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Aluminum stress impairs water transport via PIP aquaporin suppression in tomato

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ABSTRACT

Aluminum (Al) is the most abundant metal in the Earth's crust. In acidic soils (pH < 5.0), it is predominantly found as Al^{3+}, which is toxic to most plants. In Al-sensitive species such as tomato (*Solanum lycopersicum*), the first and most conspicuous symptom of toxicity is root growth inhibition, where most of the Al is covalently retained. However, Al also induces indirect symptoms in the shoot, including reduced gas exchange, low stomatal conductance (g_s), and decreased mesophyll hydration, likely due to impaired water transport from roots to shoots. These symptoms have been even observed in plants grown directly in nutrient solution containing Al. Aquaporins (AQPs), especially those of the plasma membrane intrinsic protein (PIP) subfamily, are known to function primarily as water channel proteins. We hypothesize that Al exposure downregulates specific PIPs in tomato, leading to reduced water transport, as evidenced by alterations in shoot water relations, growth inhibition, and increased Al accumulation. For this, the expression of selected AQP genes, root hydraulic conductance (Lp_r), root xylem sap pH, total plant transpiration (E_{plant}), g_s, leaf water potential (Ψ_{leaf}), and relative water content (RWC) were measured. Assessments were taken from 12 h up to 7 days after Al exposure. Biometric analyses and Al quantification were performed at the beginning and end of the experiment. As expected, PIP expression decreased in the root in response to Al, with this pattern observed for all PIP1 and PIP2 isoforms, except for *SlPIP2;11*, from 120 h onward, coinciding with reduced leaf hydration (RWC and Ψ_{leaf}). Downregulation of *SlPIP1;3*, *SlPIP1;5*, *SlPIP1;7*, and *SlPIP2;8* seems to be linked to reduced Lp_r, E_{plant}, and increased xylem sap pH. Despite the upregulation of *SlPIP2;11*, this was insufficient to maintain plant water status under toxic Al, but it could represent a target for future biotechnological initiatives on Al tolerance.

1. Introduction

Aluminum (Al) is the most abundant metal in the Earth's crust. In soils, Al is predominantly found in the form of oxides and aluminosilicates, which are inert to plants. However, when weathered, these compounds can release Al in various forms (Malavolta, 1980; Ryan et al., 2011). In acidic soils (pH < 5.0), Al is primarily available as Al $(H_2O)_6^{3+}$ or Al^{3+}, which is toxic to most plant species (Foy, 1974; Hajiboland et al., 2023). Given that acidic soils occupy approximately 30 % of the ice-free land area worldwide (von Uexküll and Mutert, 1995), Al is considered one of the major factors limiting crop productivity (Sade et al., 2016).

In Al-sensitive plants, the first and most noticeable symptom of Al toxicity is root growth inhibition (Horst et al., 2010; Silva et al., 2019) which can be detected within hours of exposure, even at low Al concentrations (<10 μM Al) (Kopittke et al., 2008). Disruption of the

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Table 1
Primer sequences for PIP aquaporin genes and reference genes used in *Solanum lycopersicum*.

Gene	Acess number	Primer sequence (Forward/Reverse)
SlPIP1;1	Solyc08g008050.3.1	F: 5′-TGCCCTTGTCTATTGTACTGCT-3′
SlPIP1;2	Solyc01g094690.3.1	R: 5′-GTCTCTGATACGGACCTTGCAT-3′
SlPIP1;3	Solyc12g056220.2.1	F: 5′-CTGAGGAACACCTTGGCCTA-3′
SlPIP1;5	Solyc08g081190.3.1	R: 5′-CACACACTTGACGTAGCAACA-3′
SlPIP1;7	Solyc03g096290.3.1	F: 5′-TTTAACAAAGACGAGGCATGGGA-3′
SlPIP2;1	Solyc09g007770.2.1	R: 5′-AAACAACAGCACCAGACAGGG-3′
SlPIP2;4	Solyc06g011350.3.1	F: 5′-CCAGCACCACTTATTGACCCT-3′
SlPIP2;5	Solyc10g084120.2.1	R: 5′- TGGTCACCATCACTTTGGCT-3′
SlPIP2;6	Solyc11g069430.2.1	F: 5′-AACAAGCATGGAAGGACCCC-3′
SlPIP2;8	Solyc01g111660.3.1	R: 5′-GCGGCAATTACATACATGCTTC-3′
SlPIP2;9	Solyc10g055630.2.1	F: 5′-GGTTCACTTGGCTACAATTCCT-3′
SlPIP2;10	Solyc09g007760.3.1	R: 5′-AAGTGCCAAACATCCAACACA-3′
SlPIP2;11	Solyc02g083510.3.1	F: 5′-TTCATTCTTGTCTACTGCACCG-3′
SlPIP2;12	Solyc05g055990.3.1	R: 5′-ACCAGCCATGACATTAACACC-3′
Actin	Solyc03g078400.3.1	F: 5′-TCCTGTATTGGCACCACTTCC-3′
GAPDH	Solyc03g111010.4.1	R: 5′GCTTATATTAATTCTTATTATTATGCAT-3′
		F: 5′-CGCAAAGGATTACACTGATCCAC-3′
		R: 5′-CCACCGCAAATATCGCCTCC-3′
		F: 5′-CATGACGCACCCATACAACG -3′
		R: 5′-GCGCTTCTCTTAGGGTCAGT-3′
		F: 5′-GAAACTCAACGCTCTAGACCAATG-3′
		R: 5′-TGACTGTGCAATTATGTAAGCAACTG-3′
		F: 5′-CACTTGGTTCATTCAGGAGCA-3′
		R: 5′-ATACTCAAAACAAAGCATAGTGA-3′
		F: 5′-CGACGCTTCTTTTCCTCTACGTT-3′
		R: 5′-CCAAAAGCCCATGCAATACCAAC-3′
		F: 5′-ATATTAATCCAGCAGTGACCTT-3′
		R: 5′-CAACGCCCCTAGTATAGCC-3′
		F: 5′-GGGATGGAGAAGTTTGGTGGTGG-3′
		R: 5′-CTTCGACCAAGGGATGGTGTAGC-3′
		F: 5′-ACCACAAATTGCCTTGCTCCCTTG-3′
		R: 5′-ATCAACGGTCTTCTGAGTGGCTGT-3′

