UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

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

INTERFERÊNCIA DO ALUMÍNIO NA HIDRATAÇÃO DA PARTE AÉREA EM *Citrus x limonia* E NO DESEMPENHO FOTOSSINTÉTICO EM *Ilex paraguariensis* e *Qualea grandiflora*

GISELLE SCHWAB SILVA

Rio Claro – SP
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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ciências Biológicas (Biologia vegetal)

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TÍTULO DA TESE: **Interferência do aluminio na hidratação da parte aérea em *Citrus x limonia* e no desempenho fotossintético em *Ilex paraguariensis* e *Qualea grandiflora***

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O que vale na vida não é o ponto de partida e sim a caminhada.

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SUMÁRIO

Resumo

Em espécies sensíveis ao Al, o primeiro sintoma de toxicidade a este elemento é a redução no crescimento da raiz. Esta inibição no crescimento tem sido associada a reduções na hidratação foliar, que por sua vez afeta negativamente a performance fotossintética, reduzindo a condutância estomática (*gs*). Em contrapartida, algumas plantas são capazes de crescer bem em solos com elevada disponibilidade de Al e algumas delas são capazes de acumular grandes quantidades de Al em seus tecidos (acumuladoras de Al), sem apresentar sintomas de toxicidade. No presente trabalho, para testar diferentes hipóteses em relação ao Al, utilizamos três espécies, uma sensível (*Citrus x limonia)*, uma tolerante (*Ilex paraguariensis*) e uma acumuladora de Al (*Qualea grandiflora*). Todos os experimentos foram realizados por 60 dias em solução nutritiva. Plantas de *C.* x *limonia* foram expostas a 0 e 1480 μM Al para avaliar se reduções na hidratação foliar ocorrem antes ou depois da inibição no crescimento da raiz. Aqui evidenciamos reduções em *gs* logo aos 3 dias de exposição ao Al e isto foi acompanhado de maior concentração do hormônio ácido abscísico (ABA) nas folhas. Reduções significativas no crescimento da raiz foram observadas aos 30 dias. Logo, reduções nos parâmetros hídricos e hidráulicos, em plantas expostas ao Al ocorreram bem antes da inibição no crescimento da raiz. Plantas de *I. paraguariensis* foram expostas a 0, 500, 1000 e 1500 μM Al para testar se o Al induz o crescimento com consequente melhora na performance fotossintética. Plantas expostas a 500 e 1000 μM Al apresentaram maior crescimento e biomassa em relação às plantas crescendo sem Al. A ausência de Al comprometeu o crescimento da raiz, reduziu a hidratação foliar e resultou em diminuição na performance fotossintética. Mudas de *Q. grandiflora* foram expostas a 0, 740 e 1480 μM Al a fim de verificar se o Al na solução nutritiva aumenta a assimilação de CO_2 devido a uma melhor eficiência aparente de carboxilação da enzima ribulose-1,5-bifosfato carboxilase-oxigenase (Rubisco). Neste experimento evidenciamos que plantas expostas aos dois tratamentos com Al apresentaram melhor desenvolvimento de raiz e maiores taxas de trocas gasosas devido a uma maior eficiência aparente de carboxilação da Rubisco. Porém essa maior eficiência da Rubisco está mais associada à melhor hidratação das folhas e trocas gasosas do que a algum efeito direto do Al sobre a performance da enzima. A ausência de Al tornou as raízes destas plantas necróticas, disfuncionais e, consequentemente, menor hidratação das folhas foi observada.

Palavras-chave: Al^{3+}; fluxo de seiva; trocas gasosas; relações hídricas; Rubisco;

Abstract

In sensitive plants, inhibition of root elongation is the first symptom of Al toxicity. This inhibition has been associated with low leaf hydration, with negative consequences for leaf gas exchange, including stomatal conductance (*gs*). On the other hand, some plants are able to grow well on acidic soils with high Al available, and some of this species accumulating high Al in their tissues (Al-accumulators), without showing toxicity symptoms. Therefore, in the present work, to test different hypotheses in relation to Al, we used tree different species, a sensitive to Al (*Citrus x limonia*), a tolerant to Al (*Ilex paraguariensis*) and an Al accumulator (*Qualea grandiflora)*. All the experiments were realized for 60 days in the nutrient solution. Plants of *C. limonia* were exposed to 0 and 1480 μM Al to evaluated whether low leaf hydration occur before or after the inhibition of root growth. Lower *gs* was observed after 3 days and was associated to a high concentration of abscisic acid (ABA) in leaves of plants exposed to Al. Root growth inhibition was evidenced at 30 days in plants exposed to Al and this occurred after hydric and hydraulic parameters are affect by Al. Plants of *I. paraguariensis* were exposed to 0, 500, 1000 and 1500 μM Al to test whether Al improves growth with consequently increases in the photosynthetic performance in this specie. Improvements in biometric and biomass parameters were observed for plants exposed to 500 and 1000 μM Al in relation to those not exposed to Al. The absence of Al affects the root growth, that consequently reduced the leaf hydration and photosynthetic performance. Seedling of *Q. grandiflora* were exposed to 0, 740 and 1480 μM Al to test whether the Al increase the carbon assimilation through enhanced apparent carboxylation efficiency of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco). Plants exposed to both Al treatments showed new white roots, increased root biomass and apparent carboxylation efficiency was higher in these plants. However, this higher efficiency is more associated with better leaf hydration and gas exchange rates instead the direct effect of Al in Rubisco performance. On the Other hand, plants not exposed to Al showed no root growth, necrotic roots, and low gas exchange rates with decreased apparent carboxylation efficiency.

Key words: Al^3; sap flow; gas exchange rates; water relations; Rubisco

INTRODUÇÃO GERAL

O alumínio (Al) é o terceiro elemento mais abundante da crosta terrestre (von Uexküll and Mutert, 1995) e ocorre naturalmente no solo nas formas não tóxicas de óxidos e aluminossilicatos. Entretanto, em solos ácidos (pH < 5.0) esses minerais são solubilizados e formas potencialmente tóxicas são liberadas para o solo (Singh et al., 2017). Dentre estas formas tóxicas, o $Al(OH)_6^{3+}$, também conhecido como Al^{3+}, é considerado a forma mais tóxica para a grande maioria das plantas (Kochian 1995; Horst et al., 2010). Uma vez que aproximadamente 30% das áreas livres de geleiras do mundo são constituídas de solos ácidos (von Uexküll and Mutert, 1995), o Al é um dos principais fatores abióticos que pode interferir no crescimento de plantas sensíveis e tolerantes ao redor do mundo.

Em plantas sensíveis, um dos primeiros e mais conhecidos sintomas de toxicidade ao Al é a rápida inibição no crescimento da raiz (Kopittke et al. 2008; Horst et al. 2010; Silva et al. 2019) que pode ser detectado em poucas horas de exposição ao Al (Koppittke et al., 2008). O Al pode se ligar a sítios do apoplasto e/ou simplasto das células da raiz (Kochian, 1995; Horst et al., 2010; Kopittke et al., 2015), levando ao enrijecimento celular e ruptura da epiderme (Koppittke et al., 2008) e, consequentemente, causando reduções no crescimento da raiz. Alguns estudos demonstram que o Al aumenta a biossíntese de auxina (ácido indolacético – AIA) nas pontas das raízes e, em relação à concentração de AIA na parte aérea, altas concentrações de AIA nas raízes pode prejudicar o crescimento da raiz (Ryan et al., 1993; Silva et al., 2019). Ainda, uma possível alteração do potencial de membrana (Ahn et al., 2002) e menor permeabilidade à água (Blamey et al., 1990; Horst et al., 2010) podem diminuir o potencial de pressão hidrostático (Ψp) e causar menor expansão celular neste órgão.

Além dos efeitos diretos e deletérios do Al no crescimento da raiz, este elemento também prejudica, indiretamente, o crescimento da parte aérea em plantas sensíveis (Vitorello et al. 2005; Gavassi et al. 2021). Alguns estudos sugerem que reduções no crescimento da raiz, causado pelo Al, resultaria em uma menor área de superfície de raiz para a absorção de água e hidratação da parte aérea (Horst, 1995; Kochian et al., 2004). De fato, reduções na condutância estomática (*gs*) (Simon et al. 1994; Konrad et al. 2005; Silva et al. 2012), no potencial da água na folha (Ψw) e no conteúdo relativo de água na folha (CRA) (Silva et al. 2018; Cavalheiro et al. 2020) já foram observados em plantas

expostas ao Al, evidenciando que reduções no crescimento da raiz resultariam em diminuições na hidratação foliar, resultando em menor *gs*. Entretanto, uma vez que a maioria dos estudos que avaliam a resposta do Al são realizados com plantas crescendo em solução nutritiva, situação em que a água não é um fator limitante, é possível que reduções na área de superfície de raiz não seja suficiente para explicar reduções na hidratação foliar e em *gs.*

Danos anatômicos ao cilindro vascular da raiz, como a presença de xilema fibroso (Banhos et al., 2016) e maior deposição de lignina no cilindro vascular (Silva et al., 2019) foram observados em plantas de *Citrus* x *limonia* quando cultivadas em solução nutritiva com 1480 μM Al. Em *Zea mays*, danos estruturais nas células do cilindro vascular também foram evidenciados quando expostas à 300 μM Al (Batista et al., 2013*)*. Além disso, diminuições na condutividade hidráulica da raiz foram observadas em plantas de *Z. mays* (Gunsé et al., 1997) e em *Solanum lycospersicum* (Gavassi et al., 2020) quando expostas ao Al. Estes estudos sugerem que o Al, além de inibir o crescimento da raiz, também pode afetar a absorção e o transporte de água para a parte aérea. Além disso, não se sabe se o impedimento no transporte de água ocorre antes ou depois do Al inibir o crescimento da raiz.

A presença do Al na raiz também pode desencadear sinais químicos para a parte aérea da planta e, uma vez que reduções em *gs* já foram evidenciadas em plantas sensíveis ao Al (Jiang et al., 2008; Silva et al., 2012; Banhos et a., 2016; Silva et al., 2018; Cavalheiro et al., 2020 Gavassi et al., 2020) é possível que o hormônio ácido abscísico (ABA) esteja diretamente relacionado com o fechamento estomático (menor *gs*) nessas plantas. O ABA tem sido amplamente estudado em plantas expostas ao Al (Shen et al., 2004; Hou et al., 2010 Kopittke, 2016), porém estes estudos associam o ABA mais com a resistência ao Al do que com a baixa hidratação foliar e *gs*. Estudos recentes evidenciaram a associação do ABA com diminuições em *gs* em plantas de *S. lycospersicum* (Gavassi et al., 2020) e em *C.* x *limonia* (Gavassi et al., 2020) expostas ao Al, entretanto estes estudos não avaliaram *gs* e crescimento da raiz ao longo do tempo.

Por outro lado, algumas espécies são capazes de crescer bem em solos ácidos com elevada saturação por Al, sem apresentar sintomas de toxicidade (Watanabe e Osaki, 2002; Fung et al., 2008; Bressan et al., 2021). Algumas espécies são consideradas "excludentes" de Al, e exsudam ácidos orgânicos (AO) (citrato, malato, oxalato e

succinato) pelas raízes para formar compostos estáveis com o Al (AO:Al) na rizosfera, podendo barrar a absorção deste metal (Ryan, 2011). Outras espécies são capazes de absorver, acumular e armazenar o Al em compartimentos celulares, como parede celular e vacúolos (Haridasan, 1982; Jansen et al., 2002; Bressan et al., 2016). É necessário enfatizar, no entanto, que para ser considerada acumuladora de Al, estudos de campo devem demonstrar que acumulam mais de 1000 mg Al por kg de massa seca de folhas (Chenery 1948). Dessa forma, plantas que crescem naturalmente em solos ácidos podem apresentar uma resistência natural ao Al (Souza et al., 2017).

Embora o Al não seja considerado um elemento essencial para as plantas acumuladoras de Al, alguns efeitos benéficos deste elemento foram relatados para algumas espécies (Watanabe et al., 2005; Muhammad et al., 2019; Liu et al., 2020). Plantas de *Melastoma malabathricum* (Osaki et al., 1997), *Camellia sinensis* (Sun et al., 2020) e *Symplocos paniculata* (Schmitt et al., 2016), quando cultivadas na presença de Al apresentaram maior crescimento da raiz e maior biomassa total em relação às plantas não expostas ao Al. Além disso, a presença de Al também aumentou a performance fotossintética de *Camellia sinensis* (Hajiboland et al., 2013) e *Camellia japonica* (Liu et al., 2020). Entretanto, o mecanismo pelo qual o Al estimula o crescimento em plantas acumuladoras de Al ainda não é totalmente compreendido.

Ilex paraguariensis é uma espécie da família Aquifoliaceae, popularmente conhecida como erva-mate, e bastante cultivada e consumida como chá em países da América do Sul, já que suas folhas e ramos são utilizados para o preparo de infusões. Diversos efeitos benéficos para a saúde humana estão associados ao consumo de infusões de erva-mate (Xu et al., 2009; Bracesco et al., 2011). Contudo, por ser uma espécie que naturalmente cresce em solos ácidos com elevada saturação por Al (Santin et al., 2013), alguns estudos apontam quantidades significativas de Al em suas folhas e infusões (Gomes da Costa et al., 2009; Pozebon et al., 2015; Barbosa et al., 2020; Olivari et al., 2020) que pode ser potencialmente tóxico para a saúde humana (Mold et al., 2020). Entretanto, apesar da raiz ser o primeiro órgão a entrar em contato com o Al e, conforme a resposta de tolerância da planta, resultar em acúmulo foliar, poucos estudos priorizam o entendimento sobre o desenvolvimento da raiz em plantas de erva-mate em presença de Al. Um desses poucos estudos foi realizado por Benedetti et al. (2017) que evidenciou maior desenvolvimento de raiz em mudas de erva-mate quando expostas a concentrações

crescentes de Al em solução nutritiva. Porém, poucas variáveis fisiológicas tem sido exploradas quando plantas dessa espécie são cultivadas em presença de Al.

Espécies acumuladoras de Al também são encontradas na vegetação do Cerrado, que tem como característica solos ácidos (pH < 4.0) com elevada saturação de Al trocável (m% > 50%) (Haridasan 1982; Lira-Martins et al. 2022). A maioria das plantas acumuladoras de Al do Cerrado pertencem às famílias Melastomataceae, Rubiaceae, Symplocaceae e Vochysiaceae (Haridasan 1982; Bressan et al. 2016; Malta et al. 2016) e podem acumular desde 4.000 até 20.000 mg Al por kg de massa seca de folhas (Haridasan 1982; Haridasan e Araújo 1988).

Alguns estudos têm atribuído funções fisiológicas ao Al em espécies acumuladoras. Em *Callisthene major*, *Qualea grandiflora* (Vochysiaceae) (Andrade et al., 2011) e *Rudgea virbunoides* (Rubiaceae) (Malta et al., 2016) espécies lenhosas do Cerrado acumuladoras de Al, foi evidenciada a presença deste elemento no cloroplasto destas espécies. Estes estudos levantaram o questionamento de que talvez o Al possa estar relacionado, diretamente, com processo fotossintético nestas espécies. No entanto, outros estudos não evidenciaram reações histoquímicas positivas para Al nos cloroplastos, nem mesmo nos tecidos fotossintéticos, sendo que o Al foi evidenciado na camada superior e inferior da epiderme (mas não no parênquima clorofiliano) de espécies acumuladoras de Al de Melastomataceae e Vochysiaceae do Cerrado (Bressan et al., 2016; Guilherme Pereira et al., 2018). Mudas de *Vochysia tucanorum* (Vochysiaceae) quando cultivadas em solução nutritiva com 1110 μM Al, apresentaram melhor performance fotossintética do que plantas cultivadas na ausência de Al, porém isto foi relacionado com um maior crescimento da raiz do que com um possível efeito direto do Al na fotossíntese (Bressan et al., 2021).

Diante do que foi exposto, na presente Tese de Doutorado foram avaliados o efeito do Al na hidratação da parte aérea em *Citrus* x *limonia*, uma espécie sensível ao Al, no crescimento e nas respostas fotossintéticas em *Ilex paraguariensis*, uma espécie tolerante ao Al, e em *Qualea grandiflora*, uma espécie acumuladora de Al nativa do Cerrado. A partir disso, três experimentos foram realizados e estão aqui apresentados no capítulo 1, 2 e 3. Para o experimento do capítulo 1, plantas de *Citrus* x *limonia* foram cultivadas por 60 dias em solução nutritiva com 0 e 1480 μM Al, para avaliar se reduções na hidratação foliar, em plantas expostas a 1480 μM Al, ocorrem antes ou depois da inibição no

crescimento de raiz. No capítulo 2, plantas de *Ilex paraguariensis* foram cultivadas por 60 dias em solução nutritiva contendo 0, 500, 1000 e 1500 μM Al, para testar se o Al induz o crescimento das raízes e da parte aérea nesta espécie com consequente aumento na performance fotossintética. Também avaliamos se reduções no crescimento da raiz, nas plantas crescendo na ausência de Al, está relacionado com maiores concentrações de IAA, uma vez que altas concentrações de IAA nas pontas das raízes podem inibir o seu crescimento. Por fim, no capítulo 3, mudas de *Qualea grandiflora* cresceram por 60 dias em solução nutritiva com 0, 740 e 1480 μM Al, a fim de verificar se o Al na solução nutritiva aumenta a assimilação de CO_2 através de uma maior eficiência aparente de carboxilação da enzima ribulose-1,5-bifosfato carboxilase/oxigenasse (Rubisco).

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CAPÍTULO I:

High abscisic acid and low root hydraulic conductivity may explain low leaf hydration in 'Mandarin' lime exposed to aluminum

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Abstract

The first symptom of Al toxicity is the inhibition of root growth, which has been associated with low leaf hydration, with negative consequences for leaf gas exchange, including stomatal conductance (g_s) observed in many plant species. Here we asked whether low leaf hydration occur before or after the inhibition of root growth of *Citrus* x *limonia* ('Mandarin' lime) cultivated for 60 days in nutrient solution with 0 and 1480 μM Al. The length, diameter, surface area and biomass of roots of plants exposed to Al were lower than control plants only at 30 days after treatments (DAT). Until the end of the study, estimated g_s (by sap flow measurements) was lower than control plants from 3 DAT, total plant transpiration (E_{plant}) and root hydraulic conductivity (Lp_r), at 7 DAT, and predawn leaf water potential (Ψpd) and relative leaf water content (RWC), at 15 DAT. Abscisic acid (ABA) in leaves was two-times higher in Al-exposed plants 1 DAT, and in roots a two-times higher peak was observed at 15 DAT. As ABA in leaves approached values of control plants after 15 DAT, we propose that low g_s of plants exposed to Al is primarily caused by ABA, and the maintenance of low g_s could be ascribed to the low Lp_r from 7 until the end of the study. Therefore, the low leaf hydration in 'Mandarin' lime exposed to Al does not seem to be caused by root growth inhibition and a simple consequence of low water uptake due to a low root system.

Key words: ABA; Al^{3+}; *Citrus*; stomatal conductance; Lp_r; root growth.

Introduction

Aluminum (Al) is toxic to nearly all plant species. The first and most conspicuous symptom of Al toxicity is the inhibition of root growth (Horst et al. 2010; Sun et al. 2010; Singh et al. 2017). This is considered a direct symptom, as the root is the first organ to get in contact with Al, which binds with pectic nets of root cell walls resulting in the first lesion in plants (Kopittke et al. 2015). Indirect symptoms involve disturbances in above-ground organs, as approximately 90% absorbed Al is retained in the roots (Vitorello et al. 2005; Gavassi et al. 2021). Among indirect symptoms, low leaf gas exchange and mesophyll dehydration could be described (Pereira et al. 2000; Chen et al. 2005; Banhos et al. 2016).

It is intuitive that Al-induced root growth inhibition could lead to decreased water (and nutrients) uptake, as reviewed by Horst (1995), Kochian et al. (2004) and Singh et al. (2017). Indeed, Al can cause low stomatal conductance (g_s) (Simon et al. 1994; Konrad et al. 2005; Silva et al. 2012; Silva et al. 2018), reduced leaf water potential (Ψw) and relative leaf water content (RWC) (Silva et al. 2012; 2018; Cavalheiro et al. 2020; Gavassi et al. 2020; 2021), reinforcing that Al-induced root growth inhibition is associated with low leaf hydration, as g_s is regulated by plant water status (Kriedemann et al. 1972; McAdam et al. 2016a). Many studies revealing association between Al toxicity and low g_s (Simon et al. 1994; Jiang et al. 2008; Silva et al. 2012; Banhos et al. 2016; Silva et al. 2018; Gavassi et al. 2020; 2021) have been performed with plants growing directly in nutrient solution, where water is permanently available. Anatomical damage to root vascular cylinder, such as fibrous xylem vessels (Banhos et al. 2016), more lignin deposition (Silva et al. 2019) and smaller protoxylem vessels (Batista et al. 2013) have been associated with less water uptake in plants exposed to Al in nutrient solution. In addition, low aquaporin (PIP family) expression, which are membrane

proteins partially responsible for water transport (Javot and Maurel 2002), was observed in *Citrus* x *limonia* exposed to Al (Cavalheiro et al. 2020). Taken together, these results suggest that Al may affect root water acquisition and transport when exposed to Al in nutrient solution. More evidence shows decreased root hydraulic conductivity (Lp_r) in maize (Gunsé et al. 1997) and, more recently, in *Solanum lycospersicum* (Gavassi et al. 2020) exposed to Al in nutrient solution. Therefore, the reduced size of roots exposed to Al may not fully explain the low leaf hydration found in plants under Al toxicity. It is not yet known whether this impairment of water transport appears before or after the inhibition of root growth caused by Al.

Roots primarily sensing Al toxicity could also trigger chemical signals to the shoots, and abscisic acid (ABA) emerges as a candidate, as ABA is directly associated with stomatal closure (Zhang and Davies 1989; Merilo et al. 2015). Endogenous ABA balance may influence its metabolism in plants under abiotic stresses, and ABA concentration is regulated by biosynthesis (Ng et al., 2014) that results from xanthophyll cleavage, and this hormone may also be degraded in the metabolism (Xu et al., 2013). 8′-hydroxy-ABA is the main compound derived from the oxidative route of ABA catabolism involving the 8′ hydroxylation (Cutler and Krochko, 1999). This compound isomerizes to phaseic acid (PA), which may be reduced to dihydrophaseic acid (DPA) (Okamoto et al., 2009). Although ABA and PA exert physiological effects, PA is the first metabolite to be degraded ending up in the inactive DPA (Schwartz and Zeevaart, 2010). ABA has already been measured in plants exposed to Al (Shen et al., 2004; Hou et al., 2010; Reyna-Llorens et al., 2015; Kopittke, 2016); however, these studies have associated ABA with Al resistance rather than low leaf hydration or reduced g_s. Few recent studies have evidenced increased ABA and low g_s in *S. lycospersicum* (Gavassi et al. 2020) and *C.* x *limonia*

(Gavassi et al. 2021) exposed to Al in nutrient solution, but these studies have not assessed g_s or root growth inhibition over time of Al exposure.

In the present study, we measured biometric data of *Citrus* x *limonia*, including the length, surface area, diameter, and biomass of roots over time of Al exposure in nutrient solution. Leaf hydration, including Ψw, RWC, and especially g_s, as well as root hydraulic conductivity (Lp_r) and ABA, PA and DPA measured in leaves and roots were also monitored throughout the study. We hypothesized that impairments on leaf hydration and Lp_r due to Al exposure occur before root growth inhibition can be observed.

Material and methods

Plant material and experimental conditions

Three-month-old *Citrus* x *limonia* Osbeck (pro. Sp.) - ITIS # 501573, also known as *Citrus limonum* Risso or *C. taitensis* Risso, popularly named 'Mandarin' lime or 'Rangpur' lime was used. The plants, obtained from a commercial Citrus nursery (Citrograf, Conchal, SP, Brazil) were 15 cm in height and showed 13 ± 1 leaves. The root system was rinsed (to remove remaining substrate from the nursery) and transferred to a hydroponic system, where the plants grew directly in an aerated nutrient solution inside opaque plastic boxes (50 cm × 30 cm × 15 cm; 20 L) for 60 days.

