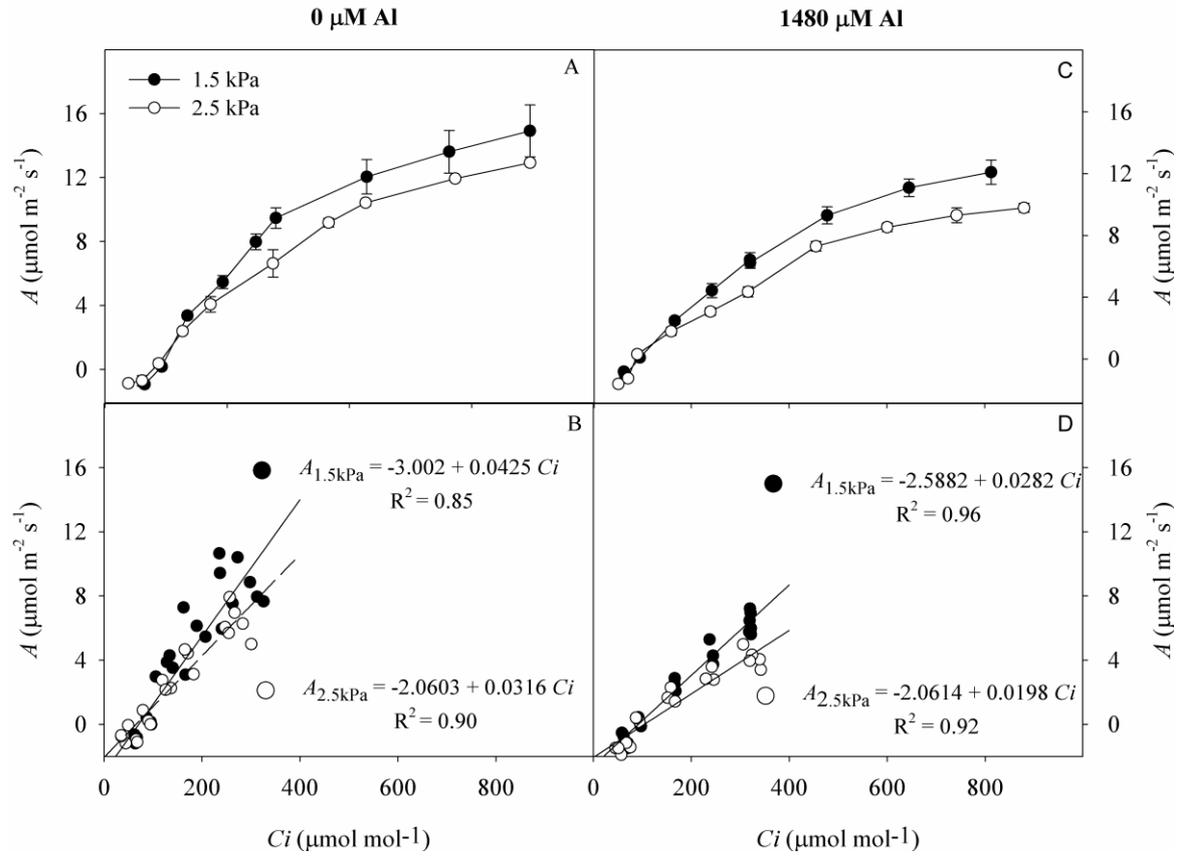


Fig 2. CO₂ assimilation rate (*A*) in response to internal leaf CO₂ concentration (*C_i*) (*A/C_i* curves) (A, C) and estimation of the apparent carboxylation efficiency from *A/C_i* curves (B, D) of 'Rangpur' lime plants at 90 DAP cultivated in nutrient solution containing 0 and 1480 μM Al and measured at 1.5 and 2.5 kPa of VPD. Bars are s.e.