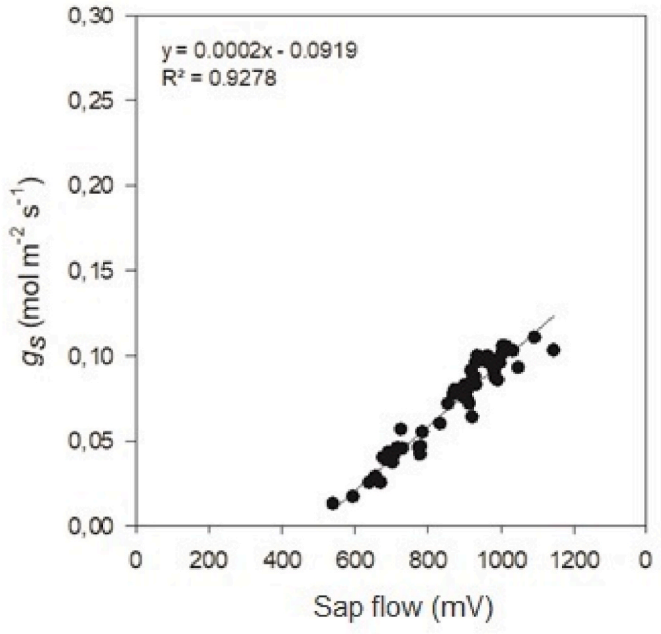

Fig. 1. Relationship between stomatal conductance (g_s), measured using a gas exchange analyzer, and sap flow (mV) in tomato plants. Measurements were taken from unstressed tomato plants (*Solanum lycopersicum* cv. Micro-Tom) grown in nutrient solution.

subapical epidermis due to cell wall stiffening (Kopittke et al., 2008), resulting from covalent Al binding to the primary cell wall (Wehr et al., 2010; Kopittke et al., 2015) is a relevant candidate to explain root growth arrest. Consequently, Al is almost entirely retained (>75 % of the absorbed Al) within the root system (Ciamporová, 2002; Vitorello et al., 2005; Singh et al., 2017; Silva et al., 2018a).

As reviewed by Kochian et al. (2015) Al-induced root growth inhibition may lead to reduced root surface area for water uptake, thereby limiting shoot hydration. However, although root surface area is naturally reduced due to inhibited growth, shoot hydration appears to depend more on the balance between functional root surface and shoot water demand — especially in studies where plants are grown in hydroponic systems, as is common in Al toxicity research (Horst et al., 1990). For instance, tomato (*Solanum lycopersycum*) exposed to 0, 25, and 50 μM Al in nutrient solution maintained a constant root-to-leaf area ratio, suggesting a decreased shoot water demand under Al stress (Gavassi et al., 2020). In addition, Al-sensitive plants show reduced specific leaf area (Santos et al., 2000) shoot growth and development (Jiang et al., 2009; Banhos et al., 2016), which may compensate for the smaller root system by attenuating the water flux for leaf transpiration. Nevertheless, even with this potential '*compensation*', leaf hydration is often reduced in Al-exposed plants, as evidenced by decreases in relative water content (RWC) and leaf water potential (Ψ_{leaf}), among other parameters (Jiang et al., 2008, 2009; Yang et al., 2011; Silva et al., 2012; Silva et al., 2018a; Cavalheiro et al., 2020; Gavassi et al., 2020). In 'Rangpur' lime (*Citrus limonia*) cultivated in nutrient solution with Al, xylem vessels became fibrous, and the stele exhibited altered anatomical structure (Banhos et al., 2016), along with increased lignin deposition in the vascular cylinder (Silva et al., 2019). Structural damage to vascular cylinder cells has also been reported in maize roots exposed to 300 μM Al (Batista et al., 2013). These findings suggest that Al retained in the root system may not only inhibit root growth but also impair the intrinsic capacity of roots to absorb and conduct water to the shoot, even in hydroponic systems.

Aluminum exposure is also associated with reduced shoot growth (Jiang et al., 2009), which is often considered a secondary or indirect symptom. Decreased CO_2 assimilation rates (A), as reported in tomato (*Solanum lycopersicum*) (Simon et al., 1994), maize (*Zea mays*) (Lidon et al., 1999), coffee (*Coffea arabica*) (Konrad et al., 2005), rye (*Secale cereale*) (Silva et al., 2012), and *C. limonia* (Banhos et al., 2016) was also associated with Al stress. Despite being linked to impaired photochemical performance (Chen et al., 2005a, 2005b; Jiang et al., 2009), Al-induced low A may also result from decreased stomatal conductance (g_s), causing 30–80 % reduction in this parameter, as evidenced in the studies mentioned above.

One factor potentially contributing to reduced root water transport is the diminished abundance of aquaporins (AQPs) (Maurel et al., 2015). AQPs are integral membrane proteins that facilitate the transport of water and small solutes through pores in the lipid bilayer—a process known as transmembrane transport (Maurel et al., 2008, 2015). This pathway may account for more than 50 % of root hydraulic conductivity in species such as tomato and *Arabidopsis* (Maggio and Joly, 1995; Sutka et al., 2011). AQP transcription and activity are known to be regulated by ABA in several species, including maize (Parent et al., 2009), tobacco (Mahdieh and Mostajeran, 2009), barley (Veselov et al., 2018), and tomato (Thompson et al., 2007; Fang et al., 2019).