The nutrient solution was based on Clark solution (Clark, 1975), and was used previously to test Al toxicity in 'Mandarin' lime (Banhos et al. 2016; Silva et al. 2018; 2019; Cavalheiro et al. 2020; Gavassi et al. 2021). It was comprised of 1372.8 µM $Ca(NO_3)_2$, 507 µM NH_4NO_3, 224.4 µM KCl, 227.2 µM K_2SO_4, 218.6 µM KNO_3, 483.2 µM $Mg(NO_3)_2$, 12.9 µM KH_2PO_4, 26.01 µM $FeSO_4$, 23.8 µM NaEDTA, 3.5 µM $MnCl_2$,

9.9 μM H_3BO_3, 0.9 μM $ZnSO_4$, 0.2 μM $CuSO_4$ and 0.4 μM $NaMoO_2$. In previous studies with 'Mandarin' lime (Banhos et al. 2016; Silva et al. 2018; Gavassi et al. 2021) and *C. grandis* (Jiang et al. 2008), Al concentrations of 1480 and 1600 μM Al, respectively, decreased the leaf gas exchange rates. Thus, in the present study, 1480 μM Al, provided through 1479.5 μM $AlCl_3$ was used. To maintain the Al soluble, the pH was kept at 4.0 ± 1 and monitored daily, and the solution was totally replaced every 15 days. Samples of the nutrient solution were also collected (n = 3) every 15 days and sent to a plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), where Al was measured by the colorimetric method (Sarruge and Haag, 1974), and nominal 1480 μM Al resulted in 1250 ± 49.8 μM Al, while the solution with no Al added resulted in trace values of Al concentration.

Expanded polystyrene (Isopor®) 50 × 30 cm plates (2-cm thick), with six holes (3 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 placed around the plant collar. The boxes were kept on benches, maintained inside a greenhouse, under semi-controlled conditions (869.7 ± 124.2 μmol photons m^{-2} s^{-1}; approximately 14 h of natural photoperiod). Air temperature and relative humidity inside the greenhouse was of 26.0 ± 2 ºC and 65.5 ± 2.8 %, between 9 h and 11:30 h.

Experimental design

Thirty-six plants were distributed to six boxes containing nutrient solution with 0 μM Al and 36 plants to six boxes with 1480 μM Al and grown for 60 days. On the first day, sensors for measuring the sap flow (estimated g_s) were placed on the main stem of five plants from one box with 0 μM Al and five other plants from a box with 1480 μM

Al, and these ten plants were measured continuously (every 15 min) until the end of the study. At 1, 7, 15, 30, 45 and 60 days after treatments (DAT), predawn (Ψpd) and midday (Ψmd) leaf water potential, relative leaf water content (RWC), total plant transpiration (E_{plant}) and root hydraulic conductance (Lp) and conductivity (Lp_r) were measured. On these same dates, leaf samples and root apices were collected for measuring abscisic acid (ABA), phaseic acid (PA) and dihydrophaseic acid (DPA). Leaf discs for RWC and leaf and root samples for measuring ABA, PA and DPA were collected within 120 s, so that these parameters interfered as little as possible in each other. The number of leaves, leaf biomass and total leaf area per plant, stem length and biomass, main root length, root surface area, root diameter and root biomass as well as the Al concentration in roots, stems and leaves were also measured on these dates. With the exception for estimated g_s, the present study did not involve repeated measurements on the same plants through time, as one box (with 6 plants) per treatment was used on each evaluation date. The ten plants (0 and 1480 μM Al) for measuring the estimated g_s were used for plant material collection and measurements at 60 DAT. On each date, three plants were used for Ψpd, Ψmd, E_{plant}, Lp and Lp_r. After measurements, organs of these three plants were separated (leaf, stem plus petiole and roots) for assessment of biometric data, biomass, and Al concentration. Three other plants were used for measuring ABA, PA, DPA and RWC.

Sap flow measurements (estimated g_s)

Sap flow measurements were based on the Heat Ratio Method (HRM) (Clearwater et al. 2009; Skelton et al. 2013). Using three ‘Mandarin’ lime plants growing in the nutrient solution without Al (pH 6.0), sap flow sensors (SF-4M, Edaphic Scientific, Port Macquarie, NSW, Australia) were placed on the main stem (5–8 mm in diameter) of each

plant. Aluminum foil was used to wrap the sensors and protect them against heat effects. The sensors were connected to a data logger (CR-1000, Campbel Scientific, Port Macquarie, NSW, Australia) that collected data (mV) every 15 min. These mV data were correlated with a reliable plant water use parameter (Forster, 2017); in the present study, stomatal conductance (g_s, mol m^{-2} s^{-1}) was used.

For this, g_s was measured with a portable gas exchange system (LI-6400xtr, LI-COR, Lincoln, NE, USA) from 5:00 h to 17:00 h, to cover daily g_s response on a typical cloudless day. The CO_2 concentration entering the leaf cuvette (LCF chamber; 2 cm^2, LI-COR) averaged 400 μmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR). The photosynthetic photon flux density (PPFD) inside the leaf cuvette (6400-40 LCF, LI-COR) was null from 5:00h to 6:00h, when light conditions in the greenhouse were also dark. Thus, PPFD and relative air humidity (RH%) in the leaf sample chamber, inside the leaf cuvette, was set according to the light environment and RH% in the greenhouse. Therefore, g_s values measured throughout the day were linearly correlated with mV data from SF-4M sensors (Supplementary material; Fig. S1), and daily values of both parameters could be observed (Supplementary material; Fig. S2). After the calibration procedure, the sensors were placed in five plants of each treatment (0 and 1480 μM Al; pH 4.0) and continuous g_s could be measured from 1 to 60 DAT.

Plant water relations

Predawn (Ψpd) and midday (Ψmd; under maximum vapor pressure deficit — VPD) leaf water potential (Ψw) were measured by the pressure chamber method (Turner, 1981), using a 3005F01 Plant Water Status Console (Soil Moisture, Santa Barbara, CA, USA) chamber.

For measuring total plant transpiration (E_{plant}), the plants were transferred to individual 300 mL cylindrical glass pots (6.5 cm in diameter, 12 cm in height) containing the same nutrient solution and made to fit into the pressure chamber (Model 3000F01; Soil Moisture Equipment Corp., USA). The plants were fixed in the glass pots with 2-cm thick foam to prevent evaporation and acclimatized for 1 h (10:00 – 11:00 h). Then, the pot was weighed at 11:00 h and 12:00 h, and the whole-plant water uptake was calculated by the difference between weights. Evaporation was measured by determining the water loss from a glass pot (without a plant) and ignored as negligible (< 3 % of the water loss of pots containing a plant). E_{plant} was calculated as the ratio between water uptake and time (mg water s^{-1}) (Puértolas et al. 2015).

Root hydraulic conductance (*Lp*) was assessed by the pressure-induced sap flow method (Jackson et al. 1996; Dodd and Diatloff, 2016). The plant was inserted into the pressure chamber with their roots in the nutrient solution (as described for measuring E_{plant}). Then, the shoot was excised, and a series of overpressures (from 0.1 MPa to 0.5 MPa at 0.1 MPa increments) were applied so that the sap flow rate was assessed at each pressure. The sap collection on the cut surface was done every 30 seconds with the aid of small portions of absorbent paper inside a microtube, whose dry mass was previously determined. The mass of the wet absorbent paper was immediately measured on a precision scale. Root hydraulic conductance measures the root permeability to the flow of water by applying increasing pressures to the root zone. The slope of the linear regression representing the relationship between exuded flow rate (*J*; mg s^{-1}) and applied pressures resulted in *Lp*. These values were also divided (normalized) by the root diameter (mm), that is a proxy of the stele diameter (anatomically, where water is transported), and resulted in root hydraulic conductivity (Lp_r).

The relative leaf water content (RWC, %) was measured between 13:00 h and 15:00 h. Disks were collected and had the fresh mass (FM) immediately measured on a scale. Then, the disks were hydrated for 24 h in 100 mL deionized water inside amber flasks (to avoid photosynthetic activity) and had the turgid mass (TM) recorded. After oven-drying the discs at 60 ºC for 48 h, dry mass (DM) was also assessed, and RWC was calculated according to Silva et al. (2018):

$$RWC\ (\%) = [(FM - DM)/(TM - DM)] \times 100$$

ABA, PA and DPA quantification

ABA and its metabolites, phaseic acid (PA) and dihydrophaseic acid (DPA) were analyzed via liquid chromatography/tandem mass spectrophotometry (LC/MS-MS) on a SciexExionLC coupled with a QTRAP 6500+ mass spectrophotometer, following the method proposed by Morris et al. (2019).

Leaf and root samples (500 mg DM) were ground to a powder (in 2 mL micro-centrifuge tubes containing two 5 mm acid-rinsed glass balls) in a Star-Beater (VWR) at 30 Hz for 2 min. The powdered material, ~20 mg for leaf samples and ~50 mg for roots, with 1 ng of internal standards added, were extracted using a Star-Beater at 30 Hz for 2 min with 1 mL of ice-cold methanol/formic acid/water solvent (60:5:35 v/v). The internal standards used were: [2H4]-abscisic acid (-)-5,8′,8′,8′ (d4-ABA); [2H3]-phaseic acid (-)-7′,7′,7′(d3-PA) and [2H3]-dihydrophaseic acid (-)-7′,7′,7′ (d3-DPA). After extraction, the samples were left on ice in the dark for 20 min and, subsequently, the plant material was pelleted by centrifugation at 24,000 × g at 4 ˚C for 10 min. The supernatant was pipetted into 15 mL conical centrifuge tubes and the solvent was evaporated overnight in a freeze-drier (-105 ˚C; Scanvac, CoolSafe 110-4 Pro). Samples were reconstituted (vortexed for

2 min at 1400 rpm, sonicated for 30 s and a final vortex at 2000 rpm for 3 min) in 1:9 methanol:water plus 1mM ammonium formate and 0.1% formic acid in order to match mobile phase. Finally, the samples were filtered through 4 mm nylon filters (0.2 µM pore size, Whatman) into silanised amber HPLC vials.

Calibration samples consisted of a series of non-deuterated compounds (from 0.1 to 500 ng mL^{-1}), each with deuterated compounds (constant 1 ng mL^{-1}). Extracts and calibration samples (20 µL) were injected with an auto-sampler into a Thermofisher Hypersil Gold 1.9 µm C18 50 x 2.1 mm (Phenomenex AJO8768) precolumn with oven temperature at 30°C. The aqueous mobile phase (A) consisted of water and 1mM ammonium formate in 0.1 % formic acid, and the organic mobile phase (B) was methanol and 1 mM ammonium formate in 0.1 % formic acid. The ratios of mobile phase A:B for separation of compounds was as follows (at a flow rate of 0.4 mL min^{-1}): gradient 0-1min hold at 5 % B, 1–3 min increase to 40 % B, 3–5.5 min hold at 40 % B, 5.5–6.5 min increase to 95 % B, 1 min hold and 7.8 min 5 % B. The column was then cleaned as follows, 10–10.5 min at 60:40; 10.5–10.6 min at 50:50 and 10.6–11.6 min at 0:100. The column was then equilibrated from 11.6–13 min at 96:4 before injection of the next sample. Analyst 1.6.3 and MultiQuant 3.0.2 (Sciex, Singapore) software was used for acquisition and quantification, respectively.

Biometric data and biomass

At 1, 7, 15, 30, 45 and 60 DAT the plant samples (n = 3) were separated into leaf, stem (plus petiole) and root. The leaves were counted and total leaf area per plant (cm^2) was measured with an area meter (LI-3100, LI-COR, USA). The stem length and main root length was measured with a ruler (cm). The root surface area (cm^2) and root diameter

were measured using a scanner (Epson perfection v700 photo, Suwa, Japan), which was coupled to a computer running the WinRHIZOTM software (Regent Instruments, Canada). The biomass of leaf, stem (plus petiole) and root (g) were measured after oven-drying the samples at 60 °C until constant mass, using a precision scale.

Al concentration in plant organs

After measuring the biometric data, the Al concentration was measured in the leaf, stem (plus petiole) and root at 1, 7, 15, 30, 45 and 60 DAT. Dry samples of these organs were sent to the plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), where these were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Al concentration was assessed by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

Data analysis

Sap flow (estimated g_s) was measured using five plants, while Ψpd and Ψmd, E_{plant}, Lp, Lp_r, RWC, ABA, PA and DPA, biometric data, plant biomass and Al concentration in plant organs were measured using three plants.

A student's t-test ($\propto = 0.05$), run separately for each evaluation date (1, 7, 15, 30, 45, and 60 DAT), was used to test differences between 0 and 1480 µM Al for each variable.

For estimated g_s, besides the daily oscillations, mean values from 10:00 h to 14:00 h (20 readings per day) were used to represent g_s assessment from the period of maximum VPD.

Results

Plant biometric data and biomass

Both treatments showed the same pattern of plant development until 30 DAT, when plants exposed to Al started exhibiting growth inhibition of roots and shoots in relation to control plants (Fig. 1). From 30 DAT, plants exposed to Al showed ten leaves less than control plants, a significant difference that was maintained up to the end of the study (Fig. 2A), and this can explain the lower values of leaf biomass (Fig. 2B) and leaf area (Fig. 2C) of plants exposed to Al, from 30 DAT. Stem length (Fig. 3A) and stem biomass (Fig. 3B) of plants exposed to Al were lower than control plants, from 30 DAT until the end of the study. The main root length (Fig. 4A), root surface area (Fig. 4B) and root biomass (Fig. 4D) of plants exposed to Al were lower and the root diameter (Fig. 4C) was higher than control plants, from 30 DAT. Therefore, until 30 DAT no difference in plant growth were observed between treatments.

Plant water relations

Calibrated sap flow (SF-4M mV x g_s curves) or estimated daily g_s of plants exposed to Al was, in general, lower than those of control plants throughout the study (Fig. 5A). When analyzed during the highest daily VPD (10:00 h – 14:00 h), plants exposed to Al showed estimated g_s lower than control plants from 3 DAT, reaching an

approximate difference of 50% between treatments at 15 DAT (Fig. 5B). From 15 DAT until the end of the study the difference between treatments oscillated considerably, and control plants showed values approximately twice as high as those exposed to Al (Fig. 5B).

Predawn leaf water potential (Ψpd) was the same between treatments over the study (Fig. 6A). Midday leaf water potential (Ψmd), however, was lower in plants exposed to Al in relation to control plants at 15 (–12%), 30 (–18%), 45 (–20%) and 60 DAT (–25%) (Fig. 6B). The relative leaf water content (RWC) and total plant transpiration (E_{plant}) followed the same response pattern, but E_{plant} started showing low values for plants exposed to Al at 7 DAT (–32, –65, –68, –50 and –62 % at 7, 15, 30, 45 and 60 DAT) (Fig. 6C), while RWC, at 15 DAT (–12, –18, –20 and –25 % at 15, 30, 45 and 60 DAT) (Fig. 6D).

Increasing the pressure applied to de-topped root systems linearly increased sap flow rates in both treatments, but the slopes of the curves were lower for plants exposed to Al (Fig. 7), from 7 DAT. This response pattern was similar when the slopes were interpreted as root hydraulic conductance (*Lp*) and even when normalized by the root diameter (root hydraulic conductivity; Lp_r) (Fig. 7). Al reduced *Lp* and Lp_r, respectively, by –42 % and –39 % (7 DAT), –45 % and –47 % (15 DAT), –53 % and –59 % (30 DAT), –54 % and –60 % (45 DAT) and –48 % and –58 % (60 DAT). Thus, Al reduced the root capacity to transport water within the first week, while leaf hydration was reduced after 15 days although stomatal pores started closing 3 days after Al exposure, especially when analyzed during the highest daily VPD.

ABA accumulation in leaves and roots

In the leaves, Al induced peaks of ABA (Fig. 8A), PA (Fig. 8C) and DPA (Fig. 8F) 1 DAT, and it was twofold higher than control plants for ABA, ninefold for PA and tenfold for DPA. These peaks decreased and approached values exhibited by control plants from 15 DAT for ABA and 30 DAT for PA and DPA, until the end of the study.

In the roots, however, Al increased ABA at 7 DAT and produced a peak at 15 DAT, when a twofold higher value was found in relation to control plants; ABA concentration in the roots of plants exposed to Al was still higher than control plants at 30 and 45 DAT (Fig. 8B). Root PA oscillated between treatments until 15 DAT, and from 30 to 60 DAT it was twofold higher than control plants (Fig. 8D). Roots of plants exposed to Al showed a peak of twofold higher DPA in relation to control plants at 7 DAT, and both treatments showed similar values from 15 to 60 DAT (Fig. 8F). Thus, in the leaves, Al induced an immediate peak of ABA, PA and DPA at 1 DAT, while in the roots the peak of ABA and DPA occurred between 7 and 15 DAT.

Al concentration in plant organs

In relation to control plants, higher Al concentration was observed in plants exposed to Al from 1, 7 and 15 DAT for roots (Fig. 9C), stems (Fig. 9B) and leaves (Fig. 9A), respectively. As expected, plants exposed to Al maintained 25-fold more Al in their roots in relation to control plants (Fig. 9C) until the end of the study. Stems and leaves of plants exposed to Al increased their Al concentration throughout the study, and it was six- and fourfold higher, respectively, in relation to control plants, at 60 DAT. Thus, in plants exposed to Al, it was significantly and immediately retained in the roots when compared to the other organs.

Discussion

Here we asked whether root growth inhibition caused by Al (Horst et al. 2010; Silva et al. 2019) occurs before or after limitations of hydric and hydraulic parameters are observed. Until 30 DAT, no difference in the visual aspect of root growth (Fig. 1), main root length (Fig. 4A), root surface area (Fig. 4B) or root biomass (Fig. 4D) was observed between treatments. In contrast, plants exposed to Al started showing low Ψmd (Fig. 6B) and RWC (Fig. 6C) at 15 DAT and low E_{plant} (Fig. 6D) at 7 DAT. Moreover, estimated daily g_s analyzed during the highest daily VPD (10:00 h – 14:00 h) were lower in plants exposed to Al from 3 DAT (Fig. 5B). These results demonstrate, as a novelty, that leaf hydration was affected by Al exposure before root growth inhibition could be observed at 30 DAT. 'Mandarin' lime exposed to 1480 μM Al in nutrient solution also showed progressive low g_s over time of Al exposure (Banhos et al. 2016). Similarly, RWC, Ψmd and g_s measured in 'Mandarin' lime started showing significant low values between 7 and 15 days after Al exposure in nutrient solution (Silva et al. 2018; Gavassi et al. 2021). *Solanum lycopersicum* showed low g_s three days after Al exposure (Gavassi et al. 2020). Nevertheless, these studies mentioned above measured biometric and biomass data only at the beginning and end of their experiments and, as far as we are aware, no studies have assessed plant growth over time of Al exposure, making the present study a novelty in this field, reiterating that low g_s found in 'Mandarin' lime exposed to Al occurs before root growth inhibition is observed.

The root diameter, however, increased in plants exposed to Al, from 30 DAT (Fig. 4C), and this is expected in plants exposed to Al, as evidenced by 20 % and 32 % increase in 'Mandarin' lime (Gavassi et al. 2021) and *S. lycopersicum* (Gavassi et al. 2020), respectively. Aluminum also caused increases in root diameter by 29 % in barley (Jaskowiak et al. 2019) and 16 % in rice (Freitas et al. 2017). Therefore, when normalizing

Lp by root diameter, Lp_r is reduced correspondingly. Indeed, Lp_r of plants exposed to Al reduced by 39 % (at 7 DAT) and it was maintained low by a difference between 47 % and 60 % in relation to control plants (Fig. 7). This indicates the root permeability to water flow was significantly compromised and could explain the low leaf hydration as evidenced by the low E_{plant} (from 7 DAT) and Ψmd and RWC (from 15 DAT), or even the low g_s from 3 DAT. Thus, the low leaf hydration observed between 3 and 15 DAT caused by low water delivery to the mesophyll is associated with low Lp_r in 'Mandarin' lime. These results could be explained by low gene expression of root aquaporins and reduced estimated hydraulic conductance from soil to the leaf (K_L) in 'Mandarin' lime exposed to 1480 μM Al (Cavalheiro et al. 2019). Fibrous xylem vessels with structural modifications of the stele (Banhos et al. 2016) and more lignin deposition in the vascular cylinder (Silva et al., 2019) have been also observed in this species exposed to Al. In addition, smaller protoxylem vessels and structural damage to the vascular cylinder was noticed in roots of maize exposed to 300 μM Al (Batista et al. 2013). Therefore, the low leaf hydration, as evidenced by low Ψmd, RWC and g_s, in plants exposed to Al could be a consequence of low Lp_r in these plants, and this seems to be associated with anatomical impairments and low expression of proteins (aquaporins) that are partially responsible for water transport in the roots (Javot and Maurel 2002). A chemical signal between roots and shoots could also influence this phytotoxic response, and ABA could be an expected candidate.

The role of ABA and its metabolites in Al-induced decrease in leaf hydration

In the leaves, Al induced an immediate peak of ABA at 1 DAT (Fig. 8A). PA (Fig. 8C) and DPA (Fig. 8E) also showed a peak one day after Al exposure. Abscisic acid is

widely known to cause stomatal closure (Kriedemann et al. 1972; McAdam et al. 2016a). The estimated daily g_s analyzed during the highest daily VPD decreased continuously during this period (Fig. 5B). Thus, immediately after exposing the roots to Al, stomata close (after 3 days) and leaf ABA shows a peak (after 1 day). This indicates that, at least at the beginning of the study, the low g_s could be induced by high leaf ABA concentration. ABA, PA and DPA peaks decreased and approached values showed by control plants between 15 and 30 DAT, until the end of the study. Therefore, only the early and not the later decrease in g_s could be explained by high leaf ABA concentration. Increased ABA in the leaves was also observed in 'Mandarin' lime (Gavassi et al. 2021) and *S. lycopersicum* (Gavassi et al. 2020) exposed to Al. However, the root rather than the leaves is the first organ to get in contact with Al (the root was the first organ to accumulate Al — Fig. 9C). Indeed, in the roots, Al rose ABA at 7 DAT and produced a peak at 15 DAT in relation to control plants (Fig. 8B). Thus, leaves of plants exposed to Al showed a peak of ABA before (1 DAT) it could be measured in the roots (7 DAT). Therefore, the peak of ABA found in the leaves is unlikely to have come from the roots. In *S. lycopersicum* exposed to increasing Al concentrations, ABA was measured in the roots, xylem and leaves, and ABA delivery to the shoots was the same between treatments (Gavassi et al. 2021), indicating that, somehow, Al induces ABA biosynthesis directly in the leaves. Moreover, in the case of plants facing drought, although ABA signaling from root to shoot has been considered an important mechanism to reduce transpiration, recent studies (Holbrook et al. 2002; Manzi et al. 2015; McAdam et al. 2016b) have demonstrated that leaves are the main site of ABA synthesis.

In the leaves, PA and DPA accompanied the immediate (1 DAT) peak of ABA induced by Al, and these metabolites also decreased and were similar to those values showed by control plants between 15 and 30 DAT, until the end of the study (Fig. 8C, E).

This indicates that catabolism of ABA occurred concomitantly to the rise of ABA in the leaves. Studies measuring ABA metabolites in plants exposed to Al are rare. In our previous study with 'Mandarin' lime, the progressive rise of ABA in plants exposed to Al also caused an increase in PA and DPA (Gavassi et al. 2021).