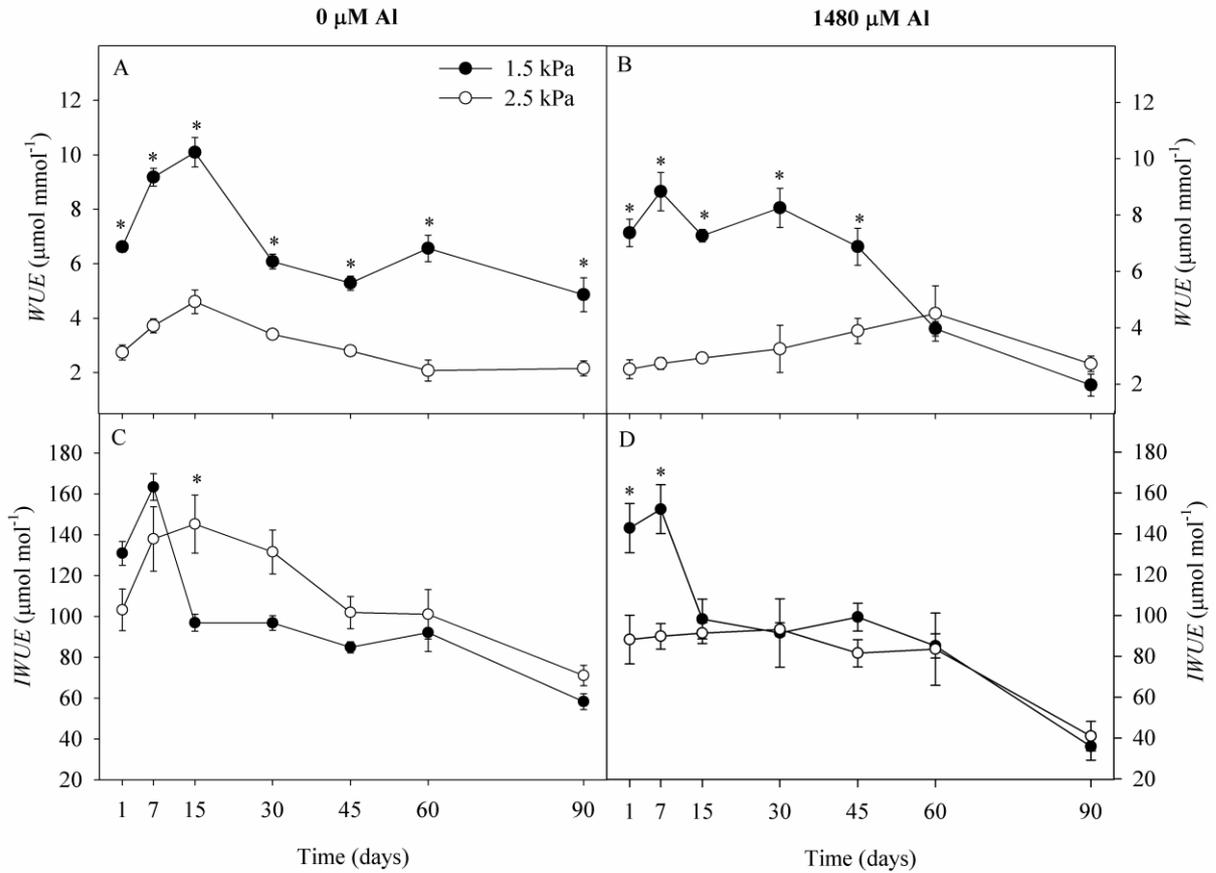


Fig 3. Water use efficiency (*WUE*) and intrinsic water efficiency (*IWUE*) of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al measured at 1.5 and 2.5 kPa of VPD. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between mean values ($n = 6$ plants) measured at 1.5 and 2.5 kPa of VPD. Bars are s.e.

