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

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

O ALUMÍNIO INFLUENCIA NA EXPRESSÃO DE TRÊS AQUAPORINAS DA SUBFAMÍLIA PIP NAS RAÍZES DE *CITRUS LIMONIA*?

MARIANA FEITOSA CAVALHEIRO

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

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TÍTULO DA DISSERTAÇÃO: O alumínio influencia na expressão de três aquaporinas da subfamília PIP nas raízes de citrus limonia?

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*Há uma hora certa e uma estação certa
para tudo. Não deixe para amanhã o que
você sabe que deve fazer agora, mas
flua com o ritmo da vida e sinta-se em
perfeita paz*

Eileen Caddy

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INDÍCE

RESUMO

O Alumínio (Al) é um dos elementos mais abundantes na crosta terrestre. Solos ácidos ($pH < 5.0$) compreendem cerca de 40% das terras agriculturáveis no mundo, e sob essas condições, o Al se encontra na forma de Al^{3+}, que é tóxico para a maioria das plantas. Na presença de Al, observa-se uma redução nos parâmetros de trocas gasosas e de hidratação foliar, que podem estar diretamente relacionadas com a diminuição no transporte de água radicular. Aquaporinas são proteínas integrais da membrana plasmática que facilitam o transporte de água transmembranar e, nas raízes, estas podem contribuir com mais de 50% na condutividade hidráulica. Portanto, uma diminuição na abundância de aquaporinas poderia acarretar em um menor transporte de água até a folha. No presente estudo, cultivamos plantas de limoeiro ‘Cravo’ (*Citrus limonia*), durante 90 dias em solução nutritiva com Al. Em seis épocas, coletamos dados de trocas gasosas, potencial hídrico, conteúdo relativo de água na folha e verificamos a expressão de três genes de aquaporinas nas raízes: *PIP1-1*, *PIP1-2* e *PIP2*. Confirmamos uma menor hidratação da folha pela redução do conteúdo relativo da água (CRA) e potencial hídrico (Ψw), bem como diminuição na assimilação de CO_2 (A), condutância estomática (gs), transpiração (E) e condutividade hidráulica estimada da raiz até a folha (K_L) em plantas expostas ao Al. Além disso, plantas submetidas ao Al apresentam uma atenuação no perfil transcricional de *PIP1-1* e *PIP2* e um aumento na expressão de *PIP1-2*, das quais apenas *PIP1-1* apresentou correlação com A e gs no tratamento com Al. Nossos dados sugerem que a atenuação no perfil transcricional de *PIP1-1* está associada à menor hidratação da folha em plantas expostas ao Al.

Palavras-chave: Estresse abiótico; limoeiro ‘Cravo’; qPCR; relações hídricas; trocas gasosas.

ABSTRACT

*Aluminum (Al) is one of the most abundant elements of the Earth's crust. Acidic soils (pH <5.0) comprise about 40% of the world arable land, and under these conditions, Al is in the form of Al^{3+}, which is toxic to most plants. In the presence of Al, there are reduction in the gas exchange parameters and leaf hydration, which may be directly related to the decrease in root water transport. Aquaporins are integral proteins of the plasma membrane that facilitate water transport and they can contribute to more than 50% of the root hydraulic conductivity by transmembrane pathway. Therefore, a decrease in aquaporin abundance in the roots may lead to lower water transport up to the leaf. In a 90-day study, we cultivated 'Rangpur' lime plants (*Citrus limonia*) in nutrient solution with Al. At six time points, we measured gas exchange, water potential (Ψw), relative water content (RWC) in the leaves and we also assessed the expression of three aquaporin genes in the roots,* PIP1-1*,* PIP1-2 *and* PIP2*. We confirm lower leaf hydration by a reduction in RWC and Ψw, as well as decreased CO_2 (*A*) assimilation, stomatal conductance (*gs*), transpiration (*E*), and estimated hydraulic conductivity from the root to the leaf (*K_L*) in plants exposed to Al. In addition, plants submitted to Al presented an attenuation in the transcriptional profile of* PIP1-1 *and* PIP2*, and an increase in the expression of* PIP1-2*, of which only* PIP1-1 *presented a correlation with* A *and* gs *in the Al treatment. Our data suggests that the attenuation in the transcriptional profile of* PIP1-1 *is associated with the low leaf hydration observed in 'Rangpur'lime plants exposed to Al.*

Keywords: *Abiotic stress; 'Rangpur' lime; qPCR; water relations; gas exchange.*

1. INTRODUÇÃO

Cerca de 40% dos solos agricultáveis do mundo são ácidos (pH < 5,0), e 60% dos solos na América do Sul têm pH < 4,0 (von Uexküll and Mutert 1995). Nessas condições, os alumino-silicatos constituintes dos solos se solubilizam, liberando íons Al^{3+} na solução do solo, os quais podem ser tóxicos e restringir o crescimento de plantas sensíveis ao alumínio (Ulrich 1986; Kinraide 1997; Kochian et al. 2004).

Um dos sintomas mais conspícuos da fitotoxicidade ao alumínio (Al) é a inibição do crescimento radicular (Barceló and Poschenrieder 2002; Horst et al. 2010). Estes sintomas já foram observados em plantas herbáceas, como o feijoeiro caupi (*Vigna unguiculata*) (Kopittke et al. 2008) e arbóreas (Brunner and Sperisen 2013), incluindo o gênero *Citrus* (Nogueira et al. 1989; Santos et al. 1999; Pereira et al. 2003). Isso se dá possivelmente pela ligação do Al às matrizes pécticas da parede celular (Wehr et al. 2010), o que pode resultar no aumento da rigidez da parede, levando a rupturas nas raízes (Kopittke et al, 2008). Além disso, uma possível alteração do potencial de membrana (Ahn et al. 2002) e menor permeabilidade da água (Blamey et al. 1990; Horst et al. 2010) poderiam diminuir o potencial pressão hidrostático e causar menor expansão celular.

O menor crescimento e desenvolvimento do sistema radicular talvez expliquem os sintomas que ocorrem na parte aérea. A diminuição da assimilação de CO_2 (*A*) e condutância estomática (*gs*) bem como menor resposta fotoquímica da fotossíntese em plantas expostas ao Al já foram observadas em cafeeiro (*Coffea arabica* L.) (Konrad et al. 2005), milho (*Zea mays* L.) (Lidon et al. 1999), tomateiro (*Lycopersicon esculentum* Mill.) (Simon et al. 1994), pomeleiro (*Citrus grandis*) (Jiang et al. 2008, 2009), tangerineira ‘Cleópatra’ (*Citrus reshni*) (Chen et al. 2005a, b) e limoeiro ‘Cravo’ (*Citrus limonia*) (Banhos et al. 2016).