After 15 days, leaf ABA concentration was the same between treatments, up to the end of the study (Fig. 8A), and ABA in the roots of plants exposed to Al also approached those values of control plants (Fig. 8B). Thus, what could explain the low g_s and low leaf hydration (low Ψmd, RWC and E_{plant}) of plants exposed to Al, from 15 up to 60 DAT? Hydraulic impairment during this period (low Lp_r — Fig. 7) emerges as a possible answer. We have been collecting evidence of compromised hydraulic performance in plants exposed to Al, as evidenced in 'Mandarin' lime (Banhos et al. 2016; Gavassi et al. 2021) and *S. lycopersicum* (Gavassi et al. 2020). Anatomical damage by Al in xylem vessels seems to be relevant (Banhos et al. 2016; Silva et al. 2019), as also observed in maize (Batista et al. 2013). Aquaporins (PIP family) expression are also decreased in the presence of Al (Cavalheiro et al. 2019). Low Lp_r in maize exposed to Al has already been observed (Gunsé et al. 1997), but these researchers have not associated this result with low leaf hydration. Therefore, we propose that Al immediately induces high ABA in the leaves, explaining the low g_s in plants exposed to Al, from 3 DAT, with concomitant decreased leaf hydration. After 15 DAT, the maintenance of low leaf hydration (including low g_s) could be ascribed to the low hydraulic performance, as evidenced by low Lp_r.

Conclusion

Root growth inhibition of 'Mandarin' lime exposed to Al occurs after hydric (Ψmd, RWC, E_{plant}) and hydraulic (*Lp* and Lp_r) parameters are affected by Al. The low g_s observed after 3 DAT are associated to a chemical signaling triggered by high concentration of ABA in leaves of plants exposed to Al, until 15 DAT. After that, the maintenance of low leaf hydration could be ascribed to low Lp_r observed from 7 DAT in plants exposed to Al. Therefore, the low leaf hydration in plants exposed to Al does not seem to be caused by root growth inhibition and a simple consequence of low water uptake, at least until 30 DAT.

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Figures

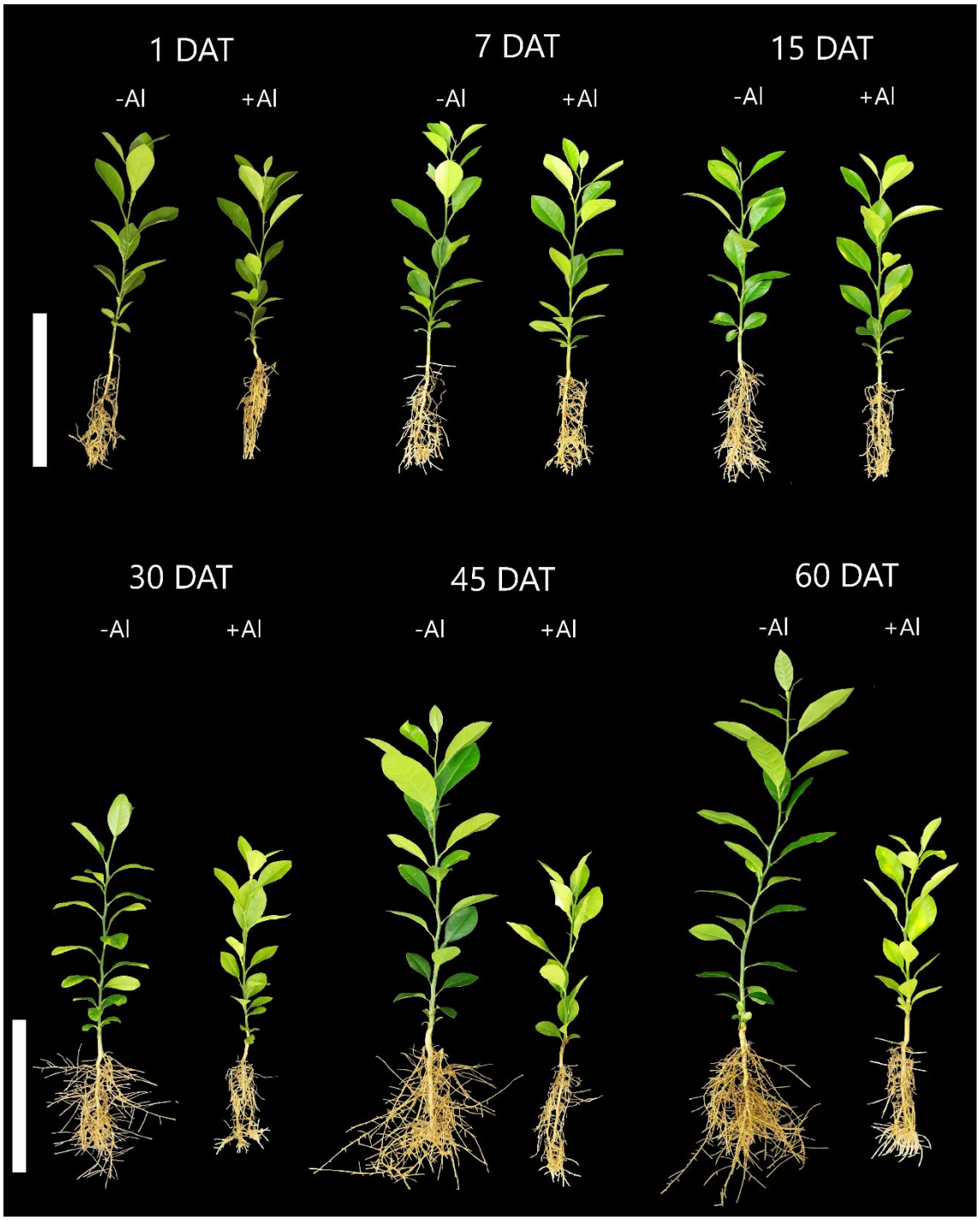

Fig 1. Morphological details of roots and shoots of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 (-Al) and 1480 μM Al (+Al) in nutrient solution. Bars = 15 cm

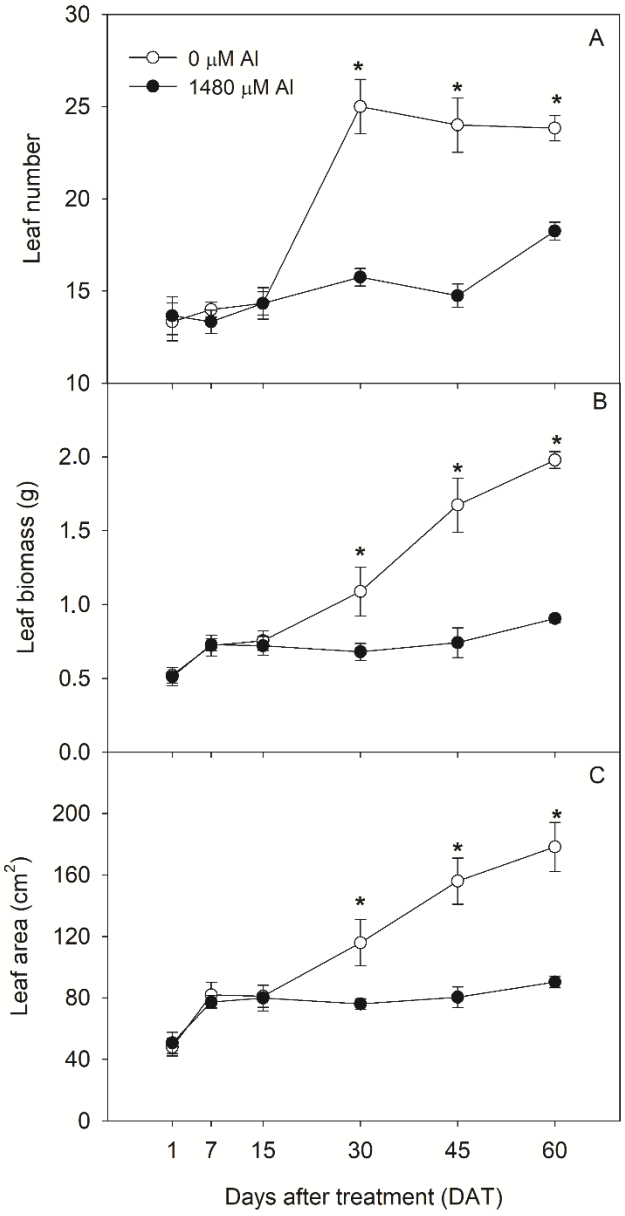

Fig 2. Mean values (n = 3 plants) of leaf number (A), leaf biomass (B) and leaf area (C) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 μM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) by Tukey test between 0 and 1480 μM Al. Bars are standard errors.

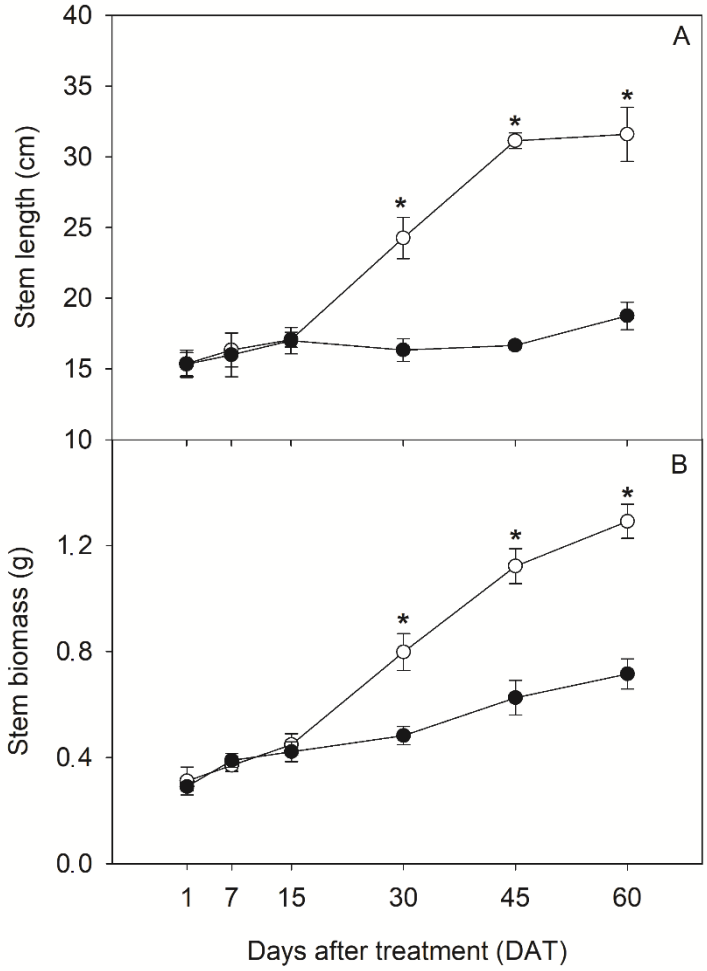

Fig 3. Mean values (n = 3 plants) of stem length (A) and stem biomass (B) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 μM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) by Tukey test between 0 and 1480 μM Al. Bars are standard errors.

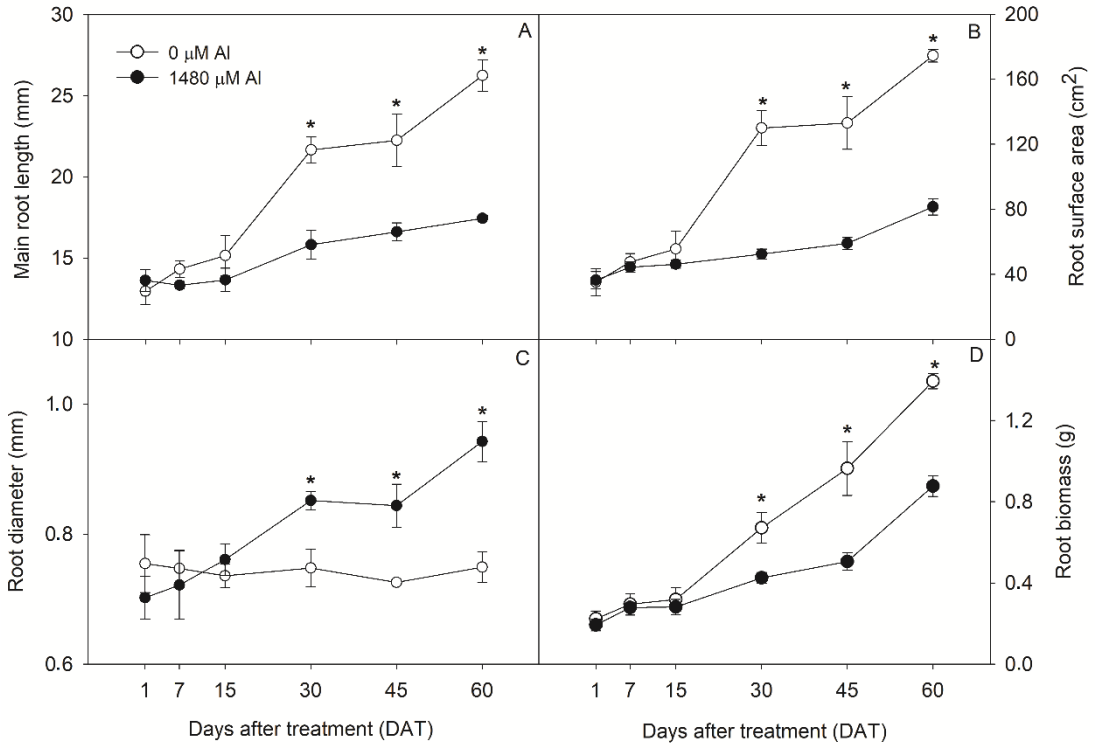

Fig 4. Mean values (n = 3 plants) of main root length (A), root surface area (B), root diameter (C) and root biomass (D) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 µM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P< 0.05$) by Tukey test between 0 and 1480 µM Al. Bars are standard errors.

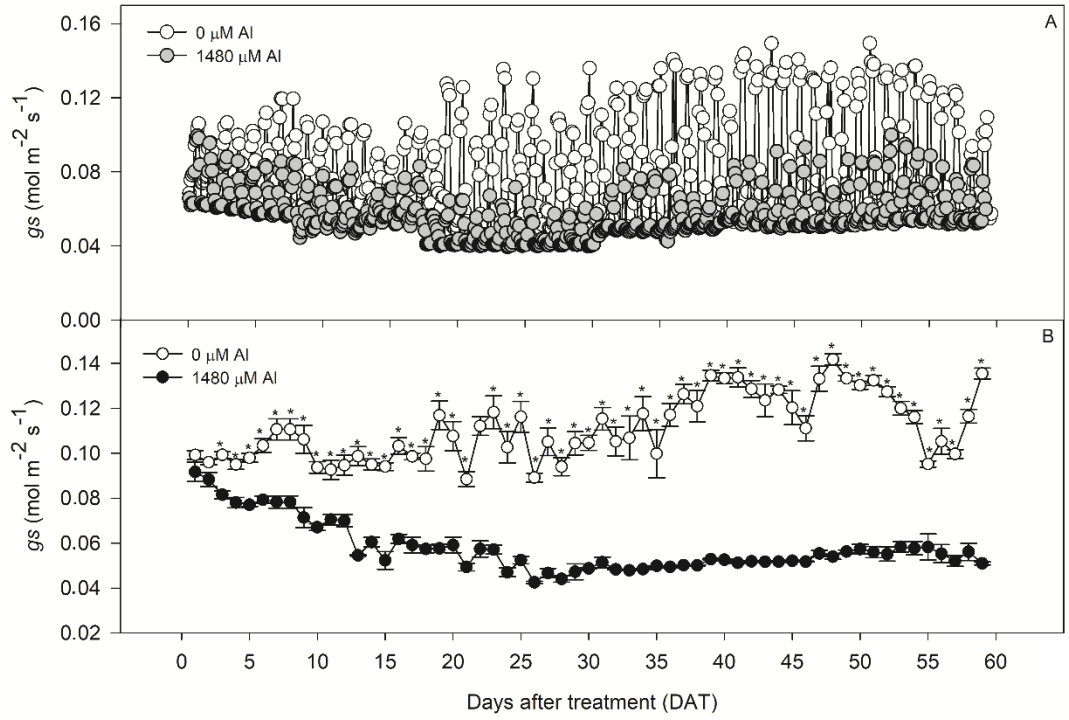

Fig. 5 Mean values (n = 5) of stomatal conductance (g_s) estimated by sap flow measurements in a daily course (24h) (A) and analyzed during the highest daily vapor pressure deficit (VPD), from 10:00 h to 14:00 h (B) in *C.* x *limonia* grown in nutrient solution with 0 and 1480 µM Al for 60 days. For each evaluation date, asterisks indicate significant differences ($P< 0.05$) by Tukey test between 0 and 1480 µM Al. In Fig. 5B, bars are standard errors.

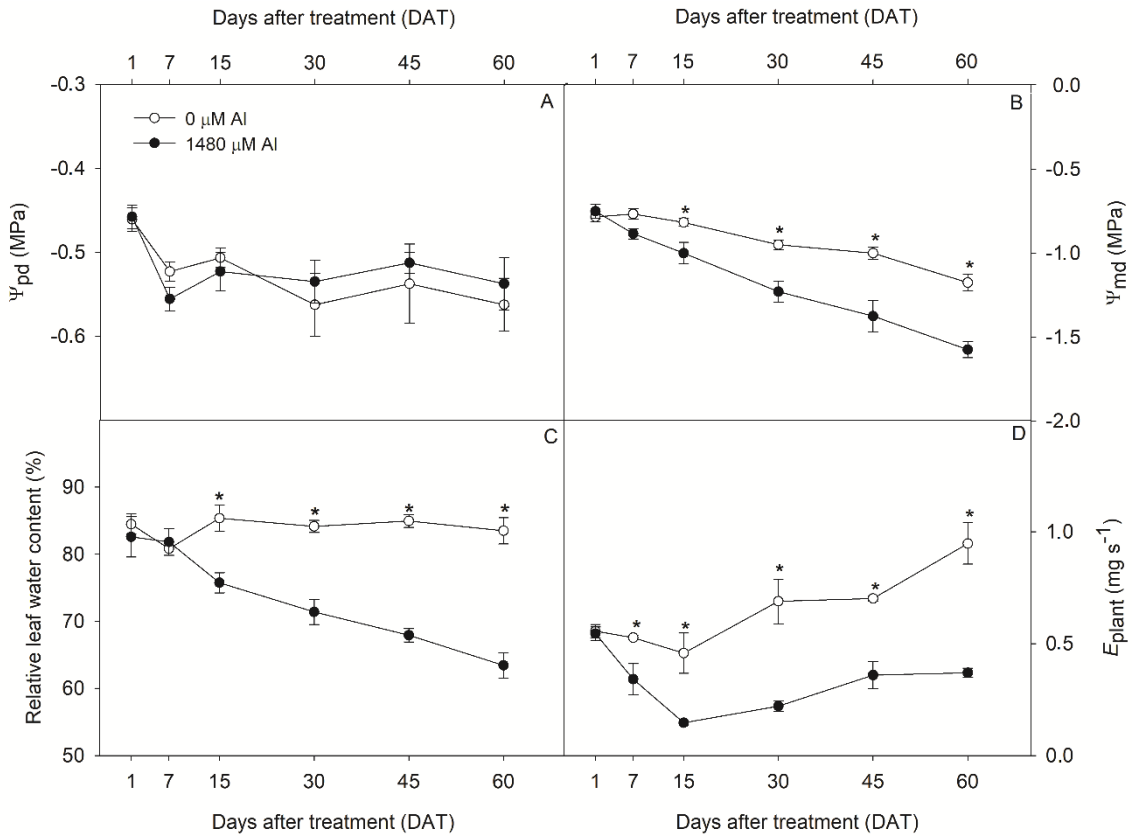

Fig 6. Mean values (n = 3 plants) of predawn (Ψpd) (A) and midday (Ψmd) (B) leaf water potentials, relative leaf water content (C) and total plant transpiration (E_{plant}) (D) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 µM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P<$ 0.05) by Tukey test between 0 and 1480 µM Al. Bars are standard errors.

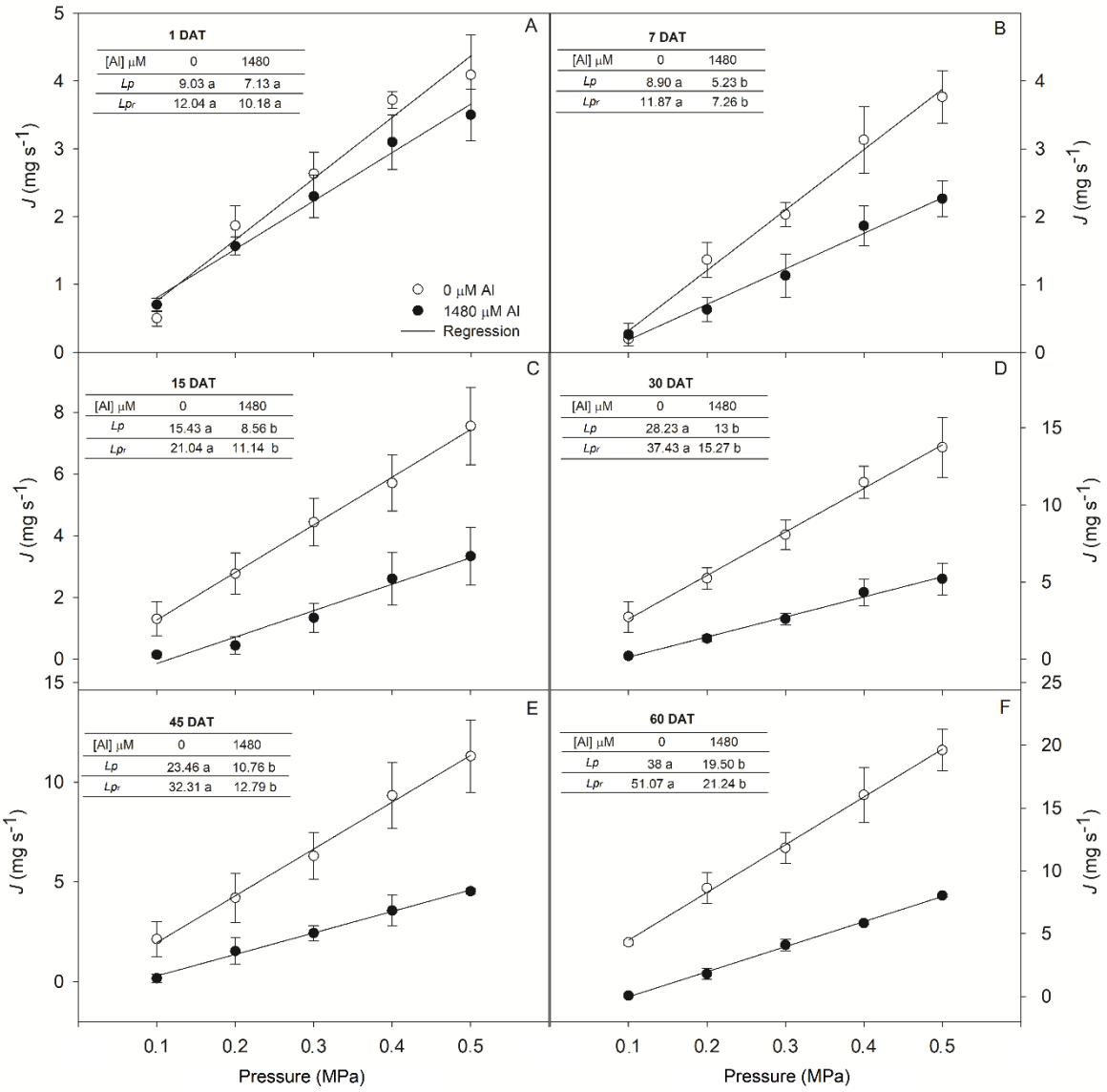

Fig 7. Relationship between xylem sap flow rate (*J)* and applied pressure (MPa) of roots of *C.* x *limonia* at 1 (A), 7 (B), 15 (C), 30 (D), 45 (E) and 60 (F) days after treatment (DAT) with 0 and 1480 µM Al in nutrient solution. The slopes of the linear regression lines indicate the root hydraulic conductance (*Lp*), and normalization by root diameter results in root hydraulic conductivity (Lp_r) Bars are standard errors. In the tables inside graphs, different letters indicate significant differences ($P< 0.05$) by Tukey test between 0 and 1480 µM Al.