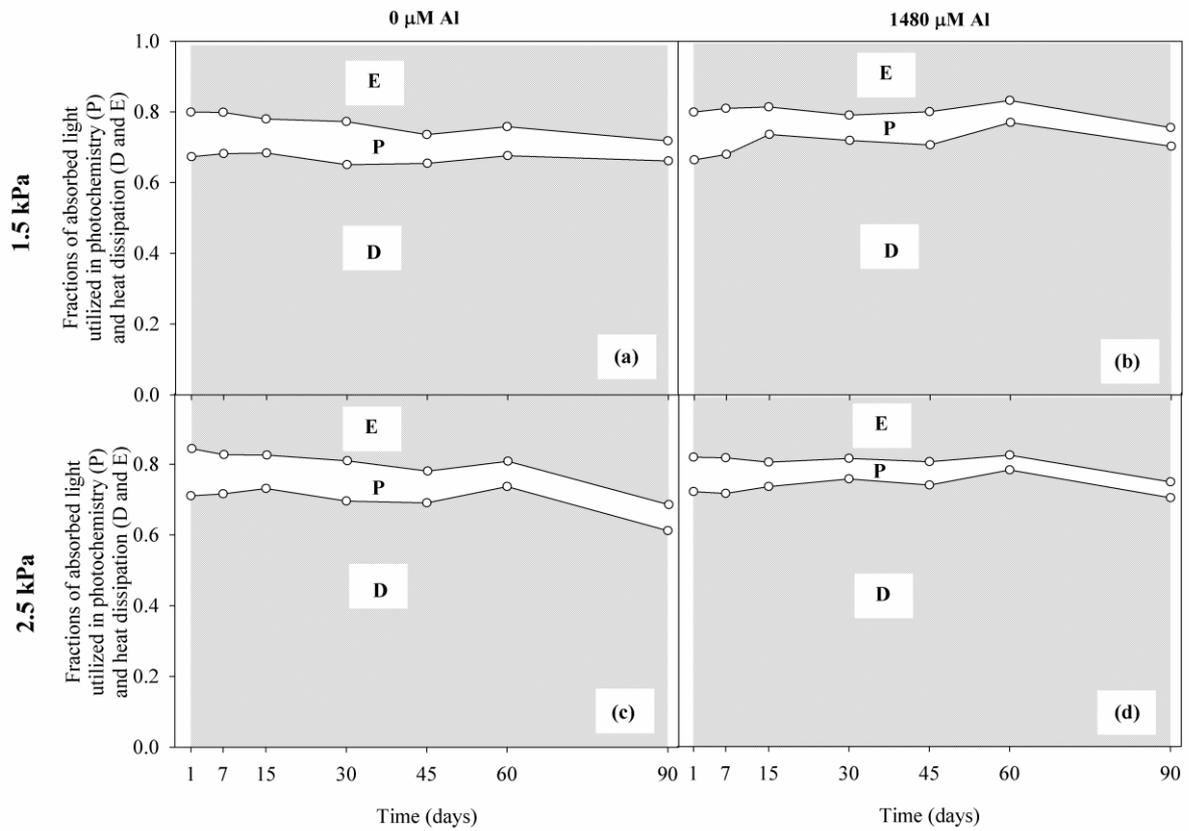


Fig 4. Variations in fractions of absorbed light utilized in photochemistry (**P**), heat dissipation in the antenna (**D**) and in reaction centers (**E**) of PSII of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al measured at 1.5 (A, B) and 2.5 kPa (C, D) of VPD.

