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

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

EFEITOS DIRETOS DO ALUMÍNIO EM FOLHAS DE PLANTAS SENSÍVEIS, MANUTENÇÃO DA HIDRATAÇÃO FOLIAR EM PLANTAS TOLERANTES E PAPEL DO ALUMÍNIO NAS RAÍZES DE PLANTAS ACUMULADORAS

BRENDA MISTRAL DE OLIVEIRA CARVALHO

Rio Claro – SP
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ORIENTADOR: PROF. DR. GUSTAVO HABERMANN

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: **"Efeitos diretos do alumínio em folhas de plantas sensíveis, manutenção da hidratação foliar em plantas tolerantes e papel do alumínio nas raízes de plantas acumuladoras"**

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Rio Claro, 31 de março de 2023

‘‘Não queremos o que pensamos que queremos.
Queremos ser felizes. Nisto está a paz do desapego’’.

Autoria desconhecida

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RESUMO

O primeiro sintoma de toxicidade ao alumínio (Al) é a inibição do crescimento radicular, que pode reduzir o crescimento da parte aérea, a hidratação foliar e a performance fotossintética. A maior parte do Al absorvido é retido no sistema radicular. Desta forma, não se sabe se os efeitos do Al na parte aérea são uma consequência da raiz atrofiada ou um efeito direto da pequena porção de Al que chega no mesofilo. Por outro lado, algumas plantas crescem na presença de altas concentrações de Al sem apresentar sintomas. Essas plantas tolerantes são divididas em acumuladoras e não acumuladoras de Al, e aquelas armazenam mais de 1000 mg Al por kg de folhas secas. Alguns mecanismos de tolerância são bem descritos na literatura, mas ainda não está claro como essas plantas mantém a hidratação foliar quando expostas ao Al. Prolina, um aminoácido osmoprotetor, reduz o potencial osmótico ($\Psi\pi$) e melhora a absorção de água em plantas expostas à seca. O acúmulo de prolina já foi evidenciado em plantas expostas ao Al, mas somente em espécies sensíveis, e a hidratação foliar não foi avaliada. Curiosamente, algumas espécies acumuladoras apresentam aumento do crescimento das raízes quando expostas ao Al. Embora o Al altere a concentração de auxinas nas raízes de plantas sensíveis, essa associação em plantas acumuladoras tem recebido pouca atenção. Dentro desse contexto, tivemos como objetivo testar as seguintes hipóteses: (H1) o Al reduz a performance fotossintética quando aplicado diretamente nas folhas de plantas sensíveis, (H2) o acúmulo de prolina está associado à manutenção da hidratação foliar em plantas tolerantes e (H3) o acúmulo de auxina está envolvido no aumento do crescimento de raízes induzido pelo Al em plantas acumuladoras. Para testar H1, cultivamos *Citrus* x *limonia* (sensível ao Al) em solução nutritiva 'sem' Al e expusemos a parte aérea a concentrações crescentes de Al por injeção no pecíolo (24 h) ou pulverização (60 dias). A performance fotossintética diminuiu e o crescimento das raízes não foi afetado em ambos os experimentos e o crescimento da parte aérea diminuiu no experimento de longo prazo. Desta forma, confirmamos que o Al reduz a performance fotossintética quando aplicado diretamente nas folhas. Para testar H2, cultivamos *Pinus sylvestris* (tolerante ao Al) em solução nutritiva com concentrações crescentes de Al por 45 dias. Como esperado, o Al não interferiu na performance fotossintética e no conteúdo relativo de água (CRA), mas a concentração de prolina aumentou nas raízes e novos ramos, e o potencial da água diminuiu, entre 7 e 14 dias. Esses resultados indicam que o acúmulo de prolina pode estar envolvido no ajuste osmótico de *P. sylvestris* expostas ao Al. Para testar H3, cultivamos *Camellia sinensis* (acumuladora de Al) em solução nutritiva contendo concentrações crescentes de Al e medimos auxina (AIA), dados biométricos, área de superfície de raiz e CRA ao longo

de 90 dias. Evidenciamos que o acúmulo de AIA nas raízes está associado ao aumento do crescimento radicular induzido pelo Al, levando à maior área de absorção das raízes e melhor hidratação das folhas.

Palavras-chave: Al^{3+}, absorção foliar de Al, auxina, prolina, transporte de Al.

ABSTRACT

The first symptom of aluminum (Al) toxicity is the root growth inhibition, and may reduce shoot growth, leaf hydration and photosynthetic performance. Most of the absorbed Al is retained in root system. Thus, it is unknown if Al effects in aboveground organs is a consequence of the stunted root or a direct effect of the minor Al concentration reaching the mesophyll. On the other hand, some plants grow in the presence of high Al concentrations showing no symptoms. These tolerant plants are divided into Al-accumulating and non-accumulating species, and the latter stores more than 1000 mg Al per kg of dry leaves. Some mechanisms of tolerance are well described in the literature, but it is still not clear how these plants maintain leaf hydration when exposed to Al. Proline, an osmoprotectant amino acid, reduces osmotic potential ($\Psi\pi$) and improves water uptake in plant exposed to drought. Proline accumulation was already observed in plants exposed to Al, but only in Al-sensitive ones, and leaf hydration was not measured. Interestingly, some Al-accumulators show increased root growth when exposed to Al. Although Al alters root auxin concentration in sensitive plants, this association in Al-accumulating plants has received less attention. Within this context, we aimed to test the following hypotheses: (H1) Al reduces photosynthetic performance when applied directly on leaves of Al-sensitive plants, (H2) proline accumulation is associated with the maintenance of leaf hydration in Al-tolerant plants, and (H3) auxin accumulation is involved in Al-induced increased root growth in Al-accumulating plants. To test H1, we grew *Citrus* x *limonia* (Al-sensitive) in nutrient solution 'without' Al and exposed the shoots to increasing Al concentrations through injection in the petiole (24 h) and spraying (60 days). Photosynthetic performance decreased and root growth was not affected in both experiments, and shoot growth decreased in the long-term experiment. Therefore, we confirmed that Al reduces photosynthetic performance when applied directly on leaves. To test H2, we grew *Pinus sylvestris* (Al-tolerant) in nutrient solution with increasing Al concentrations for 45 days. As expected, Al did not interfere in photosynthetic performance and RWC, but proline concentration increased in roots and new shoots, while water potential decreased between seven and 14 days. These results indicate that proline accumulation may be involved in osmotic adjustment of *P. sylvestris* exposed to Al. To test H3, we grew *Camellia sinensis* (Al-accumulator) in nutrient solution with increasing Al concentrations and measured auxin (IAA), biometric data, root surface area, and RWC throughout 90 days. We evidenced that IAA accumulation in roots is associated to Al-induced root growth, which leads to increased root surface area and improved leaf hydration.

Key words: Al^{3+}, Al foliar absorption, Al transport, auxin, proline.

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INTRODUÇÃO

Solos ácidos (pH < 5,0) representam aproximadamente 40% dos solos livres de gelo do globo (vonUexküll and Mutert 1995; Brunner and Sperisen 2013). Nesses solos, minerais contendo Al (como óxidos e aluminosilicatos) são solubilizados para a forma de cátion trivalente, Al^{3+}, que é toxica para a maioria das espécies (Kochian et al. 1995; Vitorello et al. 2005; Singh et al. 2017; Hajiboland et al. 2022). Desta forma, a toxicidade ao Al em solos ácidos é um dos principais fatores limitantes à produtividade vegetal (Kochian et al. 2004).

O primeiro e mais evidente sintoma de toxicidade do Al é a diminuição do crescimento radicular (Horst et al. 2010; Sun et al. 2010; Silva et al. 2019), que pode ocorrer em questão de horas (Kopittke et al. 2008). Há evidências de que a primeira lesão do Al em plantas é sua ligação às matrizes pécticas da parede celular das células das raízes (Kopittke et al. 2015), inibindo a divisão e o alongamento celular nessa região (Sivaguru & Horst 1998; Kochian et al. 2004; Doncheva et al. 2005). Outro fator que pode estar relacionado a esse sintoma é a maior biossíntese de etileno (Et) induzida por Al, atuando como um sinal para modificar a distribuição simétrica de auxina em raízes (Sun et al., 2010). Além disso, essa diminuição do crescimento radicular induzida pelo Al pode ser causada por concentrações radiculares supra-ótimas de auxina, como ocorre em ápices radiculares de *Arabidopsis* (Yang et al. 2014) e em *Citrus limonia* (Silva et al. 2019).

Além dos efeitos negativos no sistema radicular, o Al pode diminuir o crescimento da parte aérea, reduzindo o potencial da água (Ψw) e o conteúdo relativo de água na folha (CRA) (Foy et al. 1978; Delhaize and Ryan, 1995; Banhos et al. 2016; Silva et al. 2022). O Al reduz a abertura estomática, prejudicando as trocas gasosas, e diminui as reações fotoquímicas, como observado em *Citrus* spp. (Pereira et al. 2000; Jiang et al. 2008, 2009; Yang et al. 2011; Banhos et al., 2016; Silva et al. 2018; 2022), cafeeiro (*Coffea arabica*) (Konrad et al., 2005), plantas de milho (*Zea mays*) (Lidon et al., 1999) e cevada (*Secale cereale*) (Silva et al., 2012). Em plantas sensíveis, cerca de 90% do Al fica retido no sistema radicular (Kochian et al. 2004; Jiang et al. 2008; Banhos et al. 2016; Silva et al. 2018), e ainda não está claro se os efeitos do Al na parte aérea são consequência da inibição do crescimento radicular (diminuição da absorção de água e nutrientes) ou da pequena concentração de Al que chega no mesofilo. A baixa hidratação foliar evidenciada em plantas sensíveis ao Al levam à comparação dos efeitos de toxicidade do Al com os efeitos da seca (Yang et al. 2013). Considerando que as plantas dependem da divisão e expansão celular para crescerem, e que a água nas células (pressão de turgor — Ψp) é essencial nesse processo (Schopfer 2006), é intuitivo associar a inibição do crescimento da parte

aérea como uma consequência da inibição do crescimento radicular causada pelo Al, como revisado por Ma et al. (2005), Vitorello et al. (2005), Panda et al. (2009), and Yang et al. (2013). No entanto, até onde sabemos, os efeitos diretos do Al na parte aérea nunca foram testados.

Embora a fitotoxicidade do Al seja um fator limitante para a produtividade das culturas em locais com solos ácidos, algumas espécies crescem nessas regiões com saturação de Al (m%) maior que 50% sem apresentar danos estruturais e fisiológicos aos tecidos (Brunner and Sperisen 2013). Espécies que toleram o Al abrangem as não acumuladoras e as acumuladoras de Al. São consideradas acumuladoras de Al quando armazenam mais de 1000 mg Al por kg de folhas secas (Chenery, 1948; Jansen et al., 2002), incluindo espécies pertencentes a cerca de 45 famílias (sendo Melastomataceae, Rubiaceae, Symplocaceae, Theaceae e Vochysiaceae exemplos das mais estudadas) (Matsumoto et al. 1976; Haridasan et al. 1982; Jansen et al. 2002; Brunner and Sperisen 2013; Souza et al. 2015; Malta et al. 2016). Essas espécies se utilizam de mecanismos de tolerância interna para armazenar Al nas folhas, transportando o Al na forma de complexos com ácidos orgânicos e isolando-o em locais metabolicamente inativos (como os vacúolos) (Morita et al. 2008; Brunner and Sperisen 2013). Por outro lado, as espécies não-acumuladoras, em geral, detêm do mecanismo de exclusão, ou seja, exsudam substâncias orgânicas pelas raízes, formando compostos não tóxicos na rizosfera (Ryan et al. 2011; Brunner and Sperisen 2013). Nenhuma evidência de baixa hidratação foliar é conhecida nessas espécies tolerantes quando expostas ao Al.

Os motivos pelos quais plantas tolerantes ao Al mantem a hidratação foliar ainda não são elucidados. O aumento da concentração do aminoácido osmorregulador prolina (Ashraf and Foolad, 2007; Ghosh et al. 2022) é evidenciado em plantas sob vários estresses osmóticos, como seca e salinidade (Yoshiba et al. 1995; Parre et al. 2007; Sancho Knapik et al. 2017; Lopez-Delacalle et al. 2021). No estresse hídrico, por exemplo, o aumento de prolina pode reduzir o potencial osmótico ($\Psi\pi$), melhorando a absorção de água (Verbruggen and Hermans 2008; Vilagrosa et al. 2010; Sancho-Knapik et al. 2017; Ghosh et al. 2022). O acúmulo de prolina já foi evidenciado em plantas sob efeito do Al, porém apenas em espécies sensíveis ao Al (Silva and Matos 2016; Khan et al. 2000) e a hidratação foliar não foi avaliada. Desta forma, a relação do acúmulo de prolina e a hidratação das folhas de plantas expostas ao Al merece investigação, principalmente em espécies tolerantes ao Al.

Dentre as plantas tolerantes ao Al, existem algumas espécies acumuladoras que se beneficiam deste elemento (Haridasan et al. 1988; Souza et al. 2017; Schmitt et al. 2016). Curiosamente, as acumuladoras de Al *Vochysia tucanorum* (Vochysiaceae) (Bressan et al.

2021) e *Camellia sinensis* (Theaceae) (Sun et al. 2020) parecem sucumbir na ausência de Al, indicando que são dependentes deste elemento para sobreviver. Por exemplo, o crescimento das raízes de *C. sinensis* é totalmente inibido na ausência de Al, e concentrações moderadas (de 300 a 400 μM Al) induz o melhor desenvolvimento dessas plantas (Hajiboland et al. 2013; Sun et al. 2020). De alguma forma, o Al parece manter a integridade do DNA das células das raízes nesta espécie (Sun et al. 2020), mas o fator de crescimento no qual o Al está envolvido em plantas dependentes do Al permanece obscuro.

De forma geral, o crescimento de raízes é regulado por hormônios como auxina e etileno (Kang et al. 1971; Swarup et al. 2007). Embora se saiba que o Al altera a concentração de auxina e etileno nas raízes de plantas sensíveis, levando à inibição do crescimento (Sun et al. 2010; Silva et al. 2019), essa associação em plantas acumuladoras de Al tem recebido pouca atenção. Um estudo de curto prazo mostrou que o crescimento de raízes induzido pelo Al poderia estar associado ao acúmulo de ácido índol-acético (AIA) nos ápices radiculares de plântulas de *C. sinensis* (Gao et al. 2022). Além deste único estudo, que foi realizado por sete dias e com plântulas de uma semana de idade, a associação do crescimento de raízes induzido pelo Al com a concentração de AIA permanece não explorada em plantas acumuladoras totalmente desenvolvidas, a longo prazo.

Diante do exposto, é possível apontar as seguintes lacunas nessa área de investigação: (1) se os efeitos do Al em plantas sensíveis são apenas consequências da inibição do crescimento radicular, (2) de qual maneira plantas tolerantes ao Al mantém a hidratação foliar durante a exposição ao Al e (3) se o crescimento induzido pelo Al em plantas dependentes do Al é intermediado por AIA a longo prazo. Dentro desse contexto, elegemos *Citrus* x *limonia*, uma espécie sensível, para testar se o Al causa efeitos diretos na performance fotossintética e crescimento da parte aérea. Para isso, cultivamos essas plantas em solução nutritiva 'sem' Al e injetamos solução contendo concentrações crescentes de Al no pecíolo das folhas, com a ajuda de uma agulha de seringa. Em um segundo experimento, cultivamos essa espécie, também 'sem' Al na solução nutritiva, e pulverizamos, diariamente ao longo de 60 dias, concentrações crescentes de Al na parte aérea. Em ambos os experimentos, foram avaliadas a performance fotossintética, CRA, dados biométricos, biomassa e concentração de Al nos órgãos. Esse estudo está descrito no primeiro capítulo da presente Tese. No segundo capítulo, relatamos o estudo realizado no período de doutorado sanduíche pela BEPE (FAPESP) no Swiss Federal Research Institute WSL (Birmensdorf, Suíça) no qual cultivamos *Pinus sylvestris*, uma espécie tolerante ao Al, e expusemos seu sistema radicular a solução nutritiva contendo concentrações crescentes

de Al para verificar se a manutenção da hidratação foliar e performance fotossintética esperada para essa espécie está associada a um aumento da concentração de prolina nos tecidos, ao longo de 45 dias. No terceiro e último capítulo, é relatado o estudo no qual cultivamos *Camellia sinensis* em solução nutritiva contendo concentrações crescentes de Al e medimos a concentração de AIA, dados biométricos, biomassa de órgãos, área de superfície de raiz e conteúdo relativo de água ao longo de 90 dias. Desta forma, respondemos se o acúmulo de auxina nas raízes, ao longo do tempo, está associado ao aumento do crescimento radicular induzido pelo Al esperado em *C. sinensis*.

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CAPÍTULO I:

Does aluminum impair photosynthetic performance when applied '*directly*' on leaves?

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Abstract

Background and aims: The first symptom of aluminum (Al) toxicity is the inhibition of root growth, and it also reduces shoot growth and photosynthetic performance. Most of the absorbed Al is retained in the roots. Thus, it is unclear whether aboveground symptoms are consequence of the Al retained in the root or a direct effect of the minor Al concentration reaching the mesophyll. Here, we investigate whether Al 'directly' impairs photosynthetic performance.

Methods: Two experiments were performed using 'Mandarin' lime (*Citrus* x *limonia*) cultivated in nutrient solution without Al: solution absent in Al and increasing Al concentrations were (1) injected into petioles for 24 hours, and (2) sprayed daily on the leaves for 60 days. Gas exchange, photochemical responses, relative water content (RWC), biometric data, biomass, and Al concentration in the organs was evaluated.

Results: Gas exchange and photochemical parameters decreased after Al injection. Plants sprayed with Al showed low shoot growth, CO_2 assimilation (*A*), stomatal conductance (*gs*), and transpiration (*E*). Only plants sprayed with the highest Al concentration showed reduced photochemical parameters, low RWC, and increased root Al concentration. High *A* x *gs* and *E* x *gs* correlations were found at the end of both studies.

Conclusion: Aluminum reduces photosynthetic performance when applied directly on leaves. Low stomatal aperture seems to be more critical than photochemical performance to explain the low *A*, and the low *gs* could not be explained by low leaf hydration. Shoot-to-root translocation can be observed in plants sprayed with Al.

K**ey words**: Al^{3+}, Al foliar absorption, Al translocation, *Citrus* x *limonia*, shoot-to-root Al transport.

Introduction

Aluminum (Al) is toxic to most plant species (Vitorello et al. 2005; Singh et al. 2017; Hajiboland et al. 2022), and the first and most conspicuous symptom is the inhibition of root growth (Kopittke et al. 2008; Horst et al. 2010; Silva et al. 2019), resulting in limited water and nutrients uptake (Kochian et al. 2004). In addition, Al reduces shoot growth (Foy et al. 1978; Delhaize and Ryan, 1995; Banhos et al. 2016; Silva et al. 2022), low leaf gas exchange rates (Pereira et al. 2000; Chen et al. 2005b; Silva et al. 2012; Silva et al. 2018) and decreased photochemical performance (Chen et al. 2005a; Jiang et al. 2008; Silva et al. 2018).

Approximately 90 % Al absorbed by the plants is retained in the root system (Kochian et al. 2004; Chen et al. 2005a,b; Jiang et al. 2008; Banhos et al. 2016; Silva et al. 2018). Thus, only a small part is transported to the shoots, and it is not clear whether aboveground symptoms are a consequence of the Al retained in the root or just a direct effect of the minor Al concentration reaching the mesophyll. For instance, the low shoot biomass and leaf area found in plants under Al toxicity (Zhang et al. 2007; Banhos et al. 2016; Silva et al. 2018; 2022) could be associated with reduced uptake of water and nutrients as well as the low photosynthetic performance. In general, plant growth depends on cell division and expansion, and water (turgor pressure — Ψp) is essential for the latter (Schopfer 2006). Therefore, it is intuitive to associate the low aboveground growth with low water uptake caused by Al-induced inhibition of root growth, as reviewed by Ma et al. (2005), Vitorello et al. (2005), Panda et al. (2009), and Yang et al. (2013). Nevertheless, as far as we are concerned, these associations have not been tested yet.

Reduced CO_2 assimilation (*A*) in plants under Al toxicity seems to be explained more by low stomatal conductance (*gs*) than decreased photochemical performance (Banhos et al. 2016; Silva et al. 2018; Gavassi et al. 2020; 2021). Thus, leaf hydration is critical when analyzing photosynthetic impacts on plants exposed to Al. Even when plants are cultivated in

nutrient solution, where water availability is unlimited, Al reduces *gs*, leaf water potential (Ψw), and relative water content (RWC) in rye (*Secale cereale*) (Silva et al. 2012), tomato (*Solanum lycopersicum*) (Gavassi et al. 2020), and 'Mandarin' lime (*Citrus* x *limonia*) (Banhos et al. 2016; Gavassi et al. 2021; Silva et al. 2018; 2022). Thus, the Al, directly (low Al concentration reaching the mesophyll) or indirectly (Al retained in the root cells) leads to low leaf hydration. Indirectly, it could be ascribed to low root absorption or root-to-shoot transport of water. Indeed, fibrous xylem vessels (Banhos et al. 2016) and more lignin deposition in the stele (Silva et al. 2019) were found in the roots of 'Mandarin' lime exposed to 1480 μM Al in nutrient solution. Low expression of aquaporin (PIP family) — proteins found on membranes and partially responsible for water transport (Javot and Maurel 2002) — was also observed in the roots of 'Mandarin' lime cultivated in nutrient solution with Al (Cavalheiro et al. 2020). Low root hydraulic conductance (Gavassi et al. 2020) and conductivity (Silva et al. 2022) was found in tomato and 'Mandarin' lime exposed to Al, respectively. Therefore, when provided through the root environment, the Al can cause dysfunction of roots in relation to water acquisition and transport.

What would happen if the Al was provided through the shoots rather than the roots? In the present study, we applied Al directly on leaves to investigate whether it may cause a '*direct*' effect on photosynthetic performance. We conducted two experiments with 'Mandarin' lime cultivated in nutrient solution *without* Al. First, we injected Al into petioles, and leaf gas exchange rates, photochemical performance, RWC, and Al concentration in the organs were evaluated 24 h after injection. In a second long-term experiment (60 days), we sprayed four Al concentrations on the leaves of plants daily, and assessed gas exchange, photochemical responses, and biometric data throughout the study. At the end of the second study, biomass of organs and RWC were also measured. We hypothesized that Al interferes with photosynthetic

performance when applied directly on leaves. Shoot-to-root Al transport observed in the study was also discussed.