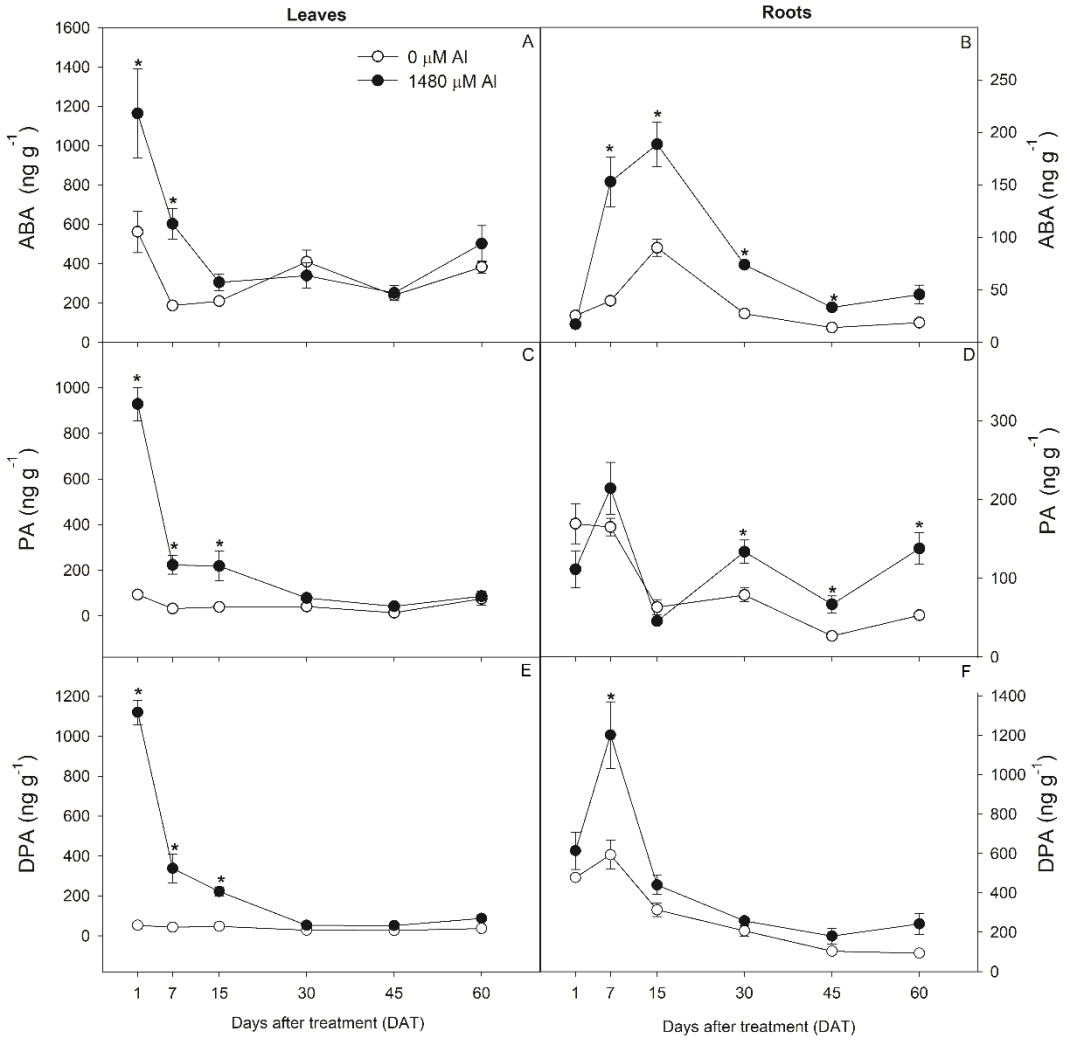

Fig 8. Mean values (n = 3 plants) of abscisic acid (A, B), phaseic acid (C, D) and dihydrophaseic acid (E, F) of leaves (A, C, E) and roots (B, D, F) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 μM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) by Tukey test between 0 and 1480 μM Al. Bars are standard errors.

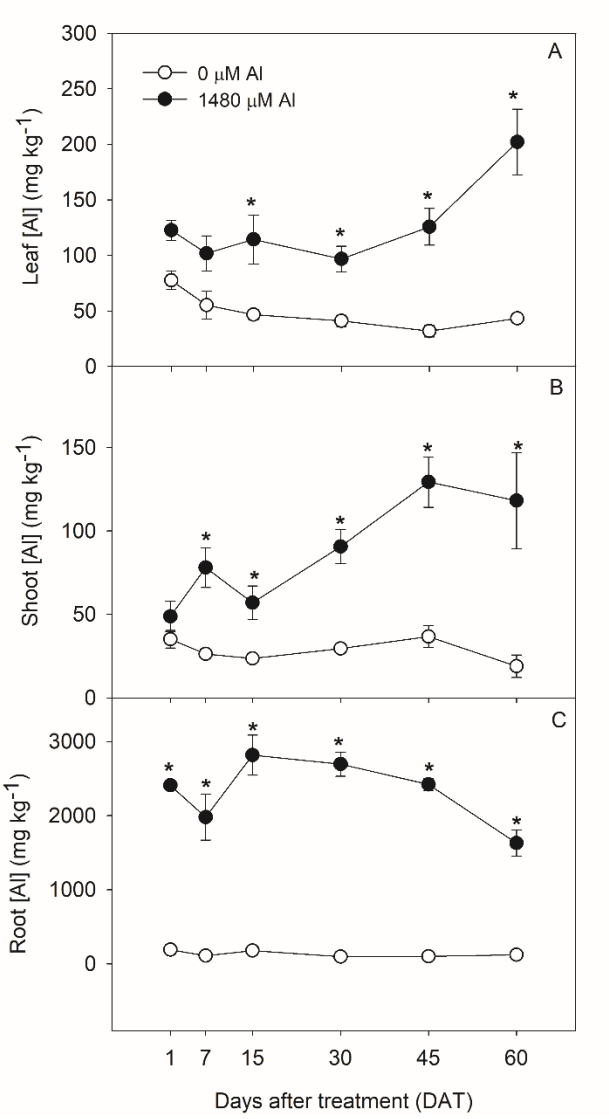

Fig. 9 Mean values (n = 3 plants) of aluminum (Al) concentration in leaves (A), stems (B) and roots (C) of *C.* x *limonia* at 1, 7, 15, 30, 45 and 60 days after treatment (DAT) with 0 and 1480 μM Al in nutrient solution. For each evaluation date, asterisks indicate significant differences ($P< 0.05$) by Tukey test between 0 and 1480 μM Al. Bars are standard errors.

Supplementary material:

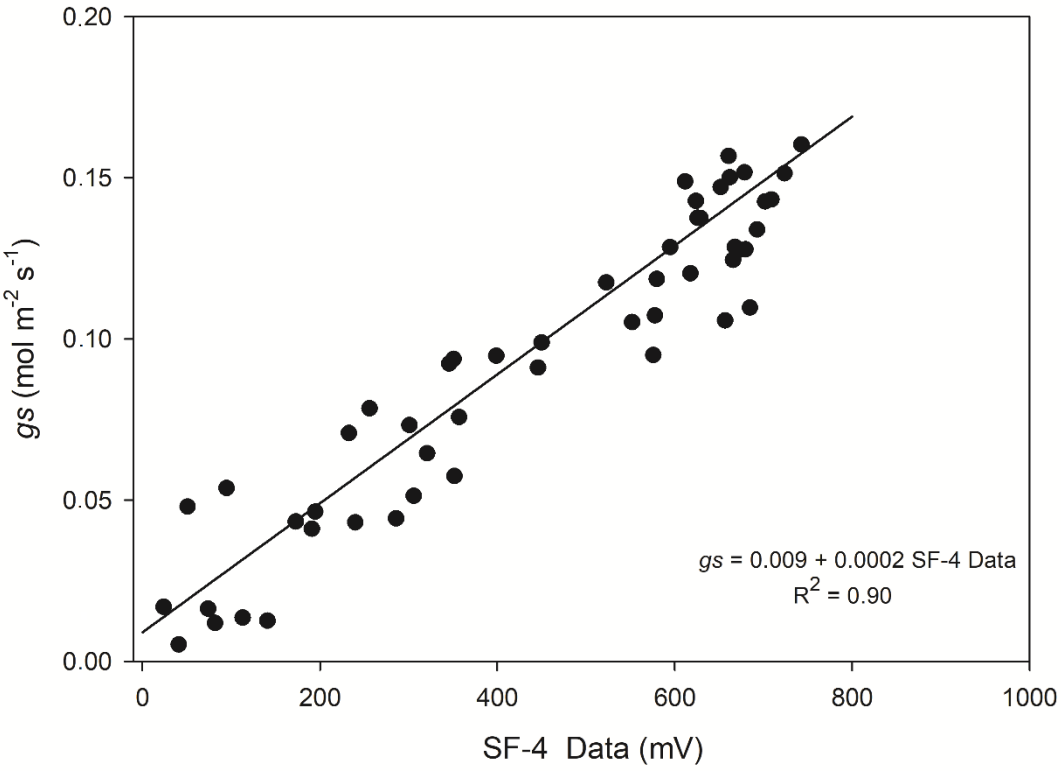

Fig S1. Linear relationship between millivolts (mV) measured by SF-4M sensors of sap flow and stomatal conductance (g_s) in *C.* x *limonia* grown in nutrient solution without aluminum. Each dot is the mean of three plants.

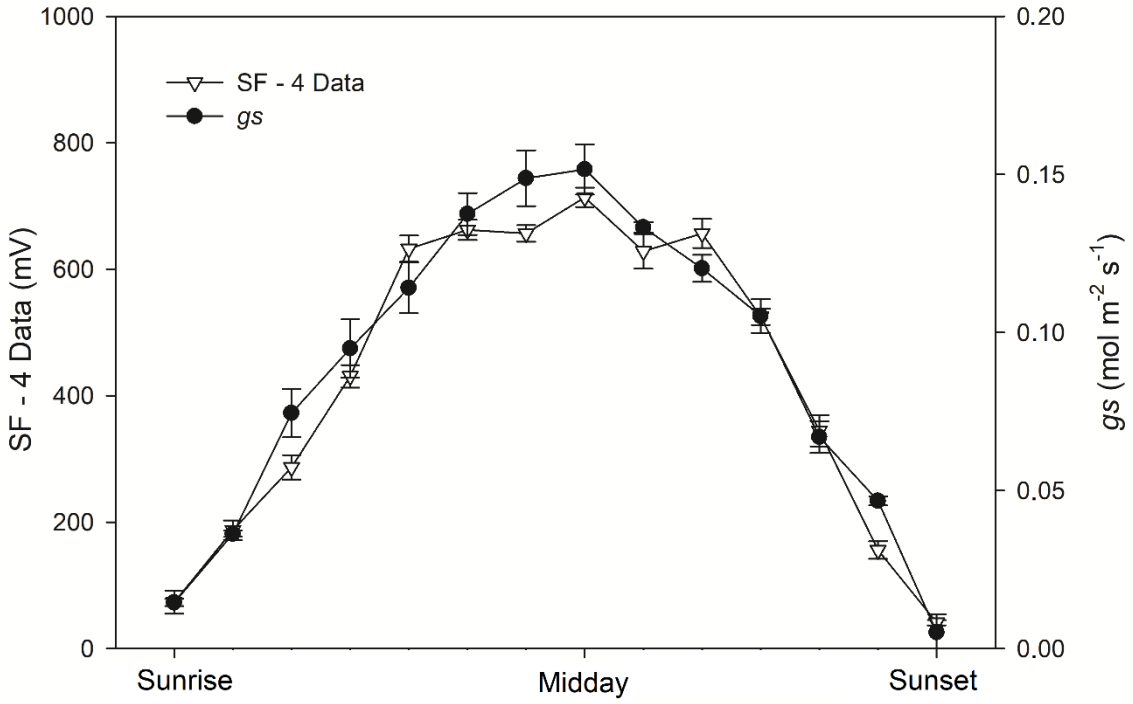

Fig S2. Mean values (n = 3 plants) of daily course of millivolts (mV) measured by SF-4M sensors of sap flow and stomatal conductance (g_s) in *C.* x *limonia* grown in nutrient solution without aluminum. Bars are standard errors.

CAPÍTULO II

Aluminum improves root growth and photosynthetic performance in *Ilex paraguariensis*

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Abstract

Aluminum (Al) is toxic to most plant species. Al-accumulating species, however, are so called because they grow well on acidic soils with high Al saturation without showing toxicity symptoms and accumulate more than 1 g per kg dry leaves. In *Ilex paraguariensis*, known as yerba mate, high concentrations of Al is observed in their leaves and infusions, but little is known about how this specie respond physiologically when exposed to Al. We asked whether the Al could increase the root growth, and, if so, it could be supported by photosynthetic performance or root auxin concentration. Seedlings of *I. paraguariensis* were cultivated in nutrient solution containing 0, 500, 1000 and 1500 μM Al for 60 days. We measured growth parameters, indole-acetic acid (IAA) concentrations in root tips, leaf gas exchange rates, photochemical variables, relative water content, leaf water potential and aluminum concentrations in plant organs. Until 60 days, Al-untreated plants showed reduced root growth in relation to plants exposed to 500 and 1000 μM Al and a peak of IAA within 15 days of study. Plants grown with 500 and 1000 μM Al showed increased root growth and a peak of IAA 7 days after Al exposure. Plants exposed to 500 and 1000 μM Al showed higher root and shoot development, which was supported by increased photosynthetic parameters. Our results demonstrate the Al is a benefic element to yerba mate plants.

Key-words: Al^{3+}; gas exchange rates; indole-acetic acid; yerba mate;

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust and it is naturally found in soils as Al silicates and oxides, all over the world (von Uexküll and Mutert, 1995). In acidic soils (pH < 5.0), Al is hydrolyzed to phytotoxic forms, being Al^{3+} the most plentiful and phytotoxic one (Vitorello et al., 2005; Singh et al., 2017). As acidic soils is a reality for approximately 30–45% of world's ice-free lands (von Uexküll and Mutert, 1995), then, Al is a critical limiting factor for plant growth and crop yields (Kochian et al., 2005; Singh et al., 2017).

In plants sensitive to Al, the inhibition of root growth is the first toxicity symptom (Ciamporová M, 2002; Vitorello et al., 2005; Horst et al., 2010). Aluminum binds with the pectic net in the apoplast of root cells, reducing cell elongation and restricting root growth (Wehr et al., 2010; Kopittke et al., 2015). Some studies, however, showed that Al increase auxin (indole-acetic acid – IAA) biosynthesis in root tips and, in relation to IAA concentration of above-ground organs, high root IAA concentration can stunt the root growth (Ryan et al., 1993; Silva et al., 2019). Aluminum also exerts indirect effects in above-ground plant organs, reducing plant height, stem, and leaf biomass (Banhos et al., 2016; Singh et al., 2017; Bressan et al., 2020), leaf gas exchange rates and mesophyll hydration (Jiang et al., 2009; Silva et al., 2018; Bressan et al., 2020; Gavassi et al., 2021).

Some species, however, are able to grow well on acidic soils with high Al saturation (m%) without showing toxicity symptoms (Watanabe and Osaki, 2002; Fung et al., 2008; Sun et al., 2020; Bressan et al., 2021). Plants that naturally grow on acidic soils with high m% can accumulate Al in their leaves and roots (Zaia et al. 2022), and when their leaf Al concentration surpasses 1000 mg kg^{-1} these are called Al-accumulating

species (Chenery 1948). Beneficial effects of Al have been reported in some Al-accumulating species (Osaki et al., 1997; Watanabe et al., 2005; Liu et al., 2020). *Melastoma malabathricum* L. (Melastomataceae) (Osaki et al., 1997), *Camellia sinensis* L. (Theaceae) (Sun et al., 2020) and *Vochysia tucanorum* Mart. (Vochysiaceae) (Bressan et al., 2021), Al-accumulating plants, showed increased root elongation and plant biomass when growing with Al in nutrient solution. Furthermore, Al also seems to improve the photosynthetic performance in some species (Liu et al., 2020; Hajiboland et al., 2013; Bressan et al., 2021). However, specific associations and interference of Al with physiological parameters explaining the beneficial effect of Al on plant growth of Al-accumulating species is not or, is rarely reported.

Ilex paraguariensis St. Hil. (Aquifoliaceae) is a native herbal tree widely consumed in Southern Latin America countries. Popularly known as 'yerba mate', their leaves are used for the preparation of the most traditional tea beverage called 'chimarrão' and 'terere' (Gaiad et al., 2006). The consumption of this beverage has many beneficial effects for human health (Bracesco et al., 2011), but since this species naturally grows in acidic soils with high m% (Santin et al., 2013), some studies point out high concentration of Al in their leaves and infusions (Gomes da Costa et al., 2009; Pozebon et al., 2015; Olivari et al., 2020), which seems to be a toxic element to humans (Mold et al., 2020). However, little attention is paid to how this plant grows and responds, from a physiological standpoint, when exposed to Al. One of these few studies demonstrated that seedlings of yerba mate increased their root growth when exposed to Al (Benedetti et al., 2017), indicating that, possibly, Al is a benefic element to this species.

In the present study, yerba mate plants were cultivated in nutrient solution for 60 days, under increasing concentrations of Al. We measured plant biomass, biometric parameters, IAA concentration in root tips, leaf gas exchange rates, photochemical

parameters, leaf hydration and Al concentrations in roots, shoots and leaves. Therefore, we ask whether the Al could increase the root growth and if it could be supported by photosynthetic performance throughout the experiment. We also assessed whether root growth differences in response to Al could be associated with root IAA concentration.

Material and methods

Plant material and experimental conditions

One hundred and twenty plants of *Ilex paraguariensis* St. Hil. (Aquifoliaceae), known as 'yerba mate', with 13 ± 0.5 cm high and exhibiting 12 ± 1.7 leaves, were used. After cleaning their roots under tap water, the plants were transferred to a hydroponic system (aerated solution), where they grew directly in nutrient solution inside opaque plastic boxes (50 cm × 30 cm × 15 cm; 20 L), for 60 days. One hundred and twenty plants per box were attached in expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick) in six equidistant holes with polyurethane foam strips placed around the plant collar. The boxes were maintained on benches, inside a greenhouse, under semi-controlled conditions (857.4 ± 112.6 μmol photons m^{-2} s^{-1} during measurements (see below)) and approximately 14 h of natural photoperiod. Air temperature and relative humidity inside the greenhouse were, respectively, 25.6 ± 2 ºC and 64.3 ± 2.2 %, between 9:00 and 11:30 h.

The nutrient solution was based on the solution proposed by Clark (1975) and it has been used to test Al resistance in different plant species (Banhos et al. 2016; Silva et al. 2018; 2019; Cavalheiro et al. 2020; Gavassi et al. 2021). It consisted of 1372.8 μM $Ca(NO_3)_2$, 507 μM NH_4NO_3, 224.4 μM KCl, 227.2 μM K_2SO_4, 218.6 μM KNO_3, 483.2 μM $Mg(NO_3)_2$, 12.9 μM KH_2PO_4, 26.01 μM $FeSO_4$, 23.8 μM NaEDTA, 3.5 μM $MnCl_2$, 9.9 μM H_3BO_3, 0.9 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.4 μM $NaMoO_2$. The nutrient

solution was completely changed every 15 days. The pH was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible. The Al treatments (500, 1000 and 1500 μM) were provided through $AlCl_3$, and these concentrations were used previously to test Al resistance in *Ilex paraguariensis* (Benedetti et al., 2017). The nominal 500, 1000 and 1500 μM Al resulted in 300 ± 18.9, 755 ± 22.5 and 1170 ± 26.4 μM Al, respectively.

Experimental design

Plants were transferred to nutrient solutions containing 0, 500, 1000 and 1500 μM Al, for 60 days. At 1, 7, 15, 30 and 60 days after transplant (DAT) the number of leaves, leaf biomass, leaf area, stem length and biomass, main root length, root surface area, root diameter and root biomass, as well as leaf gas exchange and chlorophyll fluorescence parameters were assessed. On the same evaluation dates, leaves were used to measure water potential (Ψ_{md} and Ψ_{pd}) and relative leaf water content (RWC). At the end of the experiment (60 DAT), the Al concentration in roots, stems and leaves were also measured.

Photosynthetic parameters

The CO_2 assimilation (*A;* μmol m^{-2} s^{-1}) and transpiration (*E*; mmol m^{-2} s^{-1}) rates, stomatal conductance (*gs*; mol m^{-2} s^{-1}) and intercellular CO_2 (*Ci*, μmol mol^{-1}) were evaluated in four plants per treatment 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/gs*) were calculated. The CO_2 concentration

entering the leaf cuvette (LCF chamber; 2 cm^2, LI-COR) was set to 400 µmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR). Measurements were carried out on cloudless days, between 9:00 and 11:30 h (Feistler and Habermann, 2012), period of the highest photosynthetic performance. The photosynthetic photon flux density (PPFD) was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to supply 1200 µmol photons m^{-2} s^{-1} in the leaf cuvette, according to Rakocevic et al. (2007).

Chlorophyll *a* fluorescence was measured with a portable modulated fluorometer (6400–40 LCF; LICOR), 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 F_s represents the maximum and the steady-state fluorescence in light-adapted leaves, respectively. The 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 light absorbance by the leaves. 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 from light-adapted leaves.

Leaf water potential and relative leaf water content

Four leaves per replicate (plants) were used to measure the leaf water potential at predawn (Ψ_{pd}) and midday (Ψ_{md}), and values (MPa) were obtained by the pressure chamber method (Turner, 1981), using a 3005F01 Plant Water Status Console (Soil Moisture, Santa Barbara, CA, USA) chamber.

The relative leaf water content was measured using four plants and excising eight leaf disks (two disks per plants) performed between 13:00 and 15:00, and calculated as:

$$RWC\ (\%) = [(FM-DM)/(TM-DM)] \times 100$$

where FM represents the fresh mass (the discs were immediately weighted on a scale); TM is the turgid mass after rehydration of samples for 24 h in 100 mL deionized water; and DM is the dry mass after oven-drying the discs at 60 ºC for 48h, according to Silva et al. (2018).

Biometric parameters

The plant organs were separated into leaves (plus petioles), stems and roots, at 1, 7, 15, 30 and 60 DAT. The number of leaves was counted and the shoot and root lengths were assessed with a ruler (cm). The total leaf area per plant was evaluated with an area meter (LI-3100, LI-COR, USA). The root surface area (cm^2) and root diameter were measured using a scanner (Epson perfection v700 photo, Suwa, Japan), which was coupled to a computer running the WinRHIZO^{TM} software (Regent Instruments, Canada). The plant organs samples were oven-dried at 60 ºC until constant mass, and biomass (g) was measured, using a precision scale.

Quantification of free IAA (indole-acetic acid)

Samples of root tips (420 µg fresh weight) were collected, submerged in liquid nitrogen and stored at –80 ºC before the analysis. For the extraction, samples were ground using liquid nitrogen and transferred to 2 mL microcentrifuge tubes. Sample extraction and derivatization were carried out as previously described by Rawlinson et al. (2015).

A gas chromatographer coupled to a mass spectrometer (Hewlett Packard 6890 gas chromatograph (GC) coupled to an HP5973 mass selective detector) was used for quantification of free IAA. An HP-1701 (30 m, 0.25 mm I.D., 0.5 µm film thickness)

column was used, with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The temperature program for analysis was 140 °C for 1 min, followed by an increase of 5 °C min^{-1} up to 210 °C, 1 min waiting, increase to 280 °C at 20 °C min^{-1}, then 1 min waiting at 280 °C. The ions were monitored at m/z 130 and 189, for the endogenous IAA, and m/z 136 and 195, for the labeled internal IAA standard. For calculating the endogenous amount of IAA the 130/136 ratio was used.