Plants may express multiple AQP isoforms, grouped into eight subfamilies based on subcellular localization and sequence homology: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), nodulin-26-like intrinsic proteins (NIPs), GlpF-like intrinsic proteins (GIPs), hybrid intrinsic proteins (HIPs), X intrinsic proteins (XIPs), and large intrinsic proteins (LIPs) (Johanson et al., 2001; Hussain et al., 2020). The role of AQPs in stress responses has been widely demonstrated through their differential expression under salinity, cold, and drought conditions (Jang et al., 2004; Srivastava et al., 2016; Xu et al., 2020).

The PIPs are a large and diverse subfamily of genes which can be phylogenetically divided into PIP1 and PIP2 groups, differing in both structure and function (Chaumont et al., 2000; Johanson et al., 2001; Sakurai et al., 2005). In heterologous systems, PIP2 proteins exhibit high

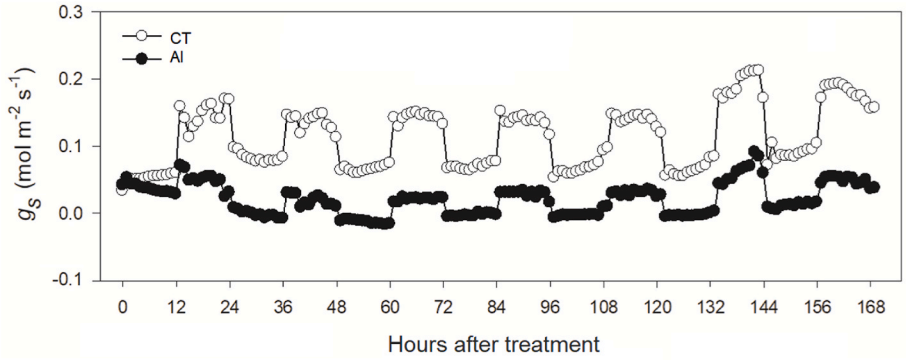

Fig. 2. Stomatal conductance (g_s) in tomato plants exposed to aluminum. Stomatal conductance (g_s, Y-axis) in tomato plants (*Solanum lycopersicum* cv. Micro-Tom) grown in nutrient solution containing 0 and 100 μM Al for 168 h (7 days). The X-axis indicates time after treatment onset (in hours). Data points represent hourly mean values.

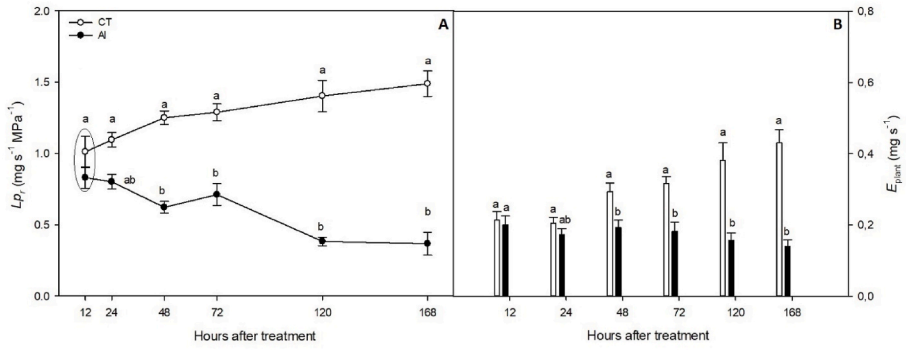

Fig. 3. Effects of aluminum on root hydraulic conductance and whole-plant transpiration in tomato. (A) Root hydraulic conductance (Lp_r) (A) and whole-plant transpiration (E_{plant}) (B) in tomato plants (*Solanum lycopersicum* cv. Micro-Tom) exposed to 0 and 100 μM Al for 168 h (7 days). The X-axis indicates different time points after aluminum exposure. Different letters indicate significant differences between treatments at each time point ($P < 0.05$). Bars represent standard error; overlapping circles denote statistically similar mean values.

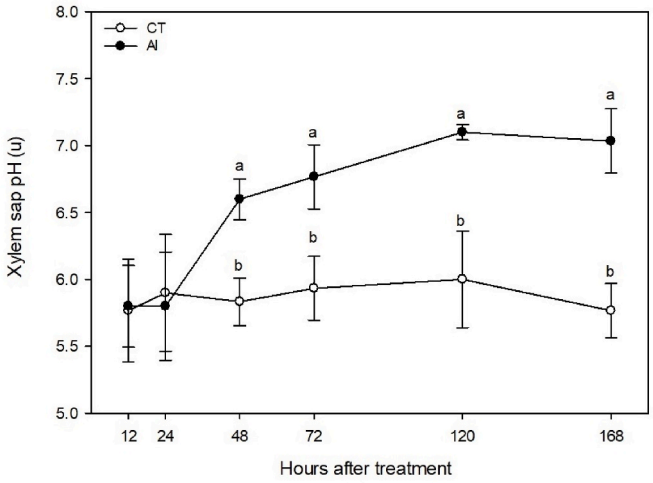

Fig. 4. Aluminum-induced changes in root xylem sap pH in tomato plants. Root xylem sap pH (Y-axis) in tomato (*Solanum lycopersicum* cv. Micro-Tom) plants grown in nutrient solution with 0 and 100 μM Al over 168 h. The X-axis represents different time points after aluminum exposure (in hours). Different letters indicate significant differences between treatments at each time point ($P < 0.05$). Bars represent standard error; overlapping circles denote statistically similar mean values.

water permeability, while PIP1 show lower transport activity (Kammerloher et al., 1994; Chaumont et al., 2000). However, co-expression of PIP1s and PIP2s enhances overall permeability, suggesting functional cooperation between them (Fetter et al., 2004). PIP2s, in particular, are more significantly associated with water stress responses and have been implicated in key physiological processes during growth, acclimation (Maurel et al., 2008; Lembke et al., 2012; Silva et al., 2013) and post-stress recovery (Alexandersson et al., 2005, 2010). Quantitative PCR has been widely used to assess the abundance of PIP2 transcripts across different organs and environmental conditions (Jang et al., 2004; Sakurai et al., 2005; Alexandersson et al., 2005, 2010; Hachez et al., 2006). For example, three highly expressed PIP2 isoforms in tomato roots (*SlPIP2;1*, *SlPIP2;5*, and *SlPIP2;7*) have been associated with high hydraulic conductivity under drought (Li et al., 2016). In the same species, *SlPIP2;10* and *SlPIP2;12* were downregulated in roots under salinity after recovery, highlighting their roles in tissue-specific and time-dependent water transport regulation (Jia et al., 2020).