Material and Methods

Plant material and experimental conditions

Three-month-old 'Mandarin' lime (*Citrus* x *limonia* Osbeck) plants with 15 ± 1 cm in height were obtained from a commercial Citrus nursery (Sanicitrus, Araras, SP, Brazil). The root system was rinsed with deionized water (to remove the remaining substrate from the nursery), and the plants were transferred to a hydroponic system, where they grew directly in an aerated nutrient solution inside opaque plastic boxes (50 cm × 30 cm × 15 cm; 20 L).

Expanded polystyrene (Isopor®) 50 × 30 cm plates (2 cm thick), with five holes (3 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in the holes with polyurethane foam strips placed around the plant collar. The boxes were kept on benches, inside a greenhouse, under semi-controlled conditions (869.7 ± 124.2 μmol photons m^{-2} s^{-1} during measurements of photosynthetic parameters, between 09:00 h and 11:00 h — see below). During the experiment, sunlight provided a photoperiod of approximately 14 h.

The nutrient solution was based on Clark solution (Clark, 1975) and was used previously in studies with 'Mandarin' lime, showing no nutrient deficiencies (Banhos et al. 2016; Silva et al. 2018; 2019; 2022; 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 $NaMoO_2$. The pH was maintained at 6.0 ± 0.2, and the nutrient solution was replaced every 15 days. In the present study, the nutrient solution did not contain Al because we aimed to test the direct effect of Al on leaves while roots are not exposed to this metal.

Two experiments were set: a short-term experiment (48 h) in which Al was injected inside the petiole of the leaves, and a long-term experiment (60 days), in which Al was sprayed on the leaves.

Aluminum injection (short-term study)

A hypodermic needle (0.45 x 13 mm) containing 10 µL of 0, 185, 370, and 740 µM Al (0, 5, 10, and 20 mg L^{-1}) solution (pH = 5.5), provided by $AlCl_3$, was fixed ("injected") into the green petiole of a healthy, mature, fully-expanded leaf, in the upward direction to enable Al uptake by the transpiration flow. As Al may be approximately 90 % less available at pH 5.5 when compared to pH 4.0 (Bertsch and Parker 1995), we estimated that, similarly, the availability of Al in the needle's solution was approximately 90% lower than added (nominal) concentrations. Thus, Al concentration inside the needles was estimated at 0, 18.5, 37, and 74 µM Al. These values could be considered low when compared to the Al concentration in nutrient solutions of studies testing Al toxicity in *Citrus* ssp. (Banhos et al. 2016; Silva et al. 2022). The Al concentrations in the needles, however, could be sufficient to study direct effects of Al on leaves because injected Al solution enters directly through the xylem, through the transpiration flow, as the solution was completely taken up within 24 h. Moreover, once Al is taken up by the cell, even low concentrations can be harmful due to the high affinity of Al for oxygen ligands on cell walls (Jones and Ryan, 2003; Kopittke et al. 2015). To check if Cl from $AlCl_3$ interfered with the results, a solution containing 1110 µM Cl (provided with $CaCl_2$) was also injected in the leaves. This Cl concentration was calculated from the highest Al treatment (740 µM Al). Therefore, both deionized water and $CaCl_2$ solution were injected into the leaves as controls. The pH of all solutions was set to 5.5 to approximate to the pH found in xylem sap (Paudel et al. 2018; Grunwald et al. 2021).

Al sprayed directly on leaves (long-term study)

A solution containing 0, 7400, 14800, and 37000 μM Al (0, 200, 400, and 1000 mg Al L^{-1}) (provided by $AlCl_3$) was sprayed on the leaves every day, using a pressure sprayer with 0.5 L min^{-1} for 30 s, over 60 days. Although these concentrations are approximately 5 to 20 times higher than those used in nutrient solutions of experiments in which plants are exposed to Al through the roots (Banhos et al. 2016, Silva et al. 2018; 2019; Cavalheiro et al., 2020; Silva et al. 2022), in these studies, the Al uptake occurs throughout the day. Moreover, *Citrus* ssp. exhibit thick leaf cuticles, which reduces solution absorption by the leaf, such as foliar fertilizers (Marschner 1995). As far as we are aware, there are no studies in which the Al is applied directly on the leaves. Here, we used spraying solutions with high Al concentrations to provoke leaf Al uptake and leaf concentrations similar or higher than those found when it is provided by nutrient solution. Besides deionized water used as a control, solutions containing 11100, 22200, and 55500 μM Cl (394, 788 e 1970 mg Cl L^{-1}) (provided by $CaCl_2$) were also sprayed as controls to test the effect of Cl present in the Al solutions provided by $AlCl_3$.

One drop of detergent per liter (5 μL L^{-1}) was added to all spraying solutions to decrease the surface tension of the droplets and increase their contact with the leaf surface. Spraying solutions pH were set to 4.0 ± 0.1, which enables Al solubility and foliar absorption. For instance, ethyl phosphonic acid (Ethrel®), sprayed as plant regulator in field crops, is prepared at pH 4.0 (as recommended by the manufacturer). This regulator, or the Al solution in the present study, will be absorbed by the cells in the mesophyll, where pH is close to 7.0.

Experimental Design

Twenty-five plants, with five replicates per treatment, were used in the short-term experiment (Al injection). The plants were distributed in five boxes (one treatment per box), where the root system was exposed to the nutrient solution '*with no*' Al. After five days of

acclimation, one needle containing 5 μL of the solutions (0, 185, 370, and 740 μM Al, and 1110 μM Cl) was fixed in the petiole of one leaf per plant, at 9:00 h. Twenty-four hours after the injection, the empty needles were removed. Then, leaves were attached to a gas exchange analyzer to evaluate the photosynthetic performance. After measurement, extra needles containing the solutions were fixed into the remaining leaves 24 h before harvesting to obtain sufficient leaf material for measuring relative water content (RWC) and Al concentration. Organ biomass and biometric parameters were measured at the beginning (with five extra plants) and at the end of the experiment (at 48 h) and, as expected, no changes were found (data not shown). Al concentration in organs was below the detection limit for all treatments and, thus, the long-term experiment was performed.

For the long-term experiment, 35 plants with five replicates per treatment were used. The plants were distributed in seven boxes containing nutrient solution '*without*' Al, in which the five replicates (plants) were acclimated for 15 days and subjected to treatments for 60 days. After acclimation, the solution containing 0, 7400, 14800, and 37000 μM Al, and 11100, 22200 e 55500 μM Cl was sprayed on the leaves for 30 seconds. The expanded polystyrene plates (of each treatment) were removed from the boxes, turned upside down, and taken out of the greenhouse for spraying, so that no spraying droplets fell on the roots or in the nutrient solutions. Then, the plate with the plants was replaced in the boxes. This procedure was performed daily for 60 days between 16:00 and 16:30 h. At 0, 1, 3, 5, 10, 20, 30, 40, and 60 days after treatment (DAT), photosynthetic performance, plant height, number of leaves, and root length were measured. Leaf area, dry mass of organs, and Al concentration in roots, stem (plus petiole), and leaves were measured at 0 (with extra plants) and 60 DAT.

Photosynthetic parameters

CO_2 assimilation (*A*) and transpiration (*E*) rates, stomatal conductance (*gs*), and intercellular CO_2 (*Ci*) were measured with an open portable gas exchange system (LI-6400xt, LI-COR, Lincoln, NE, USA). The CO_2 concentration entering the leaf cuvette averaged 400 μmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR). Measurements were taken between 9:00 and 11:00 h (Feistler and Habermann, 2012) on cloudless days, under natural fluctuation of air temperature and vapor pressure deficit (VPD) inside the greenhouse. The photosynthetic photon flux density (PPFD) was provided by an artificial led light source (6400-40 LCF, LI-COR), which was set to provide 1200 μmol photons m^{-2} s^{-1} in the leaf cuvette, as this value saturates *A* for *Citrus* ssp. (Habermann et al. 2003; Banhos et al. 2016).

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

Relative water content

Leaf RWC (%) was measured between 13:00 h and 15:00 h. Disks (1 cm in diameter) were collected and had the fresh mass (FM) immediately measured on a scale. Then, the disks were hydrated in 100 mL deionized water inside amber flasks (to avoid photosynthetic activity)

for 24 h 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):

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

Biometric data and biomass

The leaves were counted, and the stem length and main root length measured with a ruler (cm). Total leaf area per plant (cm^2) was measured with an area meter (LI-3100, LI-COR, USA). Plant samples were separated into leaf, stem (plus petiole), and root and washed three times with deionized water. Leaves from the second experiment (Al spraying) were pre-washed with water and detergent (3% v/v) to remove Al from their surface. Then, samples were placed in paper bags and oven-dried at 60 ºC until constant mass. The biomass of organs (g) was measured using a precision scale.

Al concentration in plant organs

After measuring the biomass, the samples (washed, as described above) were sent to the plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), where samples were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). The concentration of Al was assessed by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

Data analyses

All the parameters were measured using five replicates (plants). For each evaluated parameter, one-way analysis of variance (One-Way ANOVA) was performed and the differences between treatments were compared by Tukey's test ($P < 0.05$) on each evaluation date.

Results

Injection of Al solutions

Injection of 1110 μM Cl and H_2O produced similar values of *A* (Fig. 1A), *gs* (Fig. 1B), *Ci* (Fig. 1C), *E* (Fig. 1D), ΦPSII (Fig. 2A), qP (Fig. 2B), and ETR (Fig. 2C). Nevertheless, leaves injected with 185, 370, and 740 μM Al showed lower *A* (Fig. 1A), while leaves injected with 370 and 740 μM Al, lower *gs* (Fig. 1B) and *E* (Fig. 1D), in relation to 1110 μM Cl and H_2O. In leaves injected with 740 μM Al, *Ci* was higher than the other four treatments (Fig. 1C). High *A* x *gs* and *E* x *gs* correlations were noted 24 h after injections (Supplementary material; Fig. S1). Leaves injected with 740 μM Al showed lower ΦPSII (Fig. 2A), and ETR (Fig. 2C) in relation to 1110 μM Cl and H_2O, while those injected with 185, 370, and 740 μM Al showed lower qP in relation to 1110 μM Cl and H_2O (Fig. 2B). After 24 h, relative leaf water content (RWC) was not affected either by Cl or Al (Supplementary material; Fig. S2). Thus, Al directly compromised gas exchange rates and photochemical performance.

Aluminum concentration in leaves injected with 740 μM Al was higher than the other four treatments, although this was not statistically significant (Fig. 3A). As expected, Al concentration in stem (Fig. 3B) and roots (Fig. 3C) was the same between the five treatments. Thus, Al concentration in plant organs was not altered 24 h after leaf Al injection.

Spraying of Al solutions

Plants sprayed with H_2O, 11100, 22200, and 55500 μM Cl showed similar values of *A* (Fig. 4A), *gs* (Fig. 4B), *Ci* (Fig. 4C), and *E* (Fig. 4D), as well as ΦPSII (Fig. 5A), qP (Fig. 5B), and ETR (Fig. 5C) throughout the study. At the end of the study (60 days), plants sprayed with the highest Al concentration (37000 μM Al) showed lower *A* (Fig. 4A), *gs* (Fig. 4B), and *E* (Fig. 4D), and higher *Ci* (Fig. 4C) in relation to those sprayed with H_2O and the three Cl

concentrations. Carbon assimilation of plants sprayed with Al was reduced from 30 days (Fig. 4A), while *E* was reduced in plants sprayed with 14800 and 37000 μM Al from 40 days (Fig. 4D). Intermediate Al concentration decreased *gs* at 40 and 60 days (Fig. 4B). Great *A* x *gs* and *E* x *gs* correlations were found after 60 days (Supplementary material; Fig. S3). All photochemical parameters were similar between treatments throughout the study, except for the highest Al concentration, which showed lower ΦPSII (Fig. 5A) and ETR (Fig. 5C) at 60 days. Plants sprayed with the highest Al concentration (37000 μM Al) showed lower RWC than the other six treatments at 60 days (Fig. 6). Thus, Al sprayed on leaves compromised mainly leaf gas exchange that decreased earlier than photochemical parameters.

Number of leaves (Fig. 7A) and plant height (Fig. 7B) and root length (Fig. 7C) increased similarly between plants sprayed with H_2O and Cl throughout the study. In contrast, those sprayed with Al showed lower number of leaves (Fig. 7A) and shorter stems (Fig. 7B) in relation to H_2O and Cl controls, as these parameters did not increase in Al-treated plants during the study. Plants sprayed with 37000 μM Al reduced the number of leaves from 40 days and those sprayed with all Al concentrations, at 60 days (Fig. 7A). The root length increased similarly between all treatments throughout the study (Fig. 7C). At 60 days, dry mass of leaves (Fig. 8A) and stems (Fig. 8C) was lower in plants sprayed with 37000 μM Al in relation to the other six treatments. On the same date, leaf area of plants sprayed with 14800 and 37000 μM Al was lower than the other five treatments (Fig. 8B). Roots, however, showed the same biomass for all treatments at 60 days (Fig. 8D). Thus, Al inhibited stem growth and leaf production, but not root growth.

As expected, plants sprayed with H_2O and Cl did not increase Al concentration in plant organs, from 0 to 60 days. At 60 days, leaf Al concentration of plants sprayed with Al was 18-fold higher than those sprayed with H_2O and Cl (Fig. 9A). On the same day, stem Al concentration of plants sprayed with Al was higher than those sprayed with H_2O and Cl (Fig.

9B). Although stem Al concentration was statistically similar between Al treatments, plants sprayed with 37000 μM Al was two-fold higher than those sprayed with 7400 and 14800 μM Al (Fig. 9B). Interestingly, at 60 days, plants sprayed with 37,000 μM Al showed approximately 1000 mg Al kg^{-1} roots, while the other six treatments exhibited less than 200 mg Al kg^{-1} roots (Fig. 9C). Thus, plants sprayed with Al retained it in the shoot and, interestingly, Al was found in roots of plants sprayed with the highest Al concentration.

At the end of the study, roots of plants from all treatments were visually healthy and similar between them. Nevertheless, plants treated with Al were shorter, with less and smaller leaves exhibiting chlorotic spots on the adaxial and brown lesions on the abaxial leaf surface (Fig. 10).

Discussion

Aluminum is mostly retained in the root system, and only approximately 10 % absorbed Al reaches the leaves (Vitorello et al. 2005; Singh et al. 2017; Silva et al. 2022). In general, studies ascribe reductions in photosynthetic responses to an indirect effect of Al from the root. In the present study, we applied Al directly on leaves to investigate whether it may cause a '*direct*' effect on photosynthetic performance. Our results showed that, when injected in leaves, Al decreased gas exchange (Fig. 1) and photochemical parameters (Fig. 2) after 24 h. When the leaves were sprayed with Al for 60 days, *A* values decreased from 30 days until the end of the study (Fig. 4A), and *gs* was lower than the controls at 60 days (Fig. 4B). Nevertheless, only the highest sprayed Al concentration reduced photochemical parameters at 60 days (Fig. 5A and 5C), and qP was maintained unalterable throughout the study (Fig. 5B). Moreover, we found relevant *A* x *gs* and *E* x *gs* correlations at the end of both studies (injection and spraying) (Supplementary material; Figs. S1 and S3). Therefore, *gs* seems to be more critical than photochemical parameters to explain the low photosynthetic performance of plants under direct

effect of Al on leaves, as also evidenced in plants exposed to Al through the root environment (Banhos et al. 2016; Silva et al. 2018).

Although *gs* is highly influenced by leaf hydration in plants in general (Lin et al. 2015; Lawson and Matthews 2020) or those under Al toxicity, such as 'Mandarin' lime (Banhos et al. 2016; Silva et al. 2022) and tomato (Gavassi 2020), plants injected with the three Al concentrations showed the same RWC (Supplementary material; Fig. S2). Plants sprayed with Al also showed the same RWC at 60 days, except for those sprayed with the highest Al concentration (Fig. 6). Thus, in general, the low *gs* of plants injected and sprayed with Al cannot be explained by low leaf hydration. Aluminum could be triggering a chemical signal associated with stomatal closure, and, although not measured in the present study, abscisic acid (ABA) emerges as a candidate.

Abscisic acid is a plant hormone widely known to provoke stomatal closure (Zhang and Davies 1989; Merilo et al. 2015; Hasan et al. 2021). In tomato (*Solanum lycopersicum* Mill.) exposed to increasing Al concentrations in the nutrient solution, ABA rose in roots and leaves concomitant with reductions in *gs* (Gavassi et al. 2020). A previous study with 'Mandarin' lime exposed to Al through the root system also showed high ABA concentration in roots and leaves while decreasing values of *gs* were observed (Gavassi et al. 2021). More recently, ABA peaks observed in roots and leaves of 'Mandarin' lime 24 h after root Al exposure was followed by decreasing *gs* three days after, while low leaf hydration was found only between 7 and 15 days (Silva et al. 2022). In the present study, the low *gs* found in the injection (Fig. 1B) and spraying (Fig. 4B) experiments was not accompanied by low leaf hydration (Supplementary material, Fig. S2 and Fig. 6), corroborating the findings of Silva et al. (2022). Therefore, when Al is applied directly on leaves, either by injection or spraying, it seems to induce a chemical signal to stomatal closure, which could be ABA, and further investigations are needed.

On the other hand, plants sprayed with the highest Al concentration (37000 μM Al) were the only ones exhibiting low RWC at 60 days (Fig. 6). These plants, somehow, transported Al from leaves to the root system, as their root Al concentration was five- to ten-fold higher than the other six treatments at 60 days (Fig. 9C). Thus, only when Al is in the root is leaf hydration affected. As far as we are aware, this is the first study applying Al directly on leaves of entire plants, indicating a novelty in Al transport from leaves to the roots. Most studies investigating Al toxicity expose plants to Al through the root system (Vitorello et al. 2005; Singh et al. 2017; Banhos et al. 2016; Silva et al. 2018; 2022). It is noteworthy that plants from all spraying treatments, including the highest Al concentration, showed similar root length throughout the experiment (Fig. 7C) and the same root dry mass at 60 days (Fig. 8D), as also evidenced by plant's morphological view (Fig. 10). Thus, even though Al was transported from leaves to roots (in plants sprayed with the highest Al concentration), which was associated with low leaf hydration, it did not disturb the root growth. Instead, Al stunted only the stem (Figs. 7B and 8C) and leaf (Figs. 7A, 8A and 8B) growth. When plants are exposed to Al through the root system, Al binds to the root cell walls (Kopittke et al. 2015), and inhibition of root elongation can be measured within hours (Llugany et al. 1995; Kopittke et al. 2008; Horst et al. 2010). Although not tested in the present study, it is possible that shoot-to-root transport of Al was associated to ligands that formed non-toxic stable complexes avoiding Al biding with negative charges in the apoplast. Here, as a novelty, we showed that Al can be transported from shoot to root and foliar Al absorption does not inhibit root growth within 60 days.

The two experiments differed in the manner Al was delivered to the plant. Injection experiment used hypodermic needles containing 10 μL. The highest Al concentration (740 μM Al) was delivered through approximately 50 μL per plant, as 10 μL needles were used in about five leaves per plant. This would result in 0.04 mg Al kg^{-1} plant (DM). Thus, it was not expected to find differences in leaf Al concentration between plants injected with Al and those with Cl

and water, after 24 h (Fig. 3A). Although leaf Al concentration was similar between all injected treatments, it reduced gas exchange rates in this organ (Fig. 1). Plants sprayed with the three Al concentrations (7400, 14800, and 37000 μM Al), on the other hand, resulted in between 800 and 1100 mg Al kg^{-1} leaf DM (Fig. 9A), which is twofold higher than Al concentration found in leaves of 'Mandarin' lime exposed to Al through the root environment (Banhos et al. 2016). The large leaf Al concentration found in these plants could be associated with the brown spots observed on their abaxial leaf surface (Fig. 10E, 10F, and 10G) and perhaps with the leaf chlorosis found in the plants sprayed with the highest Al concentration (Fig. 10G). Aluminum-induced leaf chlorosis is common among plants under Al toxicity (Foy et al. 1978; Rahman and Upadhyaya 2021). Brown spots, however, is not so often described in plants exposed to Al through the roots. Therefore, these leaf symptoms could be associated with the high leaf Al concentration found in plants sprayed with Al, and further investigations are needed. Despite the high leaf Al concentrations found in plants sprayed with Al, plants of both experiments showed low photosynthetic performance, including the ones injected with Al, that showed leaf Al concentration below the detection limit.

Conclusion

We showed that Al causes low photosynthetic performance and reduced shoot growth when applied directly on leaves of 'Mandarin' lime. Stomatal aperture seems to be more critical than photochemical performance to explain the low carbon assimilation of these plants, and the low *gs* cannot be explained by low leaf hydration. Therefore, a chemical signal directly triggered by Al could be associated with stomatal closure, and it deserves further investigations when the Al does not come from the roots. In addition, a shoot-to-root translocation of Al occurs when plants are sprayed with high Al concentration, and root growth is not stunted when Al is delivered by the leaves.