Aluminum concentration in plant organs

The Al concentration was quantified in dried samples of leaves (plus petioles), stems and roots collected at 60 DAT. The samples were sent to the plant nutrition laboratory at the Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), where these were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Al concentration was determined by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

Data analysis

A one-way analysis of variance (ANOVA) was conducted in order to test differences between Al treatments (0, 500, 1000 and 1500 μM), in leaf (number, biomass and area) stem (length and biomass), root (length, surface area, diameter and biomass), IAA concentration, *A*, *gs*, *Ci*, *E*, *WUE*, *IWUE*, ΦPSII, ETR, qP, Ψ_{pd}, Ψ_{md} and RWC, separately, at 1, 7, 15, 30 and 60 DAT, as well as in Al concentration in plant organs at 60 DAT. The Tukey test ($P < 0.05$) was used to perform post-hoc comparisons to determine the least significant difference between mean results.

Results

Biometric parameters

Plants exposed to 500 and 1000 μM Al showed an increase in growth when compared to control plants (Fig. 1). The leaf number (Fig. 2A), leaf biomass (Fig. 2B), and leaf area (Fig. 2C) increased 40%, 60% and 50%, respectively, in plants exposed to 500 and 1000 μM Al, and 27%, 43% and 34%, respectively, in plants exposed to 1500 μM Al, when compared to control, at 60 DAT. At the end of the experiment, plants exposed to 500 and 1000 μM Al showed an increase of 27% in stem length (Fig. 3A) and 37% in stem biomass (Fig. 3B), when compared to control plants. The main root length (Fig. 4A), root surface area (Fig. 4B) and root biomass (Fig. 4D) were, respectively, 31%, 33% and 58% higher in plants exposed to 500 μM Al, and 18%, 20% and 50% more elevated in plants exposed to 1000 μM Al, in relation to control plants, at 60 DAT. On the same evaluation date, plants exposed to 1500 μM Al showed increases of 20% in root surface area when compared to control plants (Fig. 4B). Thus, plants exposed to 500 and 1000 μM Al grew more in the root and shoot in relation to control plants.

Effect of Al on root IAA contents

At 1 DAT, Al-treated plants showed higher IAA concentration in their root tips in relation to control plants (Fig. 5). A peak of IAA was observed in plants exposed to 500 and 1000 μM Al at 7 DAT, and it was higher than IAA concentrations of plants exposed to 0 and 1500 μM Al. At 15 DAT, a peak of IAA occurred in plants exposed to 0 μM Al, and it was higher than the values exhibited by Al-treated plants. After 15 DAT, IAA concentration was similar in root tips of all treatments until the end of the experiment.

Thus, Al induced peaks of IAA in Al-treated plants after 7 days, while a peak of IAA was noticed for control plants only after 15 days.

Photosynthetic parameters

Control plants showed an approximate 60% reduction in CO_2 assimilation rate (*A*) at 30 and 60 DAT when compared to Al-treated plants (Fig. 6A). The same response pattern was observed for *gs* (Fig. 6B) and *E* (Fig. 6D), and the latter was 75% and the former 67% lower at 30 and 60 DAT. The intercellular CO_2 (Fig. 6C) and *WUE* (Fig. 6E) were the same between treatments throughout the study, while IWUE (Fig. 6F) was approximately 50% (at 30 DAT) and 40% (at 60 DAT) higher in control plants in relation to Al-treated plants.

Except for qP (Fig. 7C), which was the same between treatments throughout the study, ΦPSII (Fig. 7A) and ETR (Fig. 7B) were higher in Al-treated in relation to untreated plants, at 60 DAT. On this date, ΦPSII and ETR of Al-treated plants were, respectively, 46% and 52% higher than control plants. Thus, Al seemed to have enhanced the photosynthetic performance in *Ilex paraguariensis*.

Leaf water potential and relative water content

Predawn leaf water potential (Ψ_{pd}) was the same in all Al treatments throughout the study (Fig. 8A). Midday leaf water potential (Ψ_{md}) was similar between treatments until 30 DAT (Fig. 8B). After 60 days, Ψ_{md} was 45% lower in control plants in relation to Al-treated ones (Fig. 8B). The RWC was approximately 20% lower in control plants

in relation to Al-treated plants, at 60 DAT (Fig. 9). Thus, Al-treated plants maintained leaf hydration, while the absence of Al reduced it, mainly at 60 DAT.

Aluminum concentration in plant organs

Aluminum-treated plants accumulated more Al than control plants, and plants exposed to 1500 μM Al accumulated more Al in their organs when compared to all treatments (Fig. 10). In relation to control plants, those exposed to 500, 1000 and 1500 μM Al accumulated, respectively, 66%, 84% and 87% more Al in their leaves. When compared to leaves and stem, approximately 85% of Al was retained in the root system of all Al-treated plants (Fig. 10). Thus, the more Al available in the nutrient solution, more Al was retained in plants organs.

Discussion

Plants of *Ilex paraguariensis* cultivated in nutrient solution containing 500, 1000 and 1500 μM Al showed an enhancement in growth and development when compared to those not exposed to Al. It is known that the first and most conspicuous symptom of Al toxicity is the inhibition of root growth (Horst et al. 2010; Sun et al. 2010; Singh et al. 2017; Silva et al., 2019), and this can be observed in many species, such as *Coffea arabica* (Konrad et al, 2005), *Citrus* spp. (Yang et al., 2011; Banhos et al., 2016; Silva et al., 2018) and *Zea mays* L. (Anjum et al., 2016). Here, we notice an inverse effect, with elevated concentrations of Al (especially 500 and 1000 μM Al) stimulating root growth. This was evidenced by higher values of main root length (Fig. 4A), root surface area (Fig. 4B), root biomass (Fig. 4D) and also by the release of white new roots (Fig. 1), mainly in plants

exposed to 500 and 1000 μM Al, in relation to control plants. In *Melastoma malabathricum* (Melastomataceae) (Watanabe et al., 2005) and *Vochysia tucanorum* (Vochysiaceae) (Bressan et al., 2021), Al-accumulating species, the Al caused the release of white new roots and improved the root biomass. In *Camelia sinensis* (Theaceae), 250 and 500 μM Al also stimulated the root growth (Fung et al., 2008). Therefore, plants that are tolerant to Al, including Al-accumulating ones, use the Al as a benefic element.

Besides improvements in the root growth, plants of *I. paraguariensis* exposed to all Al treatments also showed further growth and development in above-ground organs, as evidenced by larger leaf number (Fig. 2A) and leaf area (Fig 2C), resulting in higher leaf biomass (Fig. 2B) in relation to control plants. When growing without Al, Al-accumulating species show not only reduced root growth, but also morphological changes in above-ground organs. In *M. malabathricum*, the absence of Al causes chlorosis and curling in leaves (Watanabe et al., 2006). Similarly, *V. tucanorum*, a Cerrado woody Al-accumulating species, showed leaf chlorosis and shed their leaves when grown without Al (Bressan et al., 2021). In the present study, no morphological changes were observed in leaves of plants growing without Al (Fig. 1) and, although no symptoms caused by the lack of Al have been observed, it is notable that both, root and shoot growth, were stunted in the absence of Al (Fig. 1, 2, 3 and 4). In contrast, when exposed to the highest Al concentration (1500 μM Al), the plants produced more leaves and showed higher leaf biomass and leaf area in relation to control plants (Fig 2). These results demonstrate that, besides being extremely tolerant to Al, it has a beneficial effect on the growth and development of *I. paraguariensis*.

In this study, we expected that root tips of plants not exposed to Al would show high IAA concentration, and this would explain their lower root growth, since high IAA

concentration (in relation to the IAA concentration measured in leaves) can inhibit the root cell elongation (Thimann 1939; Rahman et al., 2007). Indeed, plants not exposed to Al showed a peak of IAA at 15 DAT (Fig. 5), and this could be associated with their low root growth (Fig. 4A, B and D). Nevertheless, a peak of IAA also occurred in plants exposed to 500 and 1000 μM Al, at 7 DAT (Fig. 5). Many studies demonstrate that auxin plays an important role in the lateral root development (Casimiro et al., 2001; Fukaki et al., 2007; Du and Scheres 2018). In *Arabidopsis thaliana*, auxin application increased the number of lateral roots while the inhibition of auxin transport decreased the development of lateral roots (Marchant et al., 2002). In a recent study, high IAA concentrations were observed in the roots of tea plants within 6 h to 7 days, when the plants were exposed to 400 μM Al, and this was associated with the greater root growth in these plants (Gao et al., 2022). Therefore, in the present study, it is feasible that, somehow, the Al stimulated early IAA production in roots and this could have led to the increase in root growth of plants exposed to 500 and 1000 μM Al, observed at the end of the study. Nevertheless, further studies are necessary to investigate the relationship between Al and IAA on the root growth of yerba mate plants.

In addition to stimulating growth in yerba mate plants, Al seems to improve photosynthetic performance in this species. Previous studies have already demonstrated the (benefic) effect of Al in photosynthetic parameters of plants, such as *Camelia sinensis* (Hajiboland et al., 2013), *C. japonica* (Liu et al., 2020) and *V. tucanorum* (Bressan et al., 2021). When exposed to Al, all these species showed increased CO_2 assimilation (*A*), stomatal conductance (*gs*) and transpiration rates (*E*) when compared to those not exposed to Al. In the present study, the same response pattern was observed at 30 and 60 DAT, when higher values of *gs* reflected in also higher values of *A* and *E* in Al-treated plants when compared to control ones (Fig. 6A, B and D). It is likely their increased

photosynthetic capacity could be related to their larger root surface area when exposed to Al (Fig. 4B). Larger root surface area increases water uptake, maintaining a more hydrated mesophyll, which would guarantee open stomata and better leaf carbon assimilation.

On the other hand, in Al-sensitive species, reductions in *gs*, *A* and *E* have already been reported (Simon et al. 1994; Konrad et al. 2005; Silva et al. 2012; Silva et al. 2018), and some other studies have evidenced that stomatal limitations may be related not only to root growth impairments, but also to an intrinsic difficulty for water uptake and transport when these plants are exposed to Al (Silva et al., 2018; Cavalheiro et al., 2019; Gavassi et al., 2021). Here, the opposite was observed. Ilex paragraiensis, which are tolerant to Al, when '*not*' exposed to Al, showed low *gs* (Fig. 6B), resulting in lower *A* (Fig. 6A). These results could be a consequence of low leaf hydration, since lower Ψ_{md} (Fig. 8B) and RWC (Fig. 9) were observed at 60 DAT. Furthermore, low *A* in plants not exposed to Al was also associated with reduced photochemical performances, since lower values of effective quantum yield of PSII (ΦPSII) (Fig. 7A) and apparent electron transport rate (ETR) (Fig. 7B) were observed at 60 DAT. Taken together, our results indicate the presence of Al in the roots improves, indirectly, the photosynthetic performance of yerba mate plants when compared to plants growing without Al. Similar results were found for *C. sinensis* (Hajiboland et al., 2013) and *V. tucanorum* (Bressan et al., 2021) and, and for the former, besides low ΦPSII and ETR, the absence of Al also reduced the concentrations of *a* chlorophyll and carotenoids of *C. sinensis*.

Most studies about the relationship between yerba mate and Al focus on plant Al concentrations, especially in leaves and, of course, their infusions (Wróbel et al., 2000; Costa et al., 2002; Gomes da Costa et al., 2009; Barbosa et al., 2015; Barbosa et al., 2020;

Olivari et al., 2020). In the literature, one may find leaf Al concentrations ranging from 300 (Barbosa et al., 2020) to 1,235 mg Al per kg dry leaves (Reissmann et al., 1999), and this range can be explained by management conditions, plant age and seasonal factors (Reissmann et al., 1999; Heck and Mejia, 2007). In the present study, 300, 700 and 900 mg Al per kg dry leaves were found in plants exposed to 500, 1000 and 1500 μM Al, respectively (Fig. 10). This indicates that, as expected, when the Al concentration is raised in the nutrient solution, more Al was accumulated in the leaves. Nevertheless, the largest Al concentration was found in the roots of these plants (Fig. 10), since this was the organ in direct contact with Al in nutrient solution. Similar results were found for *Citrus limonia* (Banhos et al., 2016; Silva et al., 2018) — Al-sensitive species —, *Styrax camporum* (Bressan et al., 2020) — moderate Al-accumulating species —, and *Symplocos paniculata* (Schmitt et al., 2016) — Al-accumulating species. Plants that accumulate more than 1000 mg per kg dry leaves are considered Al-accumulators (Chenery and Sporne, 1976), but studies conducted in nutrient solution are not adequate to classify plants into Al-accumulators or non-accumulators. Field studies, measuring plants growing in their native environments, are more adequate for this. In the present study, Al caused improvements in plant growth, supported by enhanced performance of physiological parameters, even in plants exposed to the highest Al concentrations (1500 μM Al). Therefore, besides being extremely tolerant to Al, this element is benefic to *I. paraguariensis*.

It is well known that infusions of yerba mate leaves naturally show high concentration of Al (Wróbel et al., 2000; Giulian et al., 2007; Bragança et al., 2011; Maiocchi et al., 2016) and several studies have focused on demonstrating how the consumption of this beverage negatively affects human health (Gomes da Costa et al., 2009; Bortoli et al., 2018). Thus, is there a balance between the use of Al to improve plant

growth and, at the same time, avoid it from being excessively accumulated in leaves? Little is known about the impact of Al on the plant growth and physiological responses of this species. As a novelty, our study demonstrated that Al enhances plant growth, which was supported by photosynthetic performance. This raises the question whether Al could concomitantly benefit the plant growth while retaining Al in their roots, avoiding its transport to the leaves. We also point out that the increased root growth in plants exposed to 500 and 1000 μM Al may be related to higher IAA concentration in root tips within the first week of exposure to Al.

Conclusion

The Al is benefic for plant growth of yerba mate, mainly when exposed to 500 and 1000 μM Al. The enhanced root growth in plants exposed to 500 and 1000 μM Al seems to be associated with increases in IAA concentration in root tips within the first week of Al exposure. The improved plant growth of Al-treated plants is supported by the increased performance of photosynthetic parameters. In contrast, the absence of Al causes a delay in root growth, with negative consequences for leaf hydration and photosynthetic performance. Nevertheless, no toxicity symptoms were observed even when yerba mate plants were exposed to the highest concentrations of Al, evidencing this species is considerably tolerant to Al.

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Figures

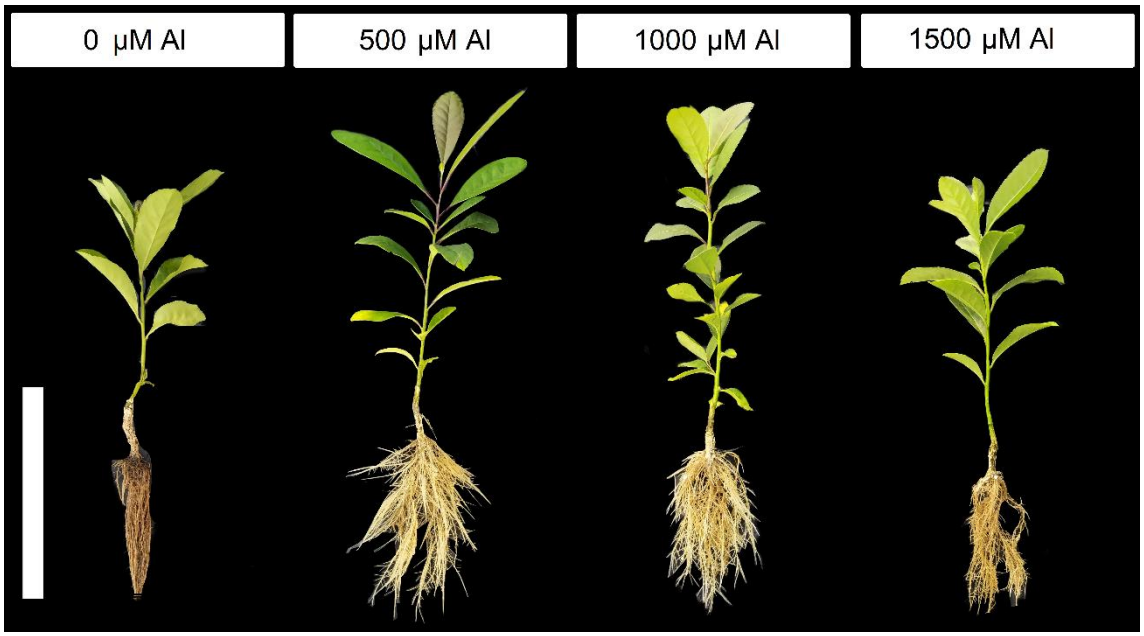

Fig 1. Morphological details of roots and shoots of *I. paraguariensis* at 60 days after treatment (DAT) with 0, 500, 1000 and 1500 µM Al in nutrient solution. Bars = 20 cm

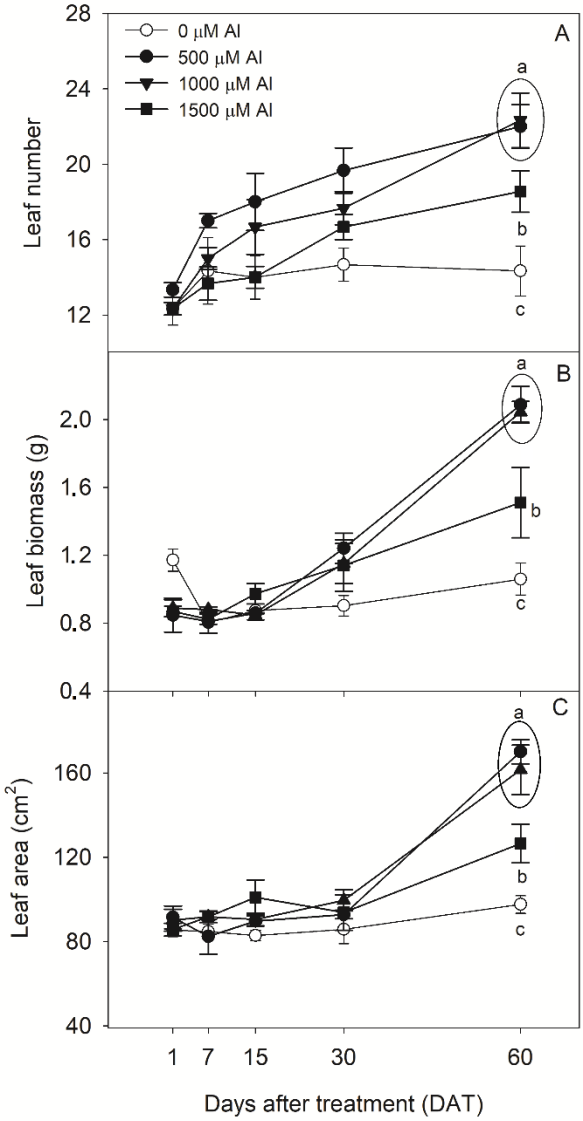

Fig 2. Mean values (n = 4) of leaf number (A), leaf biomass (B) and leaf area (C) of *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

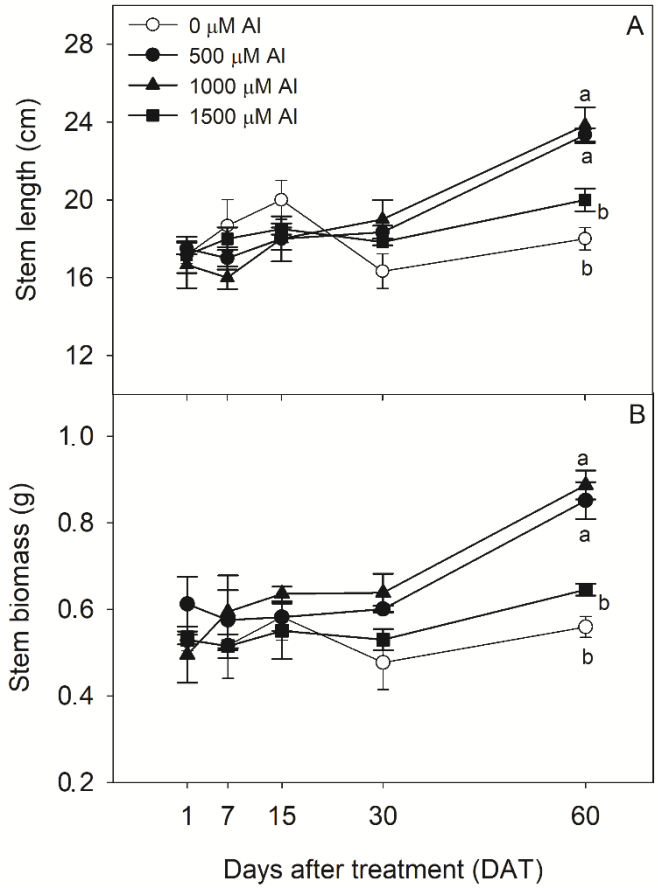

Fig 3. Mean values (n = 4) of stem length (A) and stem biomass (B) of *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

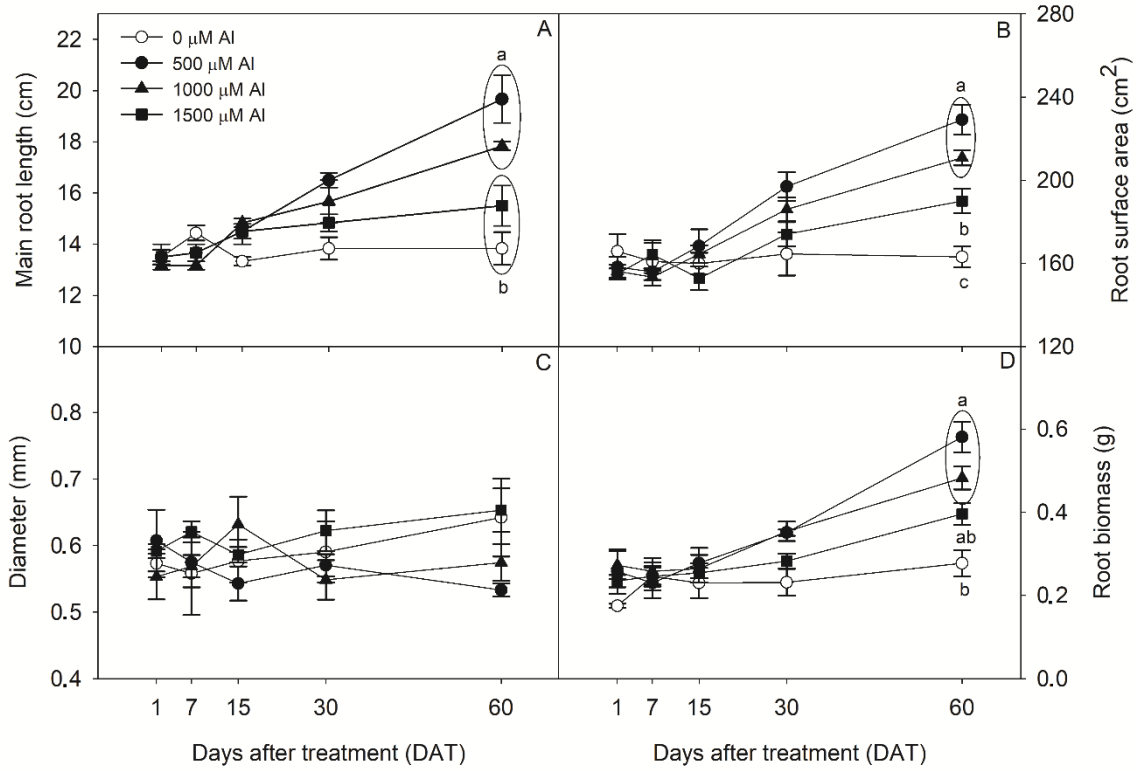

Fig 4. Mean values (n = 4) of main root length (A), root surface area (B), diameter (C) and root biomass (D) of *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