The regulation of PIP2 gene expression under Al toxicity remains poorly understood, although it is likely linked to whole-plant water status. A recent study reported decreased expression of *ClPIP1;1* and *ClPIP2* in *C. limonia* roots exposed to Al, accompanied by reductions in shoot water status parameters such as Ψw and RWC, suggesting a role for PIP-type AQPs in Al-induced dehydration (Cavalheiro et al., 2020). Transcriptomic analysis of *Arabidopsis* under Al stress revealed that out of 35 functional AQP genes, 10 were upregulated and one was downregulated, indicating that AQP-mediated water or solute transport may be an early response to Al stress (Kumari et al., 2008).

The diverse transcriptional responses of AQPs (induction, repression, or no change) suggest that PIPs can be further classified into groups with

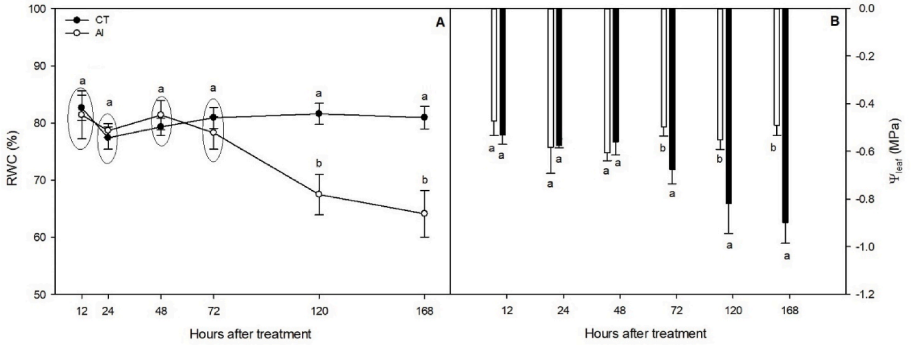

Fig. 5. Effects of aluminum on leaf hydration status in tomato plants. Relative water content (A) and leaf water potential (Ψw) (B) in tomato (*Solanum lycopersicum* cv. Micro-Tom) plants grown in nutrient solution with 0 and 100 μM Al over 168 h. The X-axis represents different time points after aluminum exposure (in hours). Different letters indicate significant differences between treatments at each time point ($P < 0.05$). Bars represent standard error; overlapping circles denote statistically similar mean values.

distinct contributions to water transport and regulation, with some isoforms being stress-responsive (Hachez et al., 2006). Accordingly, PIP aquaporins have been the focus of numerous studies in different species (Chaumont et al., 2000; Lopez et al., 2003; Jang et al., 2004, 2007; Sakurai et al., 2005; Lian et al., 2006; Azad et al., 2008; Alexandersson et al., 2005, 2010; Yooyongwech et al., 2013; Srivastava et al., 2016; Cavalheiro et al., 2020). A recent phylogenetic analysis by Rosa et al. (2020) revealed the presence of orthologous AQP genes in two Solanaceae species, *S. lycopersicum* and *Nicotiana tabacum*, with a prominent representation of PIPs. Of the 47 AQP genes expressed in *S. lycopersicum*, 14 belong to the PIP subfamily, including five *PIP1* and nine *PIP2* isoforms (Reuscher et al., 2013). Despite their known role in water uptake by roots (Javot and Maurel, 2002; Martre et al., 2002; Reuscher et al., 2013), the regulation of AQP isoforms under various stress conditions remains largely unknown (Jia et al., 2020).

Tomato is an excellent physiological model due to its rapid growth, short generation time, and well-characterized genetics and biology (Barone et al., 2008; Sant'Ana and Lefsrud, 2018). It is also an important commercial crop (FAOSTAT. FAO, 2018), highly sensitive to Al (Simon et al., 1994a,b; Zhou et al., 2009; He et al., 2019; Gavassi et al., 2020), with no cultivars proven to be Al-tolerant (Nogueirol et al., 2015a,b; Borgo et al., 2020). Thus, we propose that Al toxicity represses the expression of one or more PIP aquaporins in tomato and that this downregulation is associated with reduced water transport to the shoot, as reflected in altered shoot water relation parameters.

2. Material and methods

2.1. Plant material and experimental conditions

Forty tomato plants (*Solanum lycopersicum* Mill.) (Solanaceae) cv. 'Micro-Tom' were used. Seeds were germinated in seedling trays filled with a mixture of commercial substrate (Plantmax HT, Eucatex, Campinas, SP) and expanded vermiculite in a 1:1 ratio, supplemented with 1 g L^{-1} NPK 10:10:10 (m/m) and 4 g L^{-1} of lime. After three weeks growing in a growth chamber with the temperature set to 25 °C (500 ± 50 μmol photons m^{-2} s^{-1}; approximately 12 h photoperiod; average air temperature of 26 °C), plants with three leaves were transferred to opaque plastic boxes (43.5 x 28 x 7.7; 8 L) containing a nutrient solution with the Al treatments. Strips of hydrophobic cotton were used around the stem base to hold the plants in holes made in 1-cm-thick Styrofoam® sheets. The plants were maintained under hydroponic conditions for one week, with complete nutrient solution replacement twice, until they reached the appropriate size for sap sensor installation (approximately 30 days old).

