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

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

PAPEL DO ABA NAS RELAÇÕES HÍDRICAS EM ESPÉCIES SENSÍVEIS AO AL^{3+}

MARINA ALVES GAVASSI

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: PAPEL DO ABA NAS RELAÇÕES HIDRICAS EM METABOLISMOS CONTRASTANTES À SENSIBILIDADE AO AL^{3+}

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Try again. Fail again. Fail better.

Samuel Beckett

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SUMÁRIO

Resumo

O alumínio (Al) é o metal mais abundante na crosta terrestre. Em solos ácidos ($pH < 5,0$) é encontrado principalmente na forma de Al^{3+}, tóxico à maioria das plantas. Nas plantas sensíveis a esse metal, o primeiro e mais conspícuo sintoma de toxicidade é a inibição do crescimento das raízes, onde a maior parte do Al é covalentemente retido. Além disso, o Al também causa sintomas indiretos, reduzindo o crescimento da parte aérea. Tal redução tem sido associada à assimilação de CO_2 (A), e evidências sugerem o comprometimento das reações fotoquímicas, além da redução de 30 a 80% da condutância estomática (gs). Além disso, o Al também tem sido associado à redução na hidratação do mesofilo, como ocorre em condições de limitação hídrica. Baseado nestes fatos, testamos a hipótese de que parte dos sintomas de fitotoxicidade ao Al em plantas sensíveis, sobretudo aqueles relacionados ao baixo crescimento e desenvolvimento da parte aérea, bem como menos trocas gasosas, ocorrem em resposta à ativação de mecanismos associados à percepção de deficiência hídrica, principalmente à biossíntese de ácido abscísico (ABA). Utilizamos duas espécies sensíveis ao Al, mas cujos sintomas de toxicidade são induzidos por concentrações significativamente distintas, como *Solanum lycopersicum* (100 µM Al) e *Citrus limonia* (1480 µM Al). As plantas foram cultivadas em solução de nutrientes com e sem Al, em experimentos independentes com duração de 10 e 90 dias, para *S. lycopersicum* e *C. limonia,* respectivamente. Nos experimentos com ambas as espécies, foram avaliados parâmetros biométricos, morfologia do sistema radicular, biomassa, trocas gasosas (principalmente, gs), potencial da água na folha (Ψ_{leaf}) e quantificação de ABA em folhas e raízes. Para *S. lycopersicum*, foram adicionalmente avaliados a condutância hidráulica da raiz (Lp_r), o pH e a concentração de ABA na seiva do xilema. Para *C. limonia*, foram adicionalmente avaliados o conteúdo relativo de água na folha (CRA), a condutividade estimada da raiz até a folha (K_L) e a expressão de genes-chave para a biossíntese de ABA, *NCED1*, *NCED3* e *NCED5* em folhas e raízes. Ambas as espécies apresentaram redução na área foliar em função do menor crescimento radicular, porém, tal redução não foi suficiente para manter a hidratação foliar, evidenciada pela diminuição de Ψ_w, CRA e gs. Tanto K_L quanto Lp_r apresentaram redução significativa, indicando, portanto, prejuízo no transporte de água para a parte aérea. Além disso, em *C. limonia* a expressão de *NCED3* foi induzida em presença de Al em ambos os órgãos, enquanto *NCED1* e *NCED5* foram induzidas nas folhas, onde também foi verificado o maior acúmulo do hormônio. A sinalização feita pelo ABA provavelmente modula o fechamento estomático sob a toxicidade do Al.

Palavras-chave: expressão gênica; trocas gasosas; alumínio; relações hídricas; ácido abscísico

Abstract

Aluminum (Al) is the most abundant metal in the Earth's crust. In acidic soils ($pH < 5.0$) it is found mostly as Al^{3+}, which is toxic to most plants. In Al-sensitive plants, the first and most conspicuous symptom is the inhibition of the growth of the root system, where most of Al is covalently retained. Aluminum also causes indirect symptoms, such as reduced shoot growth. In Al-sensitive plants, Al-reduced CO_2 assimilation rate (A) is observed, and there is evidence suggesting the impairment of photochemical reactions, as well as 30 to 80% reduction in stomatal conductance (gs). In addition, Al has also been associated with a reduction in mesophyll hydration, as occurs under water-limited conditions. Based on these facts, we tested the hypothesis that part of Al symptoms in sensitive plants, especially those related to low shoot growth and development, as well as low gas exchange, occur in response to activation of mechanisms closely associated with the perception of water deficiency, especially as signalled by abscisic acid (ABA) biosynthesis. We used two Al-sensitive species, whose symptoms of toxicity are induced by significantly different concentrations, such as *Solanum lycopersicum* (100 μM Al) and *Citrus limonia* (1480 μM Al). Plants were grown in nutrient solution with and without Al, in independent experiments of 10 and 90 days, for *S. lycopersicum* and *C. limonia*, respectively. In the experiments with both species, biometric parameters, root system morphology, biomass, gas exchange (especially *gs*), leaf water potential (Ψ_{leaf}) and ABA quantification in leaves and roots were evaluated. Additionally, root hydraulic conductance (Lp_r), pH and ABA concentration in xylem sap were evaluated in *S. lycopersicum* plants. For *C. limonia*, the relative leaf water content (CRA), estimated conductivity from roots to the leaf (K_L) and the expression of a key genes for ABA biosynthesis, *NCED1*, *NCED3* and *NCED5* in leaves and roots, were also evaluated. Both species showed reduction in leaf area associated to lower root growth, but this reduction was not enough to maintain leaf hydration, as evidenced by the decrease in Ψ_w, CRA and *gs*. K_L and Lp_r decreased significantly in plants exposed to Al, thus indicating impairment in water transport from roots to the shoots. In *C. limonia*, *NCED3* expression was induced in the presence of Al in both organs, while *NCED1 and NCED5* were induced especially in leaves, where the highest accumulation of ABA was also verified. ABA signaling is likely to modulate stomatal closure under Al toxicity.

Keywords: *gene expression; gas exchange; aluminum; water relations; abscisic acid*

INTRODUÇÃO GERAL

O alumínio (Al) é o metal mais abundante na crosta terrestre, pois a maior parte dos minerais primários e secundários são aluminosilicatos, estes últimos são inertes para as plantas, mas quando solubilizados, liberam o Al em diversas formas (Malavolta, 1980; Ryan et al., 2011). Em solos ácidos (pH < 5,0) o Al é encontrado principalmente na forma de $Al(H_2O)_6^{3+}$, chamado comumente de Al^{3+}, tóxico à maioria das plantas, incluindo as cultivadas (Foy, 1974; Jansen et al., 2004). Considerando a extensão dos solos ácidos, os quais ocupam aproximadamente 30% das áreas livres de geleiras do mundo (von Uexküll & Mutert, 1995), o Al é um dos principais fatores abióticos que limitam a produtividade vegetal (Sade et al., 2016).

Nas plantas sensíveis ao Al, o primeiro e mais perceptivo sintoma de toxicidade é a inibição do crescimento radicular (Horst et al, 2010; Sun et al., 2010), o qual pode ser detectado após algumas horas de exposição ao metal, mesmo sob baixas concentrações (<10 µM Al) (Kopittke et al., 2008). Dentre as hipóteses para explicar a paralização do crescimento da raiz está o rompimento de regiões sub-apicais da epiderme, em consequência do enrijecimento celular (Kopittke et al., 2008; Blamey et al, 2011), o qual ocorre em razão da ligação (covalente) do Al à parede celular primária (Wehr et al., 2010). Assim, nas espécies sensíveis ao Al, é comum sua retenção quase que total no sistema radicular das plantas (Vitorello et al., 2005; Silva et al., 2018).

Além do efeito local, rápido e direto do Al sobre o crescimento da raiz, este elemento também está associado à redução do crescimento da parte aérea (Jiang et al., 2009), sendo comumente considerado um efeito secundário, causado pela diminuição nas taxas de assimilação de CO_2 (*A*), já observadas em plantas de tomate (*Solanum lycopersicum*) (Simon et al., 1994b), milho (*Zea mays*) (Lidon et al, 1999), cafeeiro (*Coffea arabica*) (Konrad et al, 2005), centeio (*Secale cereale*) (Silva et al., 2012) e *Citrus limonia* (Banhos et al., 2016). Apesar de o Al estar associado ao baixo desempenho fotoquímico (Chen et al., 2005a,b; Jiang et al, 2009), os valores reduzidos de *A* também podem ser explicados pela menor condutância estomática (*gs*). Os estudos em cafeeiro, *Citrus spp*, milho e centeio relatados acima apresentaram redução de 30 a 80% nos valores de *gs*, sugerindo efeito indireto do Al sobre *gs*.

Conforme revisado por Kochian et al. (2015), estudos sugerem que o Al, por reduzir o crescimento das raízes, traria como consequência uma menor superfície radicular para absorver água e hidratar a parte aérea. No entanto, a limitação de área de raíz não é exatamente limitante em condições de hidroponia, condição esta que é preferencialmente utilizada em estudos sobre os efeitos do Al (Horst et al., 1990). Além disso, as plantas sensíveis ao Al também mostram menor área foliar específica (Santos et al., 2000) e redução do crescimento e desenvolvimento da parte aérea (Jiang et al., 2009; Banhos et al., 2016), o que compensaria o menor sistema radicular, mantendo a hidratação da parte aérea. No entanto, mesmo com essa "compensação", a hidratação das folhas é menor em plantas expostas ao Al (Silva et al., 2018; Cavalheiro et al., 2020). Em limoeiro 'Cravo' (*Citrus limonia* L.) cultivado em solução de nutrientes com 1480 µM Al, os vasos de xilema se tornam fibrosos e o estelo tem sua estrutura anatômica alterada (Banhos et al., 2016), além de ocorrer deposição de lignina no cilindro vascular (Silva et al., 2019). Batista et al. (2013) também observou dano estrutural nas células do cilindro vascular de plantas de milho expostas à 300 mM Al.

Logo, é possível que o Al retido no sistema radicular, além de inibir o crescimento radicular, também altere a estrutura anatômica e funções fisiológicas do xilema a ponto de diminuir a capacidade intrínseca das raízes em absorver e conduzir água para a parte aérea. Dessa forma, a menor absorção e transporte de água pela raiz diminuiria a hidratação das folhas, possivelmente reduzindo *gs e A*. No entanto, os mecanismos envolvidos nessas respostas não estão esclarecidos.

Essas informações permitem traçar um paralelo entre os efeitos causados pelo Al em espécies sensíveis e aqueles provenientes da deficiência hídrica (Tamás et al., 2006; Yang et al., 2013), como os efeitos na hidratação foliar, por exemplo. No entanto, enquanto o Al possui efeito direto na função e crescimento radiculares, plantas sob deficiência hídrica no solo apresentam o crescimento radicular aumentado (Yang et al., 2013). De modo geral, as plantas desenvolveram mecanismos que as permitem perceber a imposição de fatores estressores e rapidamente regular seu metabolismo (Zhang et al., 2006). Um exemplo conhecido é a redução da perda de turgor através do fechamento parcial dos estômatos e/ou redução do desenvolvimento foliar antes que ocorra danos irreversíveis à planta (Davies & Zhang, 1991).

Um dos principais reguladores do movimento estomático é o hormônio ácido abscísico (ABA), cuja biossíntese ocorre em plantas sob alta salinidade (Arbona et al. 2010, Raghavendra et al., 2011), alagamento (Arbona & Gómez-Cadenas, 2008) e deficiência hídrica (Pinheiro & Chaves, 2011; Yoshida et al., 2014; Lim et al., 2015; Merilo et al., 2015). Dependendo de como esse estresse é imposto (abruptamente ou gradualmente), o ABA pode ser sintetizado na folha ou na raiz (Christmann et al. 2005, 2007; Holbrook et al. 2002). Em trabalhos que mediram a concentração de ABA, as amostras foram coletadas quinzenalmente, durante 90 dias, em videira (*Vitis vinífera* L. cv 'Cabernet Sauvignon') (Speirs et al., 2013), entre 0 e 16 dias em petúnia (*Petunia hybrida* cv 'Apple Blossom') (Kim et al., 2012), e de 0 a 4h após a imposição do estresse em *Arabidopsis* (Ikegami et al., 2009). Deste modo, a dinâmica da biossíntese e atuação do ABA é bastante variável, dependendo da espécie, do porte, das condições e da severidade do estresse (Osakabe et al., 2014).

Tem sido proposto que a intensidade da sinalização pelo ABA pode ser alterada por diversos fatores e estas modificações podem ter efeitos importantes sobre a movimentação estomática (Jiang & Hartung, 2008). Ainda não está estabelecida qual a peça chave para desencadear o fechamento estomático, se por meio de sinalização na raiz ou na folha. Alguns estudos sugerem que a sinalização ocorre em resposta ao potencial da água na folha (Rodrigues et al., 2008) ou nas raízes (Davies & Zhang, 1991), síntese *de novo* de ABA na folha (Soar et al., 2006) ou redistribuição do ABA em função do pH do xilema (Sauter & Hartung, 2002; Else et al., 2006).

Baseado nos efeitos indiretos do Al em espécies sensíveis, é possível que as alterações estruturais e fisiológicas na raiz levem a planta a apresentar respostas similares àquelas induzidas pela deficiência hídrica no solo (Batista et al., 2013; Banhos et al., 2016; Silva et al., 2019). Considerando-se que o Al causa diminuição em *gs* de forma indireta (por estar acumulado no sistema radicular), é possível que, assim como ocorre durante a deficiência hídrica, as plantas sensíveis ao Al respondam à toxicidade produzindo uma maior quantidade de ABA. O aumento de ABA poderia, então, provocar a diminuição de *gs* observada por Chen et al. (2005b), Jiang et al. (2008), Silva et al. (2012), Banhos et al. (2016) e Silva et al. (2018).

Nesse sentido, tendo como base a existência do controle molecular e bioquímico do ABA sobre nas relações hídricas de várias espécies vegetais, é inevitável levantar questões sobre a interação entre ABA e Al. Por exemplo, a biossíntese de ABA é induzida por Al em espécies sensíveis a esse metal? A expressão de genes responsáveis pela biossíntese de 9-cis-epoxicarotenóide dioxigenase (NCED), enzimas-chave na rota de biossíntese de ABA ocorre imediatamente após a percepção do Al na raiz ou é dependente da alteração na hidratação do mesofilo? A concentração de ABA nas folhas, após a indução do estresse por Al, pode ser correlacionada ao fechamento estomático? Para tentar responder a essas questões, foram estudadas a dinâmica (respostas ao longo do tempo de exposição das plantas ao Al) do ABA e as relações hídricas em duas espécies sensíveis ao Al, mas cujos sintomas severos de toxicidade são induzidos por concentrações significativamente distintas, como o *Solanum lycopersicum* (100 µM Al) e *Citrus limonia* (1480 µM Al).

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CAPÍTULO I:

Aluminum-induced decrease in stomatal conductance is related to low root hydraulic conductance and high ABA accumulation in tomato

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Abstract

Aluminum (Al) is a major constraint for crop yields in acidic soils. While many studies aim to understand how exposure to Al decreases root growth, the physiological mechanisms explaining Al-induced stomatal closure are unclear. Tomato plants (*Solanum lycopersicum* Mill.) were exposed to 0, 25, 50 and 100 μM Al in nutrient solution to understand whether Al impairs root hydraulic conductance (Lp_r), affecting leaf water potential (Ψ_{leaf}) and possibly inducing abscisic acid (ABA) accumulation in roots and/or leaves. We also investigate ABA delivery rate and xylem sap pH to explain low stomatal conductance (g_s) and the root/leaf area ratio developed in consequence of turgor adjustments. Leaf water potential (Ψ_{leaf}) was similarly decreased across all Al treatments, probably due to concentration-dependent drops in g_s acting to maintain leaf water status. Declines in root hydraulic conductance (Lp_r) were proportional to the increase in Al concentration despite increased root ABA concentrations. Al did not enhance delivery of ABA from the roots but caused root xylem sap alkalization and leaf ABA accumulation. Despite the stability of root/leaf area ratio across a range of Al concentrations (0, 25 and 50 μM Al) leaf hydration and stomatal opening was not conserved probably due to decreased Lp_r. Here we show that Al exposure triggers ABA accumulation in tomato leaves causing low g_s.

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust, and its most phytotoxic form [$Al(H_2O)^{3+}$], or Al^{3+}, occurs in acidic soils ($pH < 5.0$) (Kochian et al., 2015), which accounts for approximately 30% of the world's ice-free land (von Uexküll and Mutert 1995). Therefore, the binomial "acidic soils" and "phytotoxic Al" are worldwide challenges that limit crop yields (Maron et al., 2008) by 25 to 80% depending upon the Al sensitivity of the species (Sade et al., 2016).

The first marked and direct symptom of Al toxicity is rapid inhibition of root growth (Delhaize and Ryan 1995; Horst et al, 2010), resulting in low root area and biomass, limiting water and nutrient uptake (Kochian et al. 2004). Thus, a linear and simple cause-effect hypothesis has been sustained in the literature: less developed roots exploring low soil volume leading to low water uptake and, consequently, low leaf hydration. For instance, plants exposed to Al show low leaf relative water content (RWC) and leaf water potential (Ψ_w) (Silva et al., 2012; Silva et al., 2018; Siecińska et al., 2019), which is usually associated with low leaf area and biomass (George et al., 2012; Yang et al., 2013). These reductions in the growth of above- and belowground organs of plants exposed to Al would, in principle, maintain the root/leaf area ratio, but this parameter is not usually measured in Al toxicity studies. Among the plethora of physiological responses that enable plants to respond to changes in water availability, stomata retain a very important role in regulating leaf-level water loss to the atmosphere, thus impacting whole-plant water balance (Sperry et al., 2017; Huber et al., 2019). Actually, Al exposure decreased stomatal conductance (*gs*) was observed in *Solanum lycopersicum* (Simon et al., 1994), *Coffea arabica* L. (Konrad et al., 2005), *Secale cereale* (Silva et al., 2012), *Zea mays* L. (Anjum et al., 2016) and *Citrus limonia*

(Banhos et al., 2016). However, the mechanisms explaining Al effects on stomatal regulation remain largely unknown.