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Figures

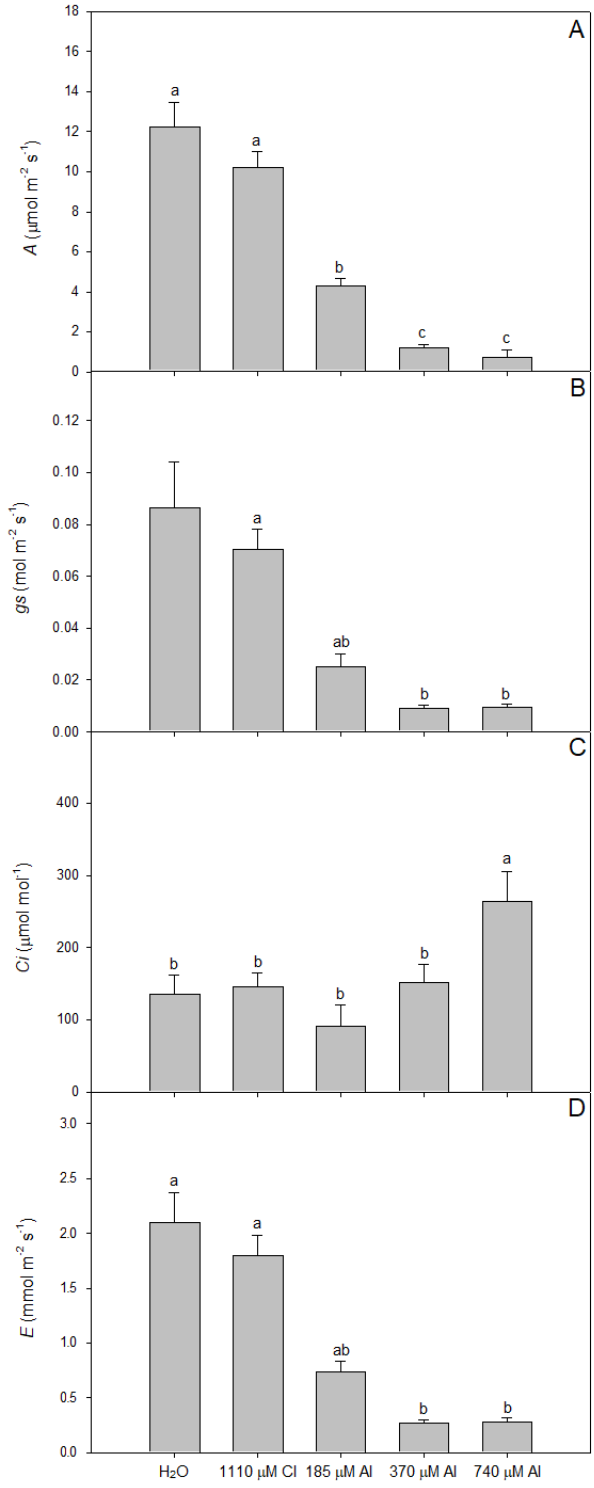

Fig 1. Mean values (n = 5) of CO_2 assimilation rate (*A*) (A), stomatal conductance (*gs*) (B), intercellular CO_2 (*Ci*) (C), and transpiration rate (*E*) (D) of leaves of *Citrus* x *limonia* injected with deionized water, 1110 µM $CaCl_2$, and 185, 370, and 740 µM Al after 24h. Distinct letters between treatments indicate significant difference by Tukey test ($P < 0.05$). Bars are s.e.

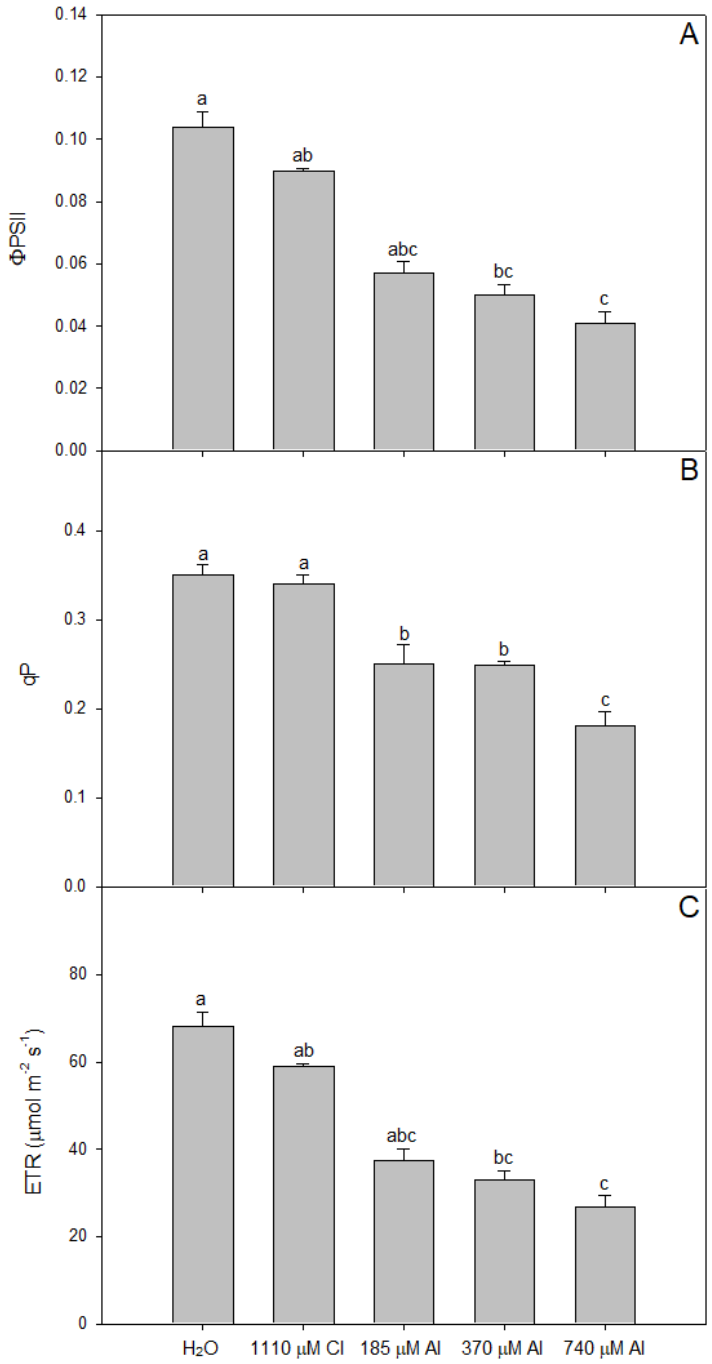

Fig 2. Mean values (n = 5) of effective quantum yield of photosystem II (PSII) (ϕPSII) (A), photochemical quenching (qP) (B), and electron transport rate (ETR) (C) of leaves of *Citrus* x *limonia* injected with deionized water, 1110 µM $CaCl_2$, and 185, 370, and 740 µM Al after 24h. Distinct letters between treatments indicate significant difference by Tukey test ($P < 0.05$). Bars are s.e.

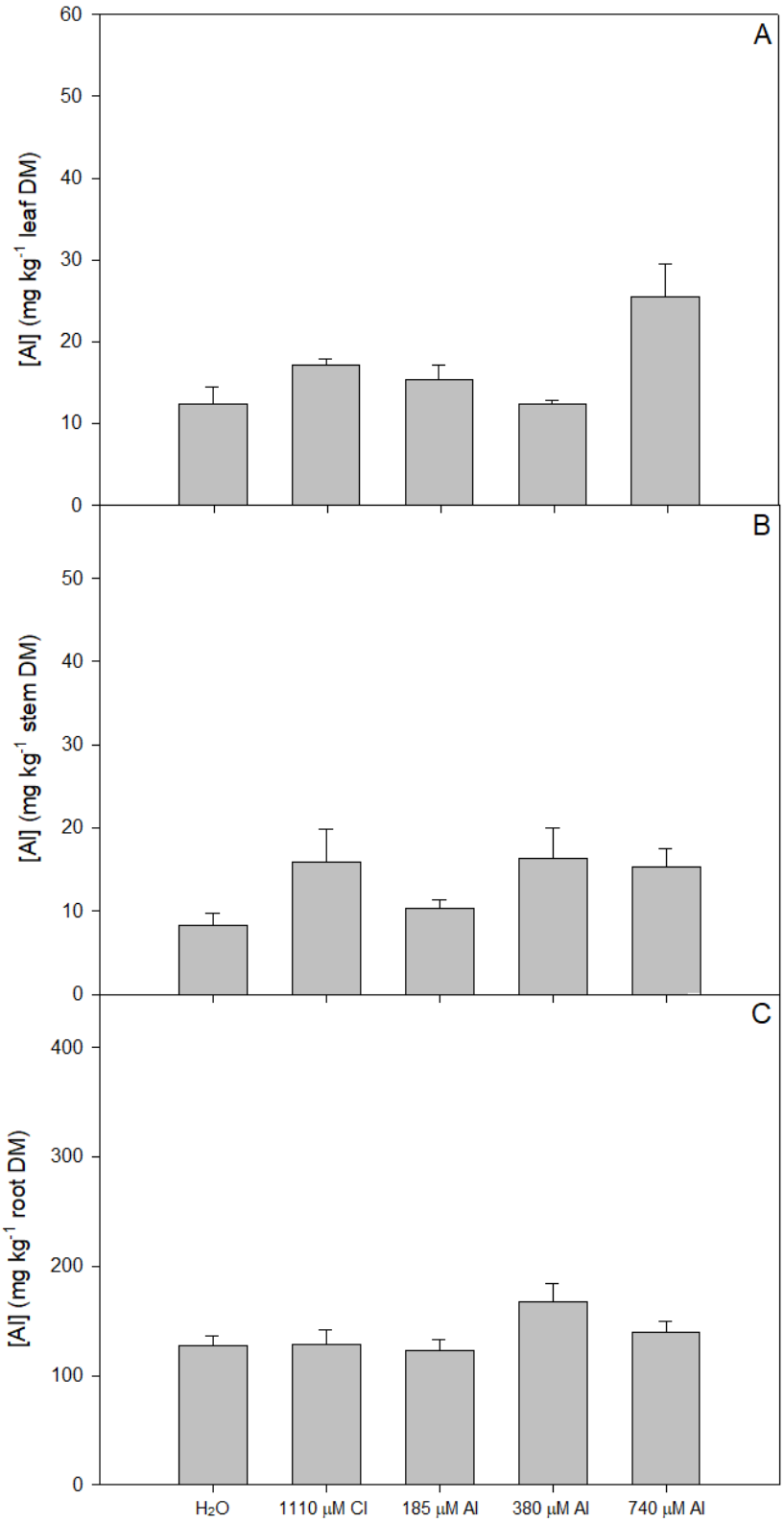

Fig 3. Mean values (n = 5) of Al concentration in leaves (A), stem (B), and roots (C) of *Citrus* x *limonia* injected with deionized water, 1110 µM $CaCl_2$, and 185, 370, and 740 µM Al after 24h. Absence of letters between treatments indicate no significant difference by Tukey test ($P < 0.05$). Bars are s.e.

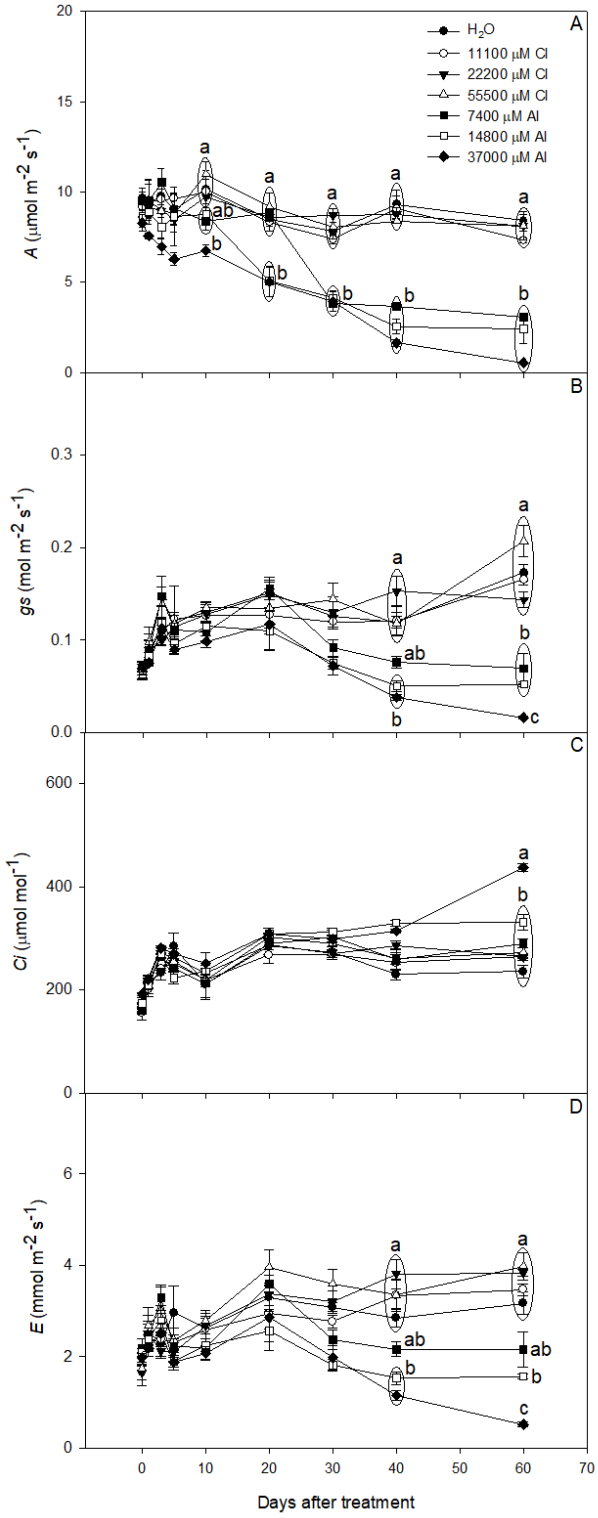

Fig 4. Mean values (n = 5) of CO_2 assimilation rate (*A*) (A), stomatal conductance (*gs*) (B), intercellular CO_2 (*Ci*) (C), and transpiration rate (*E*) (D) of leaves of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al for 0, 1, 3, 5, 10, 20, 30, 40, and 60 days. For each evaluation date, absence or distinct letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letter. Bars are s.e.

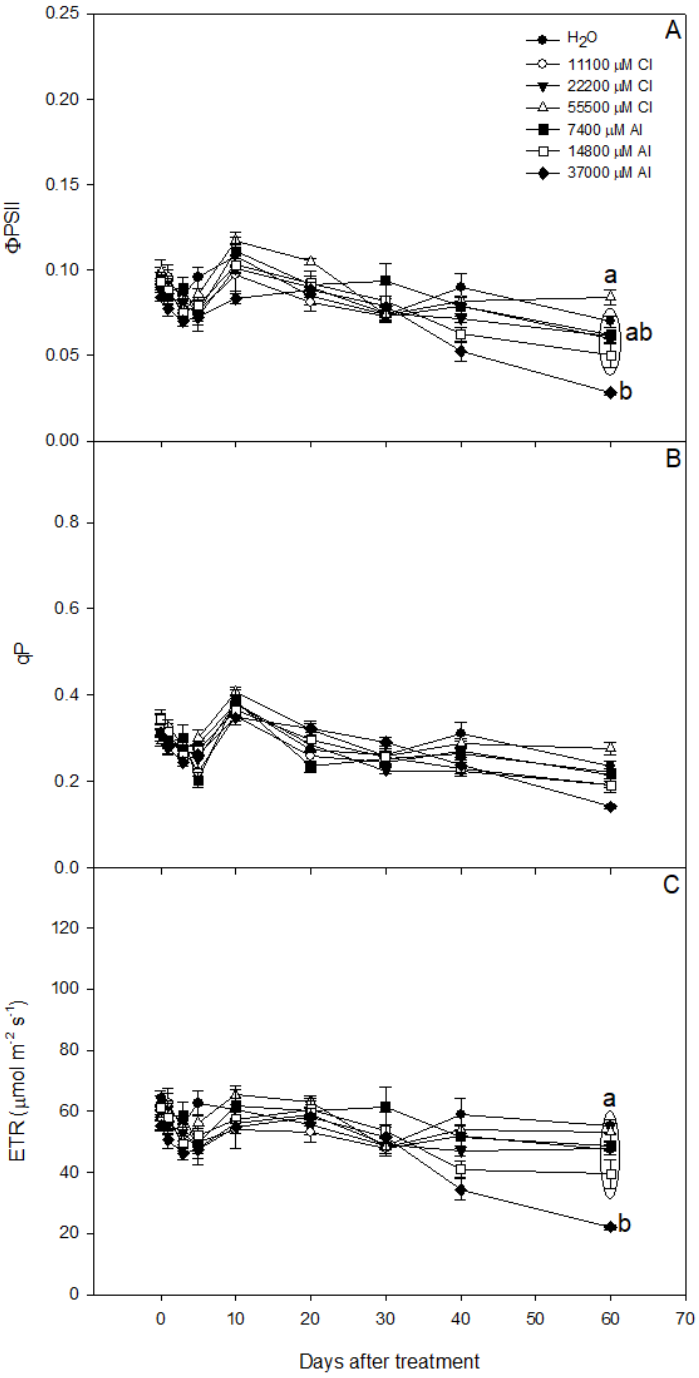

Fig 5. Mean values (n = 5) of effective quantum yield of photosystem II (PSII) (ϕPSII) (A), photochemical quenching (qP) (B), and electron transport rate (ETR) (C) of leaves of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al for 0, 1, 3, 5, 10, 20, 30, 40, and 60 days. For each evaluation date, absence or distinct letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letter. Bars are s.e.

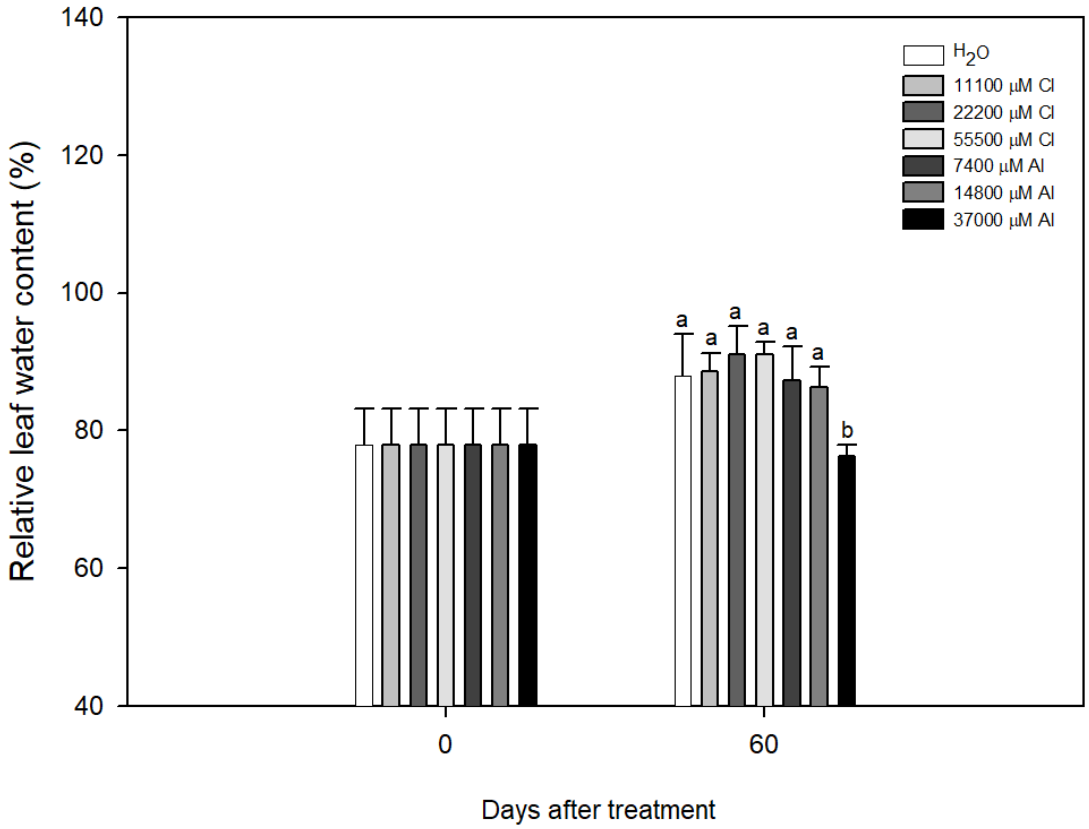

Fig 6. Mean values (n = 5) of relative water content (RWC) in leaves of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al at 0 and 60 days after treatment. For each evaluation date, distinct letters between treatments indicate significant difference by Tukey test ($P < 0.05$). Bars are s.e.

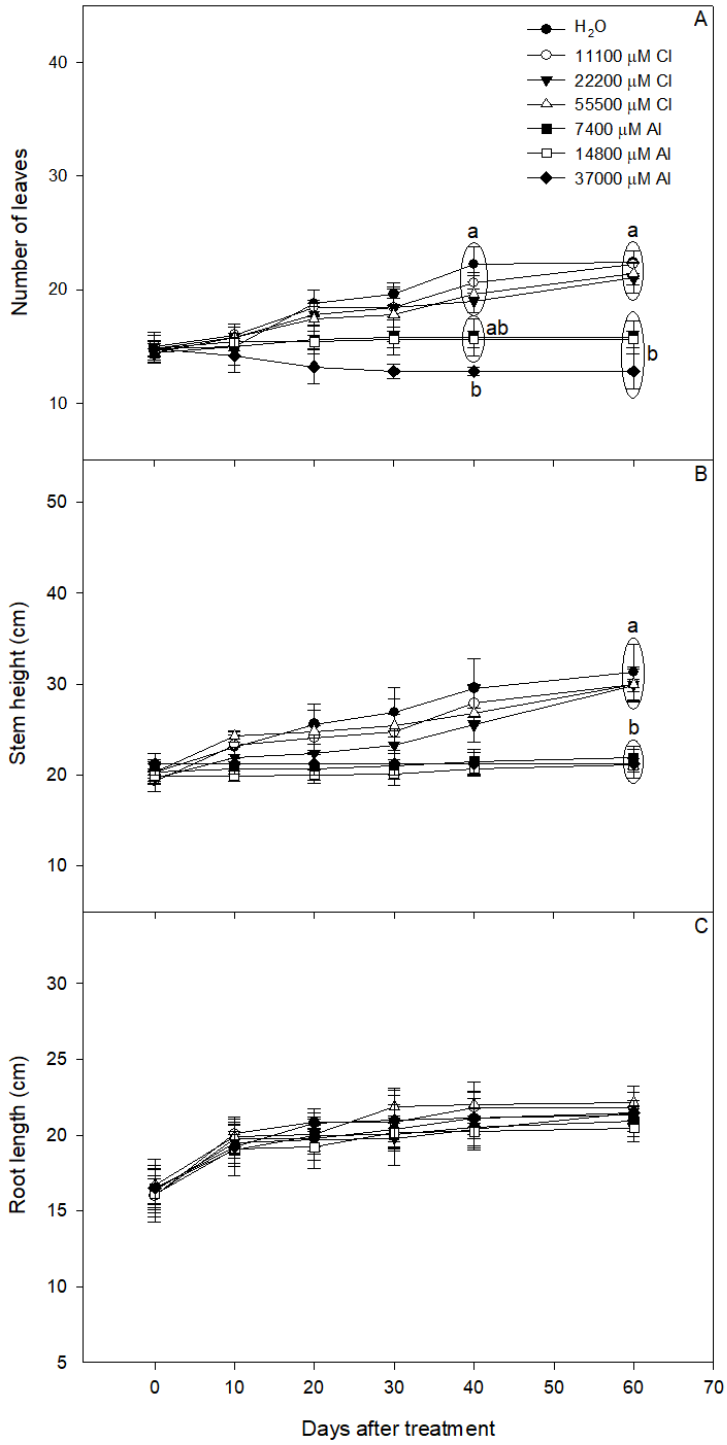

Fig 7. Mean values (n = 5) of number of leaves (A), stem height (B), and root length (C) of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al for 0, 1, 3, 5, 10, 20, 30, 40, and 60 days. For each evaluation date, absence or distinct letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letter. Bars are s.e.