The nutrient solution was based on Clark's solution (Clark, 1975), which was previously used to test Al toxicity (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 $Mg(NO_3)_2$ 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 $CuSO_4$ 5H2O, and 0.4 μM $NaMoO_2$ 2 H_2O. This solution shows high pH stability over time. In addition, it has a low phosphorus concentration compared to Hoagland's solution, which reduces the chance of precipitation of Al as $AlPO_4^-$. The nutrient solution was completely renewed every 3 days, and its pH (4.0 ± 0.1) was adjusted daily in order to minimize Al precipitation and maintain Al^{3+} in soluble form throughout the experiment. Besides macro- and micronutrients, the solution contained 0 and 100 μM Al provided through $AlCl_3$ 6 H_2O. This Al concentration was based on a previous study that reported Al toxicity symptoms in tomato plants, in which 25, 50, and 100 μM Al were tested, with the highest concentration causing the most significant growth inhibition (Gavassi et al., 2020).

2.2. Experimental design

Plants exposed to 0 and 100 μM Al were cultivated in nutrient solution for 7 days (168 h), and plant material was collected after 12, 24, 48, 120, and 168 h of treatment. At all sampling points, root tissues were collected for mRNA quantification. Stem sap flow (Q_{stem}) was measured continuously throughout the experiment. The following parameters were also assessed: total plant transpiration (E_{plant}); root hydraulic conductance (Lp_r); root xylem sap pH; leaf water potential (Ψ_w); relative water content (RWC); stomatal conductance (g_s); complete root biometric analysis; number of leaves, total leaf area, and organ biomass. In addition, Al concentration in roots and leaf blades was evaluated at the beginning and end of the study. To ensure reproducibility, samplings of Qstem, g_s, Eplant, Lp_r, root xylem sap, biometric parameters, and Al concentration were obtained from six plants (n = 6); Ψw and RWC were measured in two leaves of four plants (n = 8); and mRNA quantification was performed in four plants (n = 4), each with three technical replicates.

2.3. Analysis

2.3.1. Gene expression analysis

Leaf pieces (≈1 cm^2) and root pieces (1 cm in length), totalling 100 mg (fresh mass) for each leaf or root sample, were collected at 13h–15h, frozen in liquid nitrogen, and stored at −80 °C 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 reverse transcribed 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

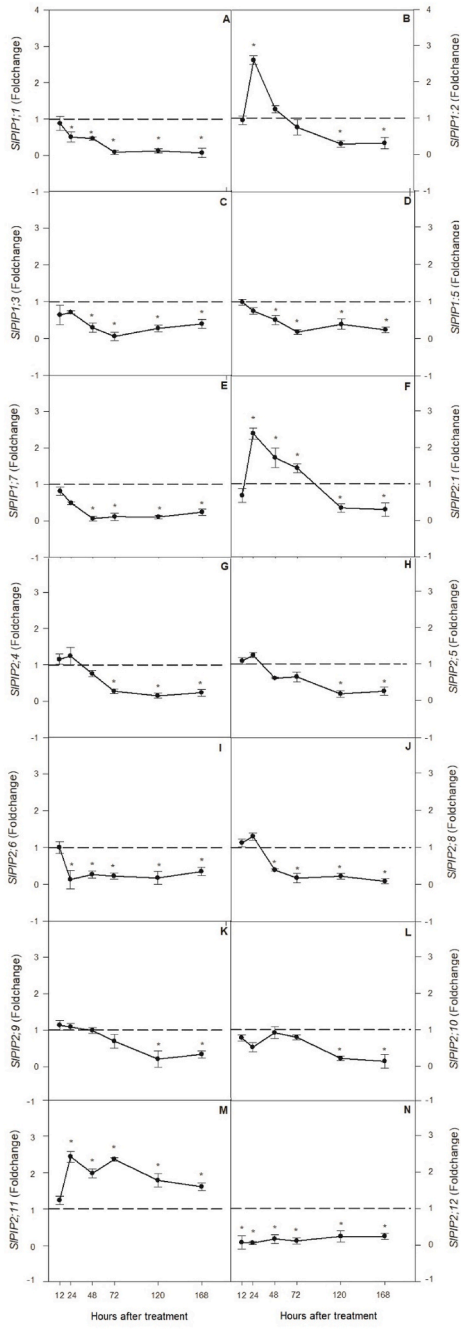

Fig. 6. Expression of PIP aquaporin genes in tomato roots under aluminum stress. Relative expression of SlPIP1 and SlPIP2 isoforms in the roots of tomato plants (*Solanum lycopersicum* cv. Micro-Tom) grown in nutrient solution containing 0 μM (control, CT) and 100 μM Al for 168 h (7 days). Panels show: *SlPIP1;1* (A), *SlPIP1;2* (B), *SlPIP1;3* (C), *SlPIP1;5* (D), *SlPIP1;7* (E), *SlPIP2;1* (F), *SlPIP2;4* (G), *SlPIP2;5* (H), *SlPIP2;6* (I), *SlPIP2;8* (J), *SlPIP2;9* (K), *SlPIP2;10* (L), *SlPIP2;11* (M), and *SlPIP2;12* (N). The Y-axis represents the relative gene expression (fold change), and the X-axis indicates time after aluminum exposure (in hours). The dashed line marks baseline expression in control plants (fold change = 1). Different letters indicate significant differences between treatments at each time point ($P < 0.05$). Bars represent standard error.

expression analysis was 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 *PIP1 and PIP2* (Table 1) used in the experiment were previously used in Jia et al. (2020). However, the primers PIP2; 5 and PIP2; 8 of their study did not amplify any product in any of our tests. Therefore, after performing a BLASTn search using the primers against the RefSeq database, we identified the accession number of the gene registered in NCBI and, based on the reference gene, two new pairs of primers for PIP2; 5 and PIP2; 8 were designed (Table 1). As reference genes, we used a*ctin* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Table 1) (Sang et al., 2017). Amplification efficiencies were calculated for each primer using Miner software (Zhao and Fernald, 2005).

GAPDH means glyceraldehyde-3-phosphate dehydrogenase.