Most studies that observed reduced root and shoot growth and low *gs* were performed using plants growing directly in nutrient solution where water is constantly available (Simon et al., 1994; Konrad et al., 2005; Silva et al., 2012; Banhos et al., 2016; Silva et al., 2018). Besides root growth inhibition, plants exposed to Al may also respond with water uptake impairment and limited transport to shoots to maintain stomata open. For instance, fibrous xylem vessels were observed in *C. limonia* grown in nutrient solution with Al and showing low Ψ_w and *gs* (Banhos et al., 2016). Al causes lignin deposition (Silva et al., 2019) and structural damage in the vascular cylinder (Batista et al., 2013). Another factor that could regulate water transport is the abundance of aquaporins (Javot and Maurel, 2002). In fact, low aquaporin (PIP family) gene expression was observed in rye (Milla et al. 2002), *Arabidopsis* (Shen et al., 2008) and *C. limonia* (Cavalheiro et al., 2020) exposed to Al. These results suggest that Al could also reduce root hydraulic conductance (Lp_r), a trait that determines root water transport capacity. Lp_r was decreased by Al in maize plants (Gunsé et al., 1997), although these authors did not measure stomatal conductance and neither associated both variables.

Besides plant hydraulics, root-to-shoot chemical signaling could also explain *gs* limitations in plants exposed to Al in nutrient solution (Dodd, 2005). Abscisic acid (ABA) is synthesized in response to multiple abiotic stresses that alter tissue water status (Zhang et al. 2006) and acts as a long-distance signal from roots to shoots (via xylem), where it restricts transpiration by closing the stomata (Schachtman and Goodger 2008; Shabala et al. 2016). Few studies have considered ABA signaling under Al toxicity. Soybean roots that accumulated ABA when exposed to Al were more Al tolerant, as they exuded organic acids, forming non-toxic Al-OAs complex in the rhizosphere avoiding excessive Al uptake (Shen et al., 2004). Al increased endogenous ABA accumulation in both roots and leaves of soybean and accelerated root ABA transport, suggesting a role of this hormone in regulating Al resistance of soybean plants (Hou et al., 2010). Independent of changes in tissue ABA concentration, xylem sap pH can induce stomatal closure by affecting the compartmentation of root-sourced ABA in the leaves, with alkalization causing apoplastic ABA accumulation and stomatal closure (Wilkinson & Davies, 1997). However, no studies have assessed if Al-induced ABA accumulation can decrease *gs*, either due to root-to-shoot signalling (xylem ABA or pH) or locally ABA synthesis in the leaf.

The present study evaluated whether low Lp_r (hydraulic function) and high ABA biosynthesis (chemical mechanisms) regulates leaf water relations of tomato plants (*Solanum lycopersicum* Mill.) exposed to increasing Al concentration in nutrient solution for 10 days. For this, biometric measurements, root system morphology, plant biomass, *gs*, whole-plant transpiration (E_{plant}), Ψ_{leaf} and Lp_r, xylem sap pH and ABA concentration in roots, xylem sap and leaves were measured.

Material and Methods

Plant material and experimental conditions

Thirty-two tomato plants (*Solanum lycopersicum* Mill.) (Solanaceae) cv. 'Ailsa Craig' were used. Seeds were germinated in seedling trays filled with rockwool cubes (2.5 x 2.5 x 4.0 cm) that were irrigated with nutrient solution (Silva et al., 2018) at ½ strength and pH 5.5 ± 0.2. Three weeks after germination under semi-controlled conditions (500 ± 50 µmol photons m^{-2} s^{-1}; approximately 14 h photoperiod; average air temperature ≈ 26°C) plants with three leaves were transferred to opaque plastic boxes

(37 cm in length, 26 cm in width and 16 cm in height; 15 L), containing the nutrient solution with the Al treatments.

The nutrient solution was based on Clark's nutrient solution (Clark, 1975), which was previously used to test Al toxicity (Villa et al., 2009; Silva et al. 2018a; 2019). It consisted of 1372.8 µM $Ca(NO_3)_2$ 4 H_2O, 507 µM NH_4NO_3, 224.4 µM KCl, 227.2 µM K_2SO_4, 218.6 µM KNO_3, 483.2 µM $Mg(NO_3)_2$ 6 H_2O, 12.9 µM KH_2PO_4, 26.01 µM $FeSO_4$ 7 H_2O, 23.8 µM NaEDTA, 3.5 µM $MnCl_2$ 4 H_2O, 9.9 µM H_3BO_3, 0.9 µM $ZnSO_4$ 7 H_2O, 0.2 µM $CuSO_4$ 5 H_2O, 0.4 µM $NaMoO_2$ 2 H_2O. This solution shows high pH stability as plants absorb water and nutrients over time. In addition, it has a low phosphorus concentration compared to Hoaglands' solution, which reduces the chance of precipitation of Al as $AlPO_4^-$. The nutrient solution was completely changed every 3 days, and solution pH (4.0 ± 0.1) was adjusted every day in order to keep the Al as soluble as possible. Besides macro and micronutrients, the solution contained 0, 25, 50 and 100 µM Al provided through $AlCl_3$ 6 H_2O. These Al concentrations were based on previous studies showing Al toxicity symptoms in tomato plants (Simon et al., 1994; Zhou et al., 2009; He et al., 2019).

The lids of the boxes containing the nutrient solution had 6 holes of 2.5 cm in diameter, and the plants growing on the rockwool cubes were fixed in these holes. The boxes were maintained on benches, inside a glasshouse, with the same conditions as previously described.

Experimental design

Plants exposed to 0, 25, 50 and 100 µM Al were cultivated in nutrient solution for 10 days to assess the effect of Al on water relations. Non-destructive traits such as leaf length, main root length, whole-plant transpiration (E_{plant}) and stomatal conductance (*gs*) were measured in eight replicates exposed to the four Al treatments at 0, 1, 3, 5, 7 and 10 days after treatment (DAT). At the end of the experiment (10 DAT), four plants were used to measure leaf water potential (Ψ_{leaf}), biometric parameters in leaves (number, area and biomass) and roots (total length, surface, diameter and biomass), and leaf and root Al concentrations. Another four plants were used to measure pressure-induced sap flow rates, root hydraulic conductance (Lp_r) and ABA concentration in roots, xylem sap and leaves.

Stomatal conductance (gs)

Stomatal conductance was measured on the middle leaflet of a fully expanded leaf (third or fourth leaf from the top of the plant) using an infrared gas analyzer (6400xt LI-COR, Lincoln, NE, USA). The leaf cuvette conditions were set to approximately match the environmental conditions in the glasshouse: CO_2 at ambient concentration (400 µmol mol^{-1}), 500 µmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD), air temperature of 25°C, and relative humidity at 40–50%.

Leaf water potential (Ψ_{leaf})

Leaf water potential (Ψ_{leaf}) was measured between 11:00h and 14:00h on the same leaf as *gs* using a pressure chamber (Model 3000F01 Plant Water Status Console; Soil Moisture Equipment Corp., USA). Detached leaves were taken to the laboratory and placed in the pressure chamber within 60s of excision. Once in the chamber, pressure was raised at a rate of 0.02 MPa s^{-1}, and Ψ_{leaf} was recorded (MPa) when xylem sap emerged on the cut surface.

Whole-plant transpiration (E_{plant})

Plants were transferred to individual 0.9-L cylindrical plastic pots (6.9 cm in diameter, 24 cm in height) designed to fit in the pressure chamber (Model 3000F01; Soil Moisture Equipment Corp., USA). The tubes contained the same nutrient solution described above with the plants fixed with 2 cm thick foam to prevent evaporation. The plants acclimatized for 1 h in the pot (9:00-10:00). Then, the pot was weighed on a 0.01g precision scale (Adventurer Pro AV4102; Ohaus, Thetford, UK). One hour later (11:00 AM), the pot was weighed again and the whole-plant water uptake was calculated by the difference between the initial and final pot weights. Evaporation was assessed by determining the water loss from a pot (without a plant) and ignored as negligible (<3% of the water loss of pots containing a plant). The whole-plant transpiration was obtained as the ratio between water uptake and the time (mg s^{-1}) (Puértolas et al., 2015).

Root hydraulic conductance (Lp_r)

Root hydraulic conductance was measured using the method of pressure-induced sap flow from roots (Jackson et al., 1996; Dodd and Diatloff, 2016). After shoot removal, a series of overpressures (from 0.1 MPa to 0.4 MPa at 0.1 MPa increment) were applied and the sap flow rate was determined at each pressure. Root hydraulic conductance quantifies the root permeability to the flow of water by applying increasing pneumatic pressures to the root zone. The slope of the linear regression representing the relationship between exuded flow rate (J) (in mg s^{-1}) and applied pressures gave the root hydraulic conductance (Lp_r).

Xylem sap pH

Following measurement of *Lp*$_r$, the overpressures (0.1–0.4 MPa) that induced the sap flow rate closest to that previously measured gravimetrically was applied to collect xylem sap (Else et al., 2006). Sap samples were collected in previously weighed 1.5 mL vials, frozen in liquid nitrogen and stored at –18°C. When the sample was defrosted, the sap pH was measured with a microelectrode (Lazar Research Laboratories, Los Angeles, CA, USA) prior to measuring root xylem sap ABA concentration ($[\text{X-ABA}]_{\text{root}}$).

ABA quantification

Leaf (the same leaf as used for *gs* measurements) and root (four root tips) samples (≈ 5-10 mg DW) were frozen in liquid nitrogen and stored at –18 °C. Leaf samples were collected prior to shoot removal to measure Lp_r while root samples were collected after this measurement, to avoid damaging the root apices for analysis. The elapsed time between excision and freezing did not exceed 20s. Leaf and root samples were freeze-dried and then ground into powder. Dry leaf and root tissues were mixed with deionized water (extraction ratio 1:30) and then shaken at 4°C overnight to extract ABA. The extracts were centrifuged at 15,000 rpm for 5 min, and the supernatant was directly used for ABA assay. ABA concentration in the leaf ($[\text{ABA}]_{\text{leaf}}$), root ($[\text{ABA}]_{\text{root}}$), and root xylem sap ($[\text{X-ABA}]_{\text{root}}$) was measured by radioimmunoassay method, using the monoclonal antibody AFRC MAC 252 (Quarrie et al. 1988). Thus, while $[\text{ABA}]_{\text{leaf}}$ and $[\text{ABA}]_{\text{root}}$ were measured in the aqueous extract, the $[\text{X-ABA}]_{\text{root}}$ was measured directly in sap samples. $[\text{X-ABA}]_{\text{root}}$ was determined in the sample with the closest sap flow rate to the whole-plant transpiration.

Biometric parameters

Immediately before Al treatment application, the smallest leaf of each plant was marked and its length as well as its terminal leaflet length were measured with a ruler (cm) at 0, 1, 3, 5, 7 and 10 DAT. The main root length (from the plant collar to the root tip) was also measured with a ruler (cm) at the same evaluation dates.

At 10 DAT, total root length, root surface area and root diameter were measured using a scanner (Epson perfection v700 photo, Suwa, Japan) which was coupled to a computer running the WinRHIZO^{TM} software (Regent Instruments, Canada). The number of leaves (considering only those at least 15 mm in length) was counted, and the leaf area (LA, cm^2) was measured with an area meter (LI-3100C, LI-COR, USA). Plants were separated into leaves and roots and dried at 60°C until constant mass. The biomass (g) of organs was weighed on a 0.01g precision scale (Adventurer Pro AV4102; Ohaus, Thetford, UK).

Aluminum quantification

Al quantification was performed according to Havlin and Soltanpour (1980) where each sample was digested with nitric acid, fortified with Al standards and analyzed using coupled plasma optical emission spectrometry (ICP-OES). Root samples were washed thrice in deionized water to avoid excess Al from the nutrient solution.

Data analysis

The data were submitted to one-way analysis of variance (ANOVA), and mean values were compared between Al treatments by LSD (least significant difference) at 0.05 confidence level using Tukey's test. Relationships between xylem sap flow rate and different pressures applied were assessed by linear regression.

Results

Within 1 day, all Al treatments limited the main root length. Thereafter, plants exposed to 100 µM Al ceased to grow (0.2 cm day^{-1}), while the 25 and 50 µM Al treatments maintained slower linear growth rates (1.9 and 0.85 cm day^{-1}, respectively) than the control (3.8 cm day^{-1}) for the rest of the experiment (Fig. 1). Main root length of the control and 25 µM Al treatments diverged at 5 DAT, as did the 25 µM and higher Al treatments, while the 50 and 100 µM Al treatments diverged at 7 DAT. After 10 DAT, the 25, 50 and 100 µM Al treatments decreased main root length by 42%, 71% and 85%, respectively, as compared to the control plants (Fig. 1). Thus, increasing nutrient solution Al concentrations proportionally decreased root elongation.

At 10 DAT, increasing Al concentration significantly decreased total root length (Fig. 2A), root surface area (Fig. 2B) and root biomass (Fig. 2C) in a concentration-dependent manner. Compared to control plants, the 100 µM Al treatment decreased total root length, root surface area and root biomass by 94, 92 and 83%, respectively. Moreover, all Al concentrations significantly increased root diameter by 36% compared to control plants, with no differences between Al concentrations (Fig. 2D). Thus Al concentration rapidly inhibited root growth and caused root thickening.

At 10 DAT, leaf number (Fig. 3A), leaf area (Fig. 3B) and leaf biomass (Fig. 3C) decreased with increasing Al concentration. For all these variables, the effects of 50 and 100 µM Al were statistically indistinguishable, with plants exposed to 25 µM Al showing intermediate values between control and higher Al concentrations. Compared to control plants, the 100 µM Al treatment decreased leaf number, leaf area and leaf biomass by 33, 82 and 64% respectively. Thus, Al treatment decreased leaf initiation, expansion and biomass accumulation.

Aluminum decreased entire leaf (Fig. 4A) and terminal leaflet (Fig. 4B) length from 5 DAT in a concentration-dependent manner. All treatments had statistically diverged by 10 DAT for the entire leaf and 7 DAT for the terminal leaflet. Compared to control plants, at 10 DAT, the 100 μM Al treatment decreased entire leaf and terminal leaflet length 55 and 48% respectively. Thus, Al treatment decreased both petiole and leaflet expansion similarly.

As Al concentrations in the root environment increased, the leaf area and the root surface area decreased proportionally (Fig. 5A, 5B), so that plants exposed to 0, 25 and 50 μM Al showed similar root/leaf area ratio. In contrast, those exposed to 100 μM Al showed a lower root/leaf area ratio (Fig. 5C). Therefore, the low leaf area compensated the decrease in root area only up to 50 μM Al.

Aluminum induced stomatal closure in plants treated with 50 and 100 μM Al from 3 DAT, and from 5 DAT to the end of the experiment in all Al treatments compared to control. Compared to the control plants, at 10 DAT the 25, 50 and 100 μM treatments decreased stomatal conductance by 30, 53 and 62% respectively (Fig. 6). Thus stomatal closure was detected immediately after root growth inhibition and earlier (by two days) than leaf growth inhibition.

Increasing the Al concentration in the nutrient solution significantly decreased the whole-plant transpiration measured at 10 DAT (Fig. 7A), being 13, 42 and 68% lower in plants treated with 25, 50 and 100 μM Al, respectively, than control plants. Increasing the pneumatic pressure applied to de-topped root systems linearly increased sap flow rates in all Al treatments. Linear regressions were fitted to these data, with the slopes representing root hydraulic conductance (Lp_r). Increasing Al concentrations decreased Lp_r from 10.78 mg s^{-1} MPa^{-1} in control plants, to 8.06 (-25%), 5.29 (-60%)

and 3.02 (-70%) mg s^{-1} MPa^{-1} in plants exposed to 25, 50 and 100 μM Al, respectively (Fig. 7B).

Generally, Al treatments increased tissue abscisic acid (ABA) concentrations in a concentration-dependent manner (Fig. 8A and 8B). Leaf ABA concentrations were more than 10 times higher than root ABA concentrations, with greater statistical divergence of the Al treatments. Root xylem sap ABA concentrations were indistinguishable between control (0) and 25 μM Al treatments, and between the 50 and 100 μM Al treatments (Fig. 8C). Sap ABA concentrations were 35% higher in the latter two treatments compared to the former. ABA delivery rate ([ABA] x transpirational flow rate), however, was indistinguishable between control (0), 25 and 50 μM Al treatments and decreased in 100 μM Al treatment (Fig. 8D). Thus, ABA concentrations increased throughout the plant in response to Al, especially in leaves, probably due to local production . Stomatal conductance declined linearly as $[ABA]_{leaf}$ increased (Supplemental Fig. S1).

All Al treatments decreased leaf water potential (Ψ_{leaf}) by 0.3 MPa (-40%) compared to control plants, with no differences between distinct Al concentrations (Fig. 9A). Root xylem sap pH increased 0.5, 0.6 and 0.7 units in plants treated with 25, 50 and 100 μM Al, respectively, when compared to the control plants (Fig. 9B).