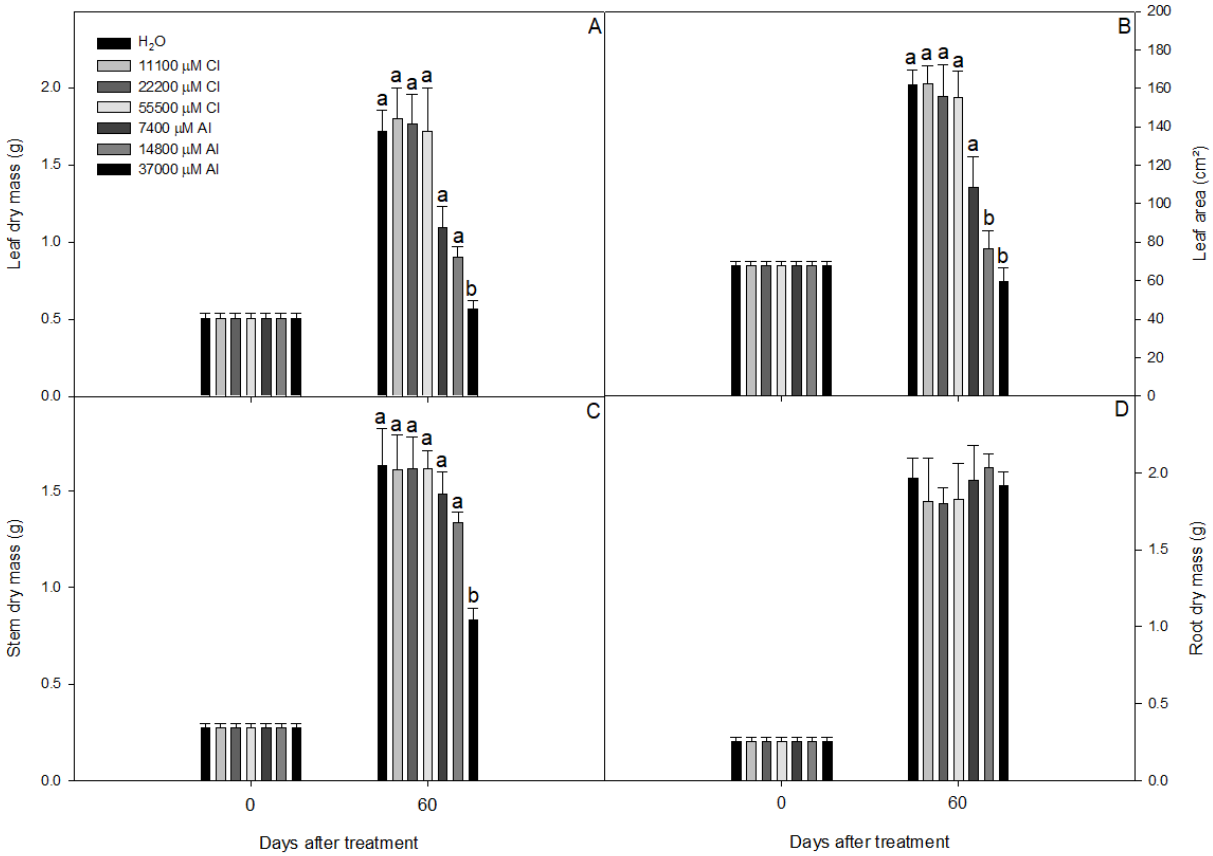

Fig 8. Mean values (n = 5) of leaf dry mass (A), leaf area (B), stem dry mass (C), and root dry mass (D) of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al after 0 and 60 days. For each evaluation date, absence or distinct letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Bars are s.e.

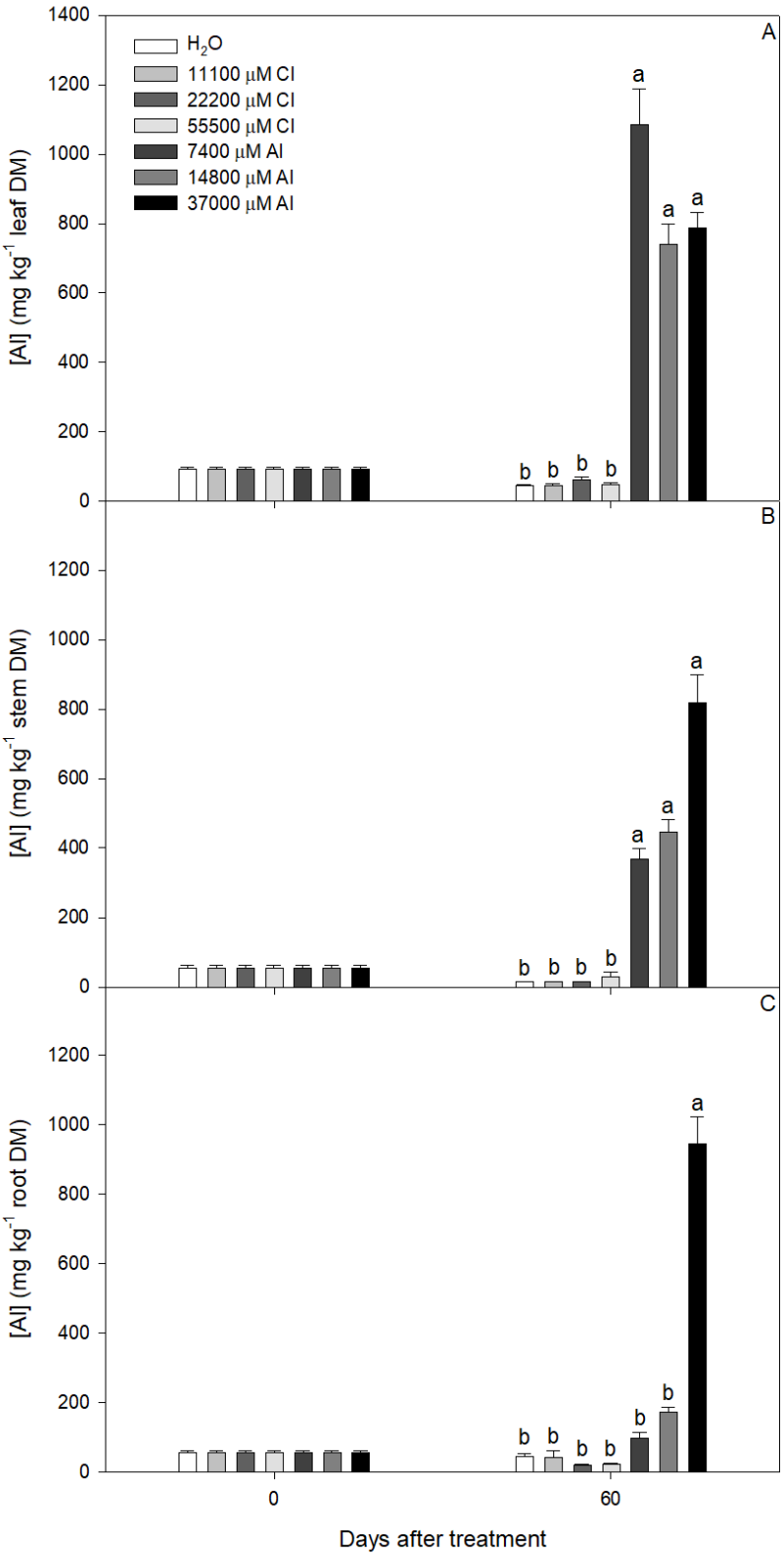

Fig 9. Mean values (n = 5) of Al concentration in leaves (A), stem (B), and roots (C) of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al after 0 and 60 days. For each evaluation date, absence or distinct letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Bars are s.e.

Fig 10. Morphological details of roots, shoot and leaves of *Citrus* x *limonia* sprayed with water (A), solution containing 11100 (B), 22200 (C), and 55500 µM Cl (D), and 7400 (E), 14800 (F), and 37000 µM Al (G) after 60 days. White (leaves) and yellow (plants) bars represent 1 and 3 cm, respectively.

Supplementary material

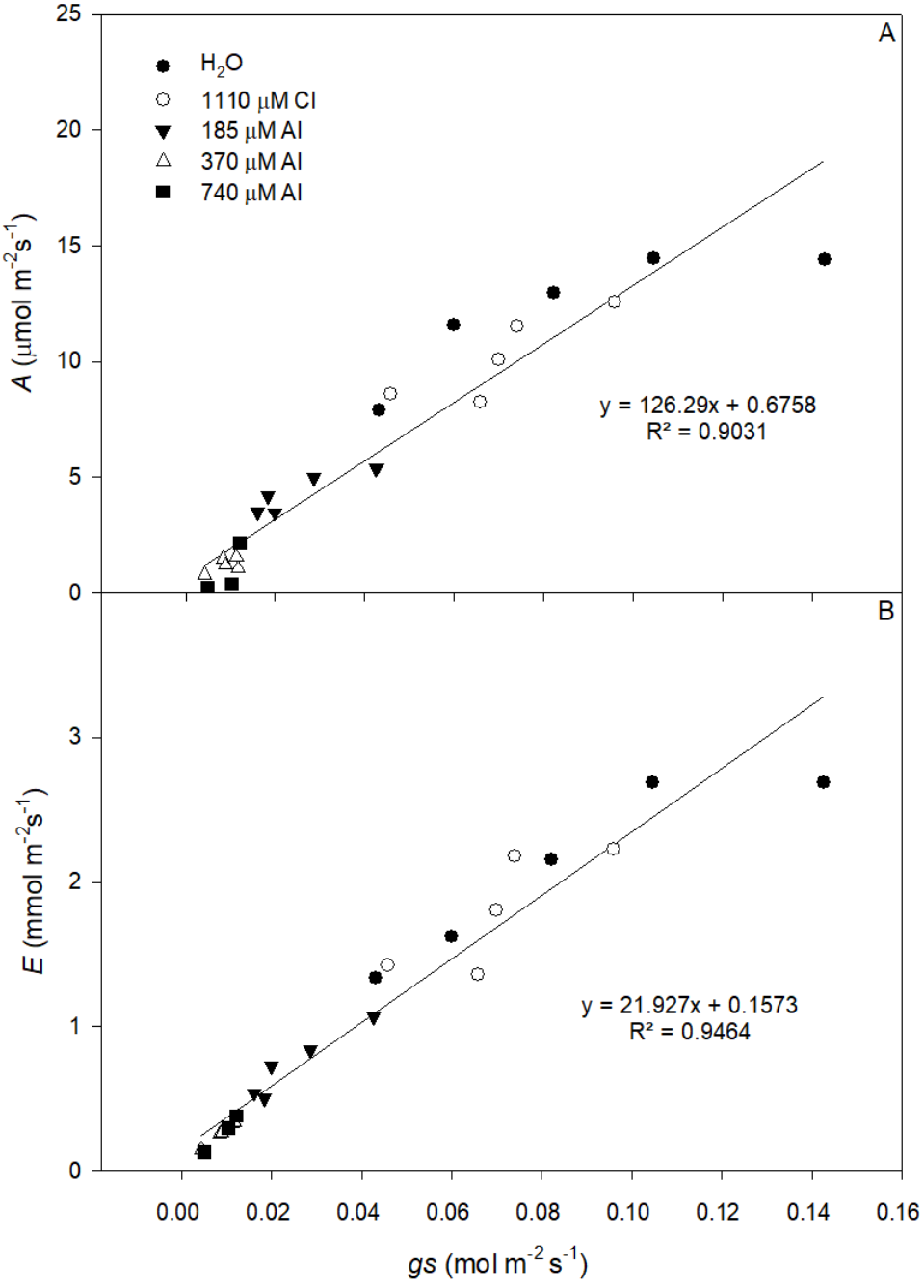

Fig S1. Individual readings (replicates) of CO_2 assimilation rate (A) and transpiration rate (B) in response to stomatal conductance of leaves of *Citrus* x *limonia* injected with deionized water, 1110 µM $CaCl_2$, and 185, 370, and 740 µM Al after 24h.

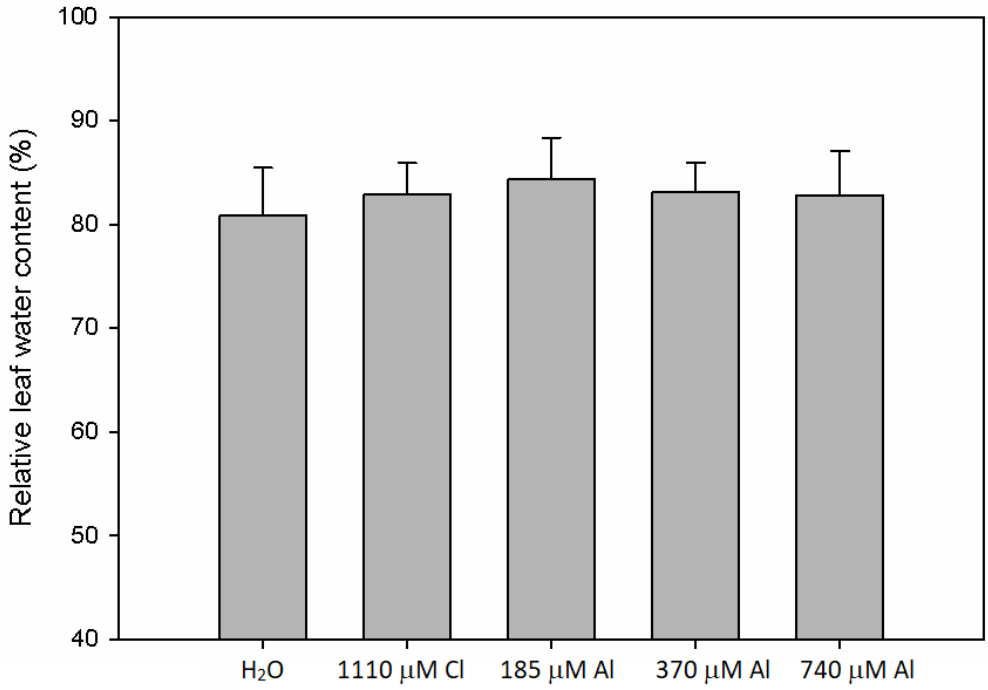

Fig S2. Mean values (n = 5) of relative water content (RWC) in leaves of *Citrus* x *limonia* injected with deionized water, 1110 µM $CaCl_2$, and 185, 370, and 740 µM Al after 24h. Absence of letters between treatments indicate no significant difference by Tukey test ($P < 0.05$). Bars are s.e.

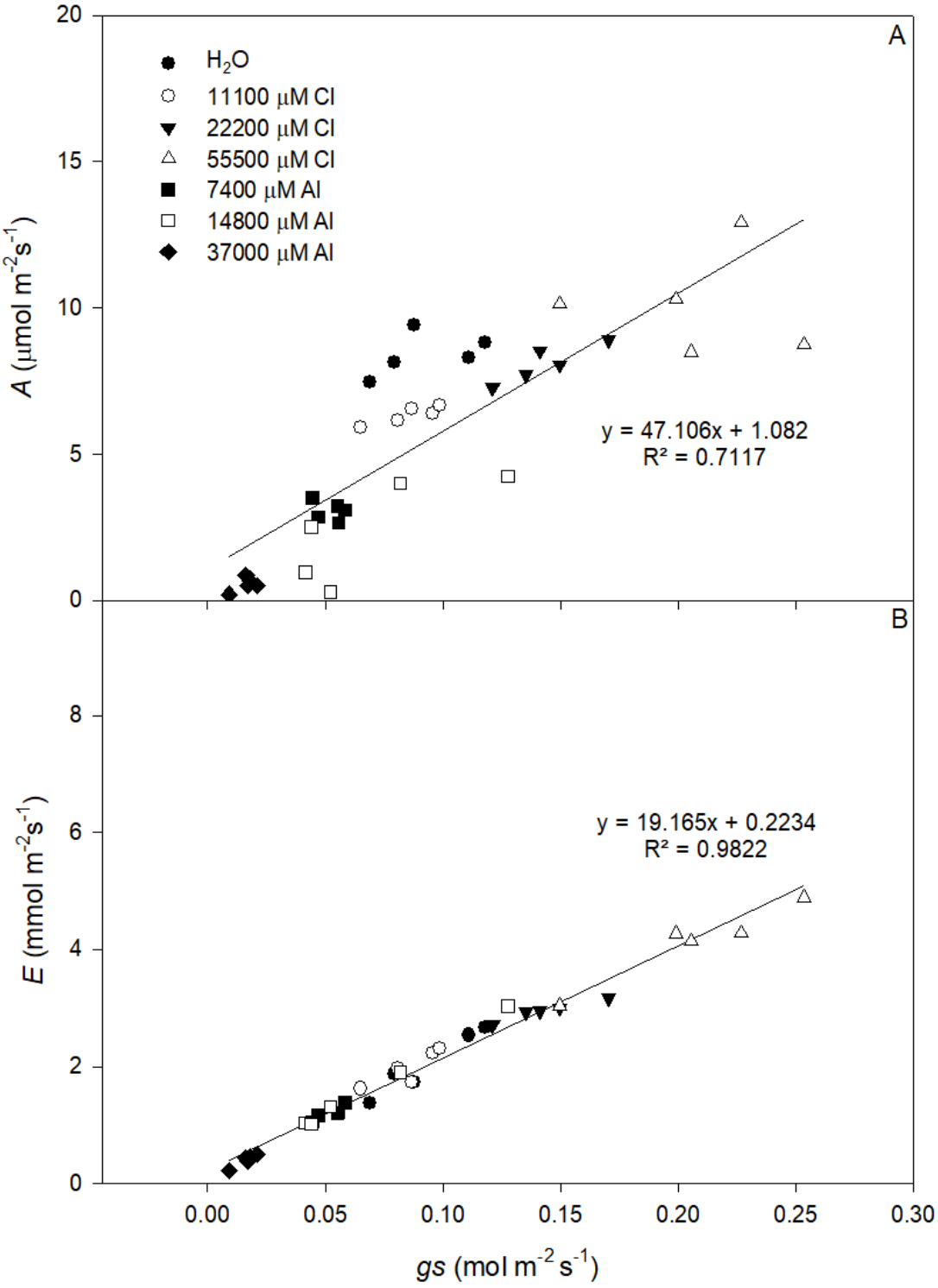

Fig S3. Individual readings (replicates) of CO_2 assimilation rate (A) and transpiration rate (B) in response to stomatal conductance of leaves of *Citrus* x *limonia* sprayed daily with deionized water, solution containing 11100, 22200, and 55500 µM Cl, and 7400, 14800, and 37000 µM Al after 60 days.

CAPÍTULO II:
Is proline involved in the aluminum tolerance of *Pinus sylvestris*?

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Abstract

Proline is an amino acid acting as osmoprotector in plants, as it reduces osmotic potential and enhances plant water uptake, especially under drought. Aluminum (Al) toxicity causes inhibition of root growth and low leaf hydration with negative consequences for photosynthetic performance. Thus, plants under Al toxicity could benefit from proline accumulation. Here we investigate whether proline could is induced by Al and could maintain needle hydration in *Pinus sylvestris*, an Al-tolerant woody species. Proline and ABA concentration, gas exchange rates, stomatal conductance (*gs*), relative needle water content (RWC), stem water potential (Ψw), biometric data and plant biomass were evaluated in plants grown in nutrient solution with 0, 250, 500, and 1000 μM Al for 42 days. Plant growth, gas exchange rates, RWC and ABA were the same between the four treatments, as expected for an Al-tolerant species. Proline increased while midday stem water potential (Ψmd) decreased in plants exposed to Al between seven and 14 days, followed by similar values between treatments for both parameters until to the end of the study. This suggests that proline could be involved in osmotic adjustment in *P. sylvestris* exposed to Al. The maintenance of needle hydration throughout the study could explain similar gas exchange values between Al-treated and untreated plants. The role of proline in Al tolerant species deserves further investigations.

Key words: Al^{3+}; ABA; gas exchange; leaf hydration; osmoprotection

Key message: Proline accumulation seems to be involved with the maintenance of needle hydration in *Pinus sylvestris* exposed to aluminum.

Introduction

Plants are often exposed to environmental fluctuations such as water and nutrients availability or the temperature, which can impact plant metabolism. In order to adjust plant's metabolic reactions to these fluctuations, chemical protectants are required. Proline is an amino acid that acts, among other roles, as osmoprotector ('osmolyte') (Ashraf and Foolad, 2007; Ghosh et al. 2022). Accumulation of proline is evidenced in plants with various osmotic stresses such as salinity and drought (Yoshiba et al. 1995; Parre et al. 2007; Sancho Knapik et al. 2017; Lopez-Delacalle et al. 2021). Under drought, for example, proline can reduce osmotic potential ($\Psi\pi$) and thus improve water uptake (Verbruggen and Hermans 2008; Vilagrosa et al. 2010; Sancho-Knapik et al. 2017; Ghosh et al. 2022) and consequently the plant water status.

Low leaf hydration leads to decreased photosynthetic performance, including reduced gas exchange due to low stomatal conductance (*gs*) and reduced photochemical reactions (Chaves et al. 2003; Mahouachi et al. 2006; Schley et al. 2022). Thus, any environmental disturbance causing low leaf hydration could trigger proline accumulation, which could mitigate physiological damage to photosynthesis and plant growth (Verbruggen and Hermans 2008; Meena et al. 2019; Ghosh et al. 2022).