To calculate the relative expression, we converted the Ct (cycle threshold) values of each sample, determined by the average of three technical replicates, 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 the 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 *actin* and *GAPDH*. The 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 the mean NRQ values that were obtained from the calibrator (root samples of plants not exposed to Al). As a result of this normalization procedure, the baseline fold change for the control group was set to 1. Four independent biological replicates (plant samples) were used to calculate the mean for each time point and treatment combination.

2.3.2. *Whole-plant transpiration (E_{plant})*

The gravimetric method was used (Edwards, 1986). 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 Plant Water Status Console; 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 h). Then, the pot was weighed on a 0.01g precision scale (Adventurer Pro AV4102; Ohaus, Thetford, UK). One hour later (11:00 h), the pot was weighed again and the whole-plant water uptake was calculated by the difference between the initial and final pot massess. 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). E_{plant} was obtained as the ratio between water uptake and time (mg H_2O s^{-1}) (Puértolas et al., 2015). This measurement assesses a hydraulic parameter that adds extra evidence to support the stem sap flow evaluations.

2.3.3. *Stem sap flow (Q_{stem}) and stomatal conductance (g_s)*

Sap flow (Q) measurement using the heat ratio method (HRM) has been successfully applied to stems—and even peduncles and petioles—with diameters as small as 2 mm, with reasonable accuracy (Skelton et al., 2013). These sensors therefore represent a viable alternative to transpiration measurements obtained via gas exchange analyzers (Silva et al., 2023). Unlike gas exchange, which is operator-dependent and typically performed at discrete time points, sap flow can be monitored continuously (e.g., every 15 min) and stored on a data logger. This is particularly advantageous when investigating stress responses, such as those triggered by Al exposure, since transient events may go undetected with punctual gas exchange measurements (Banhos et al., 2016).

To ensure HRM-derived sap flow measurements reflect stomatal conductance accurately, we calibrated the sensors (SF-4M, Edaphic Scientific) by installing them on untreated plants connected to a data logger (CR-1000, Campbell Scientific). For this purpose, HRM sensors (SF-4M, Edaphic Scientific, Port Macquarie, NSW, Australia) were installed in untreated plants and connected to a data logger (CR-1000,

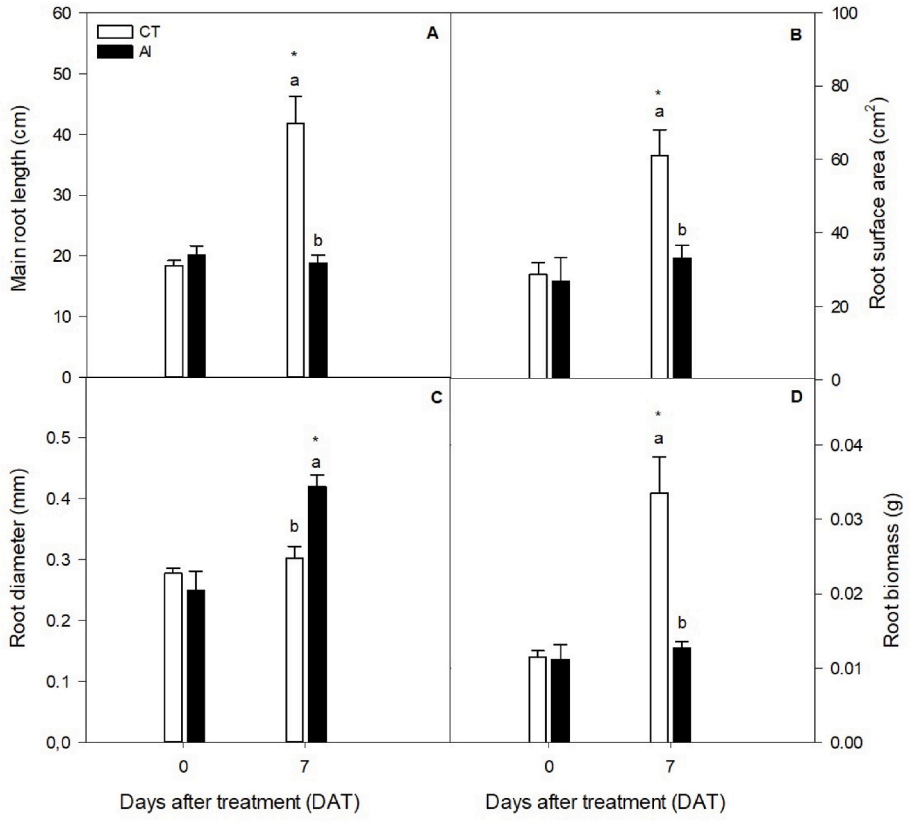

Fig. 7. Effects of aluminum on root morphology and biomass in tomato plants. Primary root length (A), root surface area (B), mean root diameter (C), and root biomass (D) of tomato plants (*Solanum lycopersicum* cv. Micro-Tom) exposed to 0 μM (control, CT) and 100 μM Al at 0 and 7 days after treatment (DAT). The Y-axis represents the respective morphological or biomass parameter, and the X-axis indicates sampling time (in days). Different letters indicate significant differences between Al treatments at each evaluation date (Tukey's test, $P < 0.05$); asterisks denote significant differences within the same treatment between 0 and 7 DAT.

Campbell Scientific) for calibration only. Gas exchange was simultaneously measured throughout the day using a portable system (Li6400xt, LI-COR, Lincoln, NE, USA) on various leaves of the plants equipped with HRM sensors. This allowed stomatal conductance (g_s) values to be linearly regressed against the voltage output (mV) recorded by the data logger (Fig. 1).

2.3.4. Leaf water potential (Ψ_{leaf})

It was measured between 11:00h and 14:00 h on the same leaf on which gas exchange rates were measured, using a pressure chamber (Model 3000F01 Plant Water Status Console; Soil Moisture Equipment Corp., USA). Detached leaves were immediately put in a plastic bag with moisturized paper and directly taken to the laboratory, where these were placed in the pressure chamber within 60 s 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.