As expected, Al concentration in the roots was approximately 100 times higher than in the leaves, and it increased as Al concentration in the nutrient solution increased (Fig. 10A). Root Al concentration was 13-, 25- and 46-fold higher in plants treated with 25, 50 and 100 μM Al when compared to the control plants. Leaf Al concentration was higher in plants exposed to Al (82%) in relation to control plants, but it showed no differences between Al treatments (Fig. 10B). Thus increasing Al in the nutrient solution increased root Al concentrations, but the roots restricted Al transport to the shoots.

Discussion

Even though Al reduce root growth and hence plant capacity to absorb water, this is unlikely to be the only factor explaining the Al-induced decrease in leaf hydration and *gs* (Banhos et al., 2016; Silva et al., 2019). In our study, we showed that the lower leaf water status experienced by plants growing in nutrient solution with high Al concentration may be caused by low Lp_r (hydraulic mechanism) and ABA accumulation in leaves, eventually explaining the low *gs* (Fig. 11).

As expected, the main (Fig 1) and total root length (Fig. 2A), root surface area (Fig 2B) and root biomass (Fig 2C) decreased as Al concentration in the nutrient solution and in root tissue increased (Fig. 10B). The reasons why root growth is inhibited under Al presence have been investigated (Zheng and Yang 2005; Horst et al., 2010; Kopittke et al., 2008; 2015; Rao et al., 2016; Silva et al., 2019), but given the complexity of the processes involved in the root growth inhibition, the exact mechanism by which Al stunt root growth remains elusive (Singh et al., 2017). In addition, less attention is paid to the Al impacts on shoot growth since these are considered indirect/long-distance effects. On the other hand, some explanations on why Al causes reduced leaf growth involve low nutrient uptake subsiding less growth aboveground (Silva et al., 2010), low biosynthesis and transport of cytokinins inducing less flushing (Mossor-Pietraszewska, 2001) and low hydraulic pressure leading to low cell elongation (Barceló et al., 1996), especially in water-limiting environments. While these mechanisms seem important in water-limiting environments, here Al toxicity was imposed hydroponically, where water is constantly available, and still leaf growth was inhibited in response to the increase of Al concentration (Fig. 3 and 4). Leaf growth of Al-exposed plants was likely regulated by some mechanisms: a non-linear relationship between Ψ_{leaf} and leaf elongation (Fig. 4 and 9A) and inhibition of leaf initiation (Fig. 3A). Irrespective of the mechanisms, plants exposed to 25 and 50 µM Al reduced their leaf area proportionally to the root surface area, so that their root/leaf area ratio was similar to control plants (Fig. 5C). Below a threshold Al concentration (between 50 and 100 µM Al), Al-induced root growth restriction was "compensated" by a low leaf area, although the coordinating mechanisms remain elusive. Though, the leaf length was reduced from 5 DAT (Fig. 4) while *gs* continuously decreased in all Al treatments (Fig. 6), suggesting that such compensation may not be sufficient to maintain leaf hydration to keep stomata open. In fact, all Al treatments showed a lower Ψ_{leaf} than control plants (Fig. 9A), being that the similarity between Al treatments could indicate that the hydraulic pressure inside xylem vessels were probably at its limit, suggesting that root-to-shoot water transport was impaired in Al-exposed plants. Indeed, increasing Al concentration in the nutrient solution decreased E_{plant} (Fig. 7A) and root hydraulic conductance (Lp_r) (Fig. 7B). Considering that plants growing hydroponically showed significant stomatal closure when exposed to Al (Fig. 6) accompanied by low leaf hydration and decreased root hydraulic conductance (Fig. 7B), water transport is certainly limited. These results indicate an Al-induced drought response as speculated by Tamás et al. (2006) and, more recently, by Yang et al. (2013). As far as we are aware, the Al-induced decrease in root hydraulic conductivity was only measured in maize (Gunsé et al., 1997), perhaps due to smaller protoxylem vessels or structural impairment in the vascular cylinder (Batista et al., 2013) or even decreased aquaporin gene expression (Milla et al. 2002, Shen et al., 2008, Cavalheiro et al. 2020). Further studies are needed to understand the effects of Al on Lp_r, as root ABA accumulation in response to drought induces root growth leading to the increase in Lp_r (Thompson et al. 2007; Dodd 2013), which is the opposite to what was observed in the present study, where Al significantly reduced root growth.

Since drought is well known to induce ABA synthesis/transport, the possibility of Al toxicity also triggering this hormone signalling was raised. However, studies investigating ABA accumulation in leaves of plants exposed to Al are rare. Soybean plants treated with 50 µM Al showed increased [ABA] in both leaves and roots and fast ABA transport, measured with [^{3}H]-ABA radioisotope technique (Hou et al., 2010), suggesting that Al may induce root-to-shoot ABA signaling. Since ABA delivery rate was equivalent in control plants and those exposed to 25 and 50 µM (the increase in $[\text{X-ABA}]_{\text{root}}$ was offset by decreased sap flow rate – Fig. 7B), it is difficult to argue that foliar ABA accumulation (Fig. 8A) was due to root-to-shoot ABA signalling. Moreover, reciprocal grafting studies with wild-type and ABA-deficient tomato plants show limited impacts of rootstock ABA status on foliar ABA accumulation under different edaphic stresses (Li et al. 2018). Thus, foliar ABA accumulation in response to increasing Al concentration in the root zone was determined by local processes. Our results showed a clear pattern of Al-dependent ABA accumulation in roots (Fig. 8C) and leaves (Fig. 8A). Increasing values of ABA in leaves in response to Al toxicity by itself is a novelty in the literature, but beyond that, the increase in ABA accumulation was proportional to the decrease of *gs* (Supplemental Fig. S1).

Some authors verified increased $[\text{ABA}]_{\text{root}}$ reducing the proton pumping of the plasma membrane of squash (Ahn et al., 2002) and Arabdopsis (Brault et al., 2004) exposed to Al. In this sense, in the present study, xylem sap pH in the root increased from 6.5 to 7.2 in response to Al (Fig. 9B). Similar pH values (6.3 to 7.2) were found in root xylem sap from water-stressed *Phaseolus vulgavis* plants (Hartung and Radin, 1989). The increase in the xylem pH is known by reducing the stomatal aperture in an ABA-dependent manner, most probably by increasing the ABA concentration of the apoplastic compartment (Wilkinson and Davies, 1997). Thus, as Al impairs proton pumps (Ahn et al., 2002, Brault et al., 2004), the apoplast (xylem sap) becomes less acid, which would maintain ABA as ABA^-, keeping it in the apoplast and limiting its return to the cells.

Conclusions

Even when plants are grown in nutrient solution, where water is constantly available, Al toxicity decreased water transport from the roots to the leaves as evidenced by low values of gs, Ψ_{leaf} and Lp_r. While root/leaf area ratio was maintained when plants were exposed to 0, 25 and 50 µM Al, leaf hydration was compromised and foliar ABA accumulation was correlated with stomatal closure in a concentration-dependent manner. Thus, ABA is likely the major cause of Al-induced decrease in gs considering the concentration-dependent responses exhibited for both variables.

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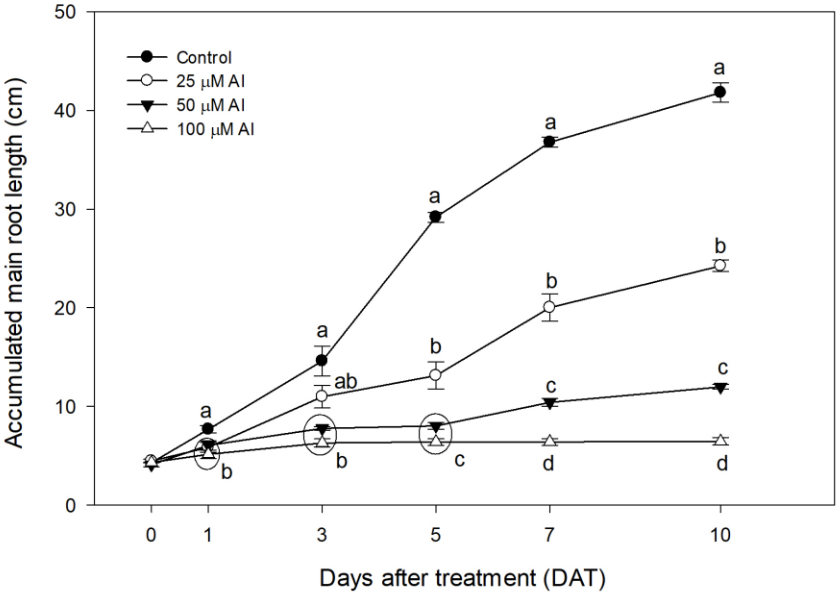


Fig 1. Accumulated growth of the main root of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments in each evaluation date. Dots are mean values (n=8 plants) and bars are standard errors. Ellipses indicate statistically similar treatments.

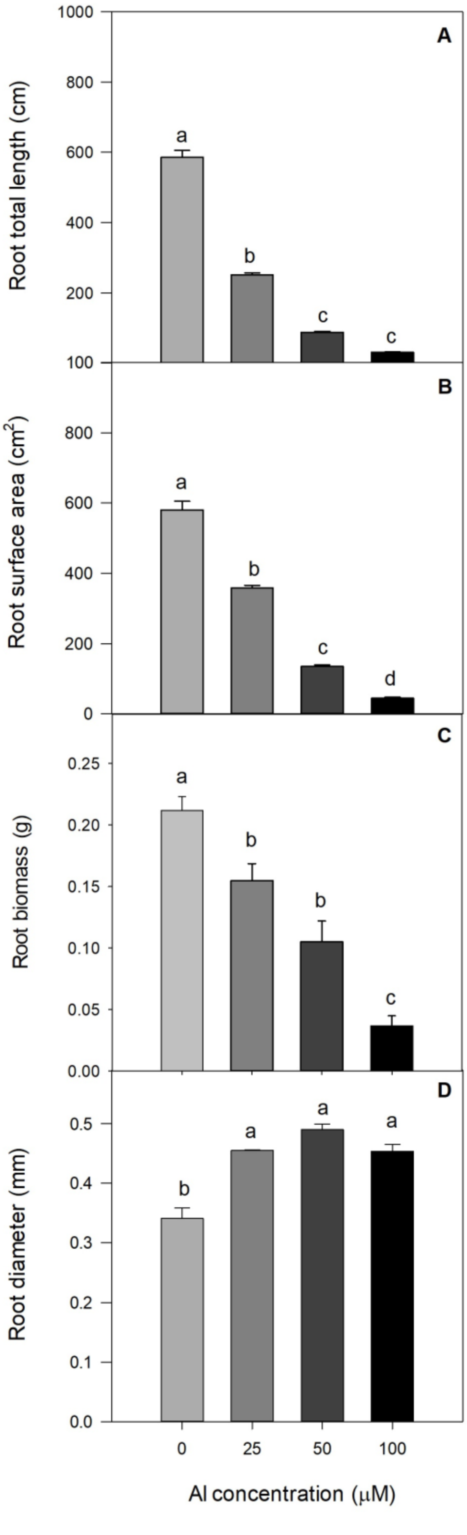


Fig 2. Root total length (A), root surface area (B), root biomass (C) and root diameter (D) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 μM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=4 plants) and bars are standard errors.

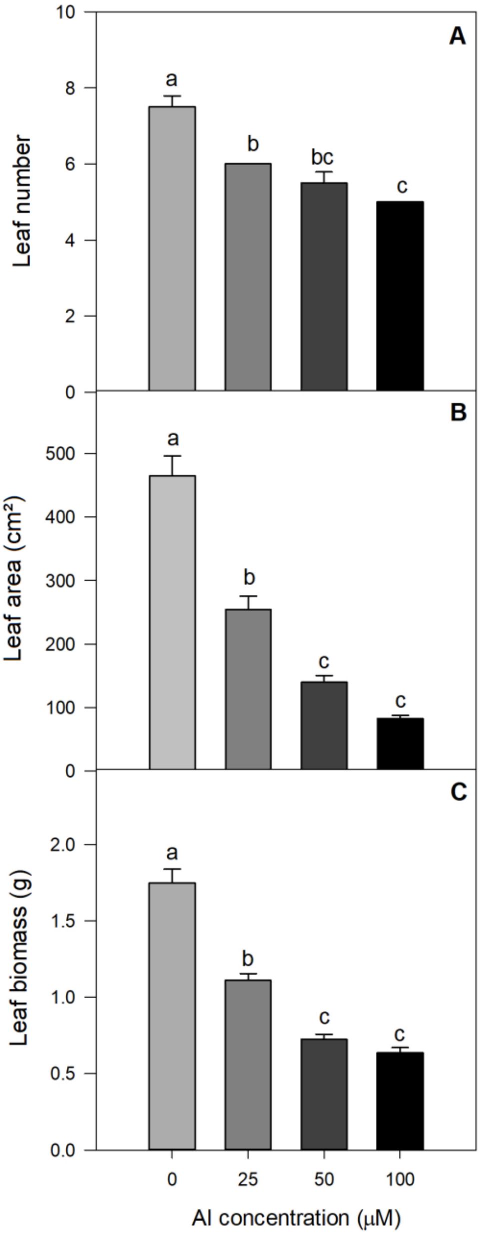


Fig 3. Leaf number (A), area (B) and biomass (C) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=4 plants) and bars are standard errors.

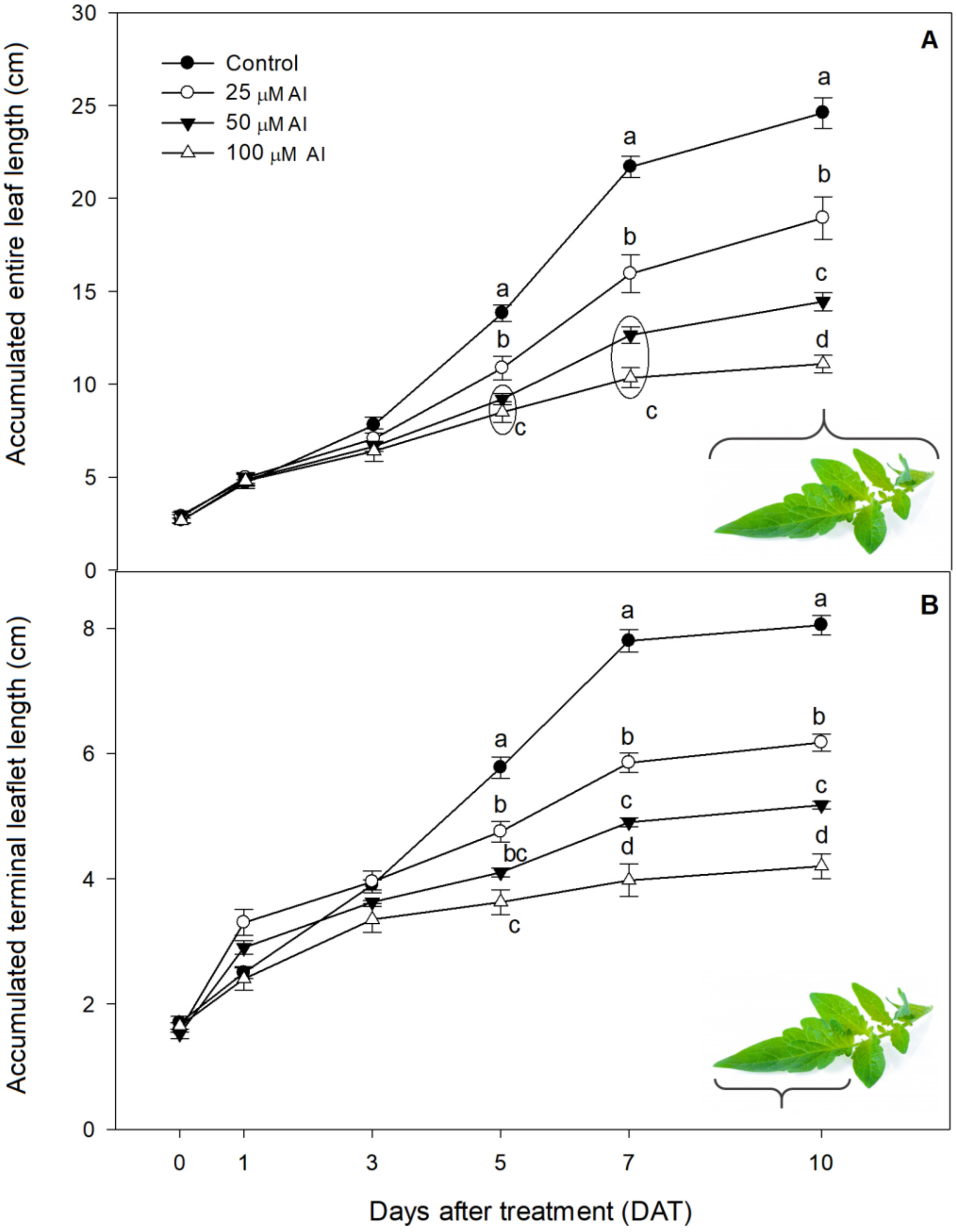
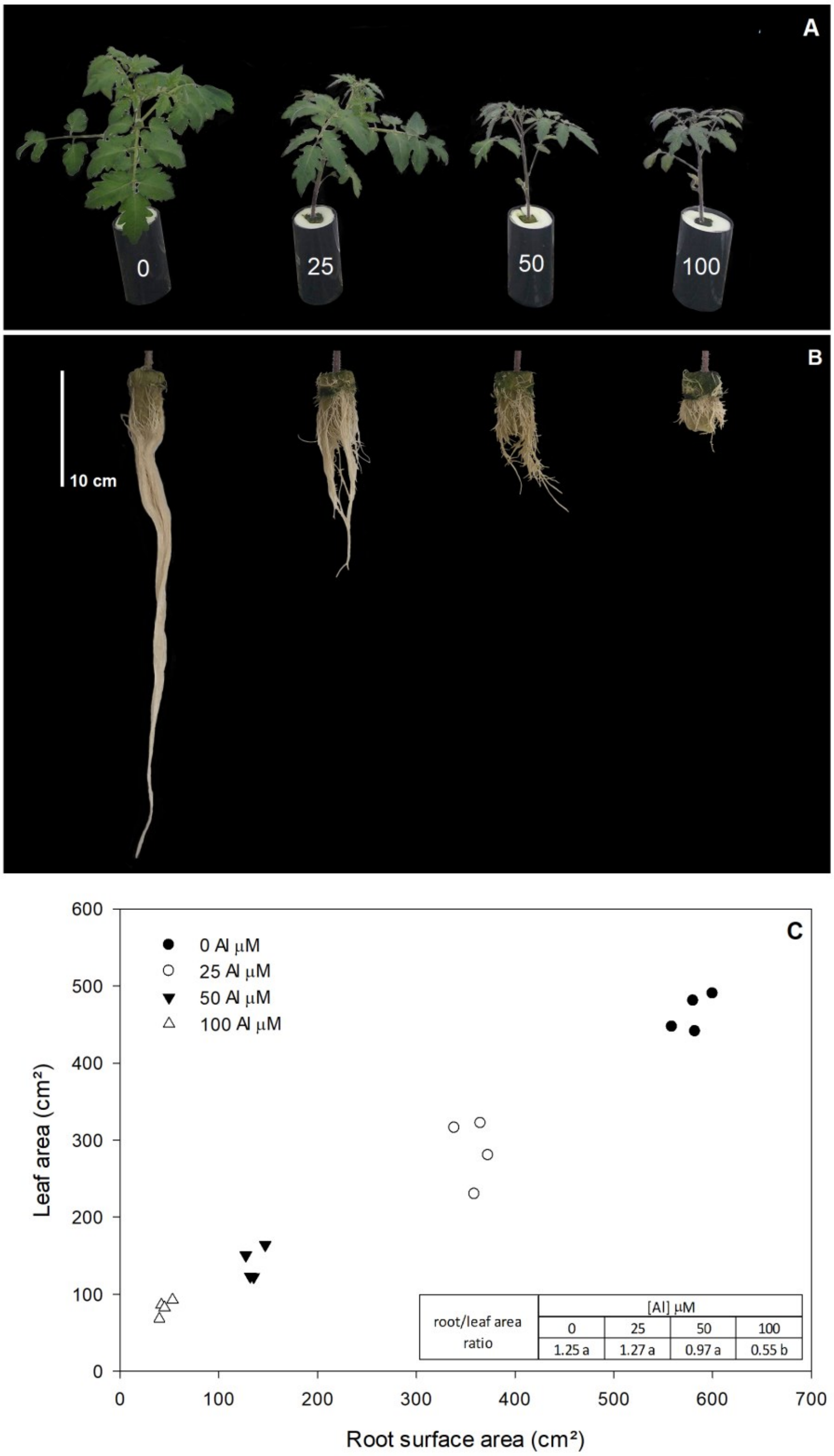