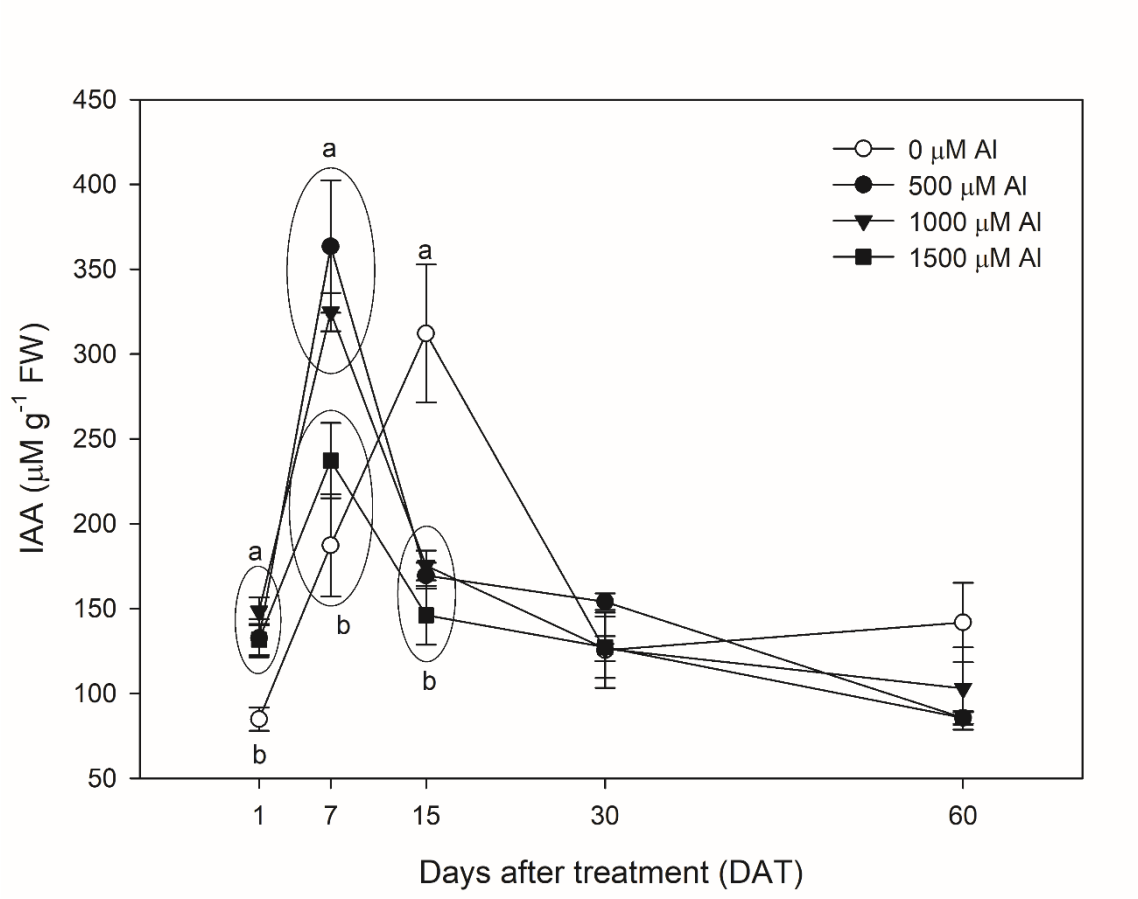

Fig 5. Indole-acetic acid (IAA) concentration measured in root tips of *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

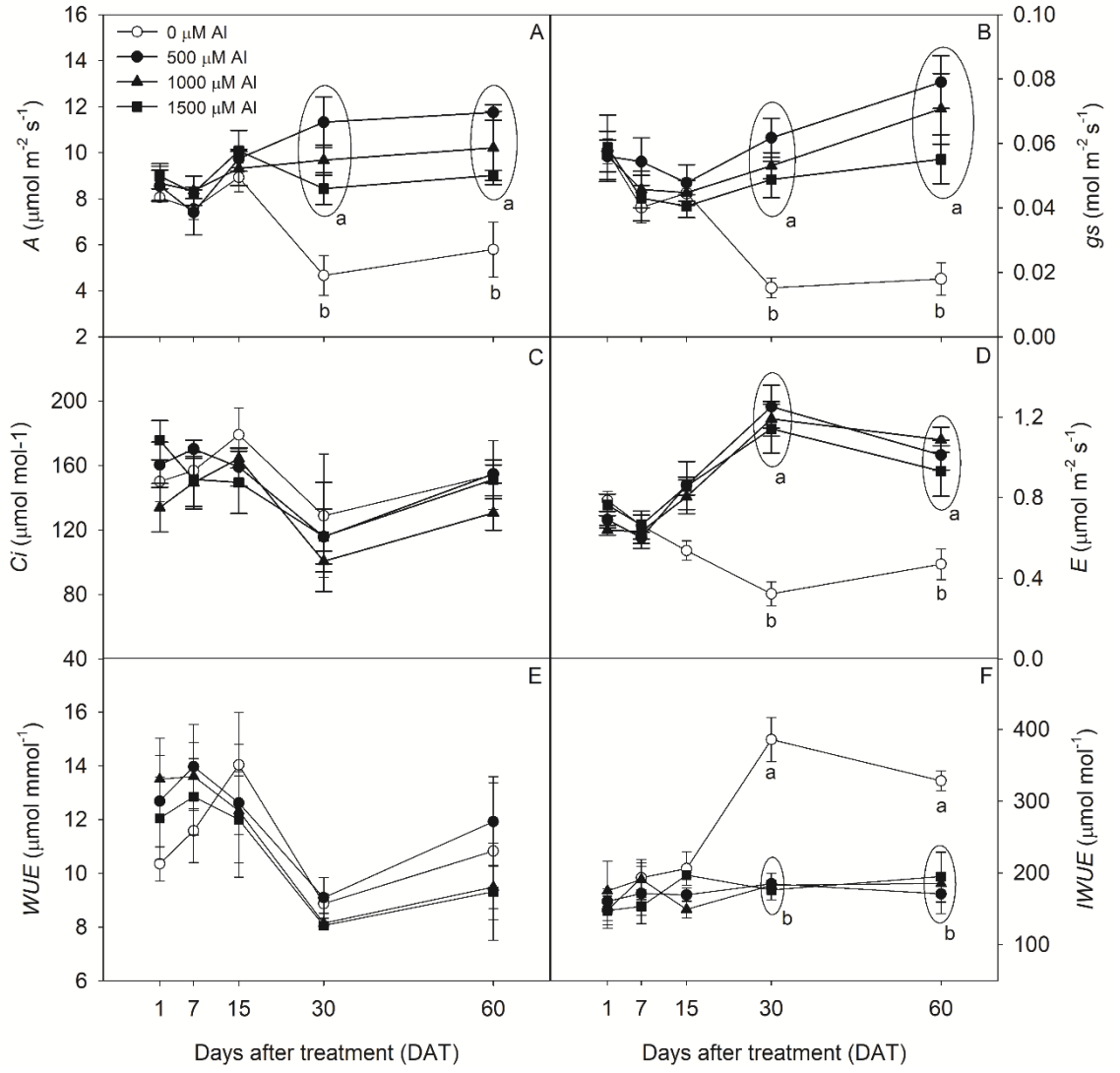

Fig 6. Mean values (n = 6) of CO_2 assimilation rates (A), stomatal conductance (B), intercellular CO_2 (C), transpiration rates (D), water use efficiency (E) and intrinsic water use efficiency (F) in *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

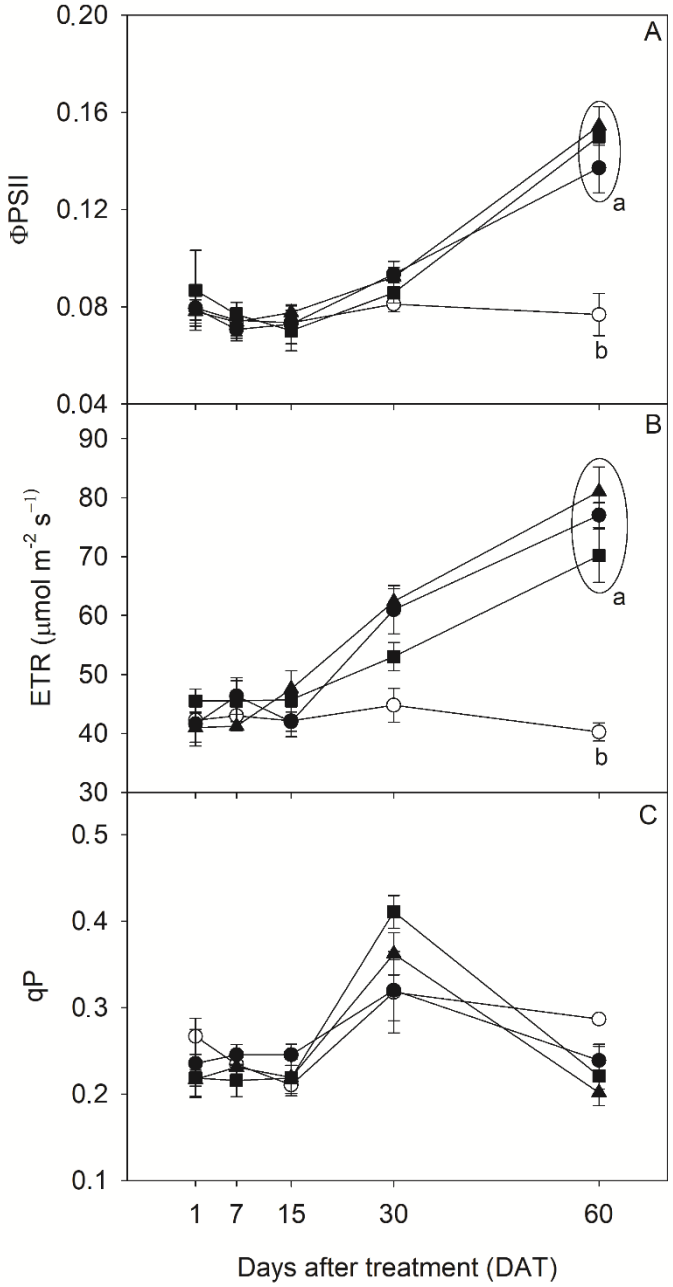

Fig 7. Mean values (n = 6) of effective quantum yield of PSII (A) apparent electron transport rate (B) and the proportion of open PSII reaction centers (C) in *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 µM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

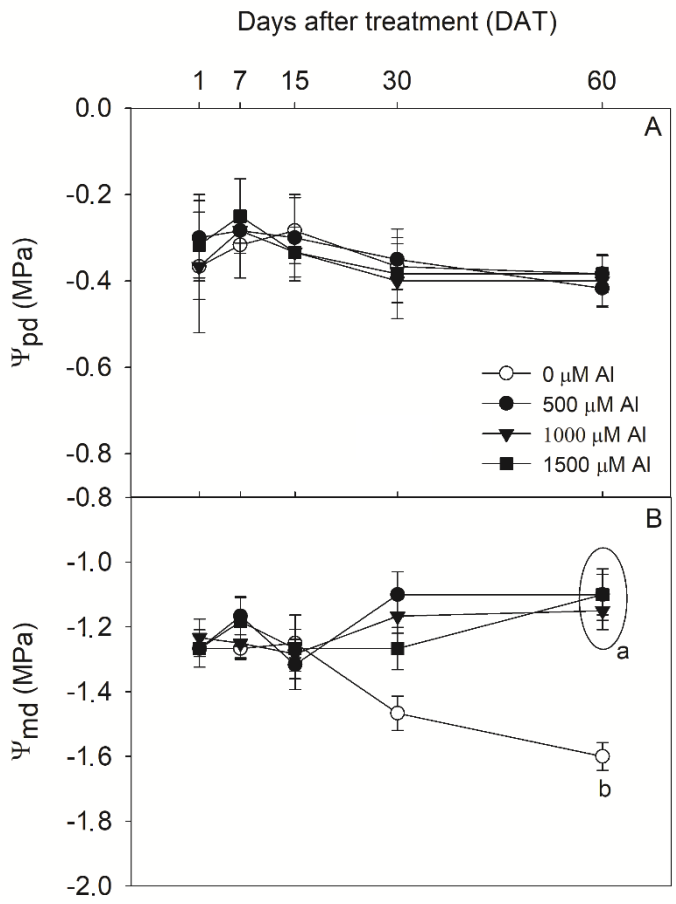

Fig 8. Mean values (n = 4) of predawn (A) and midday (B) leaf water potentials in *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

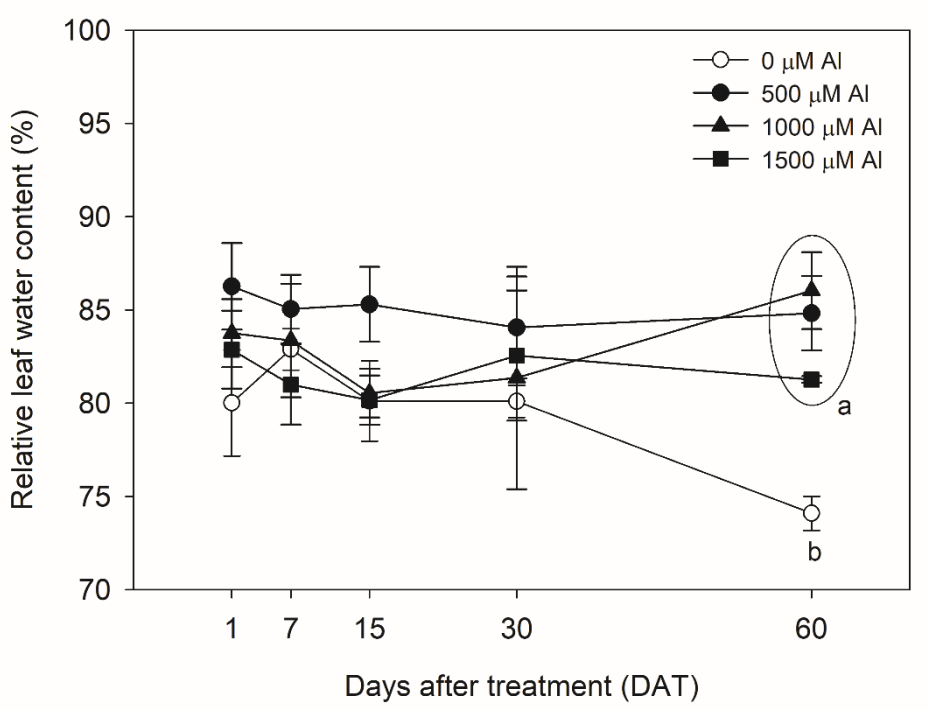

Fig 9. Mean values (n = 6) of relative water content (RWC) of *I. paraguariensis* at 1, 7, 15, 30 and 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each evaluation date, different letters indicate significant differences ($P < 0.05$) by Tukey test between Al-treatments. Bars are standard errors.

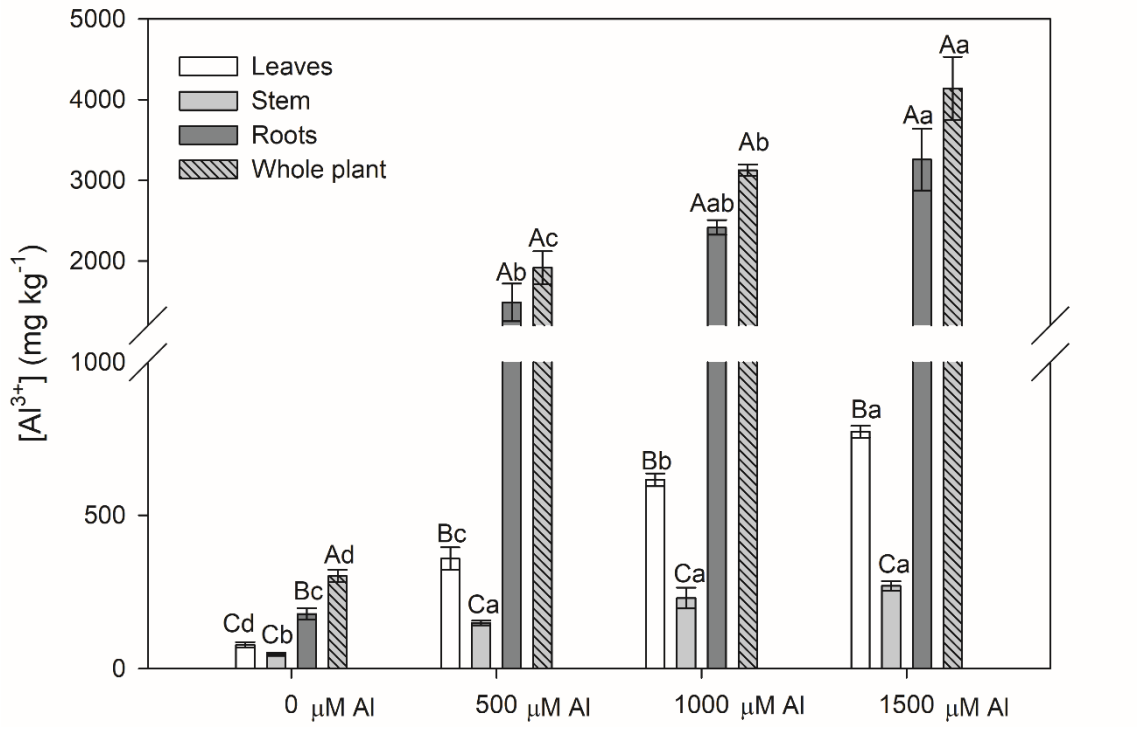

Fig 10. Mean values (n = 4) of aluminum concentrations in leaves, stem, roots and in the whole plant of *I. paraguariensis* at 60 days after treatment (DAT) with 0, 500, 1000 and 1500 μM Al in nutrient solution. For each plant organ (or the whole plant) different lower case letters indicate significant differences ($P < 0.05$) between Al-treatments. For each Al treatments, different upper case letters indicate significant difference ($P < 0.05$) between plant organs. Bars are standard errors.

CAPÍTULO III:

No direct effect of aluminum (Al) on the Rubisco performance of *Qualea grandiflora*, an Al-accumulating species from the Brazilian savanna

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Abstract

Aims Aluminum (Al) is toxic to most plant species. However, Al-accumulating species accumulate Al without showing toxicity symptoms. Previous studies have evidenced Al in chloroplasts of Al-accumulating species from the Cerrado vegetation in South America. Therefore, we ask whether the Al increase the carbon assimilation through enhanced apparent efficiency of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco).

Methods Seedlings of the Al-accumulator *Qualea grandiflora* (Vochysiaceae) were grown in nutrient solution with 0, 740 and 1480 μM Al. Plant growth parameters, relative leaf water content, Al concentration in plant organs, gas exchange and apparent carboxylation efficiency (measured from *A*/*Ci* curves) were evaluated for 60 days.

Results Al-untreated plants showed no root growth, necrotic roots, and low gas exchange rates with decreased apparent carboxylation efficiency. Al-treated plants, however, showed new white roots, increased root biomass leading to higher leaf hydration. Apparent carboxylation efficiency was higher in these plants. The more Al available in the nutrient solution, more was retained in plant organs.

Conclusion The absence of Al compromises the root integrity leading to limited leaf hydration. No positive direct effect of Al on the Rubisco performance was evidenced in Al-treated plants.

Key words: Al^{3+}. Gas exchange rates. *A*/*Ci* curves. Nutrient solution. Cerrado woody species

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust. It naturally occurs in the soil as Al silicates and oxides (von Uexküll and Mutert, 1995). Nevertheless, in acidic soils (pH < 5.0) it is hydrolyzed to phytotoxic forms, such as the metallic Al^{3+} (Kochian 1995; Singh et al. 2017), and approximately 30% of the world's ice-free soils are acidic and shows high Al saturation (m%) (von Uexküll and Mutert, 1995). Therefore, under these conditions Al is highly available to plants, including sensitive and tolerant ones.

In sensitive plants, which includes most crop species, the first symptom of Al toxicity is the inhibition of root growth (Kopittke et al. 2008; Horst et al. 2010; Silva et al. 2019). Aluminum can bind with negatively charged pectic nets in the root cell wall, decrease wall functions and inhibit root elongation (Horst et al. 2010). To explain the stunted root growth, hormonal imbalance in root tips was already reported (Sun et al. 2010; Silva et al. 2019). Aluminum can also decrease leaf gas exchange rates, lowering CO_2 assimilation (Chen et al. 2005; Silva et al. 2018), although it is considered an indirect effect as Al is mostly retained in the root system (Vitorello et al. 2005; Silva et al. 2018; Gavassi et al. 2021).

Some tolerant species rely on root exudation of organic acids (OAs) (malate, citrate, and oxalate) that form non-toxic Al-OAs complexes in the rhizosphere avoiding Al uptake by the root (Watanabe et al. 2005; Ryan et al. 2011). Nevertheless, there are plant species that accumulate Al in their leaves and roots without showing toxicity symptoms, and the most studied ones include those from Melastomataceae (Jansen et al. 2002; Timpone and Habermann, 2022), Rubiaceae (Jansen et al. 2003; Malta et al. 2016), Symplocaceae (Schmitt et al. 2016), Theaceae (Carr et al. 2003; Tolrà et al. 2011), and Vochysiaceae (Haridasan 1982; Andrade et al. 2011; Nogueira et al. 2019). To be

classified as an Al-accumulating species it must accumulate at least 1000 mg Al kg^{-1} dry leaves (Chenery 1948). Some of these species show beneficial effects of Al, enhancing plant biomass and gas exchange rates (Hajiboland et al. 2013; Liu et al. 2020; Bressan et al. 2021), although these are still uncomprehending physiological effects of Al.

The Cerrado vegetation in South America, broadly known as 'Brazilian savanna' is comprised of few Al-accumulating woody species from Melastomataceae, Rubiaceae, Symplocaceae and Vochysiaceae; the rest of its woody vegetation consists of non-accumulating ones (Souza et al. 2015), and both groups grow well on acidic (pH < 4.0) soils with m% > 50% (Haridasan 1982; Lira-Martins et al. 2022). Many studies have already tried to attribute physiological functions to Al in these plants. Aluminum was already evidenced in chloroplasts of *Callisthene major* and *Qualea grandiflora* (Vochysiaceae) (Andrade et al. 2011), and *Rudgea virbunoides* (Rubiaceae) (Malta et al. 2016), Al-accumulating woody species from the Cerrado. Thus, these findings have emerged a possible role for Al in photosynthetic processes in these plants. Other field studies, however, showed no involvement of Al in chloroplasts or even photosynthetic tissues of Melastomataceae and Vochysiaceae (Bressan et al. 2016; Guilherme Pereira et al. 2018). High CO_2 assimilation rates were found in the Al-accumulating *Vochysia tucanorum* (Vochysiaceae) seedlings grown in nutrient solution with Al, but no direct effects of Al in photosynthetic parameters could be evidenced (Bressan et al. 2021).

In the present study, *Q. grandiflora* seedlings were cultivated in nutrient solution with 0, 740, and 1480 μM Al for 60 days. We assessed plant growth, Al concentrations in plant organs, leaf hydration, gas exchange parameters, including photosynthetic responses to intercellular CO_2 concentration (*A*/*Ci* curves) to obtain the apparent carboxylation efficiency. Thus, we investigate whether the Al increase the carbon

assimilation through enhanced apparent efficiency of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco).

Material and methods

Plant material and experimental conditions

We used ninety-six four-month-old plants of *Qualea grandiflora* (Vochysiaceae) Mart. with approximately 8 ± 0.6 cm high and exhibiting 6 ± 0.5 leaves. The plants were obtained from from a native plant Cerrado nursery (Carobinha, São Simão, SP, Brazil), by seed germination directly on Cerrado soil. After removing the plants from cultivation bags containing Cerrado soil, their roots were washed with tap water and transferred to opaque plastic boxes (50 cm x 30 cm x 15 cm; 20L) with nutrient solution containing 0, 740 (20 mg L^{-1}) and 1480 (40 mg L^{-1}) μM Al, where they grew for 60 days. Eight plants per box were fixed in expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick) in eight equidistant holes with polyurethane foam strips that were placed around the plant collar.

The boxes were kept on benches (1.20 m from the ground) inside a greenhouse under semi-controlled conditions. Inside the greenhouse the air temperature and relative humidity was of 26.2 ± 2 ºC and 62.1 ± 3.5 % and photosynthetic photon flux density (PPFD) of 784.4 ± 102.9 μmol photons m^{-2} s^{-1} between 9 h and 11:30 h, and daily natural photoperiod of approximately 12 h.