Alguns autores argumentam que que a causa da baixa hidratação do mesofilo (levando à menor *A*, *gs* e respostas fotoquímicas) seja devido à menor absorção de água causado pela menor superfície e tamanho radicular causado pelo Al (Vitorello et al. 2005; Rengel et al. 2015; Kochian et al. 2015). Contudo, é possível também que o transporte de água seja prejudicado não apenas pela inibição do crescimento das raízes, mas pelas modificações causadas nos vasos do xilema que se tornam fibrosos, como observado em limoeiro ‘Cravo’ expostos ao Al (Banhos et al, 2016), o que poderia diminuir a capacidade de transporte de água. Além disso, a área, número e biomassa de folhas também é reduzida em limoeiro ‘Cravo’ exposto ao Al, reduzindo o fluxo transpiratório (Banhos et al., 2016) e compensando o menor crescimento das raízes.

As relações hídricas nos vegetais provêm de um sistema contínuo solo-planta-atmosfera (Steudle 2001). Na planta, o transporte da água pode ocorrer de maneira apoplástica, que ocorre através dos espaços intercelulares, ou simplástica, através dos plasmodesmas ou das membranas celulares (Steudle and Peterson 1998).

Um dos fatores que poderiam levar ao menor transporte hídrico nas raízes seria a redução da abundância de aquaporinas (Javot and Maurel 2002). Estas são proteínas integrais de membrana que facilitam o transporte de água e de pequenas moléculas através de poros na bicamada lipídica, conhecido como transporte transmembranar (Maurel et al. 2015). Este transporte pode representar mais de 50% da condutividade hidráulica radicular em espécies vegetais como tomateiro e *Arabidopsis* (Maggio and Joly 1995; Sutka et al. 2011).

Plantas podem apresentar mais de 30 genes de aquaporinas, que podem estar distribuídas em sete subfamílias. Nas plantas vasculares, são encontradas apenas cinco subfamílias, sendo as outras duas exclusivas de briófitas (Maurel et al. 2015). Elas derivam principalmente em estrutura e localização, sendo distribuídas da seguinte forma: as PIPs (proteínas integrais da membrana plasmática), TIPs (proteínas integrais do tonoplasto), SIPs (pequenas proteínas integrais da membrana) e NIPs (proteínas integrais da membrana similares à nodulina-6); podem-se citar também as proteínas não caracterizadas da membrana (XIPs), presentes em algumas dicotiledôneas, como laranjeira-doce (*Citrus sinensis*) (Maurel et al. 2015; Martins et al. 2015).

As PIPs geralmente compreendem a subfamília gênica com maior número de isoformas (Chaumont et al. 2000; Johanson et al. 2001; Sakurai et al. 2005). São 11 PIPs em *Citrus clementina* e apenas oito em *C. sinensis* (Martins et al., 2015), e desempenham papel intrínseco na absorção de água pelas raízes (Javot & Maurel, 2002). Elas podem ser divididas filogeneticamente em PIP1 e PIP2, divergindo não só em estrutura como em função. Em um sistema heterólogo, PIP2s demonstram alta permeabilidade à água, ao passo que PIP1s apresentam baixo ou nenhum transporte hídrico (Kammerloher et al. 1994; Chaumont et al. 2000). No entanto, quando co-expressas, há aumento na permeabilidade, sugerindo a importância da interação entre PIP1 e PIP2 (Fetter et al. 2004; Yaneff et al. 2015).

Portanto, as PIPs têm sido alvo de estudos em diversas espécies expostas à diferentes perturbações abióticas, como déficit hídrico e excesso de sal, as quais diferem em suas respostas de regulação (Maurel et al., 2015). Em *Citrus*, a diminuição da condutividade hidráulica parece estar associada à atenuação do perfil transcricional de PIPs nas raízes de plantas sob deficiência hídrica (embora haja divergências), ao passo que o estresse salino pode causar acentuação desse mesmo perfil transcricional (Rodríguez-Gamir et al. 2011; Martins et al. 2015).

Embora a diminuição da hidratação de plantas de *Citrus* seja reportada em estresse provocado por Al (Pereira et al. 2000; Chen et al. 2005b; Jiang et al. 2009; Banhos et al. 2016), pouco se sabe a respeito do mesmo em relação às PIPs. Todavia, esse assunto demonstra uma nova possibilidade de investigação para o entendimento da fitoxicidade do Al em *Citrus*. Assim, elegemos o limoeiro 'Cravo', que é uma espécie sensível ao Al (Santos et al. 1999; Pereira et al. 2000, 2003; Banhos et al. 2016) para estudar aspectos da hidratação e transporte de água. O limoeiro 'Cravo' também se faz importante junto à citricultura brasileira não irrigada. Isso é devido à grande profundidade atingida por seu sistema radicular, sendo, portanto, mais tolerante à seca (Magalhães Filho et al. 2008).

Aqui, hipotetizamos que um dos fatores que levam à diminuição do fluxo de água nas plantas expostas ao Al pode estar relacionado à atenuação do perfil transcricional das PIPs. Analisamos a expressão relativa por meio qPCR, ao longo do tempo, juntamente com medidas de relações hídricas e trocas gasosas.

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Capítulo único:

Low root aquaporin expression is associated with reduced hydration in 'Rangpur' lime plants exposed to aluminum[1]

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Abstract

In acidic soils, aluminum (Al) occurs as Al^{3+}, which is phytotoxic. One of the most conspicuous symptoms of Al toxicity is the root growth inhibition, which could lead to low water uptake and consequent reduction in leaf hydration and leaf gas exchange. However, fibrous xylem vessels have already been observed in roots of ‘Rangpur’ lime plants (*Citrus limonia*). Therefore, aquaporin gene expression could be also reduced in these plants when exposed to Al. Using three-month-old *C. limonia*, we confirmed a decrease of CO_2 assimilation (*A*), stomatal conductance (*gs*), transpiration (*E*) and relative water content (RWC) when exposed to 1480 μM Al for 90 days. The estimated hydraulic conductivity from soil to the leaf (K_L) and leaf water potential (Ψw) were not consistently reduced over time of Al exposure. The relative expression of aquaporin genes belonging to PIP family (*PIP1-1*, *PIP1-2* and *PIP2*) in root showed that *PIP1-1* and *PIP2* were down-regulated and *PIP1-2* was up-regulated in Al treatment, but only *PIP1-1* was positively correlated with of *A* and *gs* in plants exposed to Al. Our results suggest that down regulation of *PIP1-1* in roots of *C. limonia* plants could be associated with the low hydration observed in ‘Rangpur’ lime plants exposed to Al.

Key words: Abiotic stress; *Citrus limonia*; PIP; qPCR; water relations.