Fig 4. Accumulated leaf (A) and terminal leaflet (B) length of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments in each evaluation date. Dots are mean values (n=8 plants) and bars are standard errors. Ellipses indicate statistically similar treatments.



root/leaf area ratio	[Al] µM			
	0	25	50	100
	1.25 a	1.27 a	0.97 a	0.55 b

Fig 5. Morphological details of shoots and leaves (A) and roots (B) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al, respectively. Linear regression representing the relationship between root surface area and leaf area and root/leaf area ratio (table) (C), distinct lowercase letters in the table indicate significant differences ($P < 0.05$) between Al treatments (n=4).

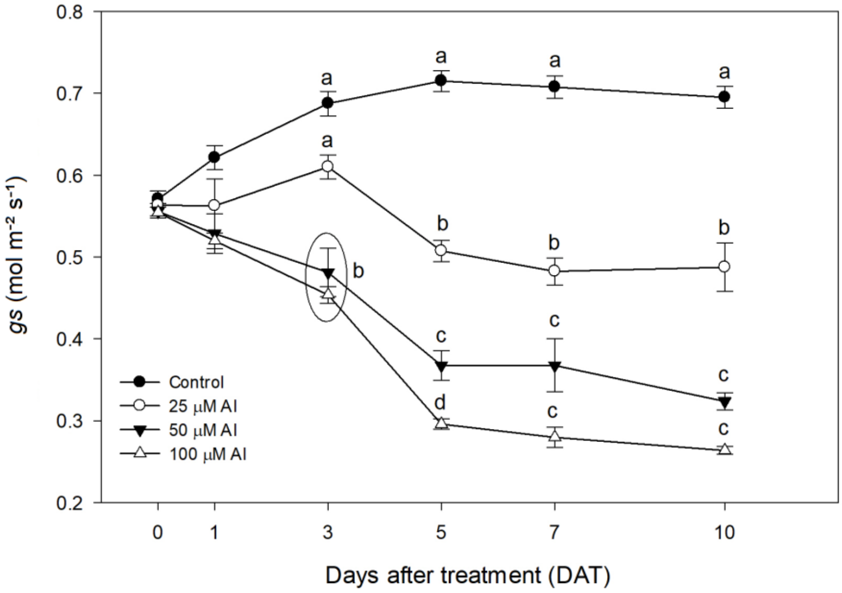
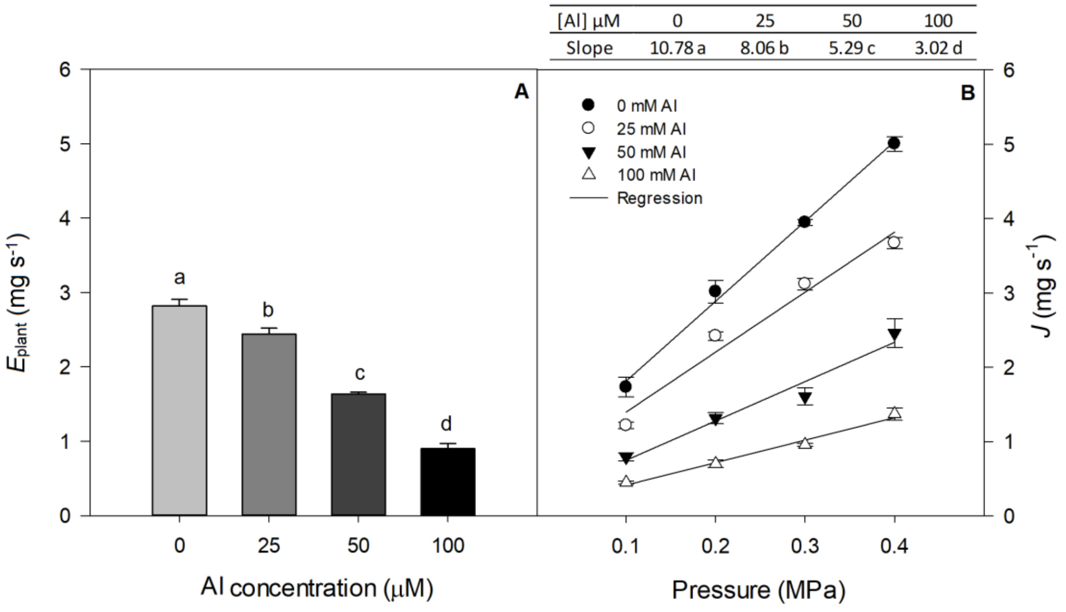


Fig 6. Stomatal conductance of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments in each evaluation date. Dots are mean values (n=8 plants) and bars are standard errors.



[Al] µM	0	25	50	100
Slope	10.78 a	8.06 b	5.29 c	3.02 d

Fig 7. Whole-plant transpiration (A) and the relationship between xylem sap flow rate and applied pressure of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al (B). The slopes of the linear regression lines indicate the root hydraulic conductivity (table). Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=8 plants for E_{plant} and n=4 for Lp_r) and bars are standard errors.

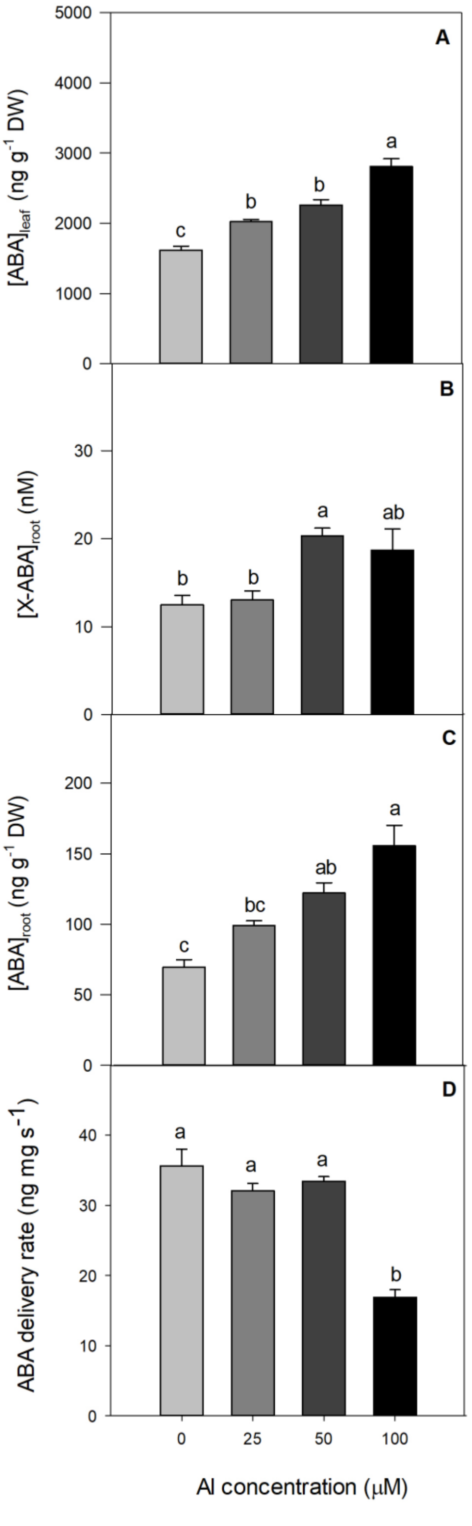


Fig 8. Abscisic acid (ABA) concentration in leaves (A), root xylem sap (B), roots (C) and ABA delivery rate (D) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=4 plants) and bars are standard errors.

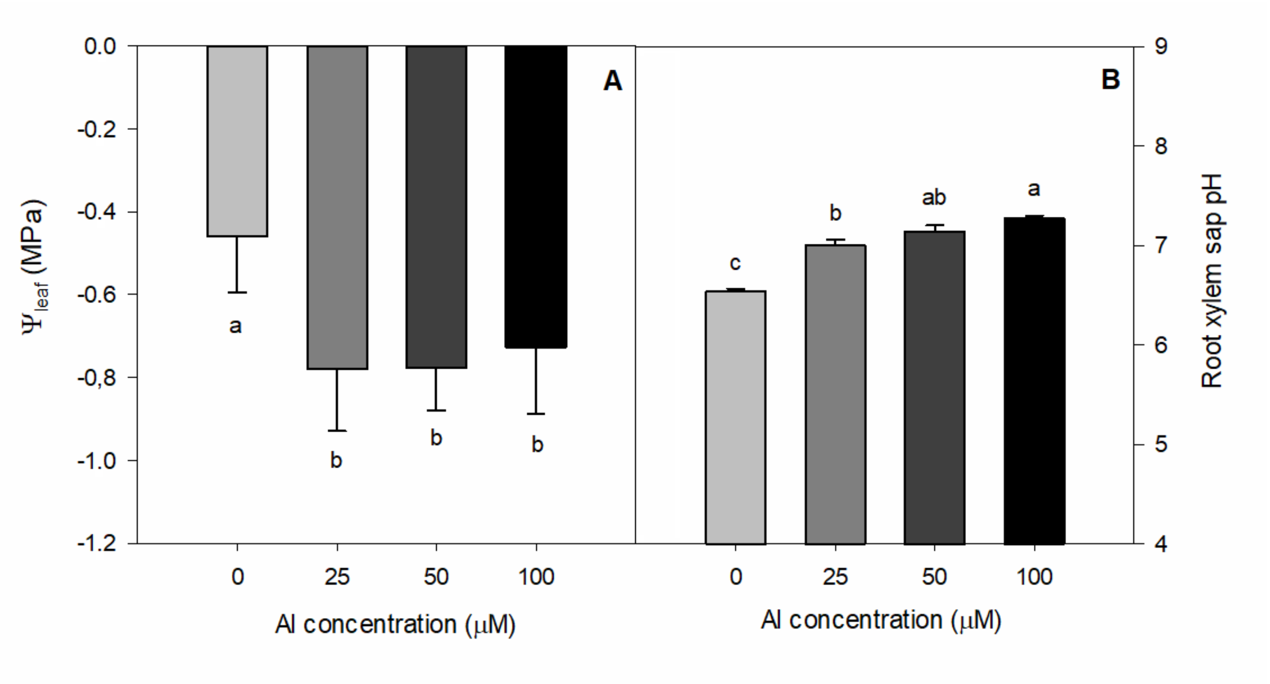


Fig 9. Leaf water potential (A) and root xylem sap pH (B) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=4 plants) and bars are standard errors.

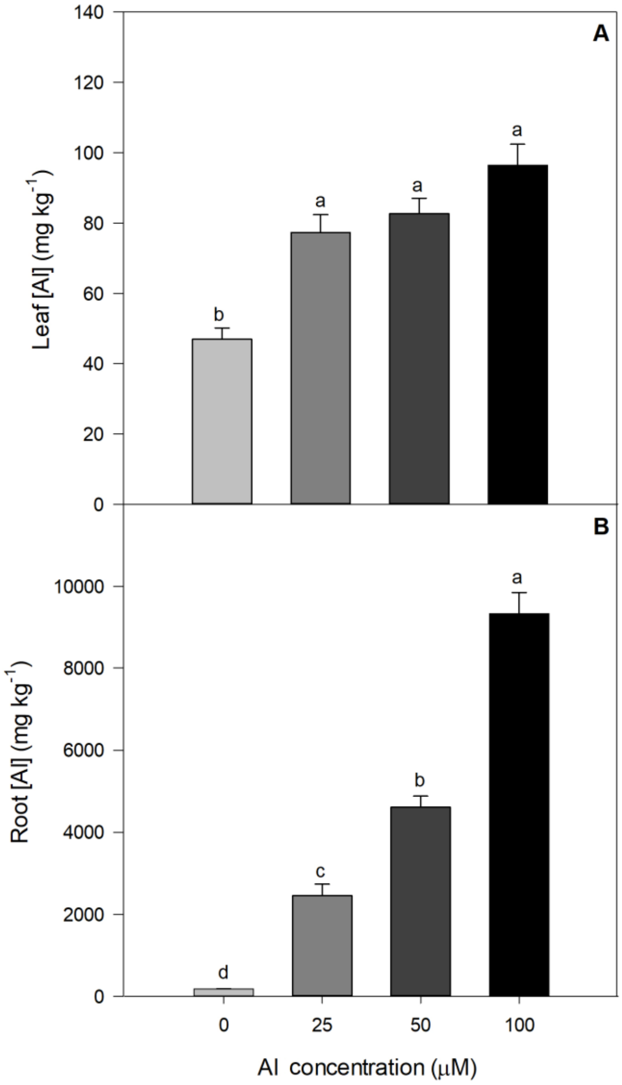


Fig 10. Aluminum concentration in leaves (A) and roots (B) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Distinct lowercase letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n=4 plants) and bars are standard errors.