Nutrient solution

The chemical composition of the nutrient solution was based on the solution proposed by Clark (1975) that has been used to test Al responses on Cerrado woody species (Banhos et al., 2016; Bittencourt et al., 2020; Bressan et al., 2020; Bressan et al., 2021). It consisted of 196.11 µM $Ca(NO_3)_2$, 72.43 µM NH_4NO_3, 32.06 µM KCl, 32.46 µM K_2SO_4, 31.23 µM KNO_3, 69.03 µM $Mg(NO_3)_2$, 4.3 µM KH_2PO_4, 3.72 µM $FeSO_4$, 3.4 µM NaEDTA, 0.5 µM $MnCl_2$, 1.41 µM H_3BO_3, 0.13 µM $ZnSO_4$, 0.03 µM $CuSO_4$, and 0.06 µM $NaMoO_2$. This solution resulted in the following macronutrients (in mM): NO_3^- 0.137; NH_4^+ 0.058; P, 0.0019; K, 0.123; Ca, 0.204; Mg, 0.047; S, 0.031; and micronutrients (in µM): Cl, 30.58; Fe (EDTA), 3.32; B, 1.19; Mn, 0.41; Zn, 0.10; Cu, 0.04; and Mo, 0.04. The Al treatments (740 and 1480 µM Al) were provided through $AlCl_3$, and these concentrations have been used to test Al responses in Cerrado Al-accumulating plants in nutrient solution (Banhos et al., 2016; Bittencourt et al., 2020; Bressan et al., 2020). The pH of the solution was monitored daily and maintained at 4.0 ± 0.1 (for all treatments) to keep the Al as soluble as possible, and the nutrient solution was totally replaced every 15 days. The nominal 740 and 1480 µM Al resulted in 659 ± 89 and 1234 ± 81 µM Al, respectively.

Experimental design

After transplant, the plants grew directly in nutrient solutions with 0, 740 and 1480 µM Al for 60 days. At 1, 15, 30 and 60 days after treatment (DAT), biometric data (leaf number, leaf area, stem and root length, and biomass of organs), leaf gas exchange rates (*A*, *gs* and *E*), apparent carboxylation efficiency (from *A*/*Ci* curves) and relative leaf water

content (RWC) were assessed. On the same evaluation dates, the Al concentration in each organ (leaf, stem, and root) were also measured.

Leaf gas exchange and apparent carboxylation efficiency

The CO_2 assimilation (*A;* μmol m^{-2} s^{-1}), transpiration rate (*E*; mmol m^{-2} s^{-1}) and stomatal conductance (*gs*; mol m^{-2} s^{-1}) were measured with an open portable gas exchange system (LI-6400xt, LI−COR, Lincoln, NE, USA) in four plants per treatment. The CO_2 concentration entering the leaf cuvette (LCF chamber; 2 cm^2, LI-COR) was set to 400 μmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR). Measurements were taken between 9:00 and 11:30h (Feistler and Habermann, 2012). The PPFD was provided by an artificial light source (6400-40 LCF, LI-COR), with 90% red and 10% blue spectra, which was set to supply 1200 μmol photons m^{-2} s^{-1}. This PPFD was established after *A*/PPFD curves for the three treatments was measured on the first day of the experiment (Fig. 1). The vapor pressure deficit (VPD) inside the leaf cuvette was 1.5 kPa and the relative humidity in the chamber was approximately 60-65%.

The CO_2 response curves of photosynthesis (*A*/*Ci* curves) were performed in four plants (replicates) on the same days that gas exchange (*A*, *gs* and *E*) was measured, under 1200 μmol photons m^2 s^{-1}, with the chamber temperature kept at 25 ºC and relative humidity maintained around 60%. The CO_2 concentrations inside the leaf 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 μmol CO_2 mol^{-1} air, according to Silva et al. (2018). As net photosynthesis approached zero, CO_2 becomes the limiting factor for photosynthesis and the initial slope of *A*/*Ci* line (angular coefficient) represents the apparent carboxylation efficiency (Farquhar and Sharkey, 1982).

Relative leaf water content

The relative leaf water content (RWC) was assessed using leaf discs obtained from four plants per treatment, collected between 13:00 h and 15:00 h and calculated as:

$$RWC\ (\%) = [(FM - DM)/(TM - DM)] \times 100$$

where FM is the fresh mass, obtained from discs immediately weighted on a scale; TM is the turgid mass after rehydration of samples for 24 h in 100 mL deionized water; and DM is the dry mass after oven-drying the discs at 60 ºC for 48 h, according to Silva et al. (2018).

Biometric parameters

At 1, 15, 30 and 60 DAT plant organs were separated, the number of leaves was counted and stem and root lengths were measured with a ruler (cm). The leaf area (cm^2) per plant was measured with an area meter (LI-3100, LI-COR, USA). After that, leaf, stems, and root samples were oven-dried at 60 ºC until constant mass, and biomass (g) was measured using a precision scale.

Aluminum concentration in plant organs

Dry samples of leaves, stems and roots were sent to the plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), for Al concentration analysis. These samples were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Al concentration was determined by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

Data analysis

Leaf gas exchange parameters (*A*, *gs* and *E*), apparent carboxylation efficiency (from *A*/*Ci* curves), RWC, and Al concentration in plant organs were measured using four plants per treatments. Biometric parameters and biomass of organs were measured using eight plants per treatment.

The data were submitted to one-way analysis of variance (ANOVA) to test differences between Al treatments (0, 740 and 1480 μM Al), separately, at 1, 15, 30 and 60 DAT. The Tukey test ($P < 0.05$) was used to perform post-hoc comparisons to determine the least significant difference between mean results.

Results

Biometric parameters

After 60 days, the plants exposed to Al exhibited healthy and new white roots in contrast to plants not exposed to Al that showed necrotic roots (Fig. 2). All treatments showed similar values of leaf number (Fig. 3A), leaf biomass (Fig. 3B), leaf area (Fig. 3C), stem biomass (Fig. 3D), stem length (Fig. 3E), root length (Fig. 3G), and total biomass (Fig. 3H), throughout the experiment. The root biomass was the only parameter that was higher in plants exposed to Al and increased 66% in plants exposed to 740 and 1480 μM Al in relation to those not exposed, at 60 DAT (Fig. 3F). Thus, the absence of Al negatively affected the root system.

Relative leaf water content (RWC)

The relative leaf water content (RWC) was similar between treatments until 15 DAT (Fig. 4). After that, reductions in this parameter were observed for plants not exposed to Al, and it was approximately 30% lower at 30 and 60 DAT when compared to Al-treated plants (Fig. 4). Thus, leaf hydration was compromised in plants grown without Al.

Leaf gas exchange and apparent carboxylation efficiency

Plants not exposed to Al showed a reduction in CO_2 assimilation rate (*A*) of approximately 65% at 30 DAT and 72% at 60 DAT (Fig. 5A), when compared to Al-treated plants. The stomatal conductance (*gs*) (Fig. 5B) and transpiration rate (*E*) (Fig. 5C) were also reduced from 30 DAT in plants not exposed to Al and, at 60 DAT, *gs* was 71% lower and *E*, 60% lower, in relation to Al-treated plants. Thus, at 30 and 60 DAT, while Al-treated plants showed grouped values of large *gs* and *A*, plants not exposed to Al showed grouped data of lower values for both parameters (Fig. 6A). When the relationship between RWC and *gs* data at 30 and 60 DAT was plotted, Al-treated plants returned high *gs* associated with high RWC, while for Al untreated plants low *gs* values were maintained under RWC below 60% (Fig. 6B). Thus, the absence of Al reduced leaf hydration with negative consequences for stomatal aperture.

A/*Ci* curves obtained for plants not exposed to Al were different in relation to plants exposed to both Al treatments, from 15 DAT until the end of the experiment (Fig 7C, E and G). The apparent carboxylation efficiency (linear regression of slopes of *A*/*Ci* curves) was 82% lower in plants not exposed to Al in relation to plants exposed to 1480 μM Al, at 15 DAT (Fig. 7D and Table 1). At 30 DAT (Fig. 7F and Table 1), this parameter

was lower in plants not exposed to Al in relation to both Al-treatments and, at the end of the study (Fig. 7H and Table 1), it was 71% and 75% lower when compared to plants exposed to 740 and 1480 μM Al, respectively. Thus, no Al in the nutrient solution reduced not only the maximum CO_2 assimilation rate but also the apparent carboxylation efficiency.

Aluminum concentration in plant organs

Aluminum concentrations in organs of plants from all treatments showed a range of 1,500 to 6,000 mg kg^{-1}, although significant differences between treatments were observed throughout the experiment (Fig. 8). Leaf [Al] was different between treatments from 15 DAT and, at 60 DAT, it was 22% and 38% higher in plants exposed to 740 and 1480 μM Al, respectively, when compared to plants not exposed to Al (Fig. 8A). Stem [Al] was similar between treatments until 30 DAT and, at 60 DAT, plants exposed to 740 and 1480 μM Al showed 54% more Al in their stem than plants not exposed to Al (Fig. 8B). Root [Al] of plants exposed to 1480 μM Al was higher than those exposed to 0 and 740 μM Al, at 1 DAT (Fig. 8C). These values oscillated from 15 to 60 DAT, when plants exposed to 740 and 1480 μM Al showed two- and four-fold more Al in their roots than plants not exposed to Al, respectively (Fig. 8C). Total [Al] was higher in Al-treated plants from 15 DAT and, at the end of study, plants exposed to 740 and 1480 μM Al showed five- and ten-fold more Al than plants not exposed to Al, respectively (Fig. 8D). At 60 DAT, Al untreated plants showed more Al in leaves, while Al-treated plants retained more Al in their roots (Fig. 9). Thus, despite plants from all treatments presented elevated Al concentrations (above 1000 mg kg^{-1}), they retained more Al with the increasing of Al in the nutrient solution.

Discussion

In this study, we investigate the physiological effects of Al on growth and photosynthetic parameters of *Q. grandiflora*. Our results demonstrated that Al-treated and untreated plants showed similar patterns of shoot development, with healthy green leaves (Fig. 2). This is expected for Cerrado woody species because plants from this vegetation invest more in root rather than shoot growth (Franco 1998; Habermann and Bressan 2011). Nevertheless, plants exposed to 740 and 1480 μM Al showed new white roots (Fig. 2) and increased their root biomass (Fig. 3F), after 60 days, while plants not exposed to Al exhibited necrotic roots (Fig. 2). *Qualea grandiflora* is an Al-accumulating species from the Cerrado vegetation (Haridasan, 1982; Nogueira et al. 2019), and Al improving root growth has also been observed in other Al-accumulators not from the Cerrado, as *Melastoma malabathricum* (Watanabe et al. 2002), *Symplocos paniculata* (Schmitt et al. 2016) and *Camellia sinensis* (Sun et al. 2020). Higher root growth was also observed for *Q. grandiflora* grown under 150 μM Al, and when grown without Al their roots were necrotic too (Cury et al. 2020). *Vochysia tucanorum* (Vochysiaceae), another Al-accumulating Cerrado woody species, showed higher root growth when grown for 60 days in nutrient solution with 1110 μM Al, and had necrotic roots under 0 μM Al (Bressan et al. 2021). Therefore, Al can improve root growth in Al-accumulating plants from different natural sites, including the Cerrado vegetation, indicating that Al may be a beneficial element for root growth in this species, and there is evidence in *C. sinensis* that Al could maintain the DNA integrity in the apical root meristem (Sun et al. 2020).

Despite healthy and green leaves exhibited by Al-treated and untreated plants, leaf gas exchange rates, in general, were higher for Al-treated plants, in relation to plants not exposed to Al (Fig. 5). The CO_2 assimilation rate (*A*) of Al-treated plants was 65–70% higher than plants grown without Al, from 30 DAT (Fig. 5A). Indeed, at the

beginning of the experiment, Al-treated and untreated plants showed the same *A*/*Ci* curve response patterns (Fig. 7A), linear regression of slopes of *A*/*Ci* curves (Fig. 7B) and apparent carboxylation efficiency (Table 1). Nevertheless, from 15 DAT, these parameters were higher for Al-treated in relation to untreated plants (Fig. 7C–H; Table 1). This indicates that from 15 DAT the higher *A* of Al-treated plants could be ascribed to higher performance of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) activity. Aluminum was already suggested to be possibly and positively involved with photosynthetic processes in Al-accumulating species from the Cerrado, as histochemical Al indicator (hematoxylin) showed positive results in the spongy and palisade parenchyma, with significant staining density between chloroplasts of *Callisthene major* and *Q. grandiflora* (Vochysiaceae) (Andrade et al. 2011). Aluminum was also localized in chloroplasts of *Rudgea virbunoides* (Rubiaceae), another Al-accumulating species from the Cerrado, by fluorescence emission, after reaction with lumogallion using confocal microscopy (Malta et al 2016). Nevertheless, this is questionable, because a previous study did not observe positive histochemical reactions of Al with the internal filling of chloroplasts in Melastomataceae or Vochysiaceae Al-accumulating species (Bressan et al. 2016). Moreover, Al was found to accumulate more in the upper and lower epidermis, and hypodermal cells of leaves in *Q. grandiflora* and *Q. parviflora*, when measured with quantitative cryo-scanning electron microscopy and X-ray energy dispersive (EDS) methods (Guilherme Pereira et al. 2018). Thus, it is uncertain that in the present study Al could have '*directly*' enhanced *A* values and the Rubisco performance in Al-treated plants from 15 DAT. The stomatal aperture had significant influence on *A* values of the three treatments. Correlation of high values of *A* and *gs* were found for Al-treated plants, while in plants without Al these parameters showed a lower range of values (Fig. 6A). Leaf hydration also had considerable influence on *gs* of plants of the three

treatments. Relative water content (RWC) and *gs* were grouped at an elevated range for Al-treated plants and a lower range for Al untreated plants (Fig. 6B). This suggests that leaves of plants grown with Al were more hydrated and this leaded to greater *gs*, consequently explaining the higher carbon assimilation of Al-treated plants, especially from 30 DAT (Fig. 5A). Therefore, the higher performance of Rubisco activity in Al-treated plants from 15 DAT is most likely related to leaf hydration than a direct effect of this metal on the enzyme activity. Indeed, water deficiency in *Citrus sinensis* L. (Rutaceae) (Vu and Yelenosky 1988a, b) and *Gossypium barbadense* (Malvaceae) (Carmo-Silva et al. 2012) leads to low performance of the Rubisco activity. In *V. tucanorum*, the absence of Al in nutrient solution also caused necrotic roots (after 7 days), with low *A* explained by *gs* values, ascribed to the dysfunctional root system (Bressan et al. 2021). Therefore, taken together, these results suggest that Al is an important mineral element for the roots of *Q. grandiflora* and other Al-accumulating plants, including those not from the Cerrado, as *Camellia sinensis* (Cury et al. 2020). The present study does not evidence any '*direct*' effect of Al on the performance of Rubisco activity. The root integrity and leaf hydration were more relevant to the higher performance of this enzyme in Al-treated plants.

The three treatments showed plant organs with high Al concentration range. High values of Al concentration (above 1000 mg kg^{-1}) for Al untreated plants (Fig. 8) is expected because all plants from the three treatments were obtained from seeds germinated directly on a Cerrado soil, that was acidic and had high Al saturation (see material and methods). Indeed, *V. tucanorum* grown on an acidic soil (3.51 ± 0.07) for 60 days also showed approximately 3,500 mg Al kg^{-1} root (Souza et al. 2017). Thus, root [Al] of Al-untreated plants maintained around 3,000 mg kg^{-1} (Fig. 8C), in the present study, might be associated with the soil on which the seedlings were cultivated in the

nursery, even though these plants have been transferred to nutrient solution without Al. Plants treated with Al, however, retained more Al in their leaves, roots and in the whole plant with the increasing of Al availability in the nutrient solution, during almost the entire experimental period (Fig. 8A, C and D). At the end of the study, these plants retained more Al in their roots than Al untreated plants (Fig. 9), and this could be associated with their root growth (Fig. 3F). Although *Q. grandiflora* is an accumulator of this metal in their leaves (Haridasan, 1982; Nogueira et al. 2019), in the field, adult plants of this species were already reported to accumulate approximately 1000 mg kg^{-1} of Al in their roots (Zaia et al. 2022). *Miconia albicans* (Melastomataceae), another Al-accumulating species from the Cerrado, also accumulates approximately 4000 mg kg^{-1} of Al in their roots (Timpone and Habermann 2022).

In this study, we demonstrate that Al can improve the root growth of *Q. grandiflora*. The absence of Al in nutrient solution compromises the root integrity and causes root necrosis. The dysfunction of this organ leads to limited leaf hydration, which negatively affect gas exchange rates and the Rubisco performance of this Cerrado woody species. We propose to deeply investigate any possible physiological role for Al in the root of Al-accumulating species.

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Tables:

Table 1

Mean values (n = 4) of the angular coefficient obtained from *A*/*Ci* curves to estimate the apparent carboxylation efficiency of *Quale grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution. Different letters indicate significant differences ($P < 0.05$) by the Tukey test between treatments.

	Carboxylation efficiency (μmol m^{-2} s^{-1})		
	0 μM Al	740 μM Al	1480 μM Al
1 DAT	0.0342 ± 0.00	0.0312 ± 0.00	0.0421 ± 0.00
15 DAT	0.0045 ± 0.00 a	0.0158 ± 0.00 ab	0.0252 ± 0.00 b
30 DAT	0.0069 ± 0.00 a	0.0180 ± 0.00 b	0.0155 ± 0.00 b
60 DAT	0.0034 ± 0.00 a	0.0136 ± 0.00 b	0.0118 ± 0.04 b

Figures

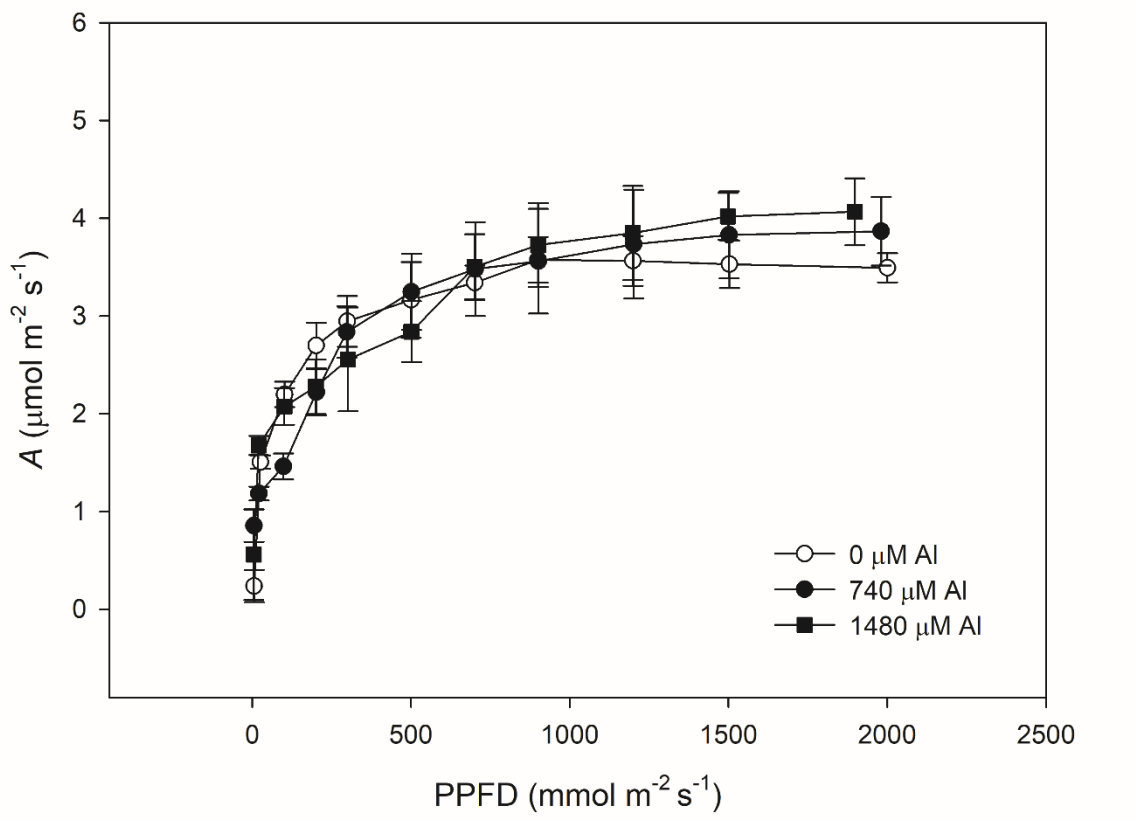

Fig. 1 Mean values (n = 4) of CO_2 assimilation (*A*) in response to the photosynthetic photon flux density (PPFD) of *Qualea grandiflora* with 0, 740 and 1480 μM Al in nutrient solution, on the first day of the experiment. Bars are s.e.