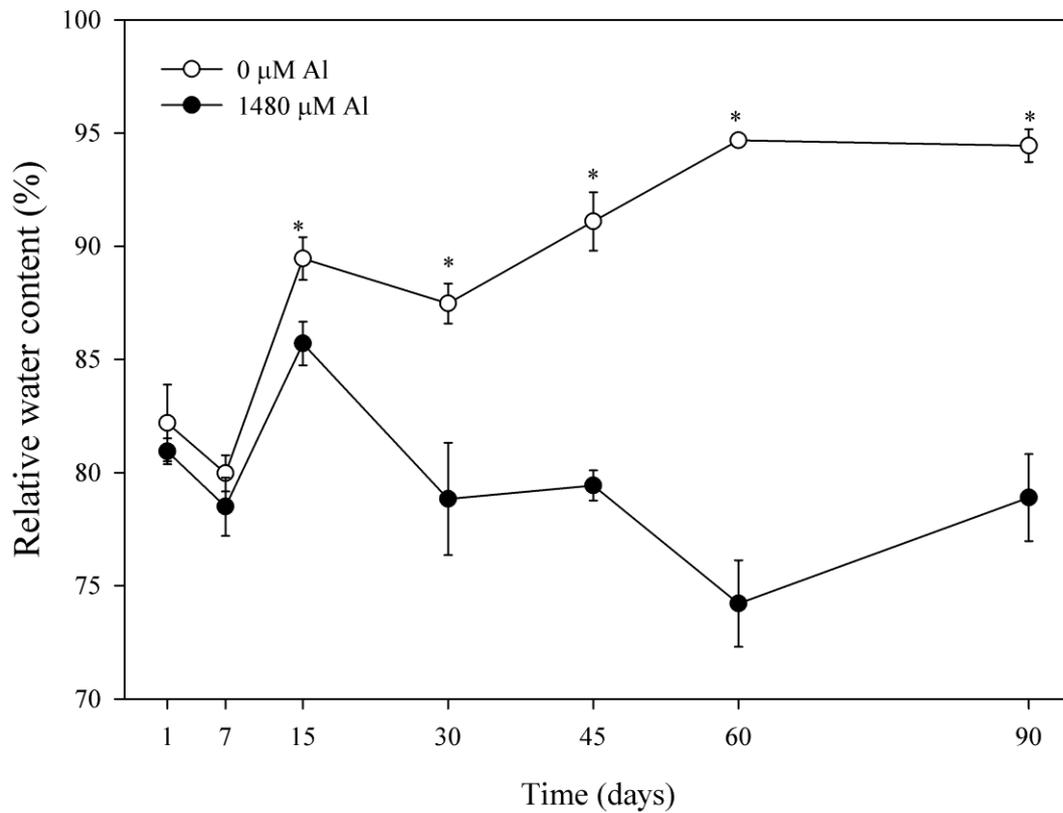


Fig 5. Relative water content of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al. For each evaluation date, asterisks indicate significant difference by the Student t-test ($\alpha = 0.05$) between mean values ($n = 6$ plants) of Al treatments. Bars are s.e.