Aluminum (Al) disturbance causes inhibition of root growth (Kopittke et al. 2008; Horst et al. 2010; Silva et al. 2019), low leaf water potential (Ψw) and relative leaf water content (RWC), with negative consequences for CO_2 assimilation (*A*) and *gs* (Chen et al. 2005; Silva et al. 2012; Banhos et al. 2016; Silva et al. 2022). Aluminum toxicity also increases the concentration of abscisic acid (ABA) (Gavassi et al. 2020; 2021; Silva et al. 2022), a plant hormone responsible for stomatal closure (Zhang and Davies 1989; Merilo et al. 2015). Aquaporin genes (PIP family), which are membrane proteins partly responsible for water transport (Javot and Maurel 2002), are also down-regulated in plants under Al toxicity (Cavalheiro et al. 2020). Thus, plants under Al toxicity and drought share similar consequences

(Yang et al. 2013) and could benefit from proline accumulation and, eventually, osmoprotection.

Nevertheless, there are Al-tolerant species, including those that accumulate Al in their tissues without showing toxicity symptoms (Al-accumulators) (Chenery 1948; Haridasan 1980; Jansen et al. 2002; Souza et al. 2015; Silva et al. 2016) and plants that exude organic acids (OA) through their roots and form non-toxic OA-Al complexes in the rhizosphere that prevent Al uptake (Al excluders) (Ryan et al. 2011; Brunner and Sperisen 2013). Plants that exhibit these Al tolerance mechanisms usually show no signs of low leaf hydration.

Proline accumulation was already observed in plants exposed to Al, but only in species sensitive to Al, such as lettuce (*Lactuca sativa* L.) (Silva and Matos 2016) and maize (*Zea mays* L.) (Khan et al. 2000). In these studies, however, leaf hydration parameters such as *gs*, RWC and Ψw were not assessed, so the relationship between proline and plant water status under Al influence remains unclear. As far as we are aware , proline was never evaluated in Al-tolerant species when exposed to Al, including woody species such as *Pinus sylvestris* L., an Al-excluder plant (Ahonen-Jonnarth et al. 2000). Under drought, this species showed proline accumulation (Sancho-Knapik et al. 2017), and therefore it is likely that *P. sylvestris* shows proline accumulation when exposed to Al.

In the present study, we grew the Al tolerant *P. sylvestris* in nutrient solution with increasing Al concentrations for 42 days. Proline and ABA concentration, needle gas exchange rates, photochemical parameters, plant water status (*gs*, RWC, and Ψw), biometric data and plant biomass were evaluated. Thus, in the present communication, we investigate whether proline explains the Al tolerance in *P. sylvestris* through the maintenance of needle hydration.

Material and methods

Plant material and experimental conditions

Two-year-old *Pinus sylvestris* L. (Pinaceae) seedlings (25 ± 0.5 cm in height) grown in a substrate of sand and soil (1:1; v:v) were obtained from the nursery of the Swiss Federal Institute for Forest, Snow and Landscape Research WSL (Birmensdorf, Switzerland). The root system was rinsed (to remove remaining substrate) and the plants were transferred to opaque plastic boxes (60 cm x 40 cm x 15 cm; 30 L) with aerated (by aquarium pumps) nutrient solution.

The nutrient solution was based on the Clark solution (Clark, 1975) and it has been used to test Al toxicity in various plant species such as *Citrus* x *limonia* (Santos et al. 2000; Banhos et al. 2016; Silva et al. 2019; 2022), *Styrax camporum* (Bittencourt et al. 2020a), *Solanum lycopersicum* (Gavassi et al. 2020) and *Vochysia tucanorum* (Bressan et al. 2021). It was composed 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 $NaMoO_2$. Besides macro- and micronutrients, the nutrient solution contained 0, 250, 500, and 1000 μM Al (added as $AlCl_3$), as *P. sylvestris* shows tolerance to these concentrations (Oleskyn et al. 1996; van Schöll et al. 2004). The pH was kept at 4.0 ± 0.1 to maintain the Al as soluble as possible. It was monitored daily, and the solution was totally replaced every week. The nominal Al concentrations resulted in 218.3 ± 8.1, 457.7 ± 10.76, and 853.9 ± 185 μM Al, measured through inductive coupled plasm optical emission spectrometry (ICP-OES). The solution with no added Al contained 4.4 μM Al.

Expanded polystyrene (Isopor®, 60 × 40 × 2 cm plates), with eight holes (3 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with Isopor® disks. The boxes were maintained in a greenhouse with semi-

controlled conditions (461.8 ± 185.2 μmol photons m^{-2} s^{-1} between 9:00 and 12:00 h; 12 h of natural photoperiod). Air temperature and relative humidity inside the greenhouse were approximately 25/20°C (day/night) temperature and 50/80% (day/night) relative humidity.

Experimental design

One hundred and sixty plants were distributed in 20 boxes (eight plants per box), consisting of one box per treatment per evaluation date (five dates). At 1, 7, 14, 28, and 42 days after treatment, samples of roots, needles, and new shoots from four plants per treatment were immediately immersed in liquid nitrogen (N_2) prior to lyophilization and used to measure proline and abscisic acid (ABA). The four remaining plants were used to measure midday stem water potential (Ψmd) and biomass of plant organs (roots, stem, needles, and new shoots). At 1, 7, 14, 21, 28, 35, and 42 days, four plants were used to measure gas exchange rates, photochemical parameters, and biometric data (root, stem, and new shoot length). At the end of the study (42 days), predawn stem water potential (Ψpd), needle relative water content (RWC), and Al concentration in plant organs were also assessed using four plants per treatment.

Proline

Root, needles, and new shoot proline concentration was measured following the procedure by Sancho-Knapik et al. (2017) that quantified proline in *P. sylvestris* based on the method of Bates et al. (1973). Lyophilized and ground (in N_2) samples (10 mg) were added to 1.8 mL of 3% (m/v) sulfosalicylic acid and centrifuged at 14,310 g at room temperature for 15 min. Subsequently, 0.4 mL of the supernatant was colored by the addition of 0.4 mL ninhydrin and 0.4 mL of glacial acetic acid. It was heated to 100 °C for 1 h, and the reaction was finished in an ice-bath. The ninhydrin-proline complex formed was added to 0.8 mL of toluene and mixed in a tube stirrer for 20 s. The supernatant (toluene phase; 150 μmol) was aspirated and

measured by a spectrophotometer (Shimadzu UV-1700; Japan) at 520 nm. Proline concentration was calculated from a L-proline standard curve, using pure toluene as blank. Values were expressed as pmol g^{-1} dry mass.

Abscisic acid (ABA)

Abscisic acid was measured according to Cocozza (2010; 2013) by enzyme-linked immunosorbent assay (ELISA) method. Roots, needles, and new shoots lyophilized and ground (in N_2) samples (5 mg) were extracted overnight in 1.5 mL deionized water in the dark, at 4 °C on a shaker (Barta and Loreto 2006). The extracts were centrifuged at 10,000 g for 25 min. The ABA content of the supernatants was then quantified using the Phytodetek-ABA kit (AGDIA, Elkhart, IN, USA), according to the manufacturer. ABA concentrations were estimated following the calculations provided by the Phytodetek-ABA kit. Data was expressed as pmol g^{-1} dry mass.

Plant hydration parameters

Stem water potential was measured at predawn (Ψpd; just at the end of the study at 42 days) and midday (Ψmd; at 1, 7, 14, 28, and 42 days) by the pressure chamber method (Turner 1981), using a Model 1000 (PMS Instruments, Albany, OR, USA) chamber.

The relative water content (RWC; %) was measured (just at the end of the study at 42 days) according to Waring et al. (1979). Eight needles were collected at 14:00 h, immediately cut to 1 cm long and weighted as fresh mass (FM) with a scale. Then, the samples were emersed in deionized water at 5°C, for 24 h. The turgid mass (TM) was obtained after blotting the samples between filter papers for 30 s. Then, the samples were oven-dried at 60 °C for 48 h, and reweighed to obtain the dry mass (DM). RWC was calculated as (FM–DM)/(TM–DM) × 100.

Photosynthetic parameters

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 measured using an open gas exchange system (LI-6800xt; LI-COR, Lincoln, NE, USA). The CO_2 concentration entering the leaf cuvette averaged 400 µmol mol^{-1}, provided by the 6800-CO_2 mixer (LI-COR) , as this is the air CO_2 concentration accepted for the experimental site where the study was performed (Birmensdorf, Zurich, Switzerland; 47° 21' N, 8° 26' E). Three fascicules (6 needles) were inserted into the cuvette (3 cm^2), avoiding overlap, and the measured needle area was calculated using ImageJ Software (Schneider et al. 2012). Measurements were taken between 9:00 h and 11:30 h on cloudless days. The PPFD in the cuvette was provided by an artificial LED light source (6800 LCF, LI-COR), which was set to provide 90% red and 10% blue spectra, and 1100 µmol photons m^{-2} s^{-1} was used inside the cuvette, as it saturates *A* in *P. sylvestris* (Oleksyn et al. 1996; Hansen et al. 1996). The vapor pressure deficit (VPD) inside the cuvette was fixed to 1.3 kPa, which was similar to the external environment (greenhouse).

Chlorophyll *a* fluorescence was measured with a portable modulated fluorometer (6800–40 LCF; LI-COR), which is integrated into the LI-6800xt gas exchange system. The effective quantum yield of photosystem II (PSII) (ΦPSII) was calculated as (Fm'–Fs)/Fm', where Fm' and Fs indicate the maximum and steady state fluorescence in light adapted needles, respectively. Apparent electron transport rate (ETR; µmol electrons m^{-2} s^{-1}) was calculated using 0.5 as the fraction of excitation energy distributed to PSII, and 0.87 as the fractional light absorbance. The proportion of open PSII reactions centers (qP) was measured as (Fm'–Fs)/(Fm'–Fo') (Bolhàr-Nordenkampf and Öquist 1993), where Fo' is the minimal fluorescence from light-adapted leaves. A saturating light pulse of 7000 µmol photons m^{-2} s^{-1} for 0.7 s was used.

Biometric data and biomass

The length of the roots, stem, and new shoots was measured with a ruler (cm). The biomass of needles, stem and new shoots was measured with a precision scale after lyophilizing the samples.

Aluminum in organs

Aluminum was quantified by digesting the lyophilized and ground samples in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, atomic absorption spectrophotometer method (Sarruge and Haag 1974) was used to calculate Al concentration. Values were expressed as mg Al per kg dry mass.

Data analysis

Analysis of variance (One-way ANOVA) was used for each evaluation date, separately, to test differences between 0, 250, 500 and 1000 μM Al for each variable. Shapiro-Wilk test was used to verify the data normality. Mean values were compared by Tuckey test ($\alpha = 0.05$) as a post-hoc multiple comparisons.

Results and discussion

After 42 days, the plants showed new shoots and roots, but growth was the same between the four treatments (Fig. 1). As expected, new shoot, stem, and root length (Supplementary material; Fig. S1) and plant organs biomass (Supplementary material; Fig. S2) were the same between treatments. A previous study showed that root growth of *Pinus sylvestris* is not affected when exposed up to 1000 μM Al in nutrient solution, resulting in an accumulation of approximately 400 mg Al kg^{-1} shoot dry mass (van Schöll et al. 2004). In the present study, plants from the three Al treatments showed most absorbed Al retained in the root

system, and only 100–300 mg Al kg^{-1} dry mass was found in the needles and new shoots (data not shown). This species is tolerant to Al because root oxalic acid exudation was evidenced (Ahonen-Jonnarth et al. 2000), suggesting an Al exclusion mechanism (Ryan et al. 2011). Thus, photosynthetic response is not expected to decrease when this species is under Al toxicity, as observed by Oleksyn et al. (1996). Our results also showed that increasing Al concentrations resulted in similar needle gas exchange (*A*, *gs*, *Ci*, and *E*) (Fig. 2) and photochemical performance (Supplementary material; Fig. S3) compared to the control, throughout the study. Similarly, Ψpd and RWC were the same between treatments after 42 days (Supplementary material; Fig. S4), which along with (the same) *gs* demonstrates that needle hydration was not affected by Al. Abscisic acid (ABA), which is known to control *gs* (Zhang and Davies 1989; Merilo et al. 2015), was also not affected throughout the study (Supplementary material; Fig. S5). Therefore, Al does not seem to interfere with plant growth, photosynthetic performance or needle hydration in *P. sylvestris*.

The present study asked whether proline explains the Al tolerance in *P. sylvestris* through the maintenance of needle hydration. Proline, which is considered an osmoprotectant (Verbruggen and Hermans 2008; Ghost et al. 2022), was already described to increase in maize (*Zea mays*) (Khan et al. 2000), and lettuce (*Lactuca sativa*) (Silva and Matos 2016) under Al toxicity. These studies, however, were not designed to elucidate the role of proline in leaf hydration or photosynthetic response. Although needle proline concentration was the same between treatments throughout the study (Fig. 3B), it was higher in new shoots of plants exposed to the three Al treatments when compared to control plants after seven days, and these results were equal between treatments from 14 days up to end of the study (Fig. 3A). Roots of plants exposed to 500 and 1000 μM Al showed higher proline concentration compared to 0 and 250 μM Al after seven days, while after 14 days, only those exposed to 1000 μM Al had higher proline concentration in relation to the other treatments (Fig. 3C). After 28 and 42 days,

however, root proline concentration was the same between treatments (Fig. 3C). Concomitantly, midday stem water potential (Ψmd) was lower in plants exposed to Al in relation to control plants after seven days, while after 14 days, only those exposed to 1000 μM Al showed lower Ψmd in relation to the other treatments (Fig. 4). Thus, the decrease in Ψmd and the increase in proline concentration observed between seven and 14 days, followed by similar values for both parameters until the end of the study, suggest that proline could have acted in maintaining needle hydration most likely through osmotic adjustment. The role of proline as an osmotic adjustment factor already demonstrated in *P. sylvestris* under drought (Sancho-Knapik et al. 2017), but we are not aware of other studies linking proline measurements, needle hydration and *gs* assessments under Al influence.

The high proline concentration observed in new shoots at seven days and roots at seven and 14 days (Fig. 3A, C) may also suggest an osmoprotection during a critical transitional growth phase. This transitional phase is characterized by the conversion of reserves (starch and lipids) into metabolically active molecules such as sugars and fatty acids (Kaufmann and Blanke 2017; Beauvieux et al. 2018). In *Picea abies*, for instance, starch is resynthesized in the buds at the end of dormancy from sugars that have acted as cold protectants during winter and degraded back to sugars during bud break (Guzicka 2001; Guzicka et al. 2017). These relations were not investigated in the present study, and it deserves attention in future studies.

The present communication points out that proline is likely to be involved with osmotic adjustment in *P. sylvestris* exposed to Al. Proline could be associated with the maintenance of needle hydration, eventually explaining similar gas exchange parameters, especially *gs*, between Al-treated and untreated plants.

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Figures

Fig. 1 Morphological details of roots, stem, needles, and new shoots of *Pinus sylvestris* exposed to 0, 250, 500 and 1000 μM Al at 0 and 42 days after treatment (DAT). Yellow bars represent 3 cm. Arrows indicate the new shoots.

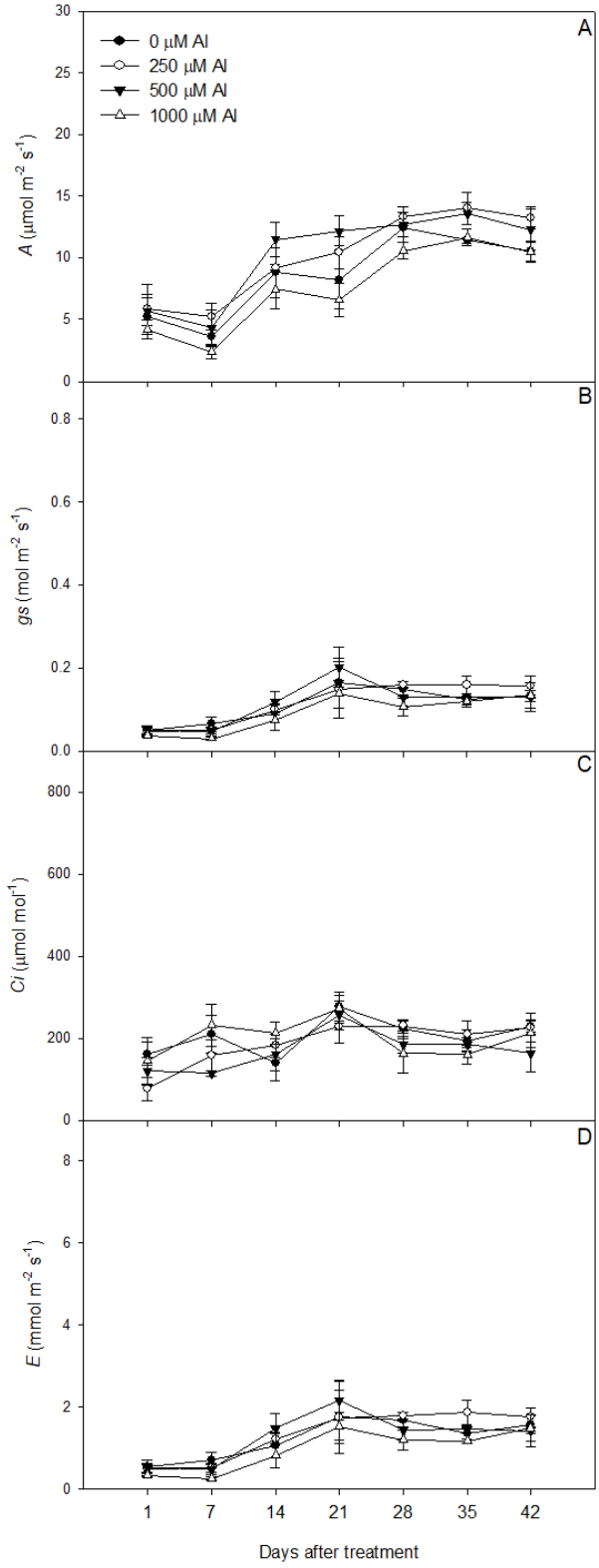

Fig. 2 Mean values (n = 4) of CO_2 assimilation rate (*A*) (A), stomatal conductance (*gs*) (B), intercellular CO_2 (*Ci*) (C), and transpiration rate (*E*) (D) of needles of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 µM Al for 1, 7, 14, 21, 28, 35, and 42 days. For each evaluation date, absence of letters between treatments indicates no significant difference by Tukey test ($P < 0.05$). Bars are s.e.

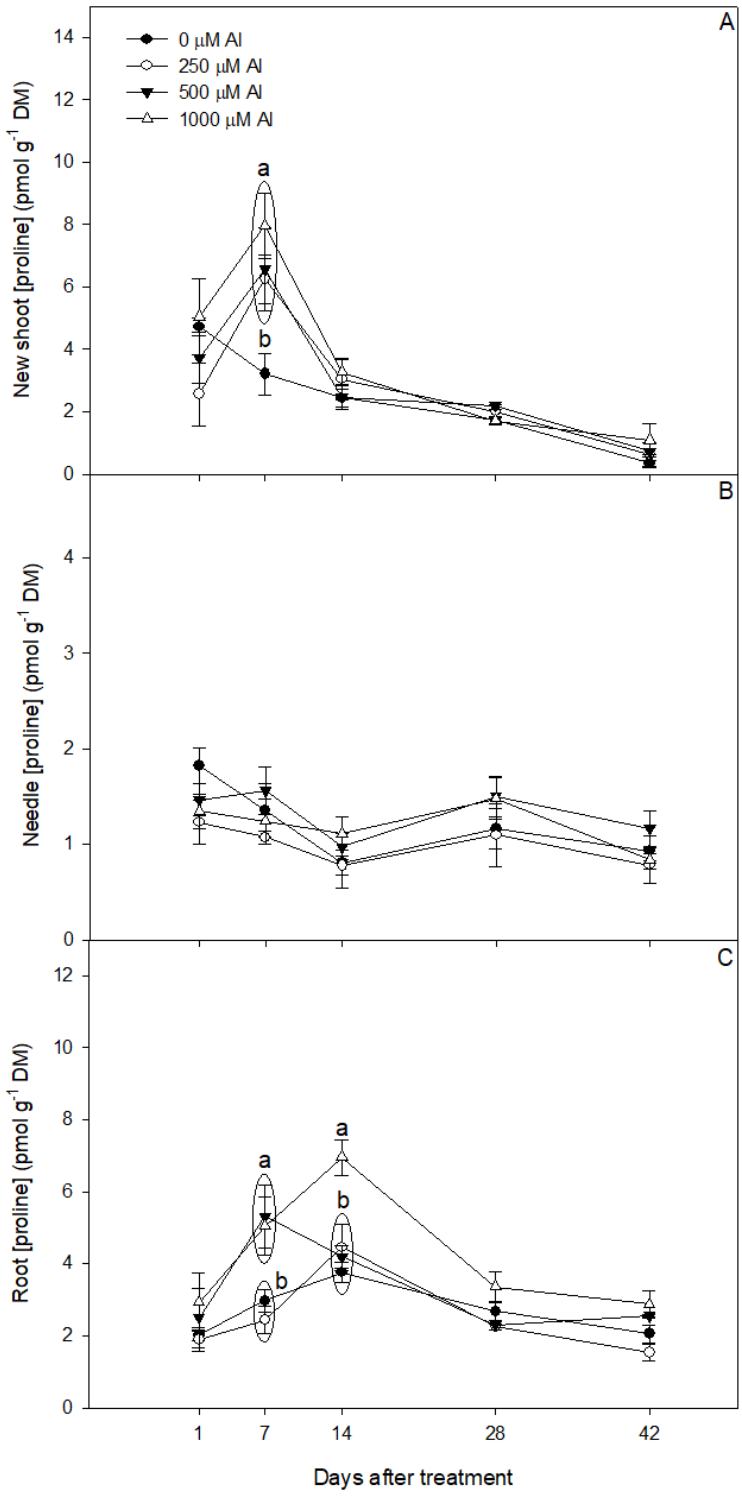

Fig. 3 Mean values (n = 4) of new shoot (A), needle (B), and root (C) proline concentration of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 µM Al for 1, 7, 14, 28, and 42 days. For each evaluation date, absence of letters or different letters between treatments indicate a non-significant or a significant difference, respectively, by Tukey test ($P < 0.05$). Ellipses show mean values with the same letter. Bars are s.e.