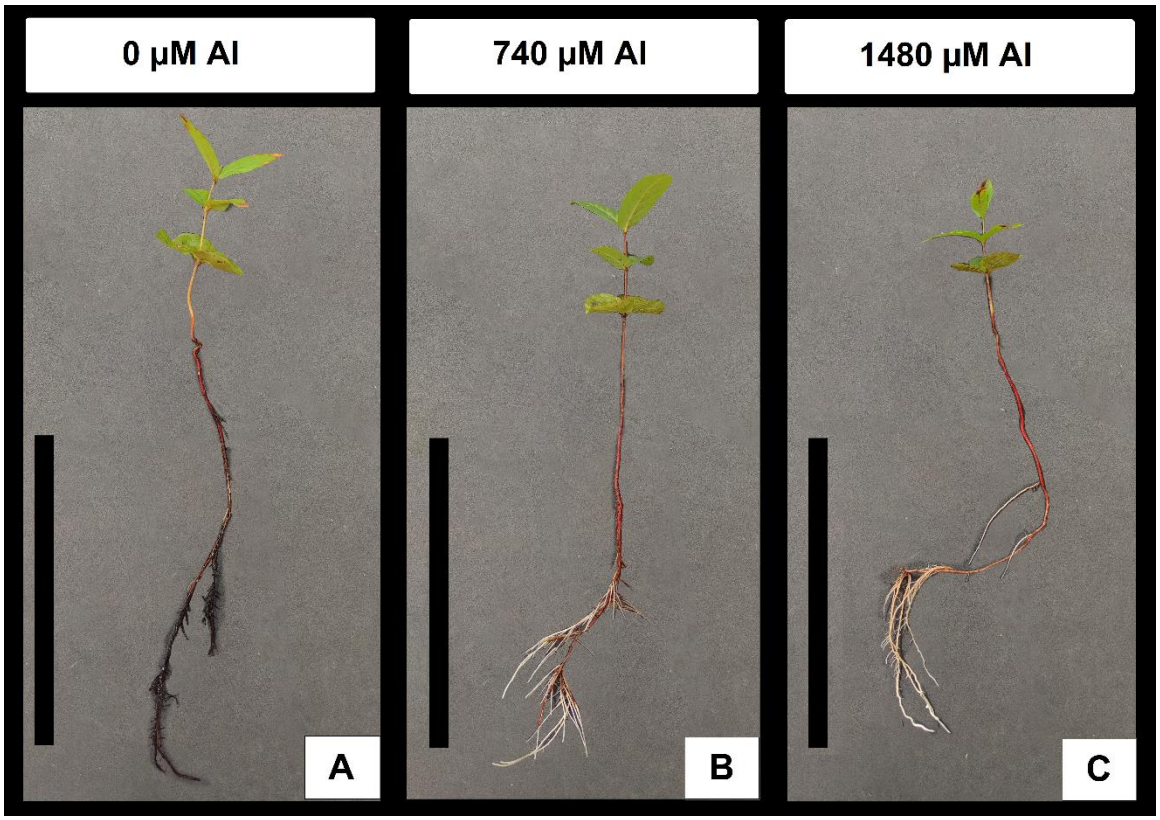

Fig. 2 Morphological details of roots and shoots of *Qualea grandiflora* at 60 days after treatment (DAT) with 0, 740 and 1480 µM Al in nutrient solution. Bars = 20 cm

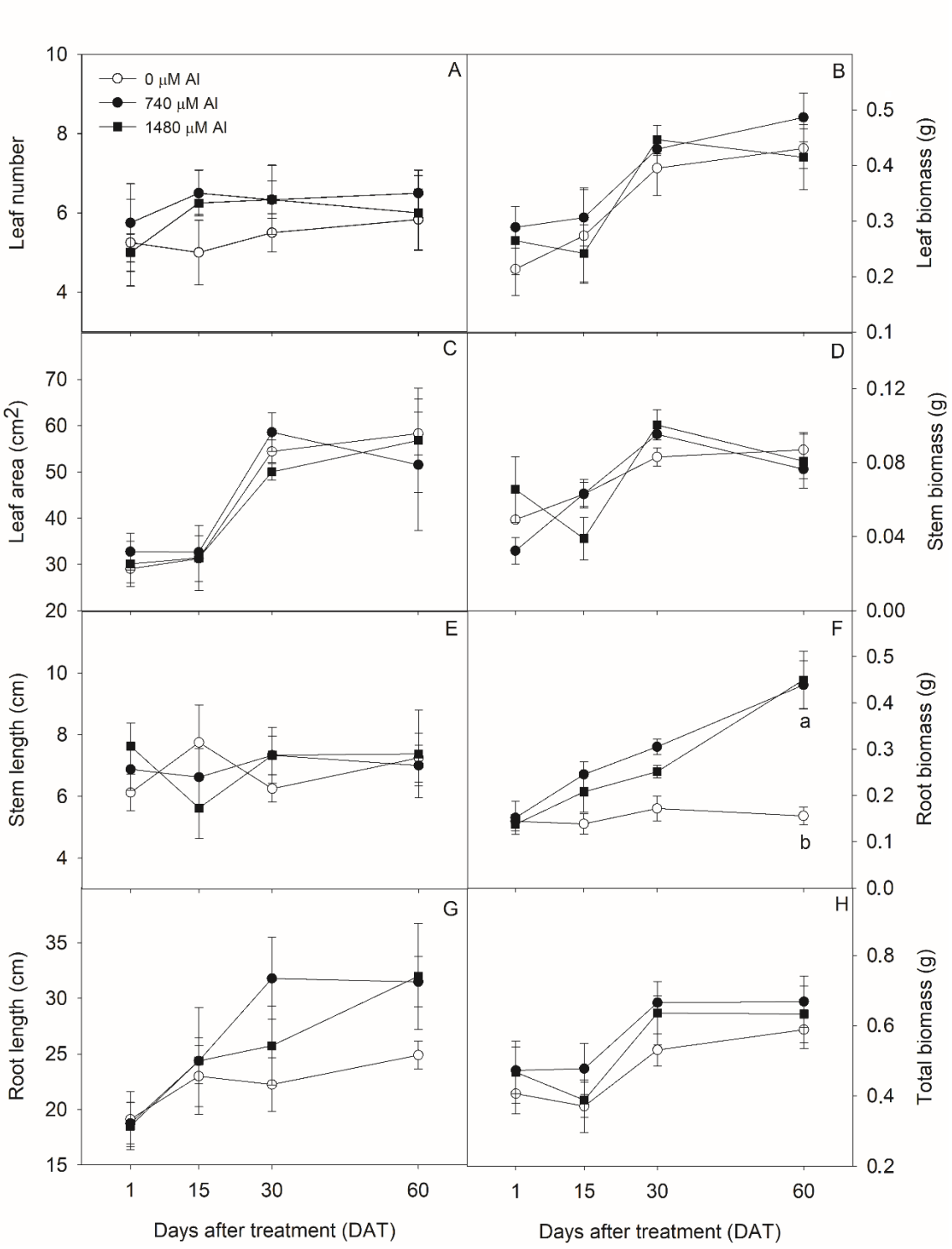

Fig. 3 Mean values (n = 8 plants) of leaf number (A), leaf biomass (B), leaf area (C), stem biomass (D), stem length (E), root biomass (F), root length (G) and total biomass (H) of *Qualea grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution. Different letters indicate significant differences ($P < 0.05$) by the Tukey test between treatments. Bars are s.e.

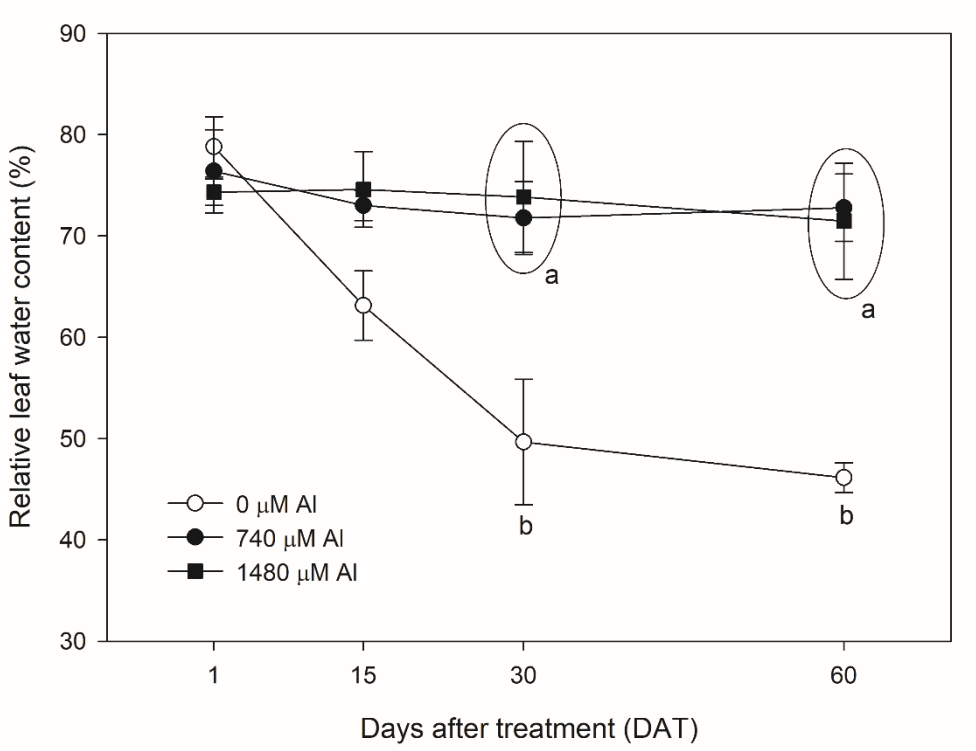

Fig. 4 Mean values (n = 4) of relative leaf water content (RWC) of *Qualea grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution. Different letters indicate significant differences ($P < 0.05$) by the Tukey test between treatments. Bars are s.e.

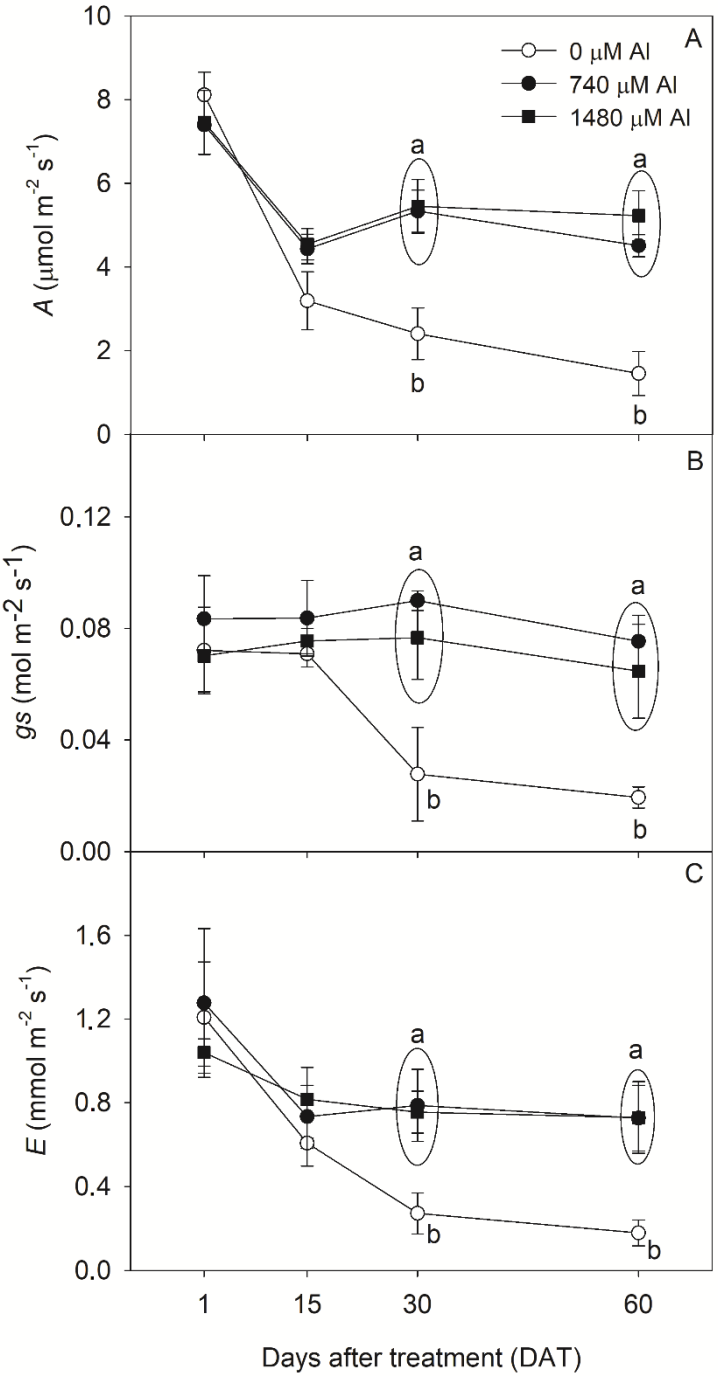

Fig. 5 Mean values (n = 4) of CO_2 assimilation (A)**,** stomatal conductance (B) and transpiration rates (C) of *Qualea grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 µM Al in nutrient solution. Different letters indicate significant differences ($P < 0.05$) by the Tukey test between treatments. Bars are s.e.

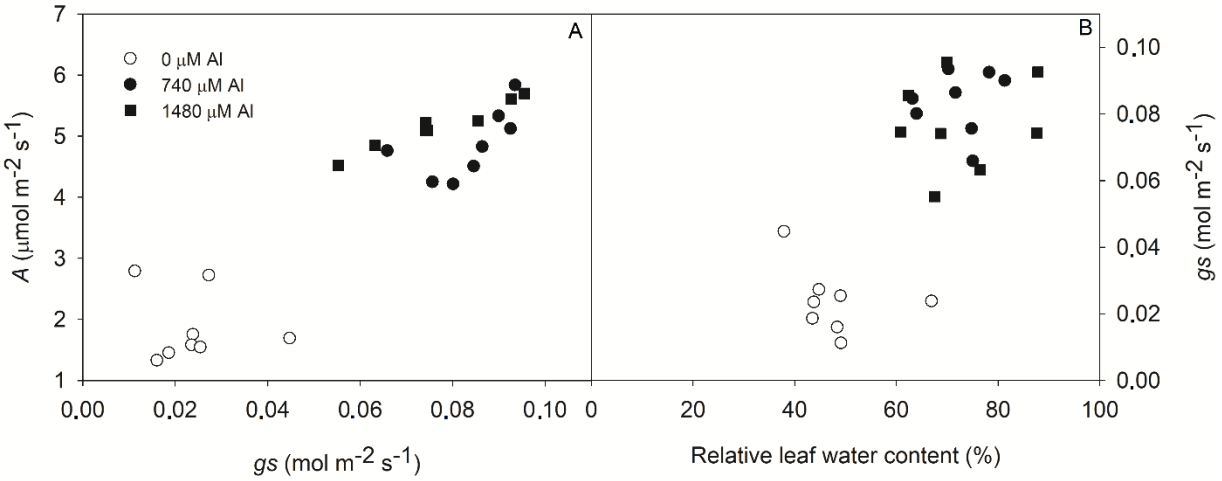

Fig. 6 Relationship between CO_2 assimilation and stomatal conductance (A) and relationship between stomatal conductance and relative leaf water content (B) of *Qualea grandiflora* at 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution.

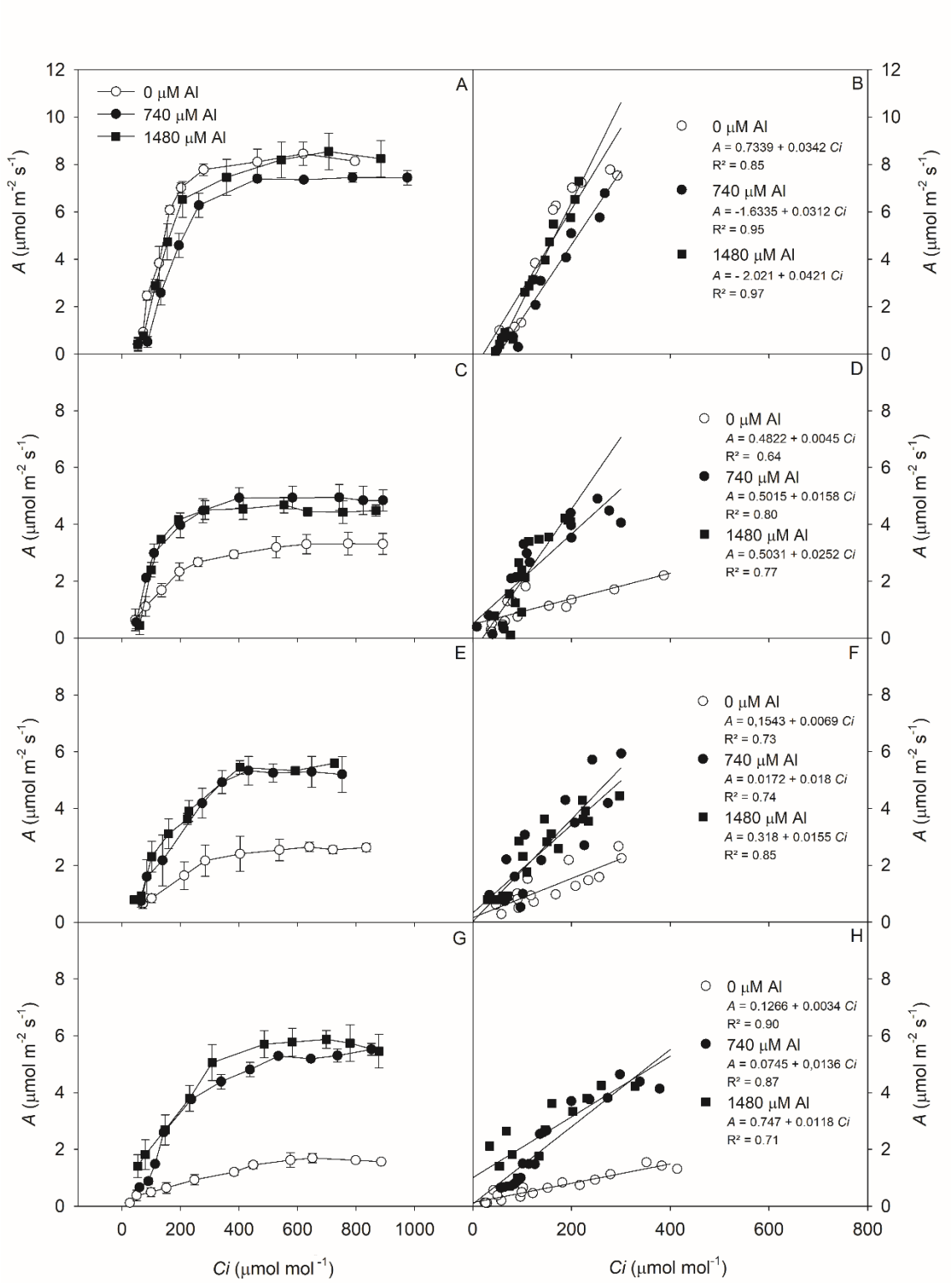

Fig. 7 CO_2 assimilation rate in response to intercellular CO_2 (*A*/*Ci* curves) (A, C, E and G) and the apparent carboxylation efficiency from *A*/*Ci* curves (B, D, F, H) of *Qualea grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution. Bars are s.e.

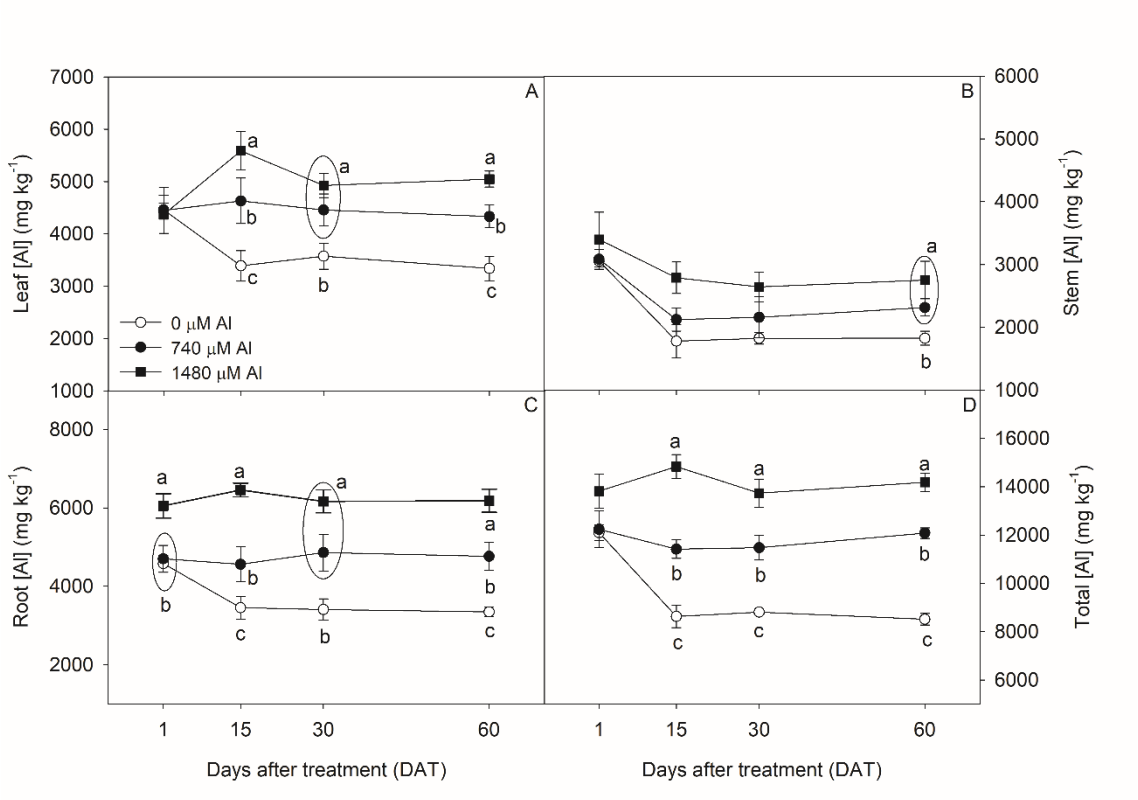

Fig. 8 Mean values (n = 4 plants) of aluminum (Al) concentration in leaves (A), stems (B), roots (C) and total (D) of *Qualea grandiflora* at 1, 15, 30 and 60 days after treatment (DAT) with 0, 740 and 1480 μM Al in nutrient solution. Different letters indicate significant differences ($P < 0.05$) by the Tukey test between treatments. Bars are s.e.

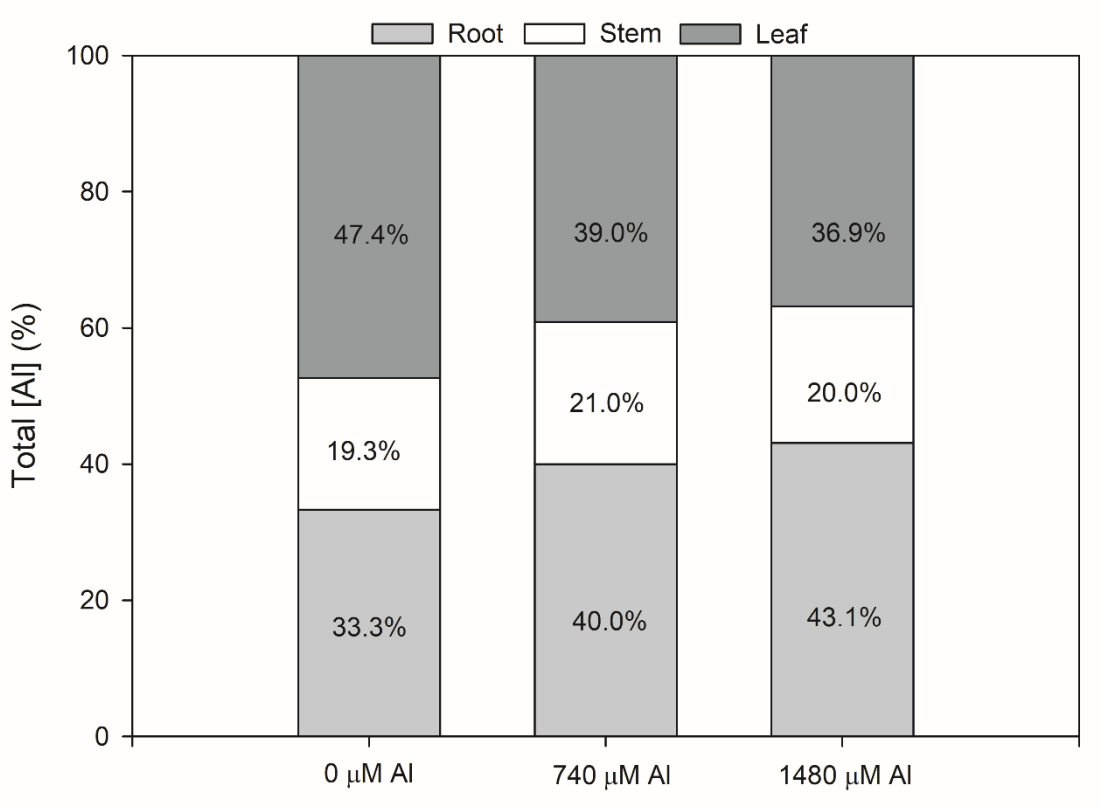

Fig. 9 Distribution of total aluminum concentration in roots, stem and leaves of *Qualea grandiflora* with 0, 740 and 1480 μM in nutrient solution at 60 days after treatment (DAT).

CONSIDERAÇÕES FINAIS

Diante do que foi exposto nos três capítulos apresentados neste trabalho, observamos que as plantas podem ter diferentes respostas fisiológicas quando expostas ao Al. Em plantas sensíveis, como é o caso de *C. limonia*, vimos que o Al afeta a hidratação foliar, através de reduções nos parâmetros hídricos e hidráulicos, sendo que estas limitações ocorreram bem antes da inibição no crescimento da raiz. Por outro lado, existem plantas que são capazes de crescer bem e até mesmo se beneficiar quando expostas ao Al, e isto foi observado em plantas de *I. paraguariensis* e *Q. grandiflora*. Plantas de *Ilex paraguariensis* expostas a 500 e 1000 μM Al apresentaram maior crescimento em relação às plantas crescendo sem Al, sendo que a ausência de Al comprometeu o crescimento da raiz, reduziu a hidratação foliar e resultou em diminuição na performance fotossintética. Já plantas de *Q. grandiflora* quando expostas ao Al apresentaram melhor desenvolvimento de raiz e maiores taxas de trocas gasosas devido a uma maior eficiência aparente de carboxilação da Rubisco. Em contrapartida, ausência de Al prejudicou o crescimento das raízes destas plantas, que se tornaram necróticas e disfuncionais. Desta forma, mais estudos são necessários para avaliar como o Al interfere na hidratação de plantas sensíveis, e também quais os mecanismos por trás do Al estimular o crescimento em plantas tolerantes e acumuladoras de Al.