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust and, it occurs in the soil as Al_3SiO_4 (von Uexküll and Mutert 1995). Approximately 30-45% of soils from the world's ice-free land are acidic ($pH < 5.0$) (Von Uexküll and Mutert, 1995) and, in low soil pH Al_3SiO_4 is solubilized to Al^{3+}, which is phytotoxic (Foy 1988; Singh et al. 2017). In sensitive plants, Al is retained in pectic nets of apoplast root cells (Horst et al. 2010; Kopittke et al. 2015), and the reduction of root growth is the first symptom evidenced in many plant species (Vitorello et al. 2005; Horst et al. 2010).

Aluminum can also cause indirect effects in the aboveground plant organs. The reduction in CO_2 assimilation (*A*) and stomatal conductance (*gs*) in plants exposed to Al have already been observed in coffee plants (*Coffea arabica*) (Konrad et al. 2005), maize (*Zea mays*) (Lidon et al. 1999), tomato (*Lycopersicon esculentum*) (Simon et al. 1994), 'Cleopatra' tangerine (*Citrus reshni*) (Chen et al. 2005a, b), pummelo (*Citrus grandis*) (Jiang et al. 2008, 2009) and 'Rangpur' lime (*Citrus limonia*) (Banhos et al., 2016; Silva et al., 2018). In addition, reduced midday leaf water potential (Ψmd) (Banhos et al., 2016; Silva et al., 2018) and low leaf relative water content (RWC) (Silva et al., 2018) have been reported in 'Rangpur' lime plants exposed to Al in nutrient solution. Thus, Al-induced symptoms in leaves seem to be associated with low mesophyll hydration. It has been suggested that the low root growth caused by Al would lead to reduced water uptake, explaining the Al-induced decrease in *gs* (Vitorello et al. 2005; Rengel et al. 2015; Kochian et al. 2015). However, Al-induced decrease in *gs* observed in 'Rangpur' lime plants exposed to Al also showed reduced number of leaves, leaf area and leaf biomass (Banhos et al., 2016; Silva et al., 2018), which would compensate for the low root growth caused by Al. Furthermore, 'Rangpur' lime plants exposed to Al presented low *gs* associated with fibrous xylem vessels (Banhos et al. 2016). Therefore, the low mesophyll hydration in plants submitted to Al treatment may be also related to a decrease in the intrinsic capacity of water transport and not only due to the reduction in root growth.

One of the factors that could influence the intrinsic capacity of water transport is the abundance of aquaporins (Javot and Maurel 2002). These are membrane integral proteins that facilitate the transport of water and small molecules through pores in the lipid bilayer known as transmembrane transport (Maurel et al. 2015). This transport may represent more than 50% of root hydraulic conductivity in plant species such as tomato and *Arabidopsis* (Maggio and Joly 1995; Sutka et al. 2011).

Plants may present more than 30 isoforms of aquaporins, which may be distributed in five subfamilies in vascular plants, and PIPs (plasma membrane intrinsic proteins) and TIPs (tonoplast intrinsic proteins) have been reported as functional water channels (Alexandersson et al. 2005). However, PIPs are likely to play a more important role in water transport than TIPs because the plasma membrane seems to be less permeable to water than the tonoplast (Javot and Maurel 2002). In *Citrus* plants, 11 PIPs have been found in *Citrus clementina* and only eight in *C. sinensis* (Martins et al. 2015). PIPs can be phylogenetically divided into PIP1 and PIP2, differing not only in structure but also in function. In a heterologous system, PIP2s demonstrate high water permeability, whereas PIP1s have low or no water transport (Kammerloher et al. 1994; Chaumont et al. 2000). However, when co-expressed, there is an increase in permeability, suggesting the importance of the interaction between PIP1 and PIP2 (Fetter et al. 2004; Yaneff et al. 2015). Thus, PIP1 and PIP2 would be critical targets when studying environmental disturbances influencing water relations and eventually leaf hydration.

Here we grew Rangpur lime plants in nutrient solution with Al in a 90-day study in order to assess leaf gas exchange, leaf water potential and estimated hydraulic conductivity from soil to the leaf (K_L) at distinct evaluation dates. We collected root tips to evaluate the relative gene expression of *PIP1-1*, *PIP1-2* and *PIP2* throughout the study. We hypothesized that plants exposed to Al show lower aquaporin expression in relation to plants not exposed to Al.

Material and Methods

Plant material and experimental conditions

Three-month-old plants and 15 ± 1 cm-high 'Rangpur' lime (*Citrus limonia* L.) plants were used for studying the hydration capacity when submitted to Al within a 90-day period. 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).

The nutrient solution was adapted from Banhos et al (2016) and shows a chemical composition based on the solution proposed by Clark (1975). This solution has been used to test Al tolerance in *C. limonia* (Banhos et al., 2016; Silva et al., 2018). 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 $CuSO_4$ $5H_2O$, 0.4 µM $NaMoO_2$ 2 H_2O. In a previous study (Banhos et al., 2016), we observed Al-induced decrease in gas exchange rates when *C. limonia* was exposed to 1480 µM for 45 days. 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 (using NaOH and HCl) to keep Al as soluble 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 stayed on benches (80 cm above the ground) inside a greenhouse with semi-controlled conditions [air temperature 28.5 ± 0.7°C; relative humidity (RH) 65.3 ± 2.1%; 862.7 ± 184.4 µmol photons m^{-2} s^{-1}; approximately 14h of natural photoperiod].

Experimental design

After acclimatizing for 14 days in the nutrient solution (without Al), the plants were grown in 0 and 1480 µM Al for 90 days, and measurements were performed in different days after planting (DAP). At 1, 7, 15, 30, 60 and 90 DAP we measured gas exchange, and water relation parameters as described below.

We use 6 plants (replicates) for each treatment and each evaluated time.

We also collected root apices for RNA extraction and further characterization as PIP gene. These were *PIP1-1*, *PIP1-2* and *PIP2*. According to PLAZA 4.0 (Van Bel et al. 2018), their orthologues in *Arabidopsis* are AT4G00430 (*AtPIP1-4)*, AT4G23400 (*AtPIP1-5)* and AT3G53420 *(AtPIP2-1)*, which act as water channels (Kammerloher et al. 1994; Weig et al. 1997; Lee et al. 2012).

Leaf gas exchange

CO_2 assimilation (A; µmol m^{-2} s^{-1}), transpiration (E; mmol m^{-2} s^{-1}), 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). Measurements were taken between 13h and 15:30h on cloudless days. Although the best period for measuring gas exchange parameters is the midmorning, values from the afternoon period were necessary to calculate the estimated hydraulic conductivity from soil to the leaf (K_L) (see below). In addition, previous studies (Banhos et al., 2016; Silva et al., 2018) already discussed midmorning photosynthetic parameters of *C. limonia* subjected to the same Al treatment used in the present study.

The photosynthetic photon flux density (PPFD) was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to provide 1500 µmol photons m^{-2} s^{-1} in the leaf cuvette, as this value saturates A for *Citrus limonia* as observed in A/Ci curves (Silva et al., 2018). The vapor pressure deficit (VPD) inside the leaf cuvette was similar to the external environment, which was not higher than 1.5 kPa (RH ~ 60%).