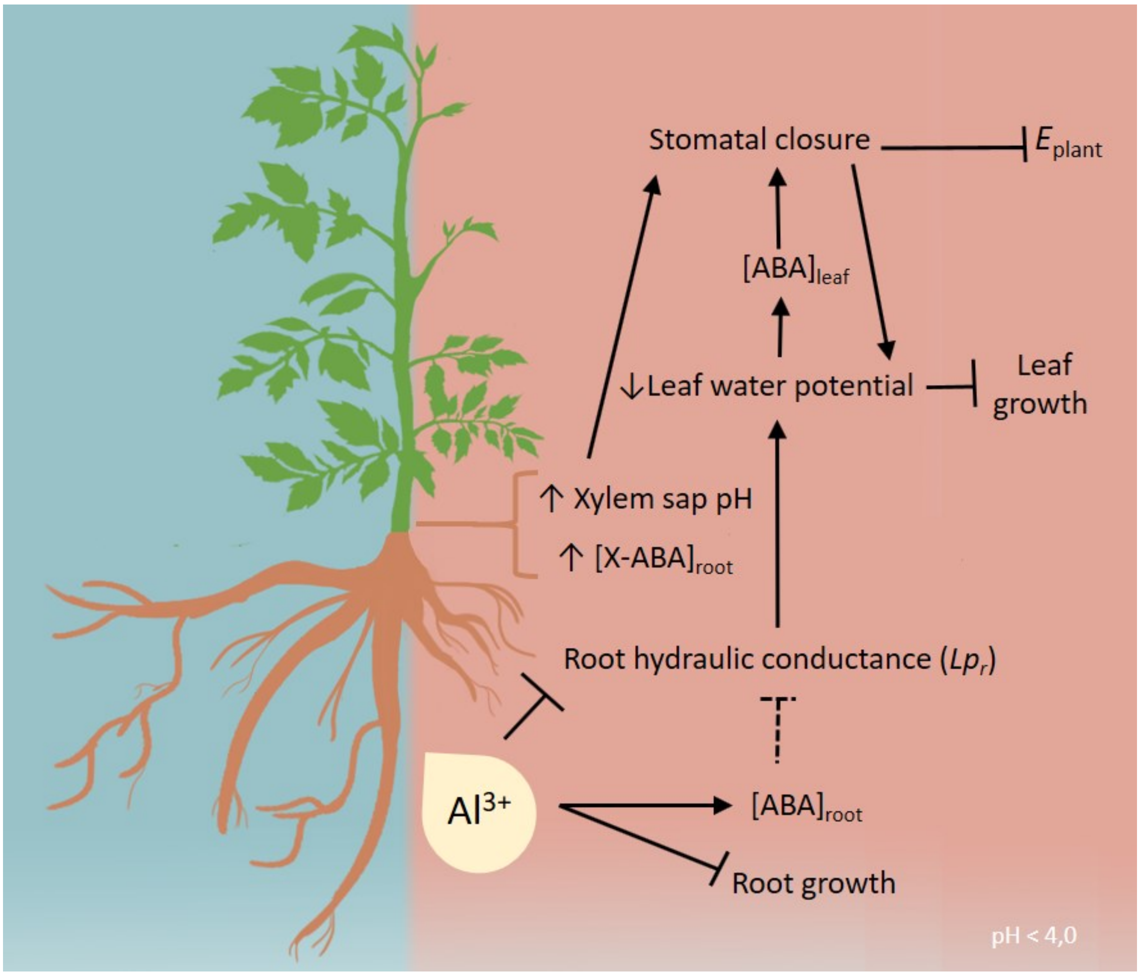
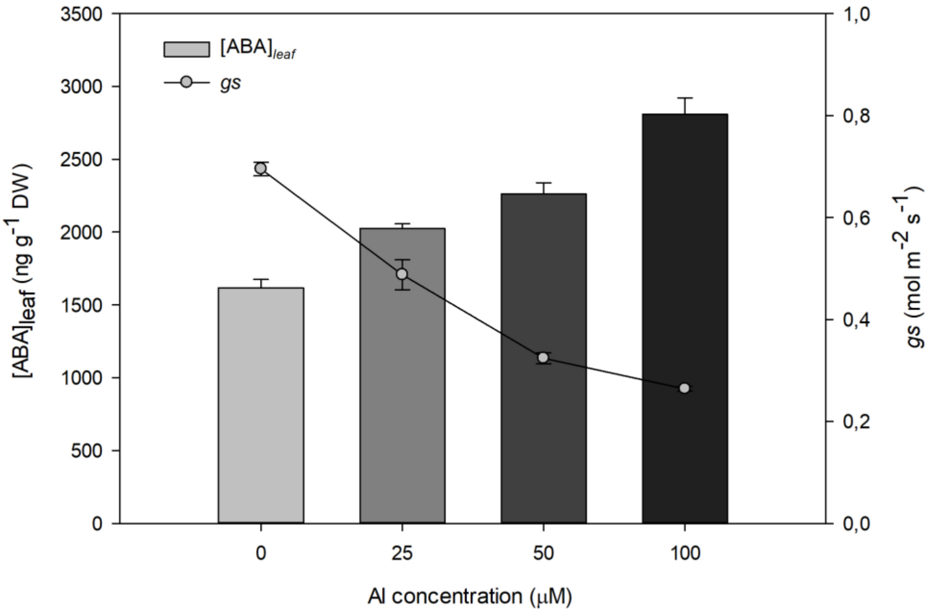


Fig 11. Model of plant hydraulics and abscisic acid (ABA) impacts on stomatal conductance of tomato plants (*Solanum lycopersicum*) exposed to Al toxicity (on the right). Lines ending in arrowheads indicate a positive impact, while lines ending in a bar indicate negative impacts. Dashed lines indicate a possible effect. Whole-plant transpiration (E_{plant}), leaf ABA concentration ($[\text{ABA}]_{\text{leaf}}$), ABA concentration in root xylem sap ($[\text{X-ABA}]_{\text{root}}$), root ABA concentration ($[\text{ABA}]_{\text{leaf}}$).



Supplemental Fig. S1. Leaf abscisic acid concentration ($[ABA]_{leaf}$) versus stomatal conductance (*gs*) of tomato plants cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM Al. Columns and dots are mean values (n=4 plants, n=8 plants, respectively) and bars are standard errors.

CAPÍTULO II:

ABA biosynthesis through differential up-regulation of NCED genes mediates stomatal closure under aluminum stress in *Citrus limonia*

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Abstract

Aluminum (Al) is a major constraint for plant growth in acidic soils. Al-induced decreased in leaf hydration has been associated with stomatal closure and low gas exchange. However, the mechanisms involved in these responses are unclear. *Citrus limonia* was exposed to 0 and 1480 µM Al in nutrient solution for 90 days to understand whether the low *gs* and leaf hydration in plants exposed to Al is associated with increased 9-*cis*-epoxycarotenoid dioxygenase (NCED) expression and ABA biosynthesis. Al triggered the expression of *ClNCED3* and ABA biosynthesis in the roots before impairments in leaf hydration (low Ψw, RWC and *gs*) could be observed in leaves. In addition, leaf ABA concentration increased from 7 to 90 days, which could be explained by the increasing expression of *ClNCED3*, *ClNCED1* and *ClNCED5* in this organ. Stomatal closure was observed concomitantly with the increase of ABA concentration and these results provide further evidence for the role of ABA modulation of plant hydration under Al toxicity.

Introduction

Low leaf gas exchange rates in plants exposed to aluminum (Al) have long been debated by the scientific community. Several studies have shown reduced stomatal conductance (*gs*) in plants under aluminum (Al) toxicity. In relation to plants not exposed to Al, the decrease in *gs* values reach -80% in *Solanum lycopersicum* (Simon et al., 1994) and *Secale cereale* (Silva et al., 2012), -44% in *Zea mays* (Anjum et al., 2016), -38% in *Hordeum vulgare* (Ali et al., 2011), -30% in *Citrus reshni* ('Cleopatra' tangerine) (Chen et al., 2005b), -40% in *C. grandis* ('Sour Pummelo') (Jiang et al., 2008) and -50% in *C. limonia* ('Rangpur' lime) (Silva et al., 2018).

As *gs* is controlled mainly by plant water status (Dodd et al., 2003; Huber et al., 2019), one explanation for low *gs* values in plants exposed to Al involves the main symptom of Al toxicity, which is the inhibition of root growth (Kopittke et al., 2008; Horst et al., 2010; Singh et al., 2017). Actually, Al-induced decrease in *gs* is considered an indirect (long distance) effect of Al, as this metal is mainly retained in negatively charged pectin nets of root cells (Kopittke et al., 2015). Thus, the low root growth caused by Al results in low root surface area (Panda et al., 2009; Szatanik-Kloc, 2016) responsible for water uptake, eventually leading to water deficiency (Tamás et al., 2006; Yang et al., 2013) and low *gs* (Vitorello et al. 2005). However, most studies in which Al induced low *gs* values (Simon et al., 1994; Jiang et al., 2008; Ali et al., 2011; Silva et al., 2012; Banhos et al., 2016; Silva et al., 2018; Cavalheiro et al. 2020) were conducted using plants growing directly in nutrient solution, where water is constantly available. In addition, fibrous xylem vessels (Banhos et al., 2016), more lignin deposition (Silva et al., 2019) and structural damage (Batista et al., 2013) in the vascular cylinder have been observed in plants under Al toxicity. Therefore, it is unlikely that low *gs* values in plants exposed to Al are to be controlled only by the reduced root growth causing low water uptake.

Abscisic acid (ABA) is another factor that strongly controls stomatal movement (Zhang and Davies, 1989; Merilo et al., 2015), being stomatal closure one of the most studied ABA roles in response to abiotic stresses such as drought, temperature and high salinity (Xiong and Zhu, 2003; Mehrotra et al., 2014). When plants are subjected to drought, ABA rapidly accumulates, inducing stomatal closure to reduce water loss via transpiration (Zhang et al., 2008; Estrada-Melo et al., 2015). Cellular ABA concentration fluctuates continuously, enabling plants to meet their needs of growth and development as well as cope with the changing environmental conditions (Ma et al., 2018). Endogenous ABA concentration is regulated by its biosynthesis (Ng et al., 2014), which originates from the catalysis of carotenoid precursors for several enzymes found in higher plants (Xu et al., 2013). Comparatively few studies have examined changes in ABA in plants exposed to Al, and these studies are totally focused on the possible role of ABA in the tolerance of roots against Al (Kopittke et al., 2016). A key enzyme involved in ABA biosynthesis is 9-*cis*-epoxycarotenoid dioxygenase (NCED), which catalyzes the rate-limiting step in the regulation of ABA biosynthesis (Iuchi et al., 2001). NCEDs belong to a small multigene family containing five members (*NCED2*, *3*, *5*, *6*, *9*) in *Arabidopsis thaliana* (Tan et al., 2003), and *NCED3* is mainly responsible for ABA accumulation under drought stress in *Arabidopsis* (Iuchi et al., 2001). More recently, *NCED3* was demonstrated to act together with *NCED5* against drought stress (Frey et al., 2012). Citrus *NCED3* ortholog, *CrNCED1*, was isolated from ‘Cleopatra’ mandarin (*Citrus reshni*) and overexpressed in transgenic tobacco plants, which exhibited higher levels of ABA under normal growth conditions and enhanced the ABA synthesis and tolerance under dehydration, drought, salt, and oxidative stresses when compared with WT (Xian et al., 2014). Although NCED genes have been well characterized in model plans under water deficiency (Xian et al., 2014), the roles of NCED in plants exposed to Al toxicity is still unknown.

Thus, considering that Al causes reduction in *gs* by a mechanism not yet elucidated, it is possible that, similarly to water deficiency, Al toxicity alters *NCED* expression and producing higher amount of ABA when compared to plants not exposed to Al. In the present study, we investigated whether 1480 µM Al provided in nutrient solution induced the expression of *NCED1*, *NCED3* and *NCED5*, as well as ABA accumulation in roots and leaves of *C. limonia* in a 90-day experiment. In addition, we seek to elucidate if ABA biosynthesis was induced before or after the decreases in leaf hydration, evidenced by leaf water potential (Ψ_w), leaf relative water content (RWC) and, mainly, *gs*.

Material and methods

Plant material and experimental conditions

Seventy-two three-month-old and 15 ± 1 cm-high 'Rangpur' lime plants (*Citrus limonia* L.) were used for studying the hydration capacity when submitted to Al within a 90-day period. At the beginning of the study, the plants had five leaves and were grown directly on an aerated nutrient solution inside opaque plastic boxes (50 cm in length x 30 cm in width x 15 cm in height; 20 L), with six plants per box.

The nutrient solution used shows a chemical composition based on the solution proposed by Clark (1975). It has been used to test Al tolerance in *C. limonia* (Banhos et al. 2016; Silva et al. 2018; Silva et al. 2019). It contained 1372.8 µM $Ca(NO_3)_2$ 4 H_2O, 507 µM NH_4NO_3, 224.4 µM KCl, 227.2 µM K_2SO_4, 218.6 µM KNO_3, 483.2 µM $Mg(NO_3)_2$ $6H_2O$, 12.9 µM KH_2PO_4, 26.01 µM $FeSO_4$ $7H_2O$, 23.8 µM NaEDTA, 3.5

µM $MnCl_2$ $4H_2O$, 9.9 µM H_3BO_3, 0.9 µM $ZnSO_4$ 7 H_2O, 0.2 µM $CuSO_4$ $5H_2O$ and 0.4 µM $NaMoO_2$ 2 H_2O. In a previous study we observed Al-induced decrease in gas exchange rates when *C. limonia* was exposed to 1480 µM (Silva et al., 2018). Therefore, the solution contained macro and micronutrients as described above and also 0 and 1480 µM Al provided through $AlCl_3$ 6 H_2O. The pH of the solution was measured daily and kept at 4.0 ± 0.1 (using NaOH and/or HCl) to keep Al as soluble/available as possible, and the solution was totally replaced every 15 days.

Expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick), with six holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar. The boxes with six plants each were randomly arranged on benches (80 cm above the ground) inside a greenhouse with semi-controlled conditions (air temperature 28.5 ± 0.7°C; relative humidity 65.3 ± 2.1%). The photoperiod of 14h of natural sunlight measured inside the greenhouse provided a photosynthetic photon flux density (PPFD) of 862.7 ± 184.4 µmol photons m^{-2} s^{-1}.

Experimental design

After transplantation, six boxes (36 plants) remained with the nutrient solution containing 0 µM Al and six other boxes (36 plants) received the nutrient solution containing 1480 µM Al. The plants grew in these conditions for 90 days, and non-destructive measurements (leaf gas exchange) and destructive measurements [leaf water potential (Ψw), relative leaf water content (RWC) and collection of leaf and root tips for ABA measurements and NCED gene expression] were performed at 1, 7, 15, 30, 60 and 90 days after treatment (DAT). Using predawn (Ψ_{pd}) and midday (Ψ_{md}) leaf water potential and transpiration rates measured in the afternoon, we also estimated the hydraulic conductivity from roots to the leaves (K_L).

The excision of leaves for measuring Ψw, leaf discs for RWC and the collection of root tips were not performed on the same plants used for measuring leaf gas exchange, so that harmed plants did not interfere in the results of gas exchange rates. Leaf discs for RWC, and the collection of leaf pieces and root tips were performed as fast as possible, so that these variables interfered as little as possible in each other. In addition, the present study did not involve repeated measurements on the same plants through time, as one box (with 6 plants) per treatment was used on each evaluation date. The leaf pieces and root tips were collected and their RNA was extracted for measuring NCED gene expression. Using six extra plants (0 DAT and 90 DAT), the biomass of leaves, stems, roots and the total plant biomass were assessed in order to check the severity of the Al treatment.

Leaf gas exchange

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-6400xt; LI-COR, Lincoln, NE, USA). The water use efficiency (WUE; A/E) and intrinsic water use efficiency ($iWUE$; A/gs) were also calculated. The CO_2 concentration entering the leaf cuvette (LCF chamber; 2 cm^2, LI-COR) averaged 400 µmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR), as this was the air CO_2 concentration accepted for the experimental site. Measurements were taken between 9h and 11:30h on cloudless days, as it is the best period for measuring gas exchange parameters (Feistler and Habermann, 2012). We also measured gas exchange in the afternoon (13h-15h) in order to calculate the estimated hydraulic conductivity from roots to the leaves (K_L).

The PPFD in the leaf cuvette was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to provide 90% red and 10% blue light at 1500 μmol photos m^{-2} s^{-1}, as this value saturates *A* for *C. limonia* as observed in *A*/*Ci* curves (Silva et al. 2018). The vapor pressure deficit (VPD) inside the leaf cuvette was similar to the external environment (inside the greenhouse), which was not higher than 1.5 kPa (RH ~ 60%) on the days of measurements.

Water relations

Leaf water potential ($\Psi_{w;}$ MPa) was measured at predawn (Ψpd) and midday (Ψmd; under maximum VPD) by the pressure chamber method (Turner 1981), using a DIK-7000 (Daiki Rika Kogyo, Tokyo, Japan) chamber. Briefly, a leaf was wrapped with plastic film (to avoid water loss), excised from the plant with its petiole and it was immediately taken to the pressure chamber that stayed in the greenhouse. When the xylem is uncut (petiole still attached to the plant), it is under tension. When the leaf petiole is cut, it causes the water to pull back into the tissue, away from the cut surface. When the leaf was inside the chamber, it was pressurized, bringing the xylem sap back to the cut surface and the pressure was annotated.

Hydraulic conductivity from roots to the leaves (K_L; mmol H_2O m^{-2} s^{-1} MPa^{-1}) was estimated by the method proposed by Hubbard *et al.* (2001), which is based on Ohm's Law. For this, the following equation was applied:

$$K_L = E_{14h} / (\Psi pd - \Psi md),$$

where E_{14h} is the transpiration rate (*E*) measured in the afternoon under maximum VPD; Ψpd is predawn leaf water potential assumed as the soil water potential (Ψ_{soil}), and

Ψmd is midday leaf water potential, measured under maximum VPD. Although the plants were grown directly in nutrient solution (not soil), the Ψpd = Ψ_{soil} principle is still valid because Ψpd is measured before sunrise (in the absence of sunlight that could open stomata and cause transpiration) in non-transpiring plants and, therefore, Ψpd represents the plant's capacity to rehydrate over night, regardless it is grown in the soil or in a nutrient solution (Turner, 1981). Furthermore, this method was already used for measuring K_L in *Citrus sinensis* grown in nutrient solution (Magalhães Filho *et al.* 2009; Cavalheiro et al., 2020).

For measuring the relative leaf water content (RWC; %), leaf discs were collected in the afternoon from plants of both treatments and calculated as:

$RWC = [(FW-DW)/(TW-DW)]\times100$,

where FW is the fresh weight (immediately measured after collected); TW is the turgid weight after rehydrating samples for 24 h in 100 mL deionized water inside amber flasks (to avoid photosynthetic activity); and DW is the dry weight after oven-drying the discs at 60ºC for 48 h, according to Silva et al. (2018).