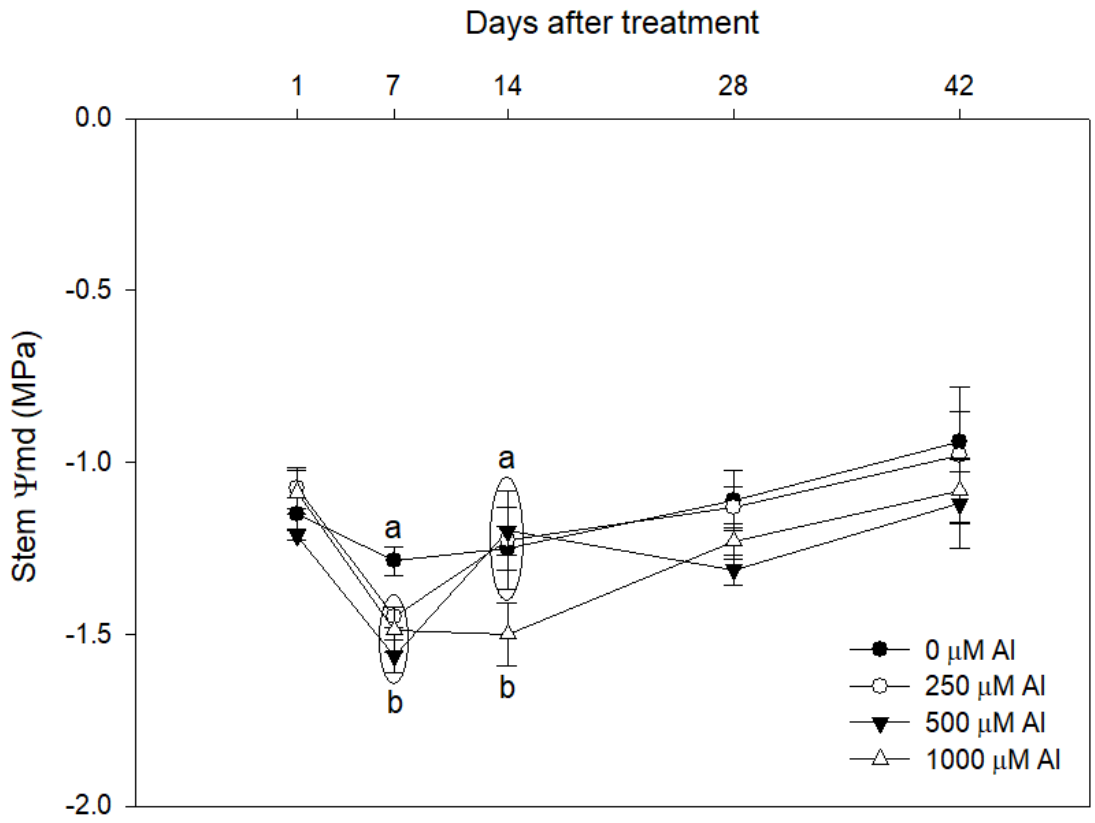

Fig. 4 Mean values (n = 4) of midday stem water potential (Ψmd) of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 μM Al for 1, 7, 14, 28, and 42 days. For each evaluation date, absence of letters or different letters between treatments indicate a non-significant or a significant difference, respectively, by Tukey test ($P < 0.05$). Ellipses show mean values with the same letter. Bars are s.e.

Supplementary material:

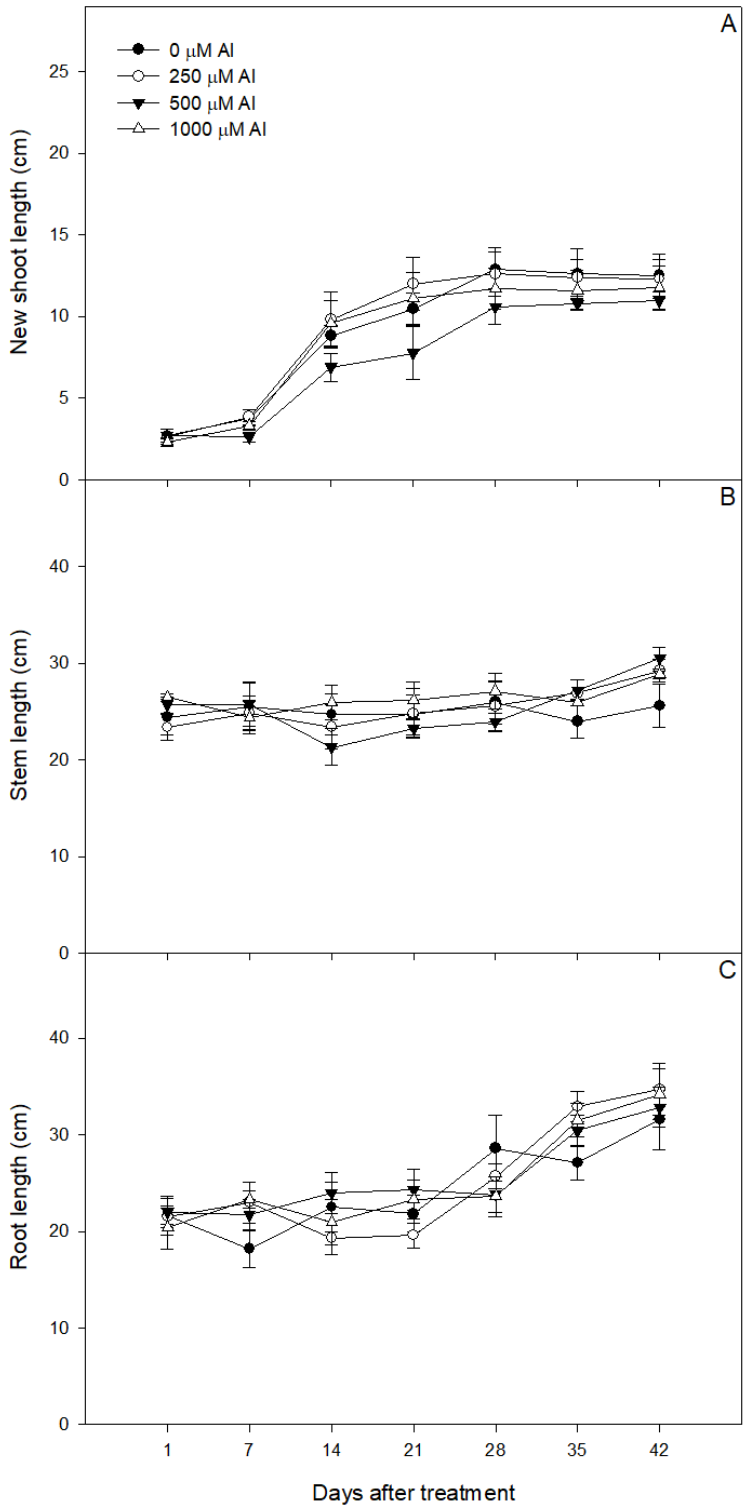

Fig. S1 Mean values (n = 4) of new shoot (A), stem (B), and root (C) length of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 μM Al for 1, 7, 14, 21, 28, 35, and 42 days. For each evaluation date, absence of letters indicates no significant difference by Tukey test ($P < 0.05$) between treatments. Bars are s.e.

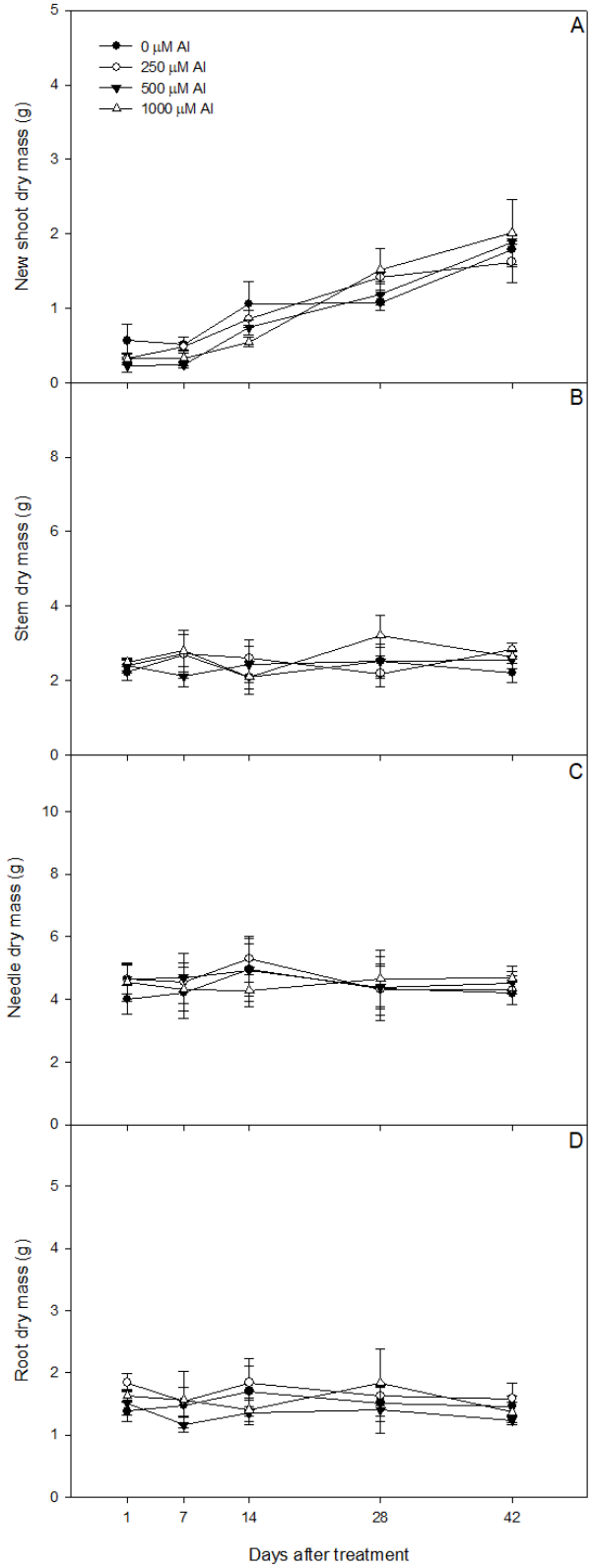

Fig. S2 Mean values (n = 4) of new shoot (A), stem (B), needles (C), and root (D) dry mass of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 µM Al for 1, 7, 14, 28, and 42 days. For each evaluation date, absence of letters indicates no significant difference by Tukey test ($P < 0.05$) between treatments. Bars are s.e.

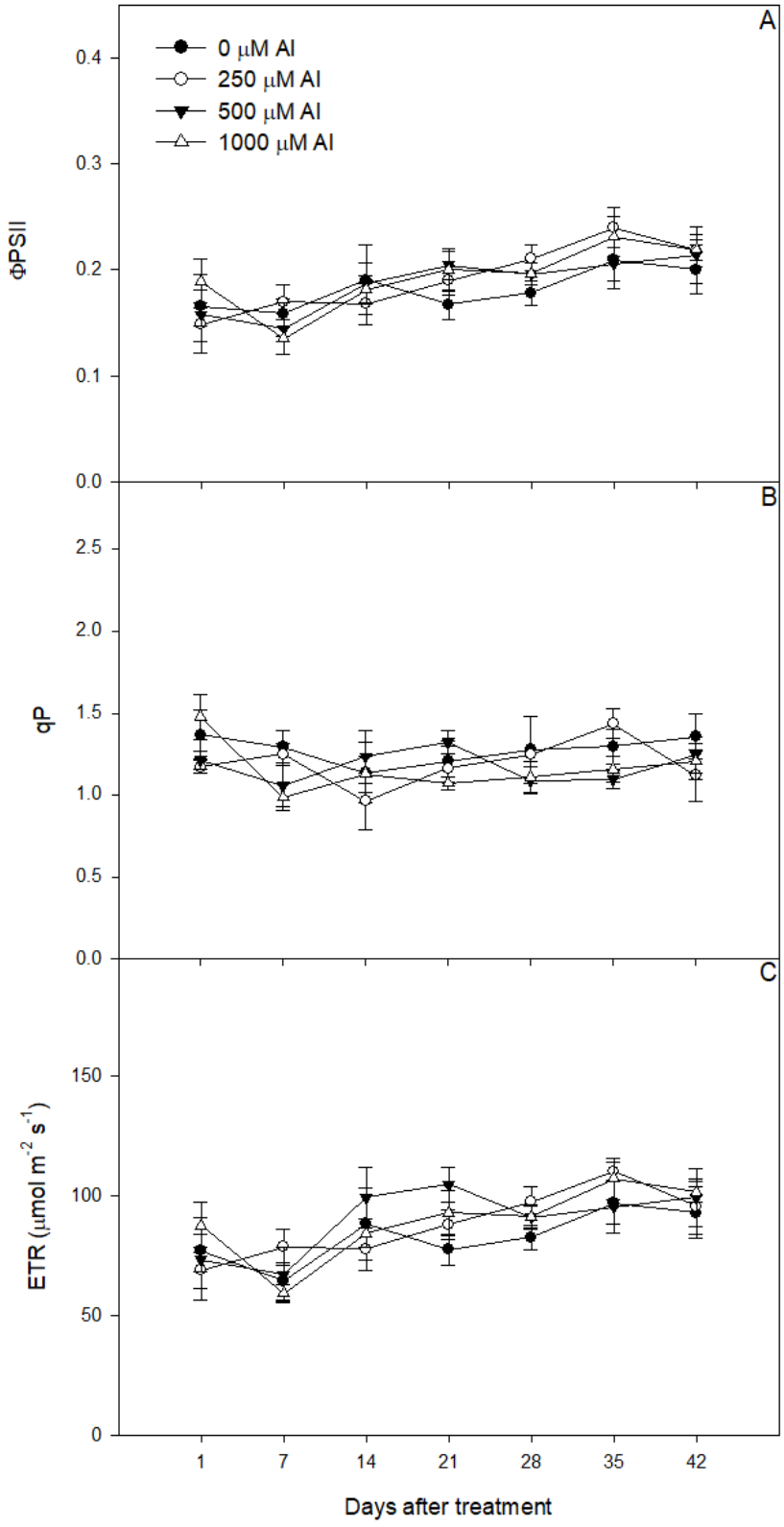

Fig. S3 Mean values (n = 4) of effective quantum yield of photosystem II (PSII) (ϕPSII) (A), photochemical quenching (qP) (B), and electron transport rate (ETR) (C) of needles of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 µM Al for 1, 7, 14, 21, 28, 35, and 42 days. For each evaluation date, absence of letters indicates no significant difference by Tukey test ($P < 0.05$) between treatments. Bars are s.e.

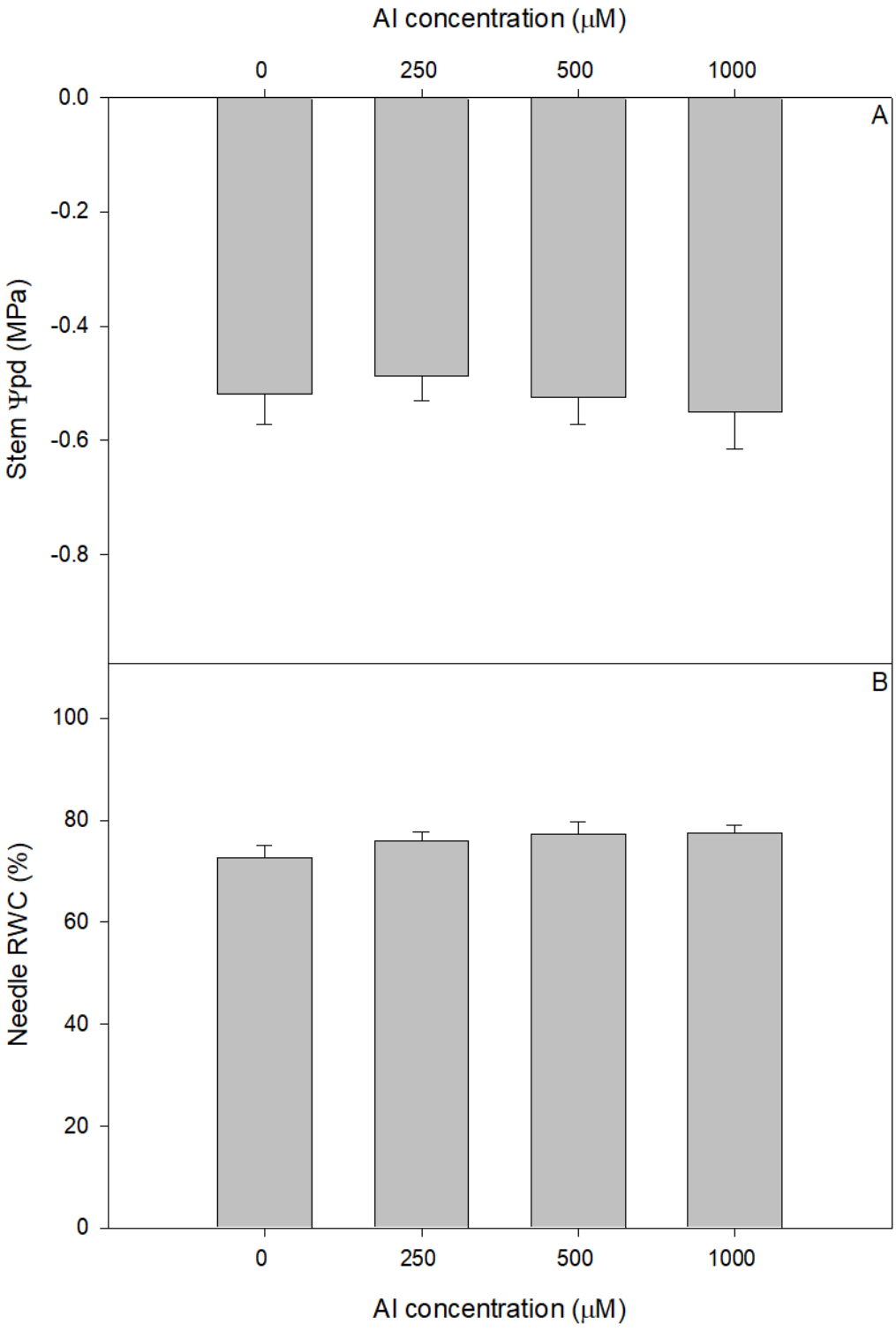

Fig. S4 Mean values (n = 4) of stem predawn water potential (Ψpd) (A) and needle relative water content (RWC) (B) of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 μM Al for 42 days. Absence of letters indicates no significant difference by Tukey test ($P < 0.05$) between treatments. Bars are s.e.

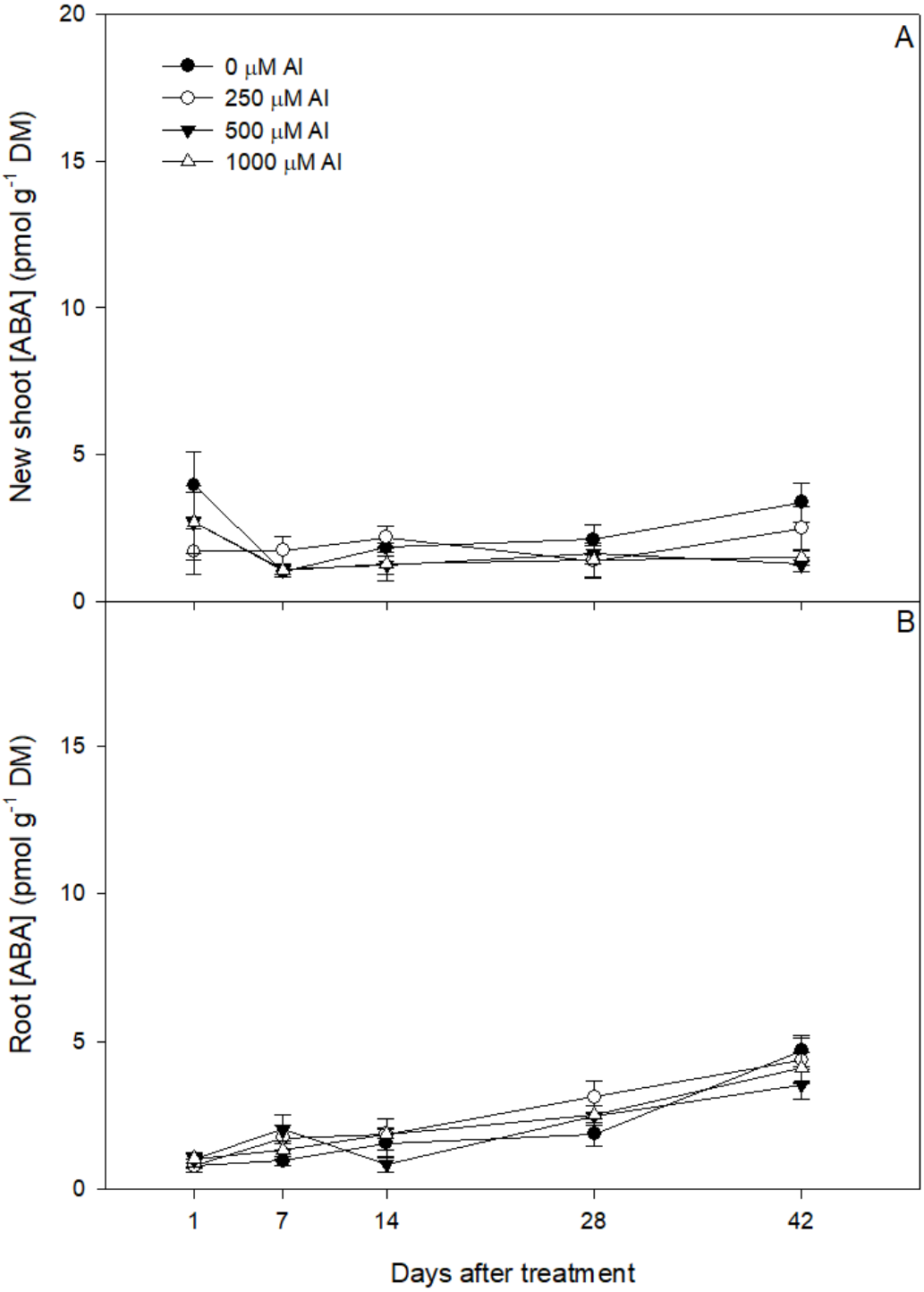

Fig. S5 Mean values (n = 4) of new shoot (A) and root (B) abscisic acid (ABA) concentration of *Pinus sylvestris* exposed to 0, 250, 500, and 1000 μM Al for 1, 7, 14, 28, and 42 days. For each evaluation date, absence of letters indicates no significant difference by Tukey test ($P < 0.05$) between treatments. ABA concentration in the needles was not showed because the values were below the detection limit for all treatments. This was likely due to high concentration of organic substances (such as resin acids) in *P. sylvestris'* needles. As the method requires the relative optical density as an inversely proportional value to calculate ABA concentration, it might have interfered with the high optical density of needles' resins. Bars are s.e.