Water relations

Leaf water potential (Ψw; 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.

Hydraulic conductivity from soil to the leaf (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 the 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 the maximum VPD. This method was already used for measuring K_L in *Citrus sinensis* grown in nutrient solution (Magalhães Filho et al. 2009).

For measuring the relative leaf water content (RWC; %), leaf discs were collected from plants of both treatments at 12 am and calculated as:

$$RWC = [(FW-DW)/(TW-DW)]\times 100,$$

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; and DW is the dry weight after oven-drying the discs at 60°C for 48 h, according to Silva et al. (2018).

RNA extraction

Root apices were collected at 12 am, frozen in liquid nitrogen (N_2) and stored at -80°C for future analysis. Total RNA was extracted from 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 an oligo-dT primer and GoScript (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) using SYBR green GoTaq q-PCR Master Mix (Promega Corp., USA), using Applied Biosystems QuantStudio 3 (Life Technologies, Carlsbad, CA, USA). We used the same primers designed by Martins et al. (2015) which are described on 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. (2018). Amplification efficiencies were calculated for each primer using Miner software (Zhao and Fernald 2005).

For calculating relative expression, Ct value of each sample, which were determined by the mean of the three technical replicates, was converted into relative quantities (RQ) according to Pfaffl (2001), using the following function:

$RQ=E^{\Delta Ct}$,

where E is the primer efficiency, and ΔCt is the difference between control Ct value for the evaluated gene and the 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.

Data analysis

Data of leaf gas exchange parameters (*A*, *gs*, *E*, *Ci*, WUE and IWUE) and RWC were measured using six replicates (plants). Leaf water potential (Ψpd and Ψmd) and K_L were assessed using four replicates, and gene expression of aquaporins was evaluated using a pool of four replicates.

For each evaluation date (1, 7, 15, 30, 60 and 90 DAP), a student's t-test ($\propto = 0.05$) was used to test for differences between 0 and 1480 µM Al for all parameters. A Person correlation analysis was performed between individual values of parameters obtained from plants exposed to 1480 µM Al.

Results

Plants exposed to Al showed approximately 45% lower CO_2 assimilation rate when compared to those not exposed to Al from 15 DAP up to the end of the experiment (Fig. 1A). Plants exposed to Al showed lower *gs* and *E* values in relation to those not exposed to Al at 15 and 60 DAP and, at 90 DAP, these parameters were higher in plants exposed to Al (Fig. 1B, C). The intercellular CO_2 was higher in plants exposed to Al at 30 and 90 DAP when compared to those not exposed to Al (Fig. 1D). Both WUE and IWUE were lower in plants exposed to Al at 30 and 90 DAP when compared to those not exposed to Al (Fig. 2A, B).

In relation to plants not exposed to Al, Ψpd was lower in those exposed to Al at 7, 15 and 90 DAP (Fig. 3A), and Ψmd was also lower at 15 and 60 DAP (Fig. 3B). The estimated hydraulic conductivity from soil to the leaf (K_L) followed the same Ψmd response pattern of plants exposed to Al (Fig. 4). The relative water content (RWC) was lower in plants exposed to Al from 15 DAP up to the end of the study (Fig. 5).

In plants exposed to Al, K_L showed positive correlations ($P < 0.001$) with *gs* ($R^2 = 0.656$) and *E* ($R^2 = 0.738$) (Table 2). In addition, RWC of these plants also showed positive correlation ($P < 0.001$) with Ψpd ($R^2 = 0.697$) (Table 2).

Plants exposed to Al showed down-regulation of *PIP1-1* and *PIP2* from 7 DAP up to the end of the study. However, these genes were not significantly different from those expressed in plants not exposed to Al at 7 and 60 DAP for *PIP1-1* (Fig. 6A) and for *PIP2* at 7 and 30 DAP (Fig. 6C). In plants exposed to Al, positive correlations were also found between *gs* x *PIP1-1* ($P < 0.001$; $R^2 = 0.988$) and *E* x *PIP1-1* ($P < 0.01$; $R^2 = 0.919$) (Table 2). The expression of *PIP1-2* was up-regulated in plants exposed to Al, being significantly different from those not exposed to Al at 1, 15, 30, 60 and 90 DAP (Fig. 6B).

Discussion

Our results confirm that *C. limonia* exposed to 1480 µM Al shows a reduction in leaf hydration, as also observed by Banhos et al. (2016) and Silva et al. (2018). In plants exposed to Al, the RWC decreased by 10% (15 DAP), 15% (30 and 60 DAP), and 5% (90 DAP) (Fig. 5). In a similar 90-day study, the RWC was reduced between 5% and 20% in ‘Rangpur’ lime plants exposed to 1480 µM Al (Silva et al., 2018). This reduction in RWC was positively correlated with Ψpd, which was also reduced at 7, 15 and 90 DAP. Indeed, a decrease in Ψpd is associated with a low capacity of nocturnal rehydration (Turner 1981). Although we also expected that K_L would diminish in plants exposed to Al, this reduction was not consistent over time of Al exposure because it was lower than control plants only at 15 and 60 DAP (Fig. 4). In plants exposed to Al, the response pattern of K_L was connected with Ψmd, but K_L showed positive correlation with *gs* and *E* and was not correlated with Ψmd (Table 2). For healthy and unstressed *Citrus* plants, it is suggested that leaf transpiration is strongly determined by the hydraulic system of the whole plant (Rodríguez-Gamir et al. 2016). Thus, for 'Rangpur' lime plants under Al stress, this may also be truth, indicating that water loss in the mesophyll (*gs* and *E*) is under the control of K_L.

We also expected that water use efficiencies (WUE and IWUE) would be reduced in plants exposed to Al in relation to those not exposed to Al. This occurred, but once more, only at two evaluation dates (30 and 90 DAP) (Fig. 2). Silva et al (2018) also reported decrease in WUE and IWUE of 'Rangpur' lime plants exposed to 1480 µM Al at three evaluation dates in a 90-day study.

Our data also demonstrate that the low hydration of plants exposed to Al may be associated with the attenuated expression of *PIP1-1* and *PIP2* in the roots of these plants, which corroborates our hypothesis. In plants exposed to Al, *PIP1-1* was down-regulated at 1, 15, 30 and 90 DAP (Fig. 6A) and *PIP2*, at 15, 60 and 90 DAP (Fig. 6C). In plants in general, the response of aquaporins to abiotic stresses are usually species-specific as well as dependent on different abiotic stresses (Aroca et al. 2012; Maurel et al. 2015). For example, under drought conditions, plants in general show inconsistent expression patterns of aquaporins (both up and down-regulation) (Alexandersson et al., 2005; Aroca et al., 2012; Maurel et al., 2015). In the case of Al stress, (Wang et al. 2017) found association between *NIP1-2* (a plasma membrane-localized member of the *Arabidopsis* NIP aquaporin subfamily) and Al-malate transport from the root cell wall into the root symplasm. Malate exudation is a very known mechanism of Al tolerance in many plant species (Ryan et al. 2011), including *C. limonia* (Silva et al., 2018). Recent evidence suggests that aquaporins in general are possibly involved in Al sequestration by roots of *Citrus grandis* and *C. sinensis* (Guo et al.