RNA extraction

Leaf pieces (≈ 1 cm^2) or root apices (0.5 cm in length), totaling 100 mg (fresh weight) for each leaf or root samples (n=4), were collected in the afternoon (13h-15h), frozen in liquid nitrogen (N_2) and stored at -80ºC for future analysis. Total RNA was extracted from leaf and root samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA (2 µg) was treated with RNase-free TURBO DNase (Ambion, Carlsbad, USA) and transcribed in reverse to cDNA using oligo-dT and the GoScript Reverse Transcription System kit (Promega Corp., Madison, WI, USA), according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

Gene expression analysis

Gene expressions were carried out by quantitative real-time PCR (qRT-PCR) with SYBR green GoTaq q-PCR Master Mix (Promega Corp., USA), using Applied Biosystems QuantStudio 3 (Life Technologies, Carlsbad, CA, USA). The primers of *Cl*NCED1, *Cl*NCED3 and *Cl*NCED5 used in the experiment were previously used in *Citrus* species (Agusti et al., 2007, Bassene et al., 2009), including in *C. limonia* (Neves et al., 2013) and are described in Table 1. As reference genes, we used GAPC2 and EFα (Table 1), which were proposed by Mafra et al. (2012) and used previously by Silva et al. (2019). Amplification efficiencies were calculated for each primer using Miner software (Zhao and Fernald 2005).

For calculating relative expression, Ct (cycle threshold) value of each sample (n=4), which were determined by the average of three technical replicates, was converted into relative quantities (RQ) according to Pfaffl (2001), using the following equation:

$RQ=E\Delta Ct$,

where E is the primer efficiency, and ΔCt is the difference between control Ct value for the evaluated gene and Ct value of the given sample. A normalization factor (NF) for each sample was calculated by the geometric mean of the RQ values of GAPC2 and EFα. Normalized-relative quantity (NRQ) of each sample was calculated as the ratio of the sample RQ and the appropriate NF. Individual fold change values were determined by dividing the sample NRQ by mean values of NRQ that were obtained from the calibrator, i.e., root samples of plants not exposed to Al. Following this, fold change in the control group always shows a mean value of 1.

ABA quantification

At least 200 mg dry powder was used for sample to quantify ABA concentration. Three technical replicates were performed for each of the four biological replicate (plant). The samples were extracted with a mixture of isopropanol and acetic acid (95:5, v/v) for 2 h under continuous shaking at 4 ◦C. Before the beginning of the extraction, 1 µg of labelled ABA ($[^2H_6]$-ABA; OlChemim, Czech Republic) were added to each sample. Further sample preparation was performed according to Zörb et al., (2013). Endogenous ABA concentrations were determined simultaneously by gas chromatography-tandem mass spectrometry-selective ion monitoring according to the principles of isotope dilution (Cohen et al., 1986), using ions at m/z 190/194 (endogenous and labelled standard; while the molecular structure of ABA would have six deuterium incorporated, during fragmentation of ABA two deuterium are lost and the fragmentation ion at m/z 194 has only four deuterium retained) for methylated ABA (Walker-Simmons et al., 2000).

Biometric parameters

At 0 and 90 DAT, after separating the plant parts into leaves (plus petioles), shoots and roots, the number of leaves was counted and the shoot length was measured with a ruler (cm). The total leaf area per plant (LA, cm^2) was measured with an area meter (LI-3100C, LI-COR, USA). Total root length, root surface area and root diameter were measured using a scanner (Epson perfection v700 photo, Suwa, Japan) which was coupled to a computer running the WinRHIZO^{TM} software (Regent Instruments, Canada). The biomass (g) of organs was weighed on a 0.001g precision scale (AR2140, OHAUS, USA) after oven-drying the samples at 60°C until constant mass.

Aluminum quantification

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

Data analysis

Data of leaf gas exchange parameters (*A*, *gs*, *E*, *Ci*, *WUE* and *iWUE*), RWC and biomass were measured using six replicates (plants). Leaf water potential (Ψpd and Ψmd), estimation of hydraulic conductivity from roots to the leaves (K_L), gene expression of NCED and ABA quantification were assessed using four replicates (plants).

A student's t-test ($\propto = 0.05$) was used, separately for each evaluation date (1, 7, 15, 30, 60 and 90 DAT), to test for differences between 0 and 1480 µM Al for each variable, as well as when testing for differences in plant biomass and Al concentration in plant organs between 0 and 90 DAT within each treatment.

Results

From 0 to 90 DAT, the main root length (Fig. 1A), root surface area (Fig. 1B), root diameter (Fig. 1C) and root biomass (Fig. 1D) increased by 23%, 46%, 185% and 81%, respectively, in plants exposed to Al and by 136%, 282%, 128% and 418%, respectively, in control plants. At 90 DAT, Al significantly limited the main root length (-48%) (Fig. 1A), root surface area (-62%) (Fig. 1B) and root biomass (-65%) (Fig. 1D), while the root diameter was enhanced in plants exposed to Al (+25%) (Fig. 1C). From 0 to 90 DAT, the leaf number (Fig. 2A), leaf area (Fig. 2B) and leaf biomass (Fig. 2C) increased by 31%, 83% and 59%, respectively, in plants exposed to Al and by 140%, 504% and 393%, respectively, in control plants. At 90 DAT, Al significantly inhibited the leaf number (-45%) (Fig. 2A), leaf area (-70%) (Fig. 2B) and leaf biomass (-68%) (Fig. 2C). These results can be observed in Supplemental Fig. S1. Thus, Al inhibited root growth, leaf initiation, leaf expansion and organ biomass accumulation, but caused root thickening.

Compared to plants not exposed to Al, values of *A* (Fig. 3A), *gs* (Fig. 3B) and *E* (Fig. 3C) decreased from 7 DAT, and at 90 DAT these parameters were 71%, 78% and 60% lower in plants exposed to Al. On the other hand, *Ci* values were higher in plants exposed to Al from 15 DAT and this difference was 55% at 90 DAT (Fig. 3D). The WUE was the same between the treatments throughout the study (Fig. 3E), while iWUE was higher in plants exposed to Al from 30 DAT and this difference was 108% at 90 DAT (Fig. 3F). Thus, limited gas exchange rates is mediated by stomatal closure under Al stress.

Predawn leaf water potential (Ψpd) was the same between plants exposed and not exposed to Al throughout the study (Fig. 4A). However, plants exposed to Al showed Ψmd (Fig. 4B) and RWC (Fig. 5) lower than control plants from 7 DAT. At 90 DAT, compared to the control, the Al treatment reduced Ψmd by 80% (Fig. 4B) and RWC by 25% (Fig. 5). Thus, Al decreased leaf hydration. The estimated hydraulic conductivity from soil to the leaf (K_L) followed the same Ψmd response pattern, being lower for plants exposed to Al from 7 DAT (Fig. 6). At 90 DAT, compared to the control, the Al treatment reduced K_L by 80% (Fig. 6). Thus, Al negatively affected water relations and leaf hydration in plants grown in nutrient solution.

In the presence of Al, NCED genes were up-regulated, mainly in leaves. In this organ, Al induced up-regulation of *ClNCED3* from 15 DAT (Fig. 7C), and *ClNCED1* and *ClNCED5* from 60 DAT (Fig. 7A and 7E). At 90 DAT, the expression of *ClNCED3* in leaves was 78-fold higher in plants exposed to Al, while *ClNCED1* and *ClNCED5* were 80 and 30-fold higher than the control plants, respectively, from 60 DAT (Fig. 7A, 7E). In roots, *ClNCED3* was up-regulated by Al at all evaluation dates, being 16-fold higher than control plants at 30 DAT (Fig. 7D). In addition, *ClNCED5* was differentially expressed (4-fold higher) in roots of plants exposed to Al only at 60 DAT, while no difference in *ClNCED1* expression was found between the treatments in the roots (Fig. 7B). Thus, Al up-regulated the key genes of ABA biosynthesis before changes in leaf hydration.

In general, Al increased ABA concentrations in leaves and roots (Fig. 8A, 8B). Leaf ABA concentrations ($[ABA]_{leaf}$) were more than 35 times higher than those found in the root ($[ABA]_{root}$) of plants exposed to Al. $[ABA]_{leaf}$ increased from 7 DAT in plants exposed to Al, being 9-times higher than the control at 90 DAT (Fig. 8A). $[ABA]_{root}$ was higher in plants exposed to Al since the first evaluation date, being 5-times higher than the control at 30 DAT (Fig. 8B). Throughout the study, $[ABA]_{leaf}$ increased as *gs* decreased in the presence of Al, which was not observed for the control plants that maintained low $[ABA]_{leaf}$ and larger *gs* values (Supplemental Fig. S2). As expected, Al concentration in the roots was approximately 10 times higher than the leaves of plants exposed to Al (Supplemental Fig. 3A and 3B). From 0 to 90 DAT, leaf and root Al concentration increased by seven- (Supplemental Fig. 3A) and 15-times (Supplemental Fig. 3B), respectively, in plants treated Al when compared to the control plants. Thus, Al increased ABA biosynthesis and accumulation mainly in leaves

Discussion

It is widely known that excessive Al in acidic soils inhibits root growth, especially in crop plants (Kochian et al., 2005; Singh et al., 2017, Wang et al., 2019). In this matter, a reduced root growth leads to low root surface area and, consequently, lower soil volume is explored, resulting in reduced water and nutrient uptake, which is particularly important under conditions of low soil moisture. Low values of Ψw, RWC and *gs* have been found in plants exposed to Al exhibiting reduced root growth, but smaller roots are unlikely to be the only factor explaining the Al-induced decrease in leaf hydration and *gs*.

Some Al toxicity studies focus on a recurrent response in plants exposed to Al, which is the decrease in CO_2 assimilation rate (*A*), but it may be considered a secondary effect in plants exposed to Al, including *Citrus* spp. (Pereira et al., 2000; Chen et al., 2005a,b; Jiang et al., 2008, 2009). One of the main causes of Al-induced decrease in *A* values is the stomatal closure, as observed in 'Cleopatra' tangerine (-30%; Chen et al., 2005b), 'Sour Pummelo' (-40%; Jiang et al., 2008) and 'Rangpur' lime (-50%; Silva et al., 2018). Accordingly, in the present study, low *A* values were associated with low *gs* and *E* from 7 DAT in plants exposed to 1480 µM Al (Fig. 3A-C) when compared to the control plants. In our previous studies using *Citrus limonia* ('Rangpur' lime plants), low *A* values are greatly explained by low *gs* rather than other factors, such as low photochemical performance (Banhos et al., 2016; Silva et al, 2018), although the latter is mentioned in other studies using *Citrus* (Chen et al., 2005a,b; Jiang et al., 2008, 2009). Thus, when investigating the low gas exchange rates in *Citrus* plants exposed to Al, but mainly in 'Rangpur' lime plants, it is the low leaf hydration leading to low *gs* that should be in the hot spot. In this regard, hydraulic and chemical factors controlling leaf hydration and *gs* should be scrutinized.

Hydraulic signals in the form of turgor changes in the leaves have been proposed to passively control stomatal aperture (Huber et al., 2019). In addition, hormonal stomatal control could have been evolved from a passive hydraulic control mechanism (McAdam and Brodribb, 2015). In the present study, plants exposed to Al showed low capacity to maintain leaf hydration, evidenced by low values of Ψmd (Fig. 4B) and RWC (Fig. 5) from 7 DAT, when compared to control plants. Banhos et al. (2016) and Silva et al. (2018) observed similar responses using the same species and conditions. On the other hand, the greatest capacity to supply water to the leaves can be represented by high K_L values (Rodríguez-Gamier et al., 2019), but the estimation of hydraulic conductance from roots to the leaves (K_L) showed the same decreasing pattern (Fig. 6) observed for Ψmd and RWC, indicating that water transport was reduced in 'Rangpur' lime plants exposed to Al. These results are supported by studies that evidenced fibrous xylem vessels (Banhos et al., 2016), more lignin deposition in the vascular cylinder (Silva et al., 2019), low expression of aquaporins (PIP family) in the roots and low K_L (Cavalheiro et al., 2020) in 'Rangpur' lime plants grown in nutrient solution with 1480 μM Al.

Even less attention is paid to chemical signals when investigating low leaf gas exchange rates and, more precisely, low stomatal conductance of plants under Al toxicity. Abscisic acid (ABA) plays a critical role in stomatal closure (Dodd et al., 2005; Munemasa et al., 2015). The quick upregulation of ABA biosynthesis upon changes in leaf turgor and water availability in the soil makes ABA a suitable candidate for actively initiating stomatal closure (McAdam et al., 2016; Sussmilch et al., 2017; Zhang et al., 2018). In the present study, root ABA biosynthesis occurred at 1 DAT in plants exposed to Al (Fig. 8B), when decreases in Ψw and RWC could not yet be observed, and $[ABA]_{root}$ kept increasing until 30 DAT (Fig. 8B). In the leaves, the increase in ABA was observed only from 7 DAT and its concentration was 10 times higher than in the roots (Fig. 8A and 8B) and its concentration kept increasing until 90 DAT (Fig. 8A). Moreover, in leaves of plants exposed to Al low *gs* values was accompanied by increased $[ABA]_{leaf}$ (Supplemental Fig. S2). Although physiologically intuitive, as far as we are concerned, the relationship between low *gs* and increased ABA had never been encountered in plants exposed to Al, even though Al symptoms have already been compared to drought (Yang et al., 2013; Pandey et al., 2014; Ahmed et al., 2016).

It has been well documented that the reaction catalyzed by the 9-cis epoxy carotenoid dioxygenase (NCED) is regarded as the rate-limiting step in the regulation of ABA biosynthesis (Thompson et al., 2000) and that ABA levels under the stresses are closely correlated with the transcript abundance of NCED genes, suggesting that NCEDs play a pivotal role in ABA accumulation (Iuchi et al., 2001; Xian et al., 2014). Here, we observed upregulation of *ClNCED3* in roots of plants exposed to Al from 1 DAT, when compared to the control, and this upregulation peaked at 30 DAT (15-fold; Fig. 7D), most likely being the key gene for the Al-induced $[ABA]_{root}$ described above. *ClNCED3* was even more expressed in leaves, reaching a 80-fold higher expression than control plants at 90 DAT (Fig. 7C) than in roots (15-fold higher than control plants at 30 DAT), also explaining the high ABA concentration in leaves (Fig. 8A) than in the roots (Fig. 8B). Taken together, these results suggest that the Al induces ABA biosynthesis primarily in root tips and, under continuous Al toxicity ABA biosynthesis is sustained in the leaves, which eventually closes the stomata. In a study testing drought in *C. limonia* plants, *ClNCED3* was highly expressed in the roots but not in the leaves (Neves et al., 2013). *ClNCED1* (Fig. 7A) and *ClNCED5* (Fig. 7E) were also up-regulated in leaves of plants exposed to Al, at 60 and 90 DAT (Fig. 7A and 7E), indicating that more than one NCED gene is required to maintain endogenous levels of

ABA under Al toxicity. NCED5 was also up-regulated in leaves of *C. limonia* submitted to 40 days of drought (Neves et al., 2013). As previously mentioned, *NCED3* is predominantly induced by drought and controls endogenous ABA content in this condition (Endo et al., 2008; Hao et al., 2009). *AtNCED5* and *AtNCED3* participate together in water deficit response of *Arabidopsis thaliana* (Frey et al., 2012), and *NCED1* is up-regulated by drought in leaves of *C. sinensis* (Rodrigo et al., 2006; Xian et al., 2014) and *C. reshni* (Zandalinas et al., 2016), as also observed here in the leaves, but not in the roots of plants exposed to Al, where *ClNCED1* was upregulated (Fig. 7B). Taken together, our results could suggest that Al triggers ABA biosynthesis in root tips of 'Rangpur' lime plants that also increases ABA biosynthesis and accumulation in the leaves, which eventually induces stomatal closure and limit leaf gas exchange. The low *gs* could cause a decrease in water flux from the roots to the leaves, as evidenced by the low K_L in the presence of Al (Fig. 6). However, Al also caused low leaf hydration, as evidenced by the low Ψmd (Fig. 4B) and decreased RWC (Fig. 5). Then, the increased ABA, shown here in plants exposed to Al, explains great part of the low *gs*, but stomata could also close due to a lack of appropriate mesophyll hydration caused by impaired water transport from roots to the leaves, and whether Al directly impair water transport through decreased root water conductivity as suggested by Banhos et al. (2016), Silva et al. (2019) and Cavalheiro et al. (2020) remains to be better elucidated.

One could still argue that the conspicuous decrease in root growth parameters caused by Al at 90 DAT (Fig. 1A-D) could have acted as a physical limitation for water uptake, which could not maintain the water vapor loss by leaves, explaining the low *gs* values. However, this low root growth was followed by reduced leaf number (Fig. 2A), leaf area (Fig. 2B) and leaf biomass (Fig. 2C), which could compensate for the decrease in their root growth. But even if such compensation occurs, here we demonstrate that low *gs* was already observed at 7 DAT (Fig. 3), when low Ψmd (Fig. 4), RWC (Fig. 5) and increased *ClNCED3* expression in roots and more $[ABA]_{root}$ were also noted. We have measured biometric parameters only at 90 DAT, but it seems unlikely that reduced root growth at 7 DAT could have caused low gs values due to less root responsible for water uptake.

Conclusions

In the present study we showed that in *C. limonia* Al triggered *NCED3* expression and ABA biosynthesis in the roots ($[ABA]_{root}$) at 1 DAT, before impairments in leaf hydration (low Ψw, RWC and *gs*) could be observed. In addition, $[ABA]_{leaf}$ increased from 7 to 90 DAT and this result could be explained by the increasing expression of *ClNCED3*, *ClNCED1* and *ClNCED5* in this organ. Finally, stomatal closure was observed concomitantly with increasing ABA concentration and this results provides further evidence about the role of ABA modulation of plant hydration under aluminum stress.