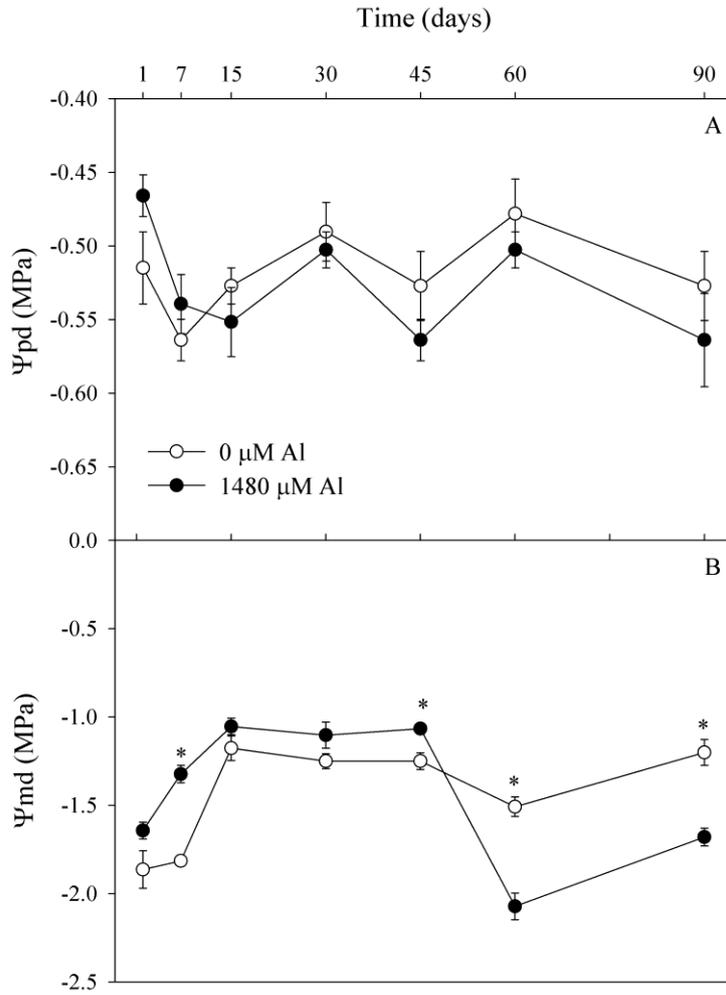


Fig 6. Leaf water potential at predawn (Ψ_{pd}) (A) and midday (Ψ_{md}) (B) of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 $\mu\text{M Al}$. For each evaluation date, asterisks indicate significant difference by the Student t-test ($\alpha = 0.05$) between mean values ($n = 6$ plants) of Al treatments. Bars are s.e.