2017). As far as we are aware, the present study is the first report of down-regulation of aquaporins (PIP subfamily) in response to Al in *Citrus* plants, and this response was associated with reduction in leaf hydration. Thus, it is reasonable to believe that channels responsible for water transport, like aquaporins, would be associated with low water conductance in the plant. In fact, studies for many plant species have shown that most PIP genes are down-regulated in the roots of plants submitted to soil water deficit (Afzal et al. 2016). In the present study, 'Rangpur' lime plants exposed to Al showed positive correlation between *PIP1-1* with *gs* and *E* (Table 2), indicating that these aquaporin genes were down-regulated in response to the Al presence in the nutrient solution.

On the other hand, *PIP1-2* was up-regulated in the roots of plants exposed to Al, mainly at 30 DAP (Fig. 6B), and that *PIP1-2* expression was not associated with the other water relation parameters (Table 2). In addition, *PIP1-2* was negatively correlated with *PIP2* (Table 2), indicating either that the Al presence could induce opposite responses in each of these genes or that *PIP1-2* could inhibit *PIP-2*. In the case of translated aquaporins, it has been reported that *PIP1* could be modulated by *PIP2* in a posttranslational process involving trafficking, heteromerization and adjust of channel activity (Yaneff et al. 2015). Furthermore, although PIP genes are usually correlated with water transport (Maurel et al. 2015), there is evidence that some PIP genes are also associated with the transport of other metabolic compounds, such as H_2O_2 (Bienert et al. 2006; Hooijmaijers et al. 2012; Bienert and Chaumont 2014), CO_2 (Uehlein et al. 2003; Maurel et al. 2015) and small neutral solute such as glycerol and glycine (Biela et al. 1999; Kaldenhoff and Fischer 2006; Maurel et al. 2015). Therefore, in the present study, *PIP1-2* could not be associated with water transport and we cannot rule out its interactions with aquaporins that were not explored in the present study, and this merits further investigations in plants exposed to Al. Here we studied only three PIP genes from a gene family composed most probably of 10-15 genes (Martins et al., 2015; Maurel et al., 2015), but it can be a starting point for analysis of how PIP genes are modulated by Al.

One may still argue that the reduction in leaf hydration in plants exposed to Al is mostly due to Al-induced root growth inhibition leading to an impairment of water uptake (Vitorello et al., 2005; Rengel et al., 2015). However, besides presenting reduced root growth, 'Rangpur' lime plants exposed to 1480 µM Al also show reduced leaf number, leaf area, leaf biomass, shoot length and shoot biomass compensating for the Al-induced root growth inhibition (Banhos et al., 2016; Silva et al., 2018; Silva et al., 2018). The present study evidenced down-regulation of *PIP1-1* and *PIP2*, and this may be associated with the low hydration in this species when exposed to Al, suggesting that this is not just a matter of root growth inhibition.

We confirmed that 'Rangpur' lime plants exposed to 1480 µM Al decrease their leaf hydration, mostly evidenced by the RWC. The estimated hydraulic conductivity from soil to the leaf (K_L) was not consistently reduced over time of Al exposure, but aquaporins (*PIP1-1* and *PIP2*) were down-regulated in the presence of Al, suggesting that the intrinsic capacity for water transport in roots of this species is modified when exposed to Al, which could be associated with the Al sensitivity in *C. limonia*.

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Tables:

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

Gene name	Foward	Reverse
GAPC2	TCCTATGTTTGTTGTGGGTG	GGTCATCAAACCCTCAACAA
EFα	TCAGGCAAGGAGCTTGAGAAG	GGCTTGGTGGGAATCATCTTAA
PIP1-1	TCACTCCCGTAGCAAGATCA	TTTCGCTCGCTCTTCTTCA
PIP1-2	CATTCTCATCACAACATCAAACG	CTGCTAGTCCCTCAAAAACACAA
PIP2	GCAAACACAACAGTCGTAGCTCT	CTTCAACATCCTTCCCCATTT

Table 2. Pearson correlations between individual values of parameters obtained from plants exposed to 1480 µM Al.

	gs	***Ci***	***E***	**WUE**	**WIUE**	**RWC**	**Ψpd**	**Ψmd**	**K_L**	**Foldchange PIP1-1**	**Foldchange PIP1-2**	**Foldchange PIP2**
A	0.137	-0.349	0.348	0.363	0.333	-0.517	-0.674	-0.522	0.174	0.735	-0.232	0.695
	0.427	0.0372	0.0377	0.0298	0.0475	0.00123	0.000306	0.00885	0.415	0.0961	0.658	0.125
gs		0.78	0.925	-0.77	-0.789	-0.0131	-0.241	-0.318	0.656	0.988	0.35	0.147
		2.08E-08	7.73E-16	4.03E-08	1.05E-08	0.94	0.256	0.13	0.000502	0.000202	0.497	0.781
Ci			0.574	-0.923	-1	0.25	0.123	-0.0711	0.574	0.376	0.693	-0.699
			0.000253	1.15E-15	8.09E-55	0.141	0.567	0.741	0.00335	0.463	0.127	0.123
E				-0.671	-0.592	-0.306	-0.572	-0.413	0.738	0.919	-0.0458	0.505
				7.67E-06	0.000143	0.0691	0.00346	0.0447	0.0000386	0.00961	0.931	0.307
WUE					0.929	-0.0325	0.0304	0.0847	-0.723	-0.492	-0.43	0.388
					3.12E-16	0.851	0.888	0.694	0.0000658	0.322	0.395	0.447
WIUE						-0.23	-0.0989	0.0824	-0.592	-0.404	-0.677	0.669
						0.177	0.646	0.702	0.00229	0.427	0.14	0.146
RWC							0.697	0.575	-0.425	-0.689	0.205	-0.635
							0.000154	0.0033	0.0386	0.13	0.697	0.175
Ψpd								0.322	-0.499	-0.788	0.173	-0.64
								0.125	0.0131	0.0629	0.743	0.171
Ψmd									-0.116	-0.649	-0.274	-0.158
									0.591	0.163	0.599	0.765
K_L										0.72	0.211	-0.00327
										0.107	0.689	0.995
Foldchange PIP1-1											0.317	0.175
											0.54	0.74
Foldchange PIP1-2												-0.819
												0.046