2.3.5. Relative water content (RWC)

Leaf discs were collected at 13h–15h from plants of both treatments and calculated as:

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

where FM is the fresh mass (immediately measured after collected); TM is the turgid mass after rehydrating samples for 24 h in 100 mL deionized water inside amber flasks (to avoid photosynthetic activity); and DM is the dry mass after oven-drying the discs at 60 °C for 48 h, according to Silva et al. (2018a).

2.3.6. 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 the plant was inserted into the pressure chamber with its roots in a nutrient solution, as described for measuring E_{plant}, the shoot was removed, and a series of overpressures (from 0.1 MPa to 0.4 MPa at 0.1 MPa increments) were applied so that the sap flow rate was determined at each pressure. The sap collection on the cut surface was done every 30 s with the aid of small portions of absorbent paper inside a microtube, whose dry mass was previously known. After collecting the sap, the mass of the wet absorbent paper was immediately measured on an analytical scale. 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 resulted in Lp_r.

2.3.7. 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 were applied to collect xylem sap (Else et al., 2006). Sap samples were collected in previously weighed 1.5 mL vials, frozen in liquid nitrogen (N_2) 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).

2.3.8. Biometric parameters

Immediately before applying Al treatments, the smallest leaf of each plant was marked, and its length, as well as its terminal leaflet length, was measured with a ruler (cm) at 0, 1, 3, 5, 7 and 10 days after treatment (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.

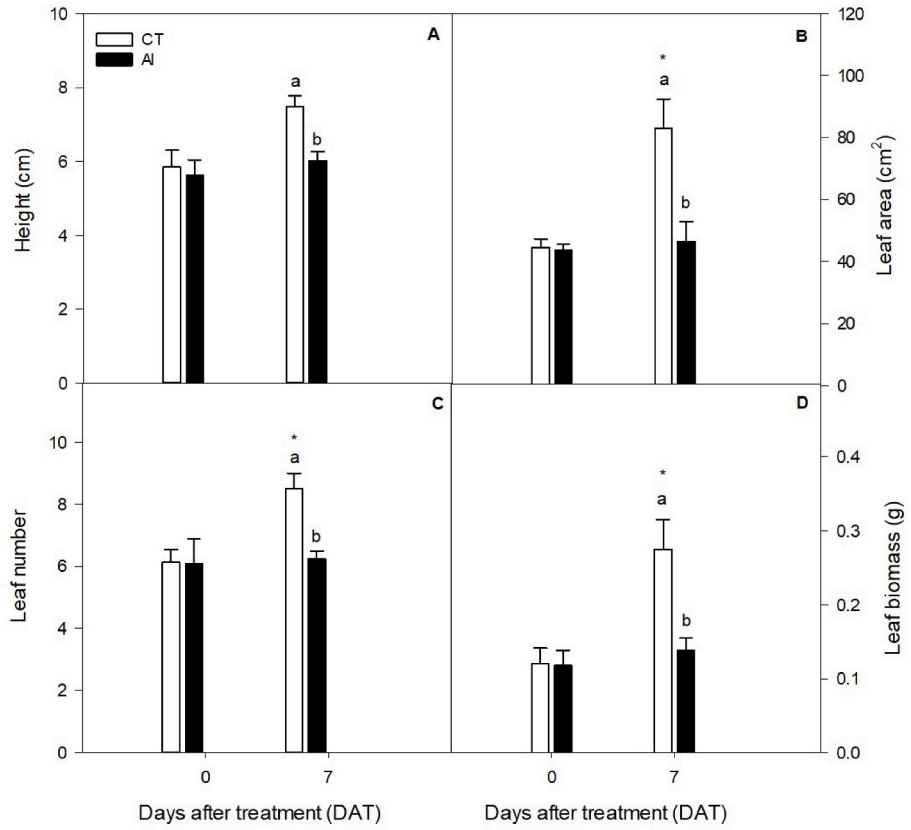

Fig. 8. Effects of aluminum on shoot growth and biomass in tomato plants. Stem length (A), leaf area (B), number of leaves (C), and leaf biomass (D) of tomato plants (*Solanum lycopersicum* cv. Micro-Tom) exposed to 0 μM (control, CT) and 100 μM Al at 0 and 7 days after treatment (DAT). The Y-axis represents each shoot growth parameter, and the X-axis indicates sampling time (in days). Different letters indicate significant differences between Al treatments at each time point (Tukey's test, $P < 0.05$); asterisks denote significant differences within the same treatment between 0 and 7 DAT.

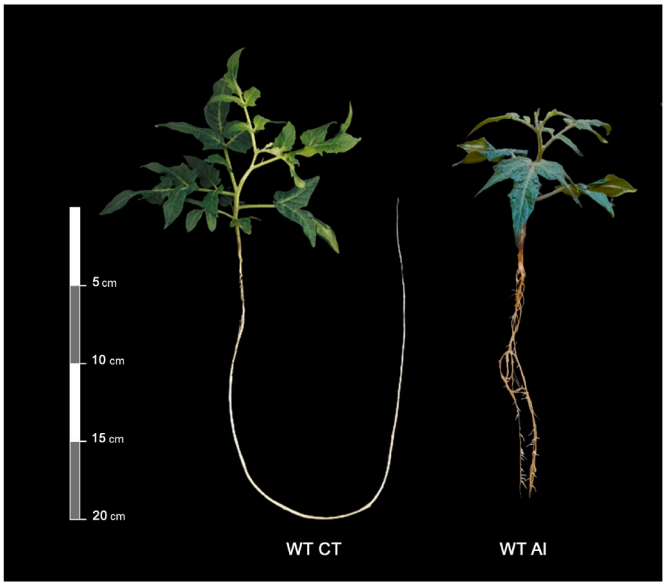

Fig. 9. Morphological details of tomato (*Solanum lycopersicum* cv. Micro-Tom) plants grown in nutrient solution containing 0 (control, CT) and 100 μM Al for 168 h (7 days).