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Table 1. List of gene primers used for qRT-PCR analysis.

Gene name	Forward	Reverse
GAPC2	5’-TCCTATGTTTGTTGTGGGTG-3’	5’-GGTCATCAAACCCTCAACAA-3’
EFα	5’-TCAGGCAAGGAGCTTGAGAAG-3’	5’-GGCTTGGTGGGAATCATCTTAA-3’
NCED1	5’-GACCAGC AAGTGGTGTTCAA-3’	5’-AGAGGTGGAAACAGGAGCAA-3’
NCED3	5’-GGAGAATGAGGATGATGGCTAC-3’	5’-CTTTCGCGCTTATGAACGTG-3’
NCED5	5’-CTTCCCAACGAAGT CCATAG-3’	5’-GGATTCCATTGTGATTGCTG-3’

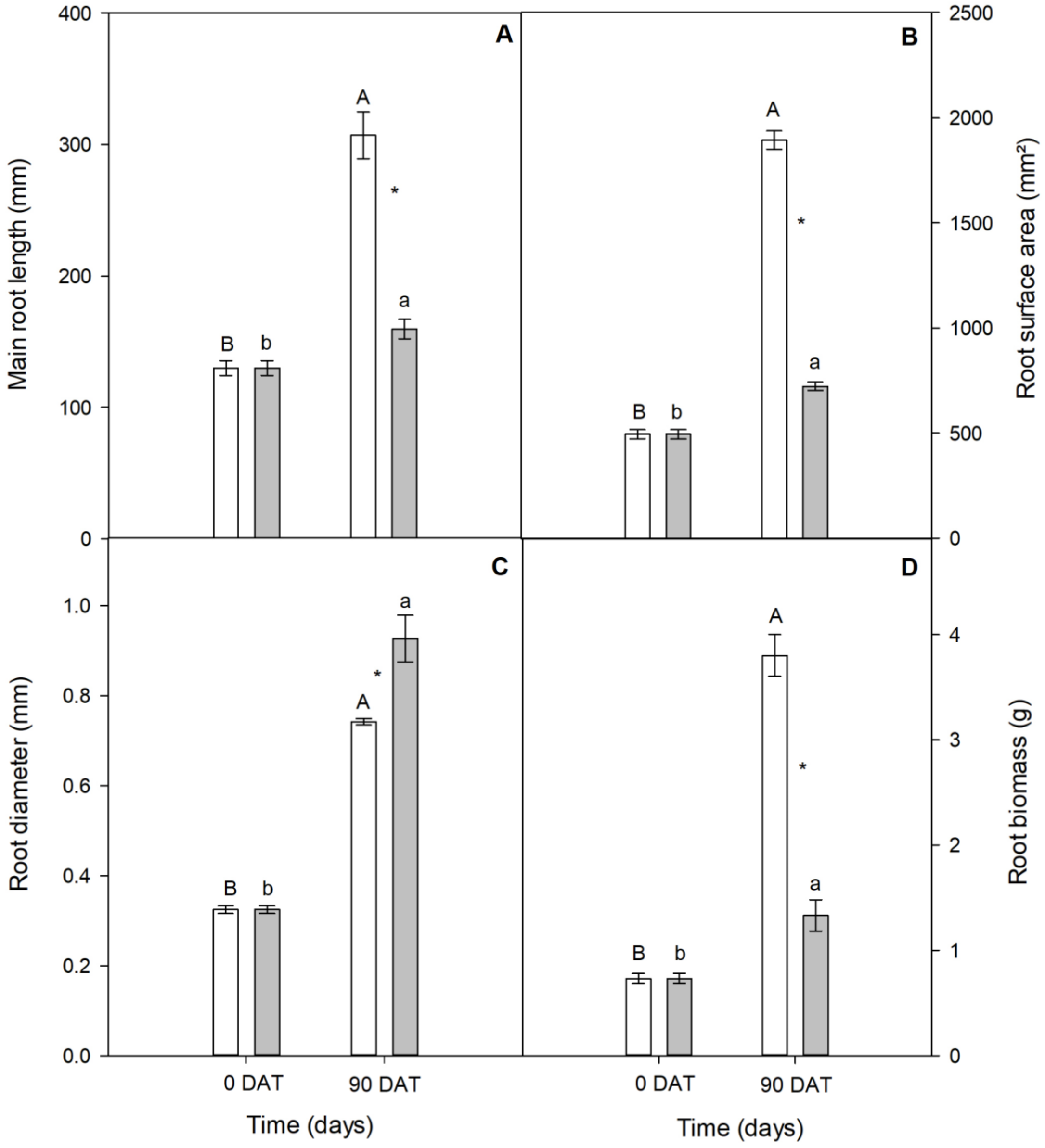


Fig. 1. Main root length (A), root surface area (B), root diameter (C) root biomass (D) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al.For plants not exposed to Al, distinct uppercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP; for plants exposed to Al, distinct lowercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Columns are mean values (n=6 plants) and bars are standard errors.

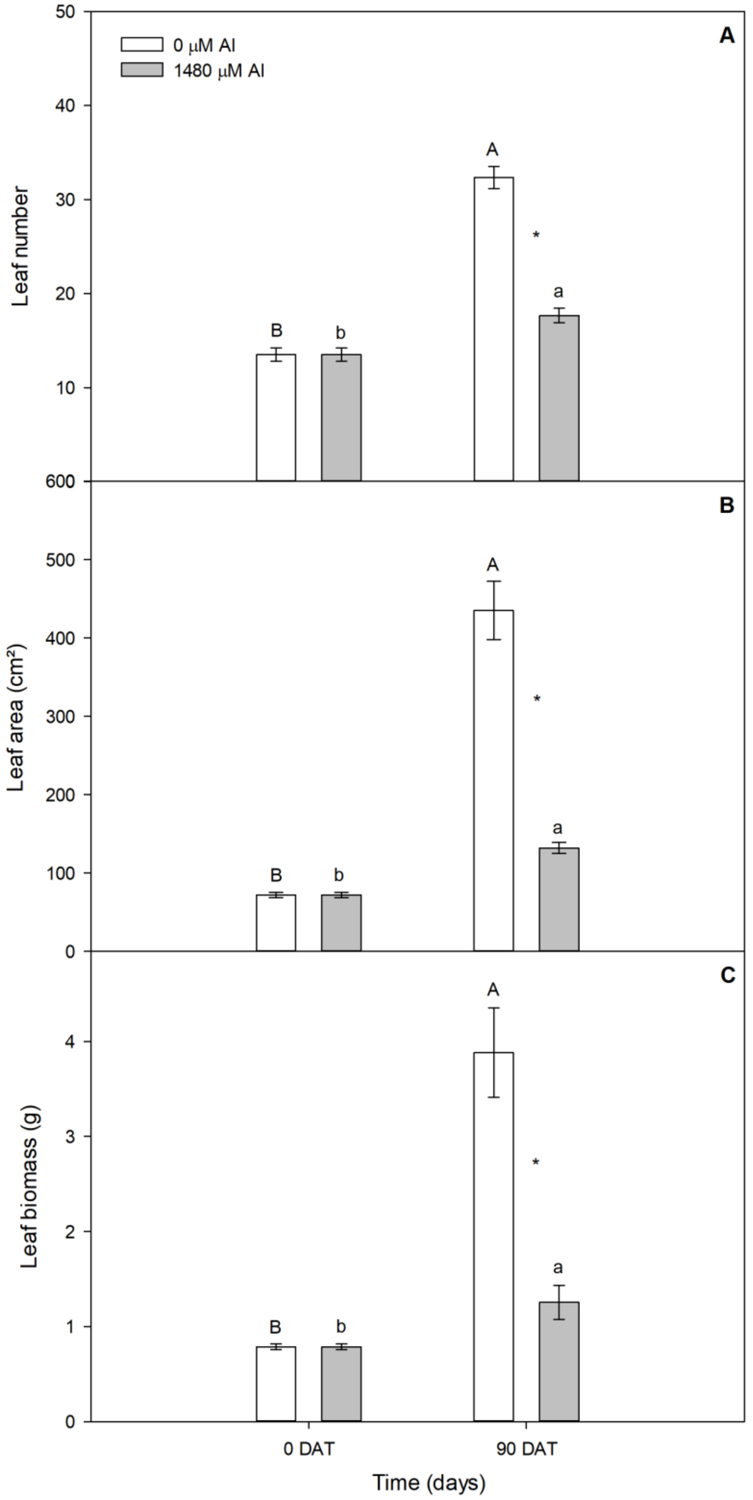


Fig. 2. Leaf number (A), area (B) and biomass (C) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. For plants not exposed to Al, distinct uppercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP; for plants exposed to Al, distinct lowercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Columns are mean values (n=6 plants) and bars are standard errors.

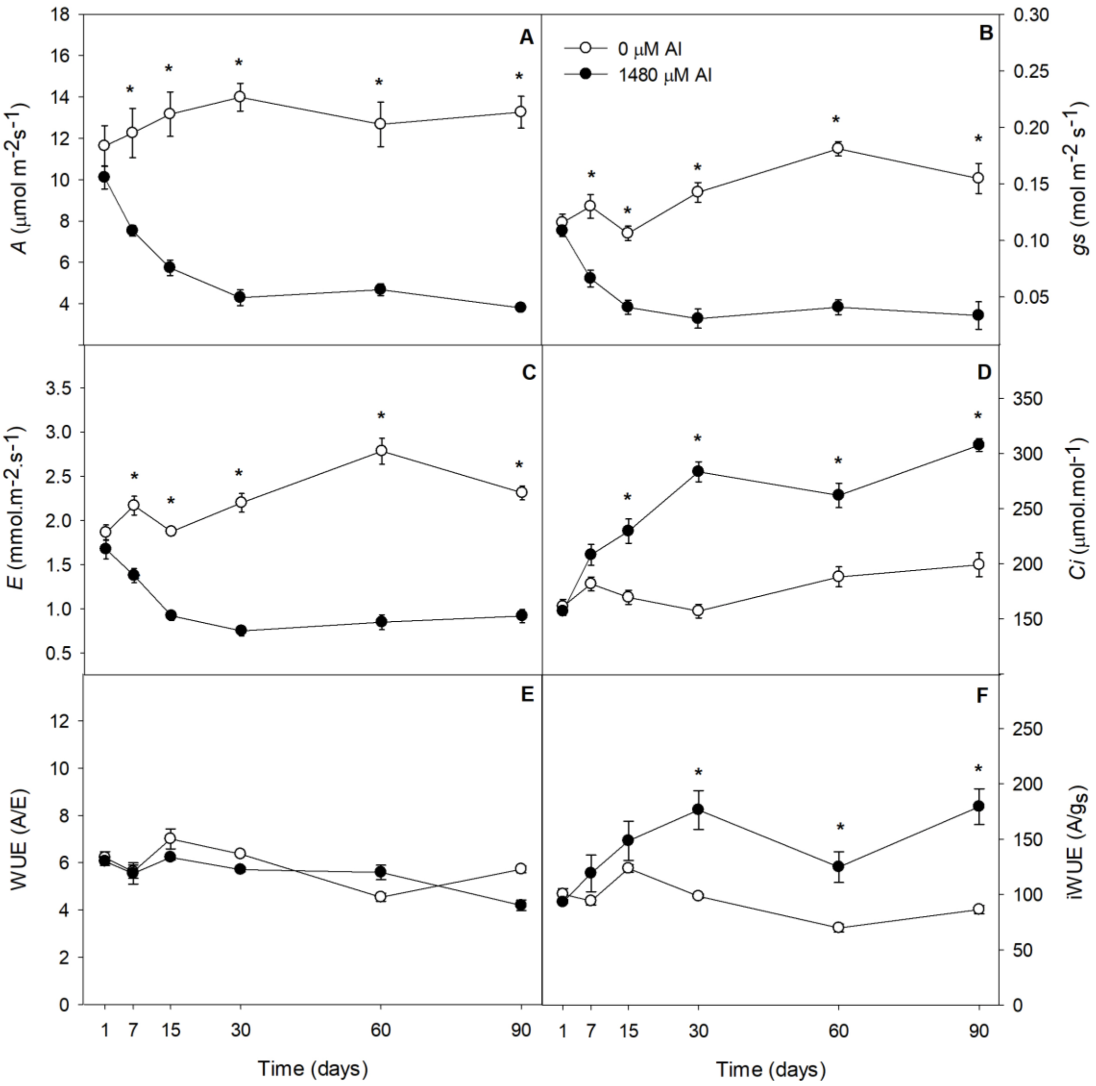


Fig. 3. Leaf gas exchange and water use efficiency of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. (A) CO_2 assimilation, (B) stomatal conductance, (C) transpiration, (D) intercellular CO_2, (E) water use efficiency and (F) intrinsic water use efficiency. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Dots are mean values (n=6 plants) and bars are standard errors.

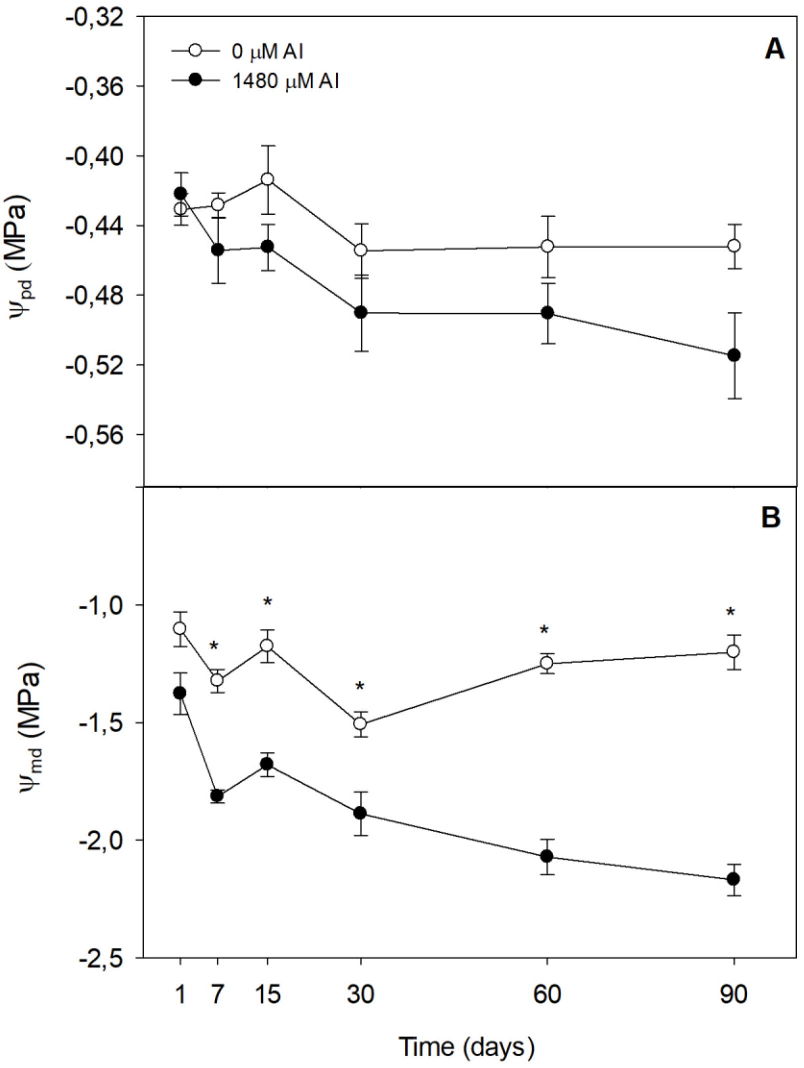


Fig. 4. Leaf water potential at predawn (Ψpd) (A) and midday (Ψmd) (B) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Dots are mean values (n=4 plants) and bars are standard errors.

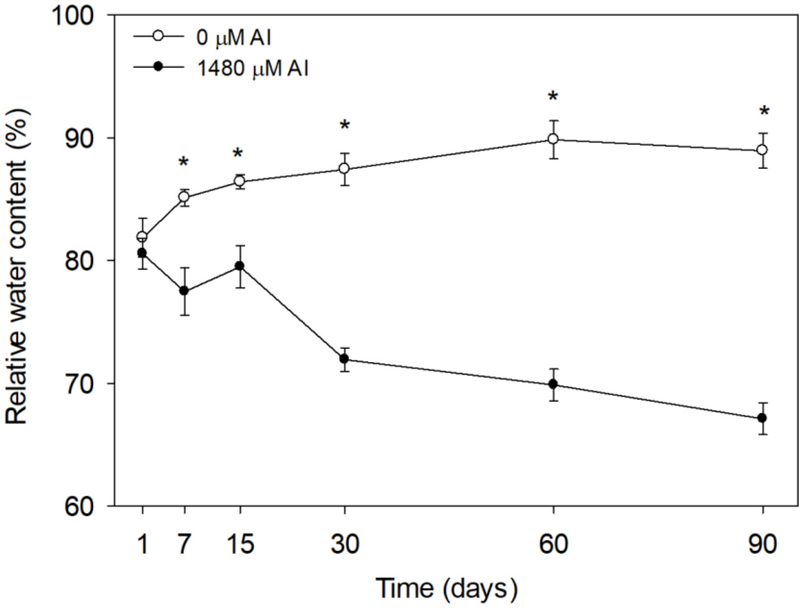


Fig. 5. Relative water content of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Columns are mean values (n=6 plants) and bars are standard errors.