CAPÍTULO III:

Is aluminum-induced plant growth of *Camellia sinensis* associated with root auxin accumulation?

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Abstract

Some plants accumulate aluminum (Al) in their organs without showing toxicity symptoms, and others may benefit from Al or even show Al dependence, and the reasons are still unclear. Auxins (and ethylene) are plant hormones known to regulate the root growth, and the association between Al and these hormones is not explored in Al-accumulating and -dependent species, like *Camellia sinensis* (Theaceae). Here, we grew *C. sinensis* in nutrient solution with 0, 500, 1000 and 2000 μM Al for 90 days, and quantified root indole-acetic acid (IAA), measured biometric data and biomass of organs, root surface area, and relative leaf water content (RWC) over time. We tested whether root IAA accumulation is associated with Al-induced increased root growth expected for *C. sinensis*. Plants exposed to 500 μM Al showed the highest root growth when compared to those exposed to higher Al concentrations, and plants grown without Al shed their leaves and showed inhibited root growth. From 60 days, more root IAA was found in plants treated with 500 μM Al when compared to the other treatments, which was followed by increased root growth. The higher root growth observed in plants exposed to 500 μM Al was likely due to ideal IAA concentration; more and longer roots resulted in higher root surface area, which led to higher RWC from 30 days in these plants. We demonstrate, as a novelty, that better root growth of *C. sinensis* induced by Al in a long-term study is mediated by IAA, which eventually improves shoot growth, supported by adequate plant water status.

Key words: Al-accumulating, aluminum dependence, indole-acetic acid, tea, theaceae.

Introduction

Aluminum (Al) is toxic to most plant species, causing inhibition of root growth (Kopittke et al. 2008; Horst et al. 2010; Silva et al. 2019). Nevertheless, some plants accumulate Al in their organs without showing toxicity symptoms. These are called Al-accumulating species, classified as such when accumulate at least 1000 mg Al kg^{-1} dry leaves (Chenery 1948; Jansen et al. 2002). Despite being few species from few families (Melastomataceae; Rubiaceae; Symplocaceae; Vochysiaceae; Theaceae) (Matsumoto et al. 1976; Haridasan et al. 1982; Jansen et al. 2002; Brunner and Sperisen 2013; Souza et al. 2015; Malta et al. 2016), they grow well on acidic soils (pH < 5.0) with Al saturation (m%) higher than 50% (Brunner and Sperisen 2013). Therefore, these plants benefit from mechanisms that allow them to grow despite acidic soils with high m%.

Aluminum accumulation in these plants is feasible due to internal tolerance mechanisms, mainly chelation of Al with organic acids (OA) and phenolic compounds, and Al sequestration into metabolically less sensitive compartments (e.g., vacuoles) (Brunner and Sperisen 2013; Hajiboland et al. 2022). Despite isolating Al from their metabolism, Al-accumulating species may benefit from Al to grow (Haridasan et al. 1988; Souza et al. 2017; Schmitt et al. 2016) or may be even dependent on Al, succumbing to death in its absence (Sun et al. 2020; Bressan et al. 2021). Thus, Al is a growth factor for these plants, and the reasons are under debate in the literature.

In *Vochysia tucanorum* (Vochysiaceae), an Al-dependent and -accumulating plant from the Brazilian savanna, the absence of Al causes brown and necrotic roots, compromising its function, especially water acquisition for leaf hydration (Bressan et al. 2021). In *Camellia sinensis* (Theaceae), also an Al-dependent and -accumulating species, Al increases plant growth, and it is suggested that this metal maintains DNA integrity in root meristematic cells

(Sun et al. 2020). Thus, Al is critical for root growth of Al-dependent species, and chemical signals mediating this process might be involved.

In plants in general, plant hormones auxins and ethylene (Et) are known to regulate the root growth (Kang et al. 1971; Swarup et al. 2007). In plants sensitive to Al, however, Al was evidenced to induce Et production in the roots, which alters the symmetric distribution of the auxin indole-acetic acid (IAA), leading to the arrest of root growth (Sun et al. 2010). In Al-accumulating species, studies testing the association between Al and plant hormones to explain the Al-induced root growth enhancement receives less attention. It was evidenced that auxins improve root growth in *C. sinensis* (Wei et al. 2019), although Al effects was not tested in this study. A previous short-term study (up to seven days) with *C. sinensis* seedlings showed that Al-induced root growth could be related to auxin accumulation in root tips (Gao et al. 2022). Besides this only observation, IAA and Al-induced root growth is not explored in entire fully developed plants under long-term experiments.

Here we grew *C. sinensis* plants in nutrient solution with increasing Al concentrations for 90 days. We measured root IAA concentration, biometric data and biomass of organs, root surface area, and relative leaf water content throughout the experiment. We tested whether root IAA accumulation over time is associated with Al-induced increased root growth expected for *C. sinensis*.

Material and methods

Plant material and experimental conditions

Tea (*Camellia sinensis* L. (Kuntz)) (Theaceae) seedlings with 20 ± 3 cm in height obtained from cutting were purchased from a commercial nursery (Viveiro Santa Maria, Pariquera-Açu, SP, Brazil). The root system was rinsed with deionized water to remove the remaining substrate. Then, the plants were transferred to a hydroponic system (opaque plastic

boxes — 50 cm × 30 cm × 15 cm; 20 L) where they grew directly in an aerated nutrient solution containing 0, 500, 1000 e 2000 μM Al.

Expanded polystyrene (Isopor®) 50 × 30 x 2 cm plates, with five holes (3 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in the holes with polyurethane foam strips placed around the plant collar. The boxes were kept on benches, inside a greenhouse, under semi-controlled conditions. The air temperature, photosynthetic photon flux density (PPFD), and relative humidity inside the greenhouse was 25 ± 3 °C, 753.5 ± 118.7 μmol photons m^{-2} s^{-1}, and 65.8 ± 2.5 %, between 9:00 and 12:00 h, with a photoperiod of approximately 13 h provided by natural sunlight.

The nutrient solution was based on Clark solution (Clark, 1975) and was used previously in studies with other Al-accumulating species (Banhos et al. 2016. Bittencourt et al. 2020; Bressan et al. 2020; 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 $NaMoO_2$. The Al treatments were provided through $AlCl_3$, and the solution pH was kept at 4.0 ± 0.1 to maintain the Al as soluble as possible. Nominal 500, 1000, and 2000 μM Al resulted in 398.5 ± 21, 855.5 ± 30, and 1648.1 ± 44 μM Al, respectively. The solution with 0 μM Al contained only trace Al concentration. The nutrient solutions were completely replaced every 15 days.

Experimental Design

Eighty plants were distributed in 16 boxes (5 plants per box), consisting of one box per treatment per evaluation date (four dates). At 15, 30, 60, and 90 days after treatment, root tips (0.5 cm in length) were collected to measure indole-acetic acid (IAA) concentration. At the same dates, Al concentration and biomass of organs (root, stem, and leaves), and relative leaf

water content (RWC) were measured. At 0, 7, 15, 30, 45, 75, and 90 days, five plants per treatment were used to measure biometric data (root and stem length, and number of leaves). Before treatments were stablished (day 0), five extra plants were used to assess the above-mentioned parameters.

Indole-acetic acid quantification

Root tips (0.5 cm in length) were washed in deionized water and stored at −80 °C prior to analysis. Samples (500 µg fresh mass) were extracted using isopropanol:acetic acid (95:5; v:v) solution at 4 °C. A solution containing 0.5 µg of a labelled standard [13C6]-IAA (Cambridge Isotopes, Inc.) was added as internal standard (IS). After 2 h, the solvent was separated by centrifugation at 14,000 × g at 4 °C for 10 min. The supernatant was concentrated to 50 µL, acidified to pH 2.5, and IAA was extracted three times with ethyl acetate, according to Rawlinson et al. (2015). After purification, the solution was evaporated to dryness and re-suspended in 30 µL ethyl acetate for methylation with diazomethane.

Quantification of free IAA was performed using gas chromatography coupled with mass spectrometry (Hewlett Packard 6890 gas chromatograph coupled to an HP5973 mass selective detector) (GC-MS). The GC column was HP-1701 (30 m, 0.25 mm I.D., 0.5 µm film thickness), with helium as the carrier gas at flow rate of 1 mL min^{-1}. The temperature program for analysis was 140 °C for 1 min, followed by increase of 5 °C min^{-1} up to 210 °C, 1 min hold, increase to 280 °C at 20 °C min^{-1}, then, 1 min hold 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 IS. The ratio 130:136 was used to calculate the endogenous amount of IAA, adapted according to Chen et al. (1988). Values were expressed as ng IAA g^{-1} fresh mass.

Relative leaf water content

RWC (%) was measured between 13:00 h and 15:00 h. Disks (1 cm in diameter) were collected and had the fresh mass (FM) immediately measured on a scale. Then, the disks were hydrated in 100 mL deionized water inside amber flasks (to avoid photosynthetic activity) for 24 h 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 following equation.

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

Biometric data and biomass

The leaves were counted, and the stem length and main root length measured with a ruler (cm). The root surface area (cm^2) was measured using a scanner (Epson perfection v700 photo, Suwa, Japan) coupled to a computer running the WinRHIZOTM software (Regent Instruments, Canada). Plant samples were separated into leaf, stem (plus petiole), and root, and washed three times with deionized water. Then, samples were placed in paper bags and oven-dried at 60 ºC until constant mass. The biomass of organs (g) was measured using a precision scale.

Al concentration in plant organs

After measuring the biomass, the samples were sent to the plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil), where they were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). The concentration of Al was assessed by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

Data analyses

All the parameters were measured using five replicates (plants). For each evaluated parameter, one-way analysis of variance (One-Way ANOVA) was performed, and mean values were compared between treatments by Tukey test ($P < 0.05$) on each evaluation date.

Results

After 90 days, plants exposed to Al showed new white lateral roots, and the degree of root branching decreased with the increase of Al concentration in the nutrient solution (Fig. 1B, C, D). In contrast, plants grown without Al showed necrotic and brown roots (Fig. 1A).

Plants exposed to 1000 and 2000 μM Al increased root IAA concentration in relation to those exposed to 0 and 500 μM Al, at 15 days (Fig. 2). From 30 days, plants exposed to 0, 1000 and 2000 μM Al showed similar values until the end of the study (Fig. 2). Plants exposed to 500 μM Al showed higher root IAA concentration in relation to the other treatments, at 60 and 90 days (Fig. 2). Therefore, the two highest Al concentrations induced IAA peak on a short-term basis, while the lowest Al concentration increased root IAA on a long-term basis.

Stem length (Fig. 3B), and stem dry mass (Fig. 4B) was the same between treatments throughout the study. Nevertheless, plants exposed to 500 μM Al showed higher number of leaves (Fig. 3A) and leaf dry mass (Fig. 4A) in relation to the other treatments, from 60 days. Similarly, their roots were longer (Fig. 3C) and exhibited higher dry mass (Fig. 4C), at 90 days. Root surface area of plants grown without Al was maintained low throughout the study, and this parameter in those exposed to 500 μM Al was higher than plants exposed to 0 μM Al (at 60 days) and higher than all treatments at 90 days (Fig. 5). Thus, plants exposed to 500 μM released more leaves and produced more and longer roots with higher root surface area.

Relative leaf water content (RWC) was higher in plants exposed to 500 μM Al in relation to the other treatments from 30 days (Fig.6). Plants exposed to 1000 μM Al showed

higher RWC in relation to those exposed to 0 and 2000 μM Al, but lower than 500 μM Al, at 90 days. Thus, plants exposed to 500 μM Al showed higher leaf hydration than the other treatments throughout the study.

As expected, plants exposed to 2000 μM Al accumulated more Al in their leaves (Supplementary material; Fig. S1A) and roots (Supplementary material; Fig. S1C) in relation to the other treatments, from 60 days. Stems of plants exposed to 1000 and 2000 μM Al accumulated more Al in relation to the other treatments, from 60 days (Supplementary material; Fig. S1B). Thus, the more Al in nutrient solution, more Al accumulation, mainly in leaves and roots.

Discussion

In the present study, plants were exposed to increasing Al concentrations, and 500 μM Al induced the highest root growth, which was associated with the highest root IAA concentration from 60 days. As a novelty, we demonstrate, in a long-term study (90 days), that better root growth of *C. sinensis* induced by Al is mediated by IAA.

Camellia sinensis is an Al-accumulating species (Matsumoto et al. 1976; Ruan and Wong 2004), and it is known to grow better in the presence of Al (Konishi et al. 1985; Hajiboland et al. 2013; Sun et al. 2020). In the present study, plants were exposed to increasing Al concentrations, and a moderate value (500 μM Al) induced the highest root growth (Fig. 1B, 3C) and more leaves (Fig. 3A). Increasing Al concentration were already tested in *C. sinensis* (Hajiboland et al. 2013 — 0, 50, 100, and 300 μM Al; Sun et al. 2020 — 0, 400, 1000, and 2000 μM Al), and moderate values (300 and 400 μM Al, respectively) were reported as ideal for plant growth and development, corroborating our results. These and other studies (Konishi et al. 1985; Fung et al. 2008) point out that absence of Al stunts plant growth. In the present study, plants grown without Al shed their leaves (Fig. 3A), showed inhibited root growth along

the study (Fig. 3C, 4C) and, after 90 days, brown and necrotic roots became apparent (Fig. 1A). Other Al-accumulating species, such as *Vochysia tucanorum* (Vochysiaceae) also succumbs to the lack of Al, showing compromised root integrity (Bressan et al. 2021). Therefore, root growth of Al-accumulating plants seems to benefit from Al, and metabolic root signals could be involved in these benefits.

In the present study, we investigate whether root accumulation of indole-acetic acid (IAA) would be associated with Al-induced root growth of *C. sinensis* in a long-term experiment. Plants treated with 500 μM Al showed more root IAA accumulation when compared to those treated with 0, 1000, and 2000 μM Al from 60 days (Fig. 2), which was followed by increased root growth (Fig. 3C, 4C). IAA has a systemic effect in plants. IAA concentration promoting sprout and growth of shoot apical meristem is higher than IAA concentration promoting bud growth, which in turn is higher than IAA concentration promoting root growth (Scott 1972; Reinhardt et al. 2003). In the present study, we measured IAA only in roots, meaning that our results show no relationship with IAA in shoots. Thus, 500 μM Al seems to induce sufficient IAA concentration for root growth of *C. sinensis* — approximately 40 ng IAA g^{-1} root fresh mass (Fig. 2). A short-term study (up to 7 days) also showed that 40 ng IAA g^{-1} root tips of *C. sinensis* seedlings exposed to 400 μM Al promoted root growth (Gao et al. 2022), corroborating our results. In contrast, plants exposed to 0, 1000, and 2000 μM Al showed 20 ng IAA g^{-1} root (Fig. 2) and lower root growth in relation to those exposed to 500 μM Al (Fig. 3C, 4C). Therefore, 40 ng IAA g^{-1} root seems to be ideal to induce better root growth in the presence of Al. Despite measuring genes associated with IAA accumulation (YUC8 and AMI1) and transport (PIN2 and PIN5) in roots of *C. sinensis*, Gao et al. (2022) was not able to demonstrate '*how*' Al mediates IAA action in root tips of this species, and this mediation remains to be revealed.

In plants sensitive to Al, in contrast, this metal inhibits root growth (Vitorello et al. 2005; Horst et al. 2010; Singh et al. 2017). It was demonstrated that Al toxicity induces ethylene production in root tips of *Arabidopsis*, which acts as a signal to change IAA distribution through polar IAA transport genes, leading to stunted root growth (Sun et al. 2010). IAA concentration above ideal for root growth was evidenced in root tips of *Citrus limonia* under Al toxicity leading to the inhibition of root growth (Silva et al. 2019). In contrast to inhibition of root growth, in the case of Al-accumulating species, like *C. sinensis*, there are no studies demonstrating how Al could enhance root growth beyond IAA involvement. Thus, the association between Al, IAA, and root growth remains to be revealed in Al-accumulating species.

Although the mediation of IAA by Al is not clear in *C. sinensis*, the higher root growth observed in plants exposed to 500 μM Al was likely due to ideal IAA concentration. More and longer roots (Fig. 3C, 4C) resulted in higher root surface area (Fig. 5), which led to higher relative leaf water content (RWC) from 30 days (Fig. 6). Thus, Al ends up enhancing leaf hydration in *C. sinensis*. Similarly, in the Al-accumulator *V. tucanorum*, Al increased root growth with high stomatal conductance (*gs*), indicating sufficient leaf hydration (Bressan et al. 2021). In contrast, in the Al-sensitive *C. limonia*, Al caused low root surface area associated with lower RWC, *gs* and leaf water potential (Ψw) (Silva et al. 2022). Leaf hydration is essential for plant growth, as it depends on cell division and expansion in the shoot apical meristem, and cell expansion is compromised under water shortage. Indeed, the number of leaves, which represents the functioning of the shoot apical meristem, was higher in plants exposed to 500 μM Al from 60 days (Fig. 3A). Therefore, in *C. sinensis*, Al improves root growth, which is mediated by IAA, and eventually improves shoot growth, supported by adequate plant water status.

Conclusion

Here we confirmed that Al improves plant growth in the Al-accumulator *C. sinensis*, and 500 μM Al could induce the highest root development. Auxin (IAA) highly explains the better root performance, evidenced in a long-term study. Although the mediation of root growth by Al through IAA is unclear in Al-accumulating plants, the Al-induced root growth guarantees adequate plant water status and, eventually, plant growth.

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Figures

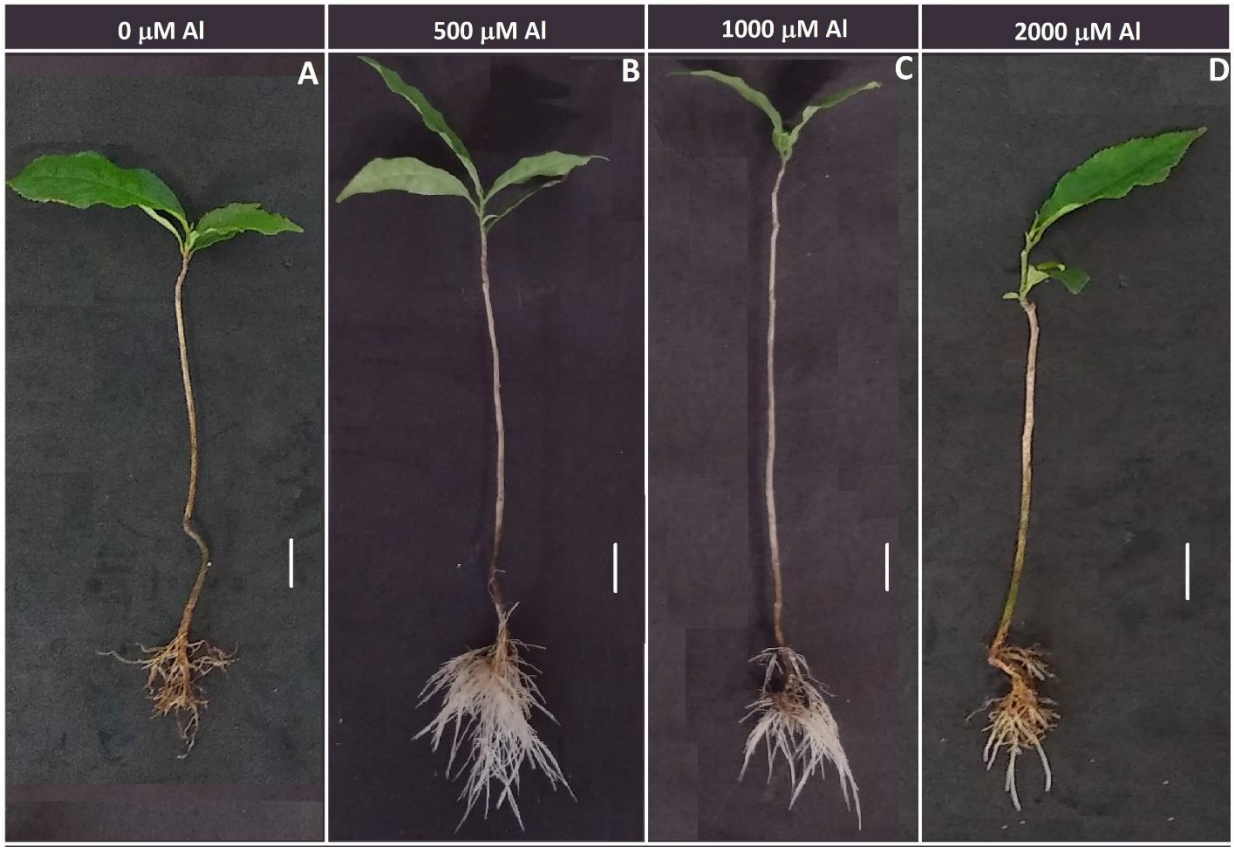

Fig 1. Morphological details of *Camellia sinensis* exposed to 0 (A), 500 (B), 1000 (C), and 2000 (D) μM Al in nutrient solutions after 90 days. Bars represent 3 cm.