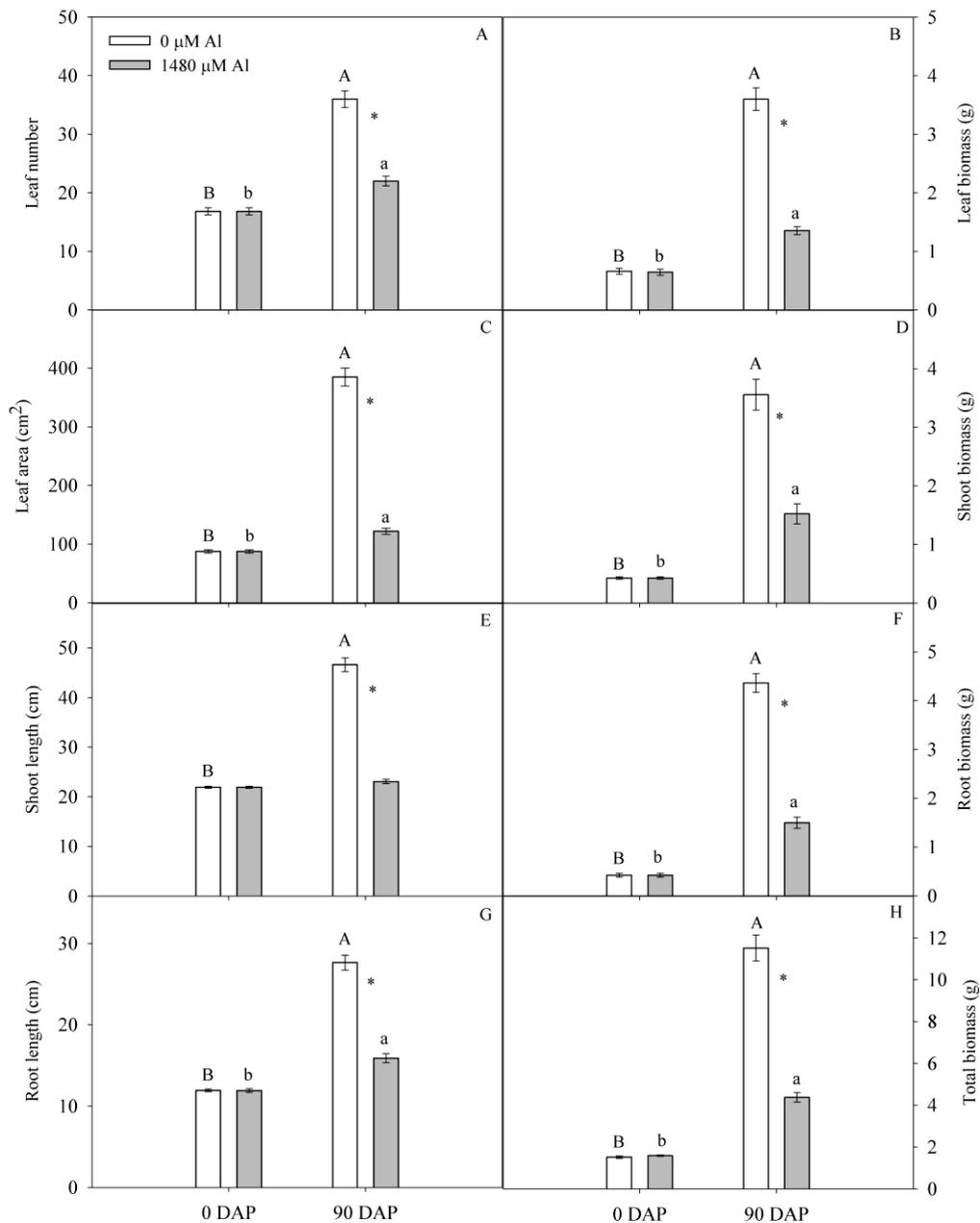


Fig 7. Biometric parameters of organs of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 μM Al. For plants not exposed to Al, distinct uppercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP; for plants exposed to Al, distinct lowercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Columns are mean values ($n = 6$ plants) and bars are s.e.

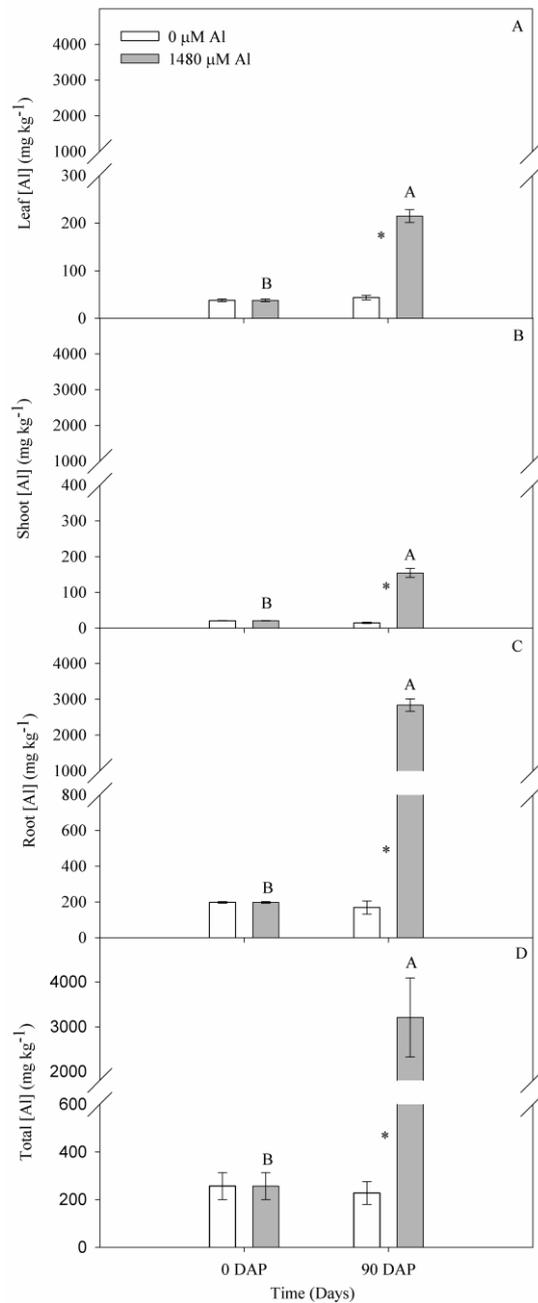


Fig 8. Aluminum concentration in leaves (A), shoots (B), roots (C) and in the whole plant (D) of 'Rangpur' lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 $\mu\text{M Al}$. Asterisks represent significant differences ($P < 0.05$) between 0 and 1480 $\mu\text{M Al}$. For each treatment, distinct letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Columns are mean values ($n = 6$ plants) and bars are s.e.