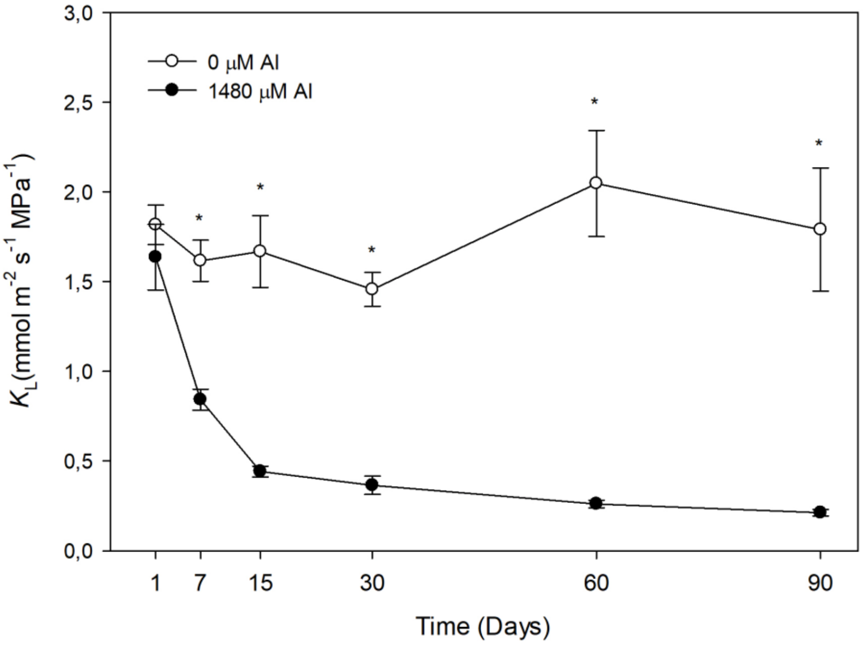


Fig. 6. Estimated hydraulic conductance from roots to the leaf in *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Dots are mean values (n=4 plants) and bars are standard errors.

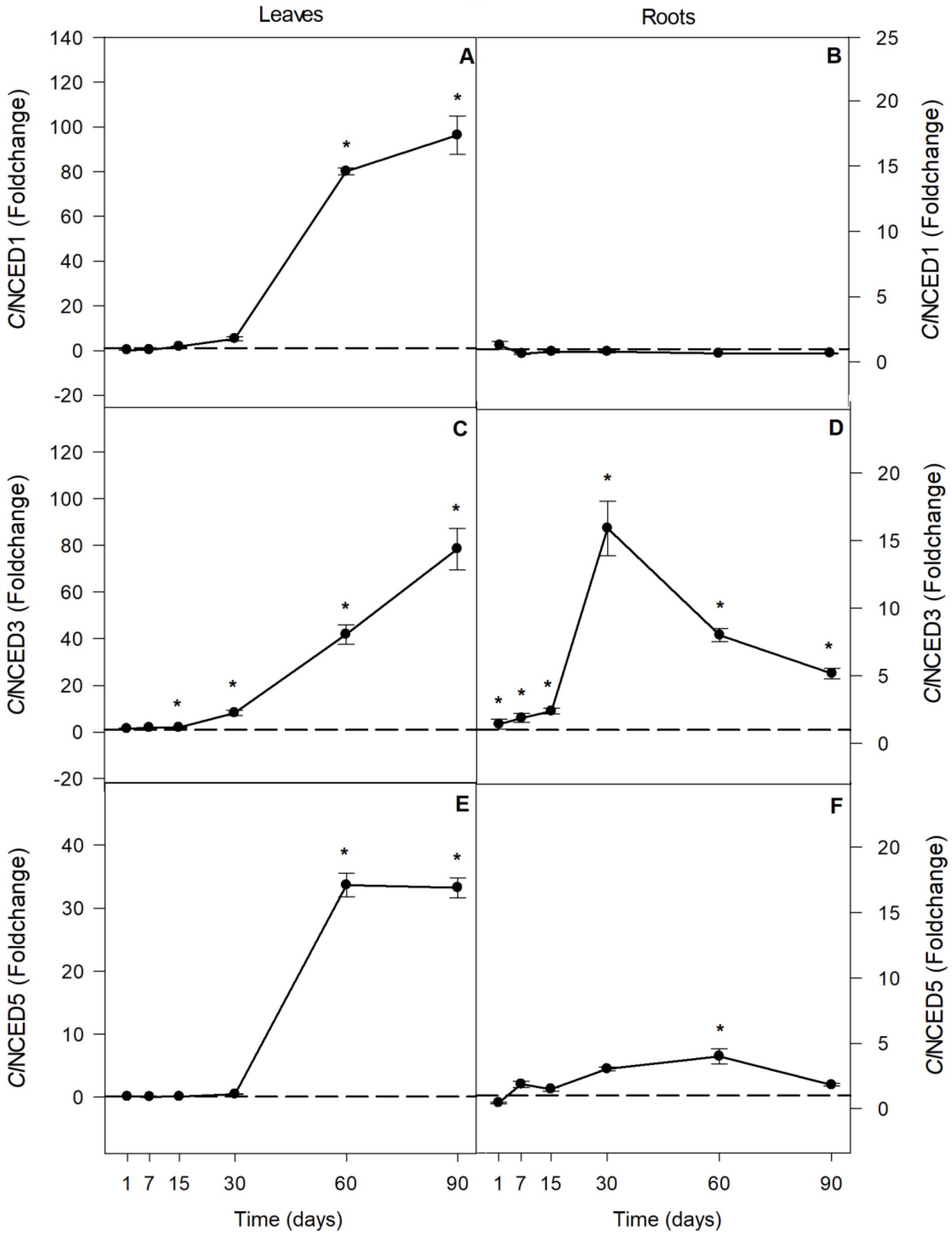


Fig. 7. Foldchange of normalized expression level of *ClNCED1*, *ClNCED3* and *ClNCED5* in leaves (A, C, E, respectively) and root tips (B, D, F, respectively) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Dots are mean values (n=4 plants) and bars are standard errors.

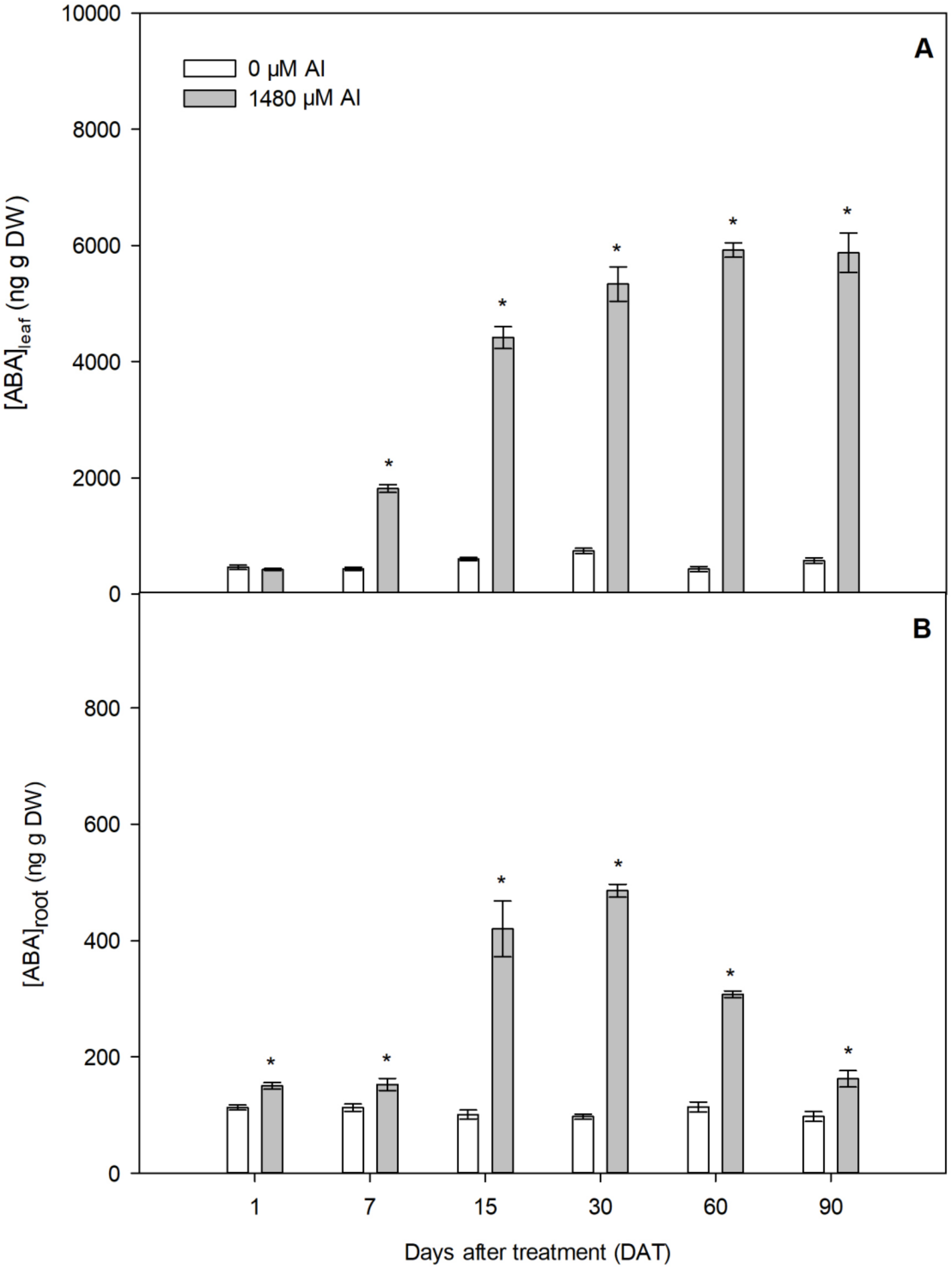
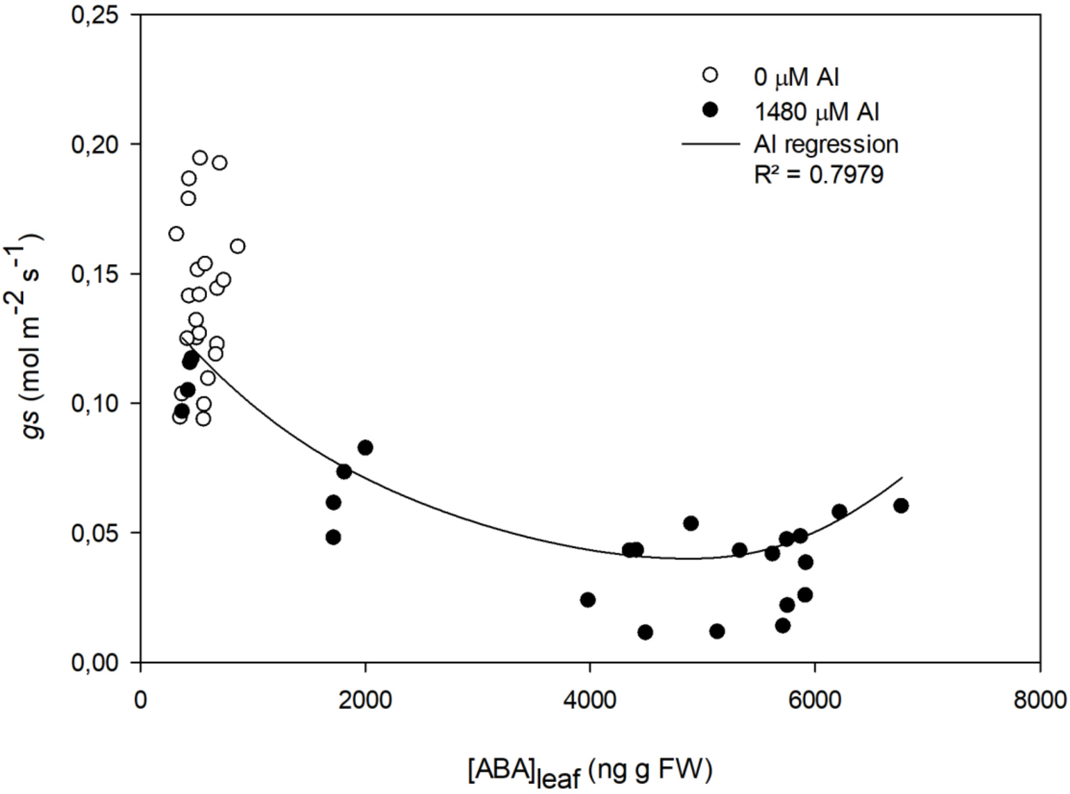


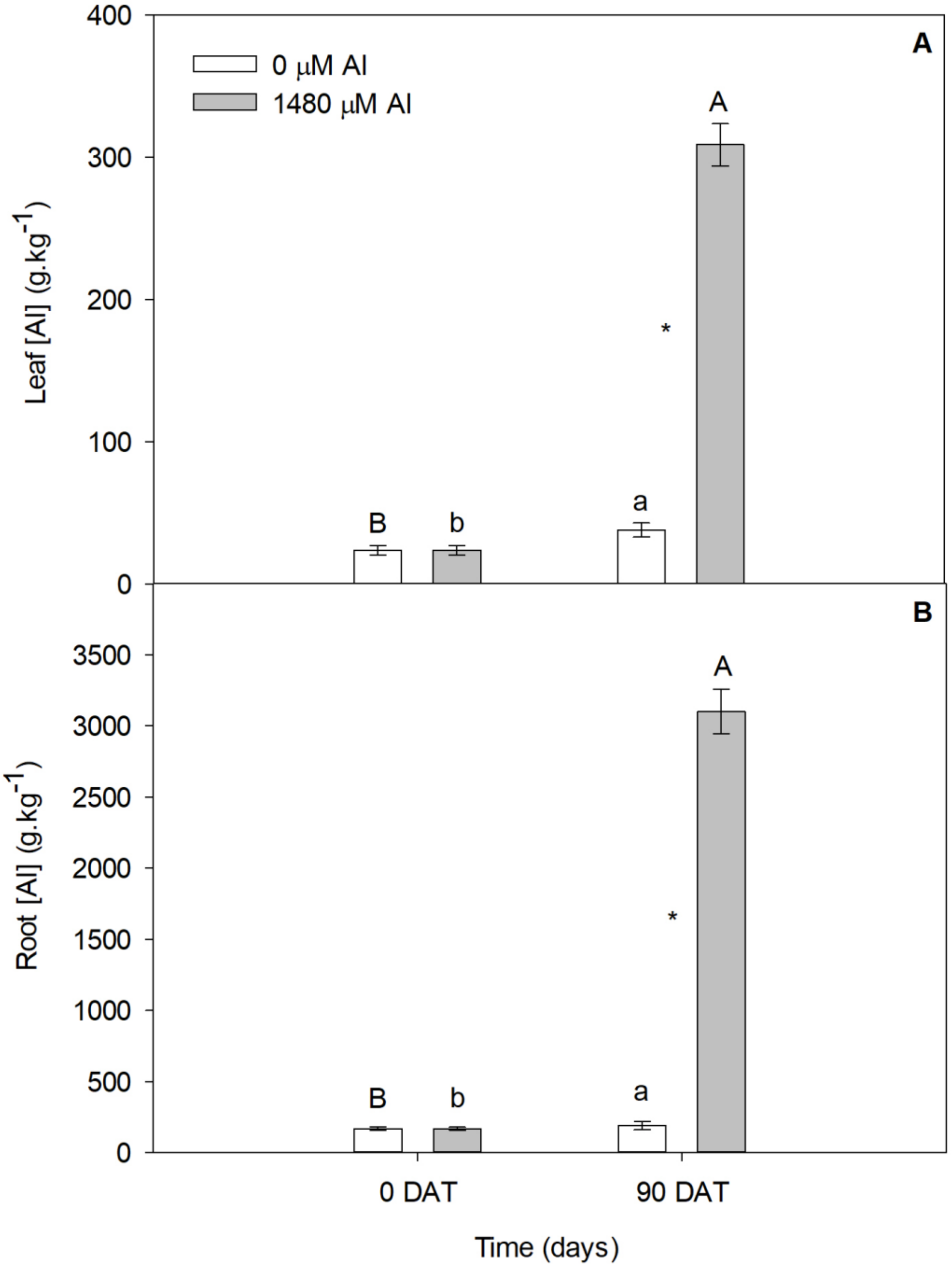
Fig. 8. Abscisic acid (ABA) concentration in leaves (A) and roots (B) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. Columns are mean values (n=4 plants) and bars are standard errors.

Supplemental Data

Supplemental Fig. S1. Morphological details of shoots and leaves and roots of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 (on the left) and 1480 µM Al (on the right).



Supplemental Fig. S2. Leaf abscisic acid concentration ($[ABA]_{leaf}$) versus stomatal conductance (*gs*) *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. Dots are mean values (n=4 plants).



Supplemental Fig. S3. Aluminum concentration in leaves (A) and roots (B) of *C. limonia* plants cultivated for 90 days in nutrient solution containing 0 and 1480 µM Al. For each evaluation date, asterisks indicate significant differences ($P < 0.05$) between 0 and 1480 µM Al. For plants not exposed to Al, distinct uppercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP; for plants exposed to Al, distinct lowercase letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Columns are mean values (n=6 plants) and bars are standard errors.

CONCLUSÃO GERAL

A biossíntese de ácido abscísico faz parte da sinalização ao alumínio tóxico, sendo o fechamento estomático uma das principais respostas induzidas por esse hormônio, a fim de modular o status hídrico da planta sob estresse. Além disso, o Al parece diminuir a capacidade intrínseca de transporte de água para a parte aérea, fato que demanda estudos mais detalhados.