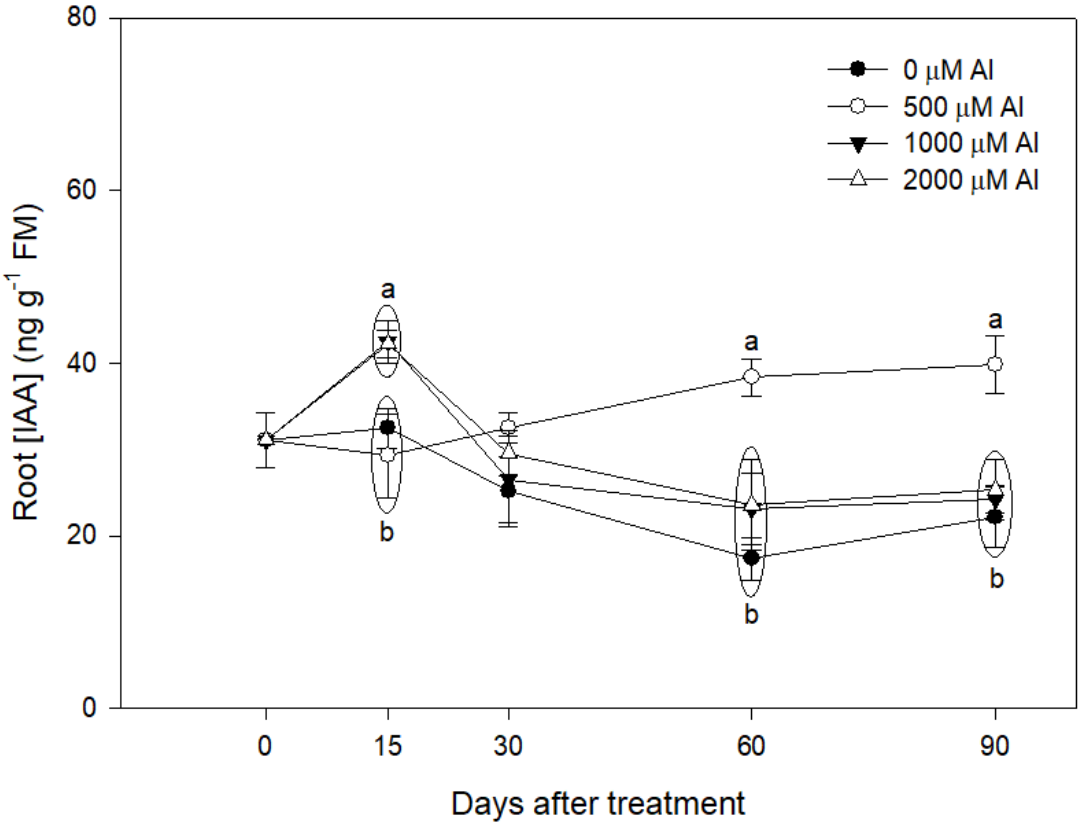

Fig 2. Mean values (n = 5) of indole-acetic acid (IAA) concentration in root tips of *Camellia* sinensis exposed to 0, 500, 1000, and 2000 µM Al at 0, 15, 30 and 60 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letters. Bars are s.e.

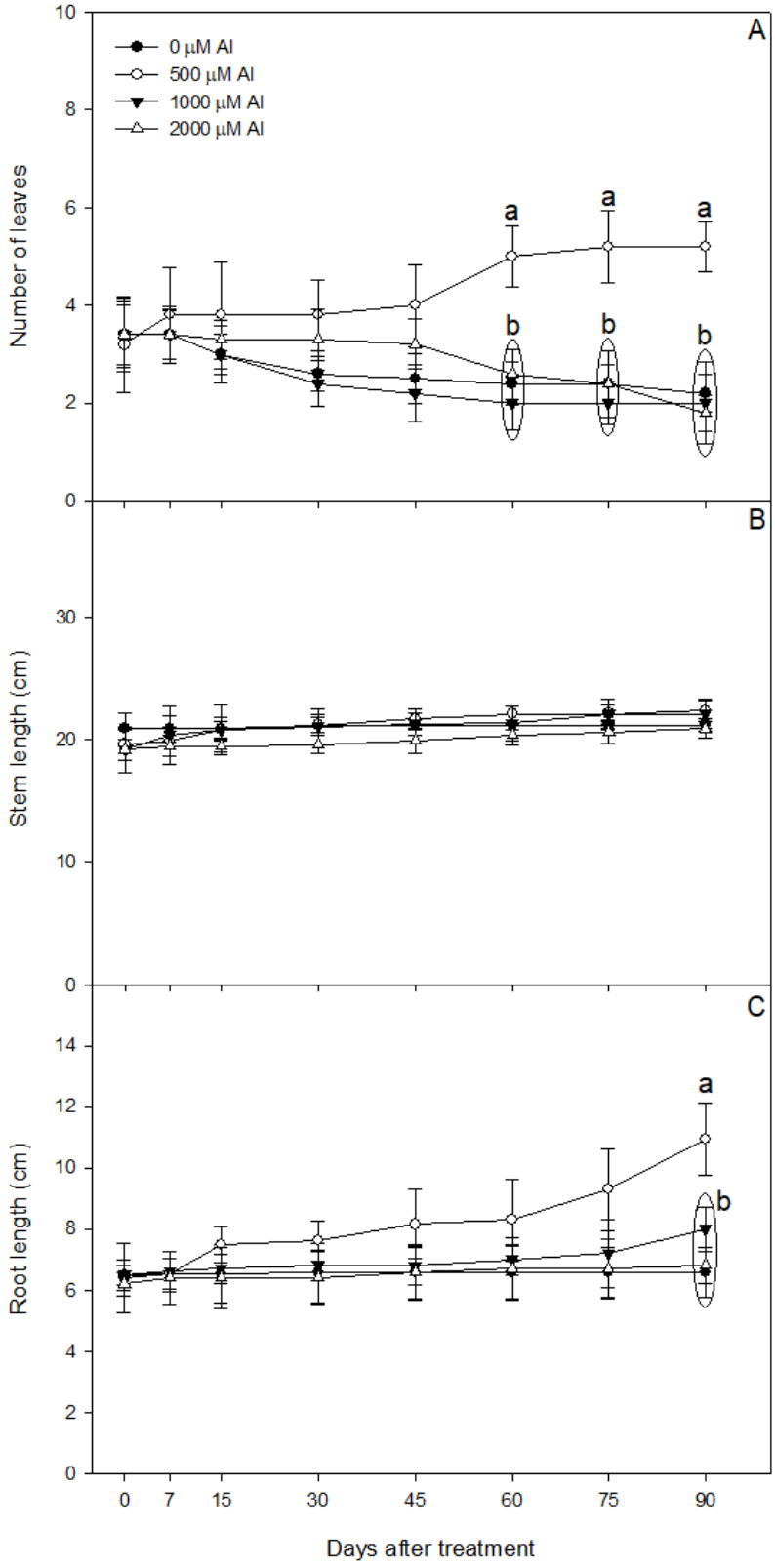

Fig 3. Mean values (n = 5) of number of leaves (A), stem length (B), and root length (C) of *Camellia sinensis* exposed to 0, 500, 1000, and 2000 µM Al in nutrient solution at 0, 7, 15, 30, 45, 60, 75, and 90 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letters. Bars are s.e.

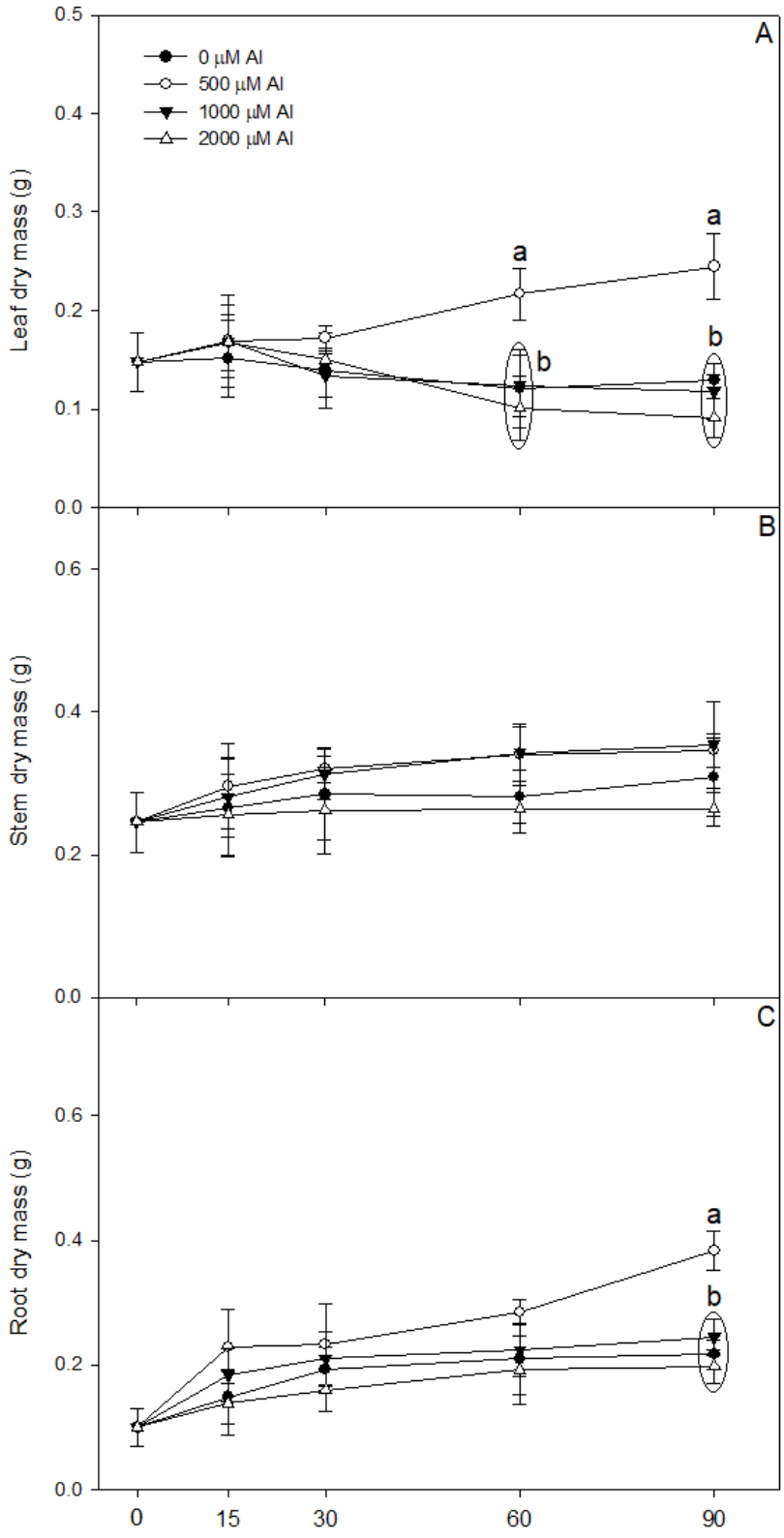

Fig 4. Mean values (n = 5) of leaf dry mass (A), stem dry mass (B), and root dry mass (C) of *Camellia sinensis* exposed to 0, 500, 1000, and 2000 µM Al in nutrient solution at 0, 15, 30, 60, and 90 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letters. Bars are s.e.

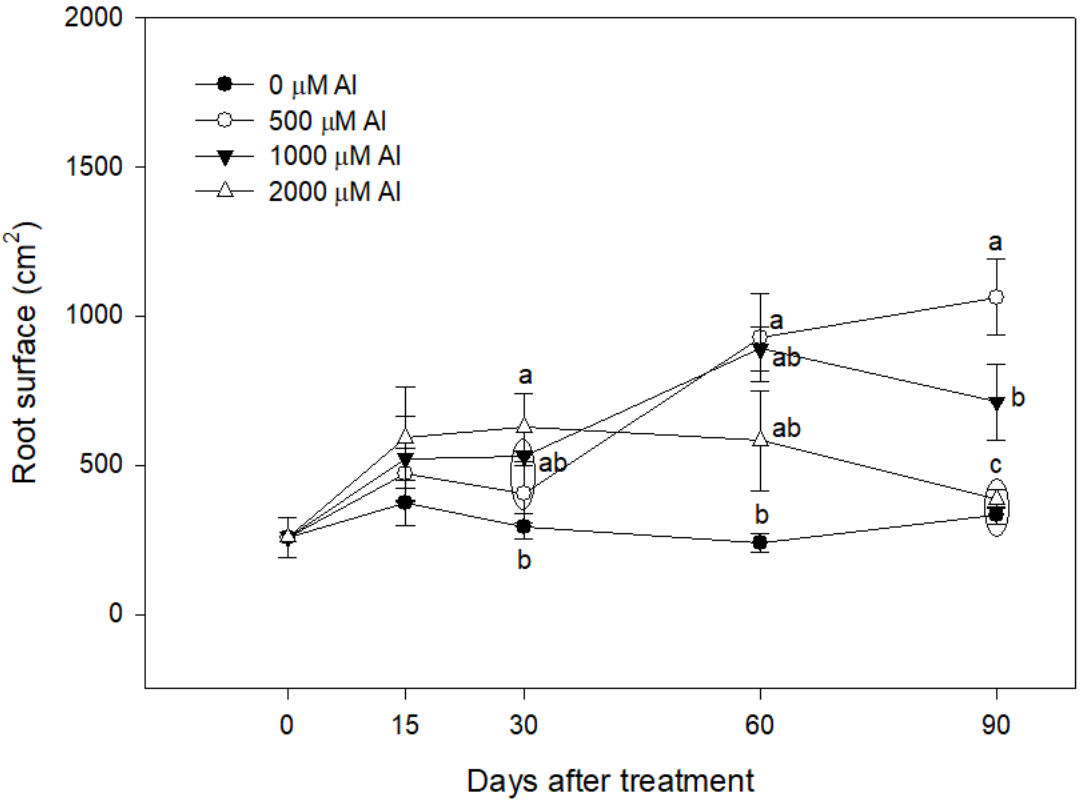

Fig 5. Mean values (n = 5) of root surface (cm²) of *Camellia sinensis* exposed to 0, 500, 1000, and 2000 µM Al in nutrient solution at 0, 15, 30, 60, and 90 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letters. Bars are s.e.

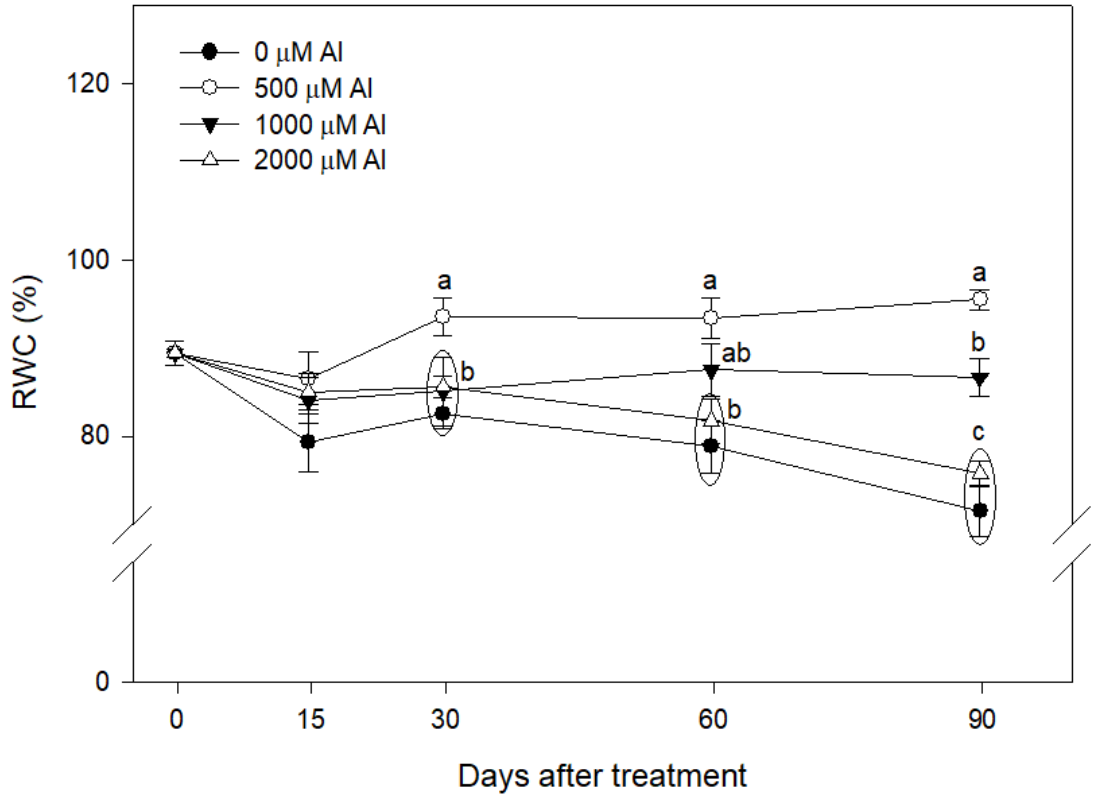

Fig 6. Mean values (n = 5) of relative water content (RWC) in leaves of *Camellia sinensis* exposed to 0, 500, 1000, and 2000 μM Al in nutrient solution at 0, 15, 30, 60, and 90 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letter. Bars are s.e.

Supplementary material

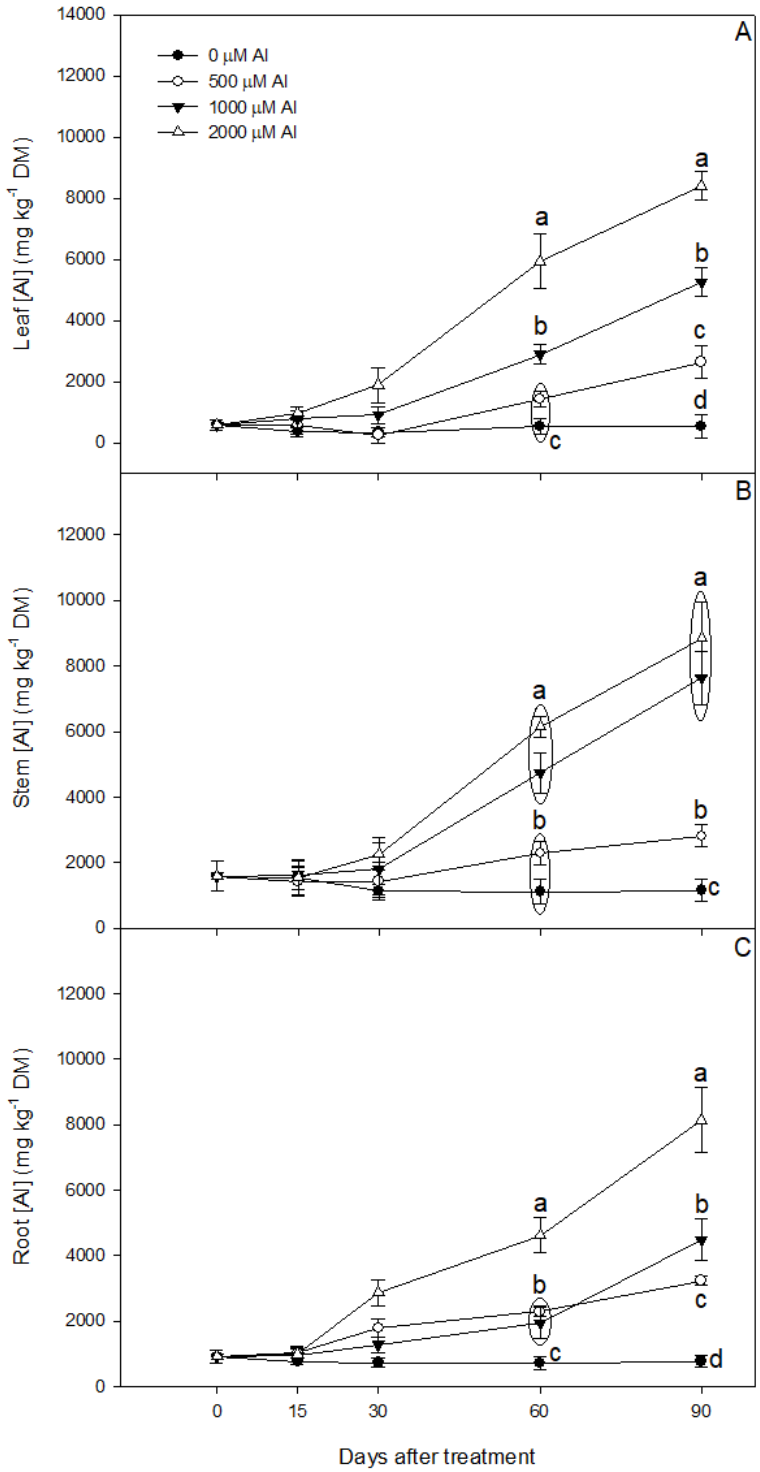

Fig S1. Mean values (n = 5) of Al concentration in leaves (A), stem (B), and roots (C) of *Camellia sinensis* exposed to 0, 500, 1000, and 2000 µM Al in nutrient solution at 0, 15, 30, 60, and 90 days after treatment. For each evaluation date, distinct or absence of letters between treatments indicate, respectively, significant or no difference by Tukey test ($P < 0.05$). Ellipses contain mean values with same letters. Bars are s.e.

CONSIDERAÇÕES FINAIS

Evidenciamos que o Al causa danos à performance fotossintética quando aplicado diretamente nas folhas de plantas sensíveis, indicando que os efeitos de toxicidade ao Al nessas plantas podem ocorrer mesmo quando as raízes não são expostas a esse elemento. Essa conclusão se opõe à sugestão, até então nunca testada, de que os efeitos da toxicidade ao Al observados na parte aérea de plantas sensíveis são apenas consequência dos efeitos no sistema radicular.

Além disso, mostramos que o acúmulo de prolina pode estar envolvido na manutenção da hidratação foliar de plantas tolerantes ao Al. O papel da prolina como osmoprotetor já havia sido sugerido para plantas sob estresse por seca e salinidade, mas nunca havia sido testado em plantas tolerantes expostas ao Al.

Por fim, evidenciamos que o crescimento de raízes induzido pelo Al em plantas acumuladoras é intermediado pelo acúmulo de auxina ao longo de três meses. Embora a associação entre o acúmulo de auxina e o crescimento de raízes induzido pelo Al tenha sido proposta por um estudo anterior, essa única investigação foi feita com plântulas (2 cm de altura) ao longo de apenas sete dias.

Como confirmado no presente estudo, o Al apresenta uma conjuntura de efeitos completamente diferente em espécies sensíveis, tolerantes e acumuladoras. A presente Tese mostra avanços científicos nas três frentes de investigação e, como ciência de base, poderá compor o conhecimento necessário para avanços futuros no melhoramento genético por meio de ferramentas biotecnológicas que visam implementar os mecanismos observados em plantas tolerantes e acumuladoras de Al nas plantas sensíveis que são comercialmente importantes.