For each variable, the first line represents the correlation coefficient (R^2) and the second line, the *P* value

Figure captions:

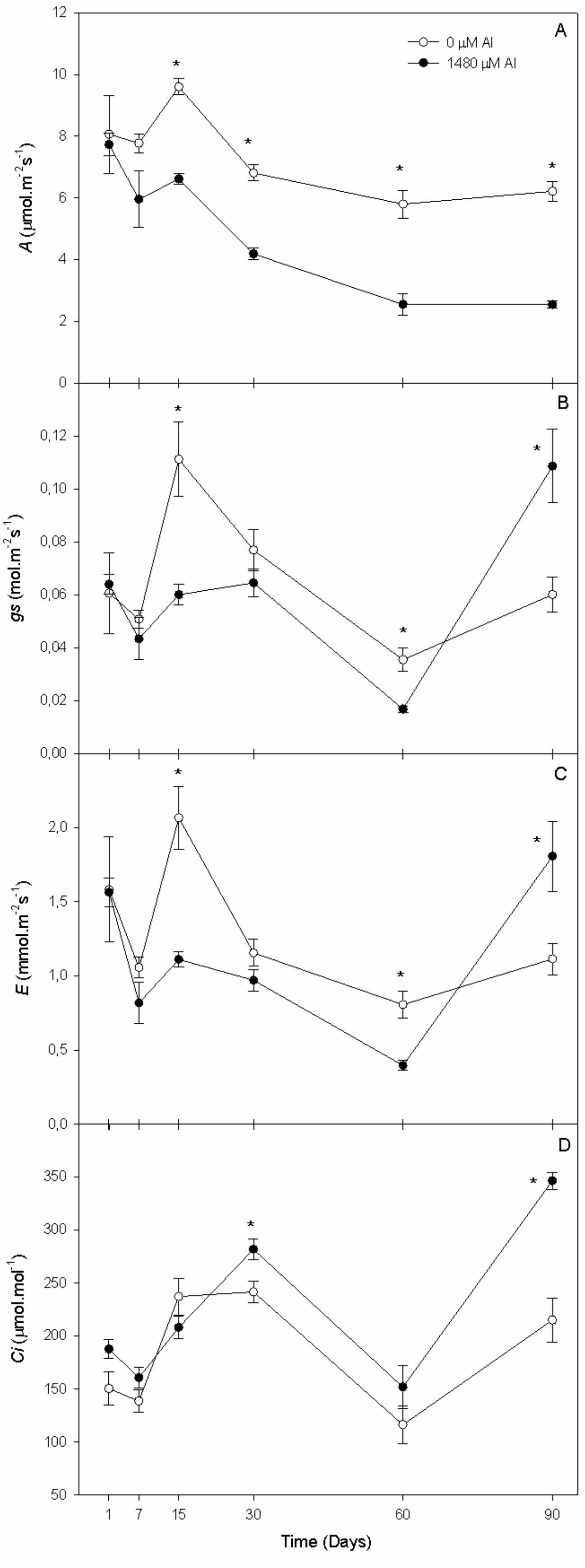

Fig. 1 Changes in leaf gas exchange characteristics in *C. limonia* plants subjected to 0 and 1480 uM Al in nutrient solution. CO_2 assimilation (*A*) (A), stomatal conductance (*gs*) (B), transpiration rates (*E*) (C) and intercellular CO_2 (*Ci*) (D) at 1, 7, 15, 30, 50 60 and 90 DAP. Values are mean ± standard errors. Values with asterisks indicate significant difference ($P < 0.05$).

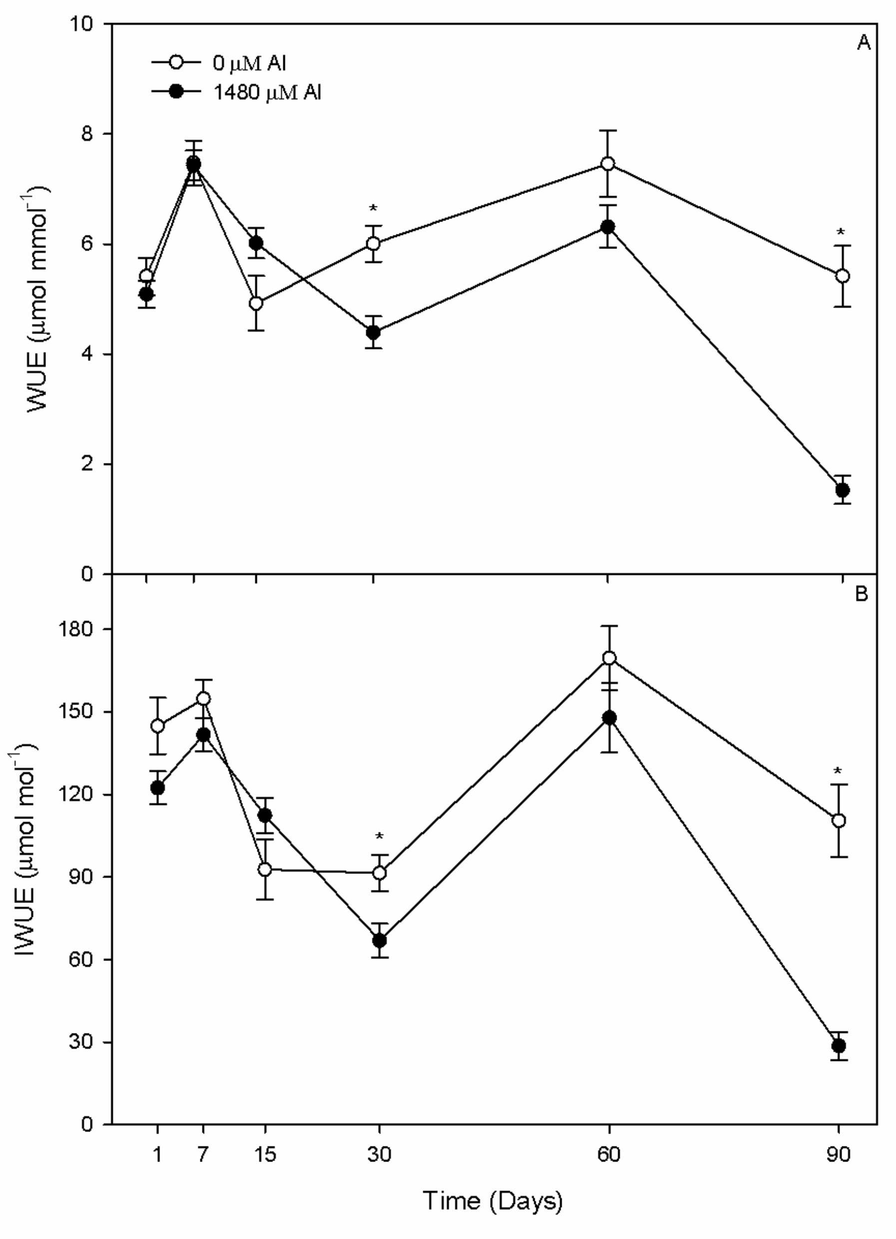

Fig. 2 Changes in water use efficiency (WUE) (A) and water intrinsic use efficiency (WIUE) (B) in *C. limonia* plants subjected to 0 and 1480 mM Al in nutrient solution at 1, 7, 15, 30, 60 and 90 DAP. Values are mean ± standard error of the mean of six plants. Values with asterisks indicate significant difference (P < 0.05).