Supplementary material

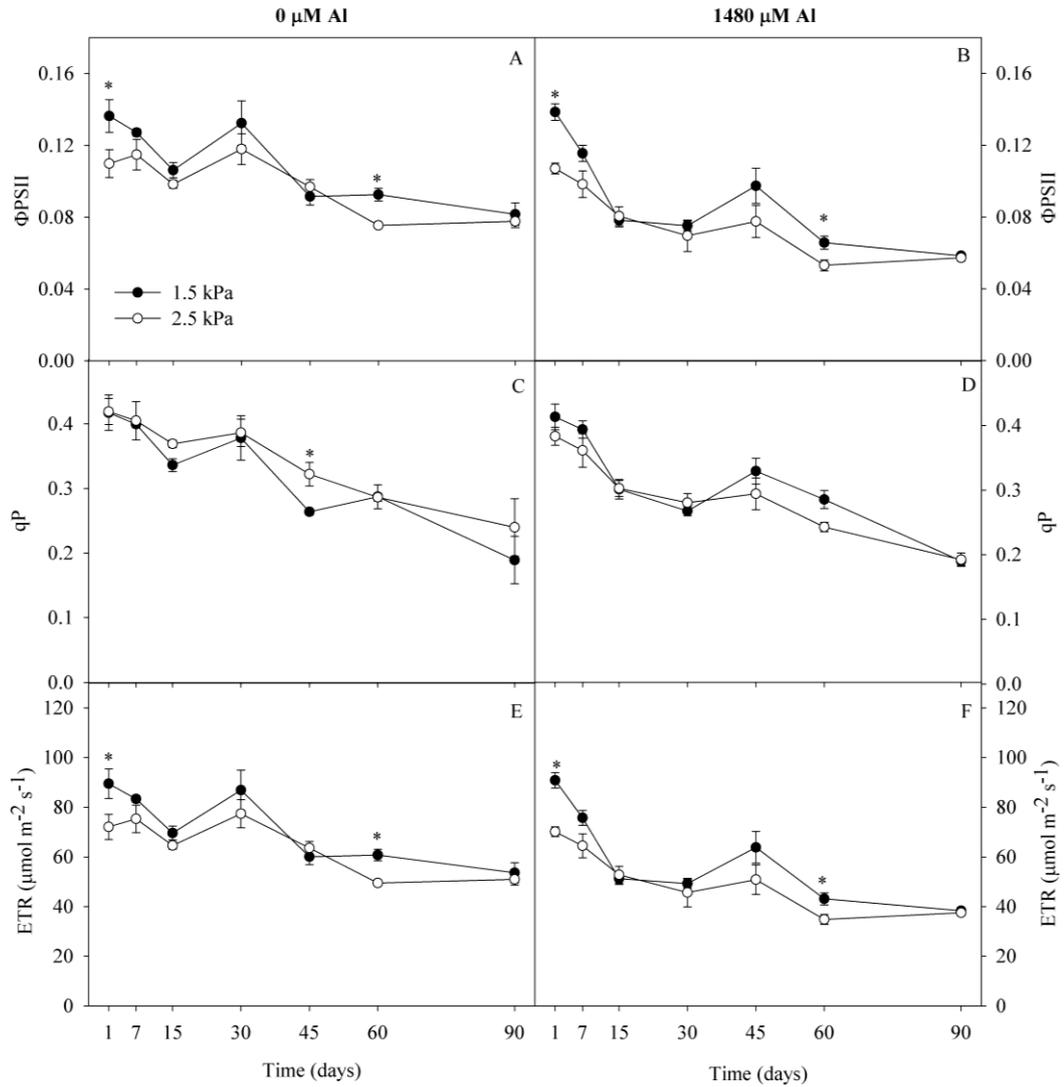


Fig S1. Photochemical parameters in leaves of ‘Rangpur’ lime plants cultivated for 90 days in nutrient solution containing 0 and 1480 μM Al, measured at 1.5 and 2.5 kPa of VPD. Asterisks indicate significant differences ($P < 0.05$) between mean values ($n = 6$ plants) measured at 1.5 and 2.5 kPa of VPD. Bars are s.e.