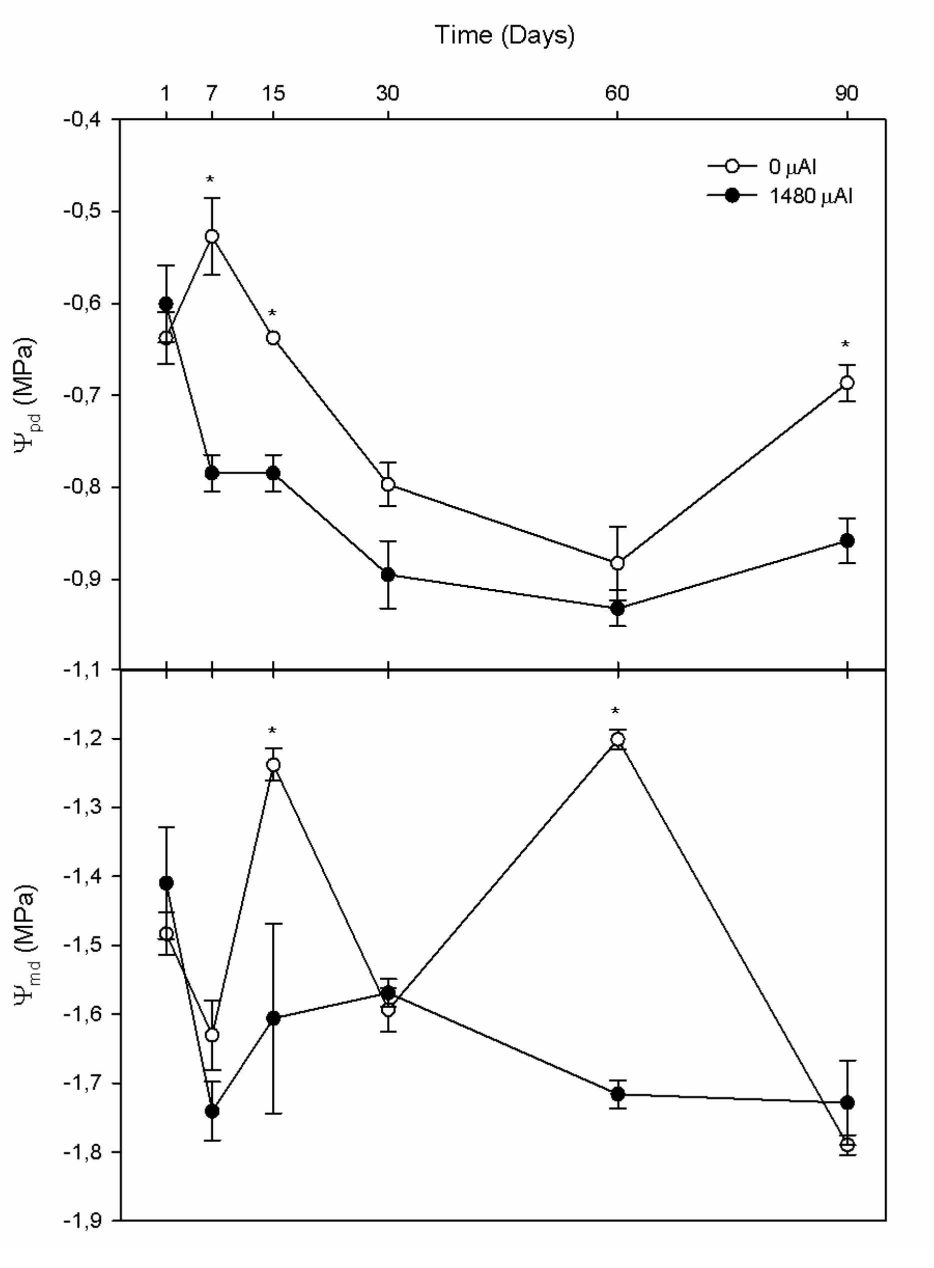

Fig. 3 Predawn leaf water potential (Ψpd,) (A), midday leaf water potential (Ψmd,) (B) in *C. limonia* plants submitted to 0 and 1480 mM Al at 1, 7, 15, 30, 60 e 90 DAP. Values are mean ± standard error of the mean of six plants. Values with asterisks indicate significant difference ($P < 0.05$).

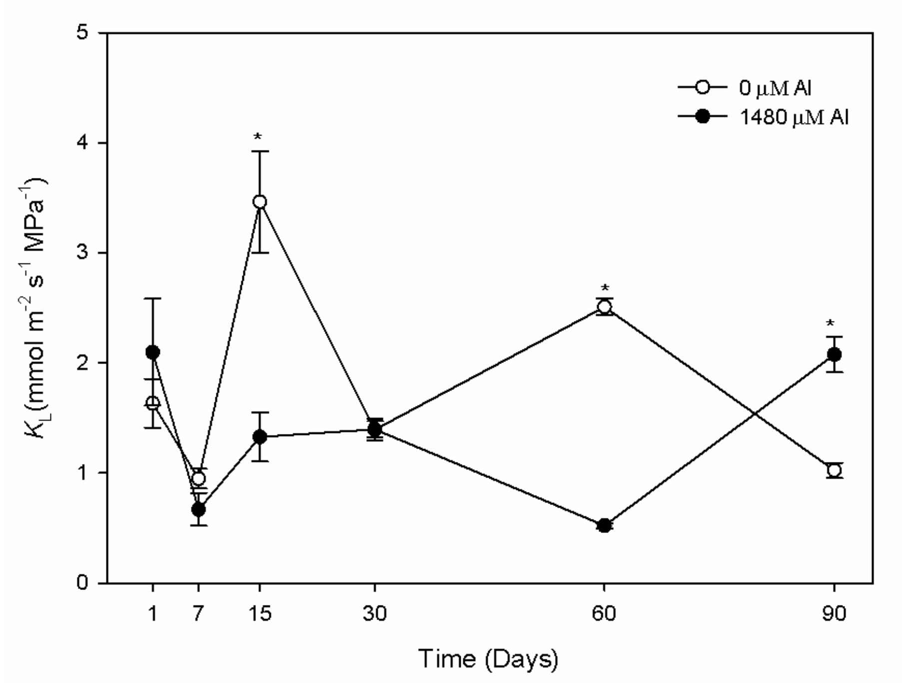

Fig. 4 Changes in estimated hydraulic conductance from soil to the leaf (K_L) in *C. limonia* plants exposed to 0 and 1480 µM Al throughout 90-day study. Values are mean ± standard error of the mean of six plants. Values with asterisks indicate significant difference ($P < 0.05$).

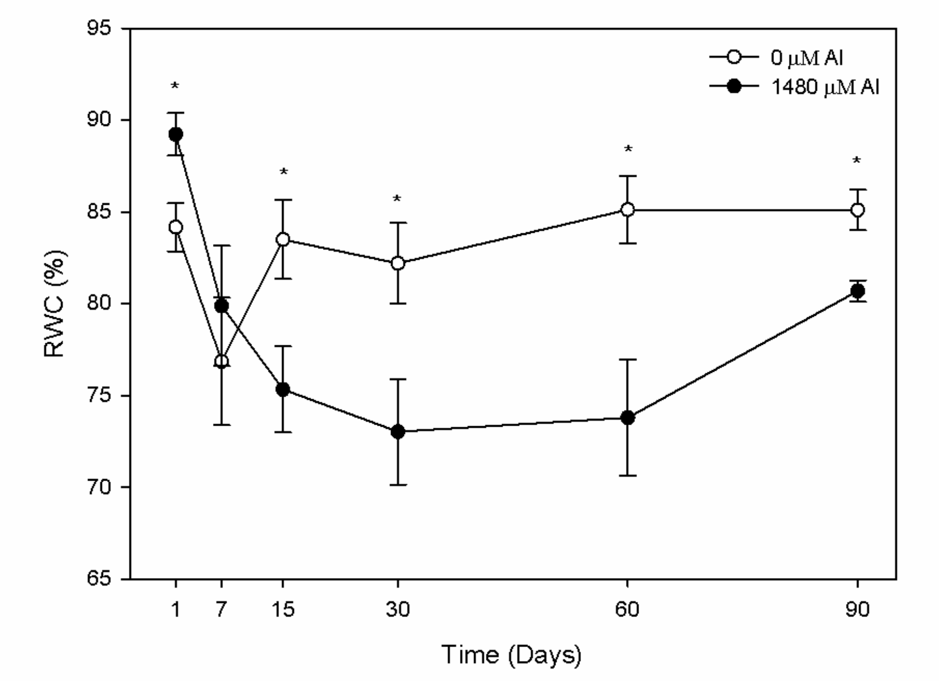

Fig. 5 Changes in leaf relative water content (RWC) in *C. limonia* plants exposed to 0 and 1480 µM Al throughout 90-day study. Values are mean ± standard error of the mean of six plants. Values with asterisks indicate significant difference ($P < 0.05$).

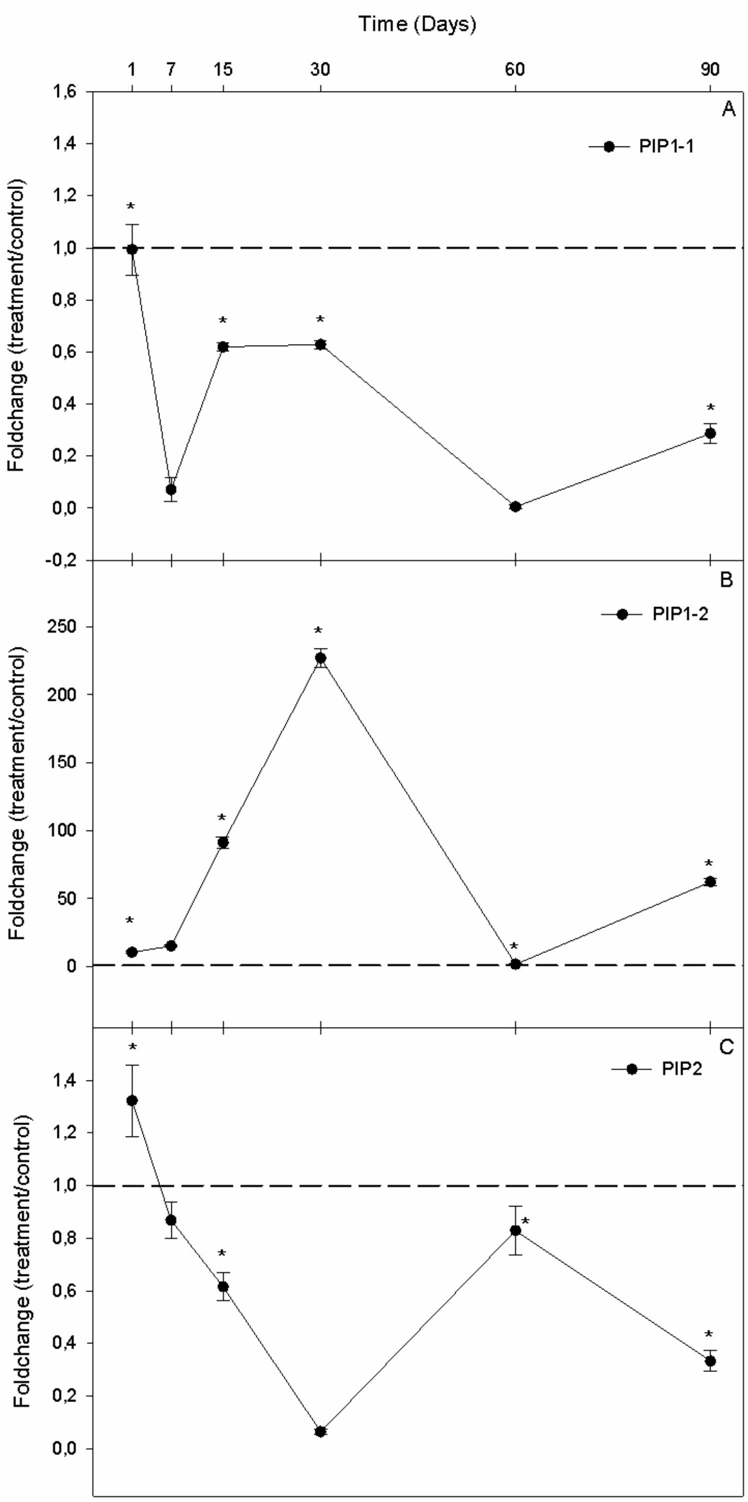

Fig. 6 Foldchange of normalized expression level of three aquaporin genes in root tissue of *C. limonia* plants. Samples were collected at 1, 7, 15, 30, 60 and 90 DAP. Values are the ratio of gene normalized expression level between treatments (1480 µM Al) and control (0 µM Al). Values are mean ± standard error of replicates and asterisks indicate significant difference ($P < 0.05$).