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™ 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 oven-dried at 60 °C until constant mass. The biomass (g) of organs was measured on a 0.01g precision scale (Adventurer Pro AV4102; Ohaus, Thetford, UK).

2.3.9. Aluminum quantification

Aluminum quantification was performed according to Sarruge and Haag (1974). Root samples were washed thrice in deionized water to avoid excess Al from the nutrient solution. Each sample was digested with nitric acid, fortified with Al standards and analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES).

2.3.10. Data analysis

The data were submitted to one-way analysis of variance (ANOVA), and mean values were compared, separately for each DAT, between Al treatments by least significant difference (LSD) at 0.05 confidence level using Student's T test ($P < 0.05$).

3. Results

3.1. Al-induced impairment of water relations over time

From the linear calibration equations (Fig. 1), g_s values were calculated over 168 h (Fig. 2). The g_s curves showed clear patterns of stomatal opening and closure following the light cycle in the growth chamber. Stomatal closure occurred a few hours after Al treatment began, and between 11:00 h and 13:00 h, Al-treated plants exhibited lower g_s values as early as 24 h after treatment (Fig. 2).

Al significantly reduced *Lpr* and E_{plant} from 48 h onwards, and at 168

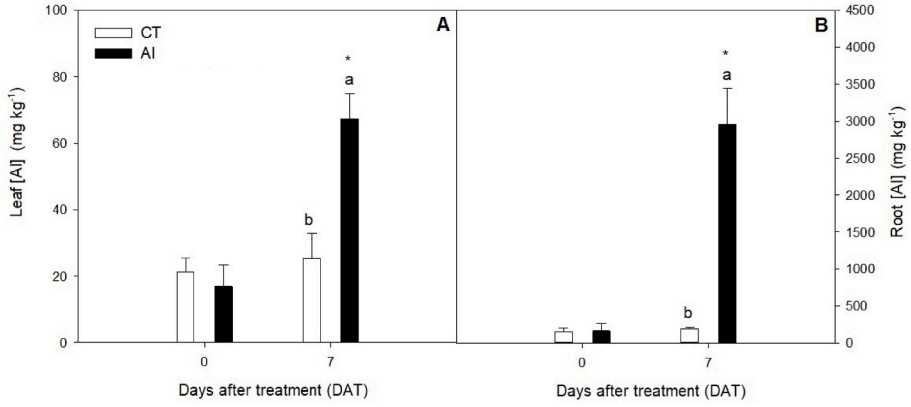

Fig. 10. Aluminum concentration in leaves (A) and roots (B) of tomato (*Solanum lycopersicum* cv. Micro-Tom) plants exposed to 0 (control, CT) and 100 μM Al at 0 and 7 days after treatment (DAT). Different letters indicate significant differences between Al treatments at each time point (Tukey's test, $P < 0.05$); asterisks denote significant differences within the same treatment between 0 and 7 DAT.

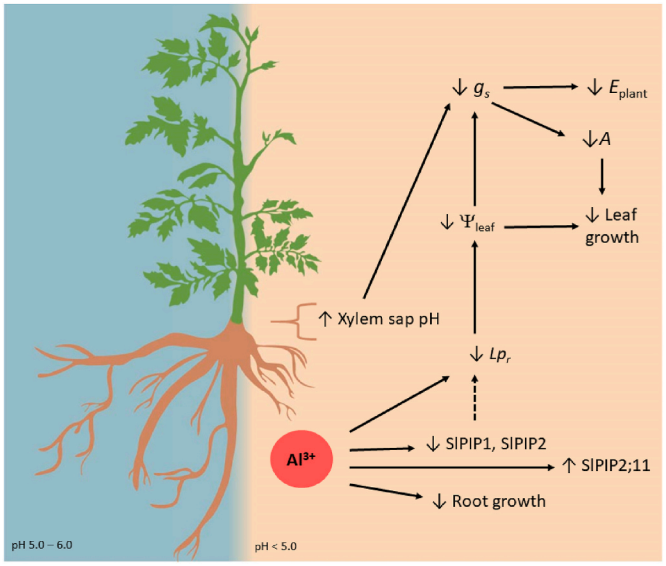

Fig. 11. Model of plant hydraulics in tomato (*Solanum lycopersicum*) exposed to aluminum toxicity (on the right). Solid lines ending in arrowheads indicate causal or stimulatory effects, while dashed lines represent suggested or indirect effects.

h they were 75 % and 67 % lower, respectively than in the CT (Fig. 3A and B). Xylem sap pH increased by more than 20 % in Al-treated plants compared with the CT over the same period (Fig. 4).

RWC decreased significantly only after 120 h of Al treatment, reaching 64.1 %, which was 20.8 % lower than in the CT at 168 h (Fig. 5A). Ψ_{leaf} decreased from 72h after Al exposure and was nearly twice as negative as in the CT at 168 h (Fig. 5B).

3.2. PIP expression in roots over time

SlPIP1;1 and *SlPIP2;6* were downregulated from 24 h (Fig. 6A–L); *SlPIP1;3*, *SlPIP1;5*, *SlPIP1;7*, and *SlPIP2;8* from 48 h (Fig. 6C–E, J); and *SlPIP2;4* from 72 h (Fig. 6G). *SlPIP2;12* was consistently downregulated in all sampling periods (Fig. 6N). *SlPIP1;2* and *SlPIP2;1* showed transient upregulation before downregulation after 120 h (Fig. 7B–F). *SlPIP2;11* was upregulated from 24 h to the end of the experiment (Fig. 6M).

3.3. Biometric parameters

At 7 DAT, main root length, surface area, and biomass were 55 %, 45 %, and 62 % higher, respectively, in the CT in relation to the Al treatment (Fig. 7A–B, D; Fig. 9), whereas root diameter was 38 % higher in Al-treated plants, in relation to the CT (Fig. 7C). The shoot growth, height, leaf area, leaf number, and leaf biomass were reduced by 26 %, 44 %, 19 %, and 49 %, respectively, in response to Al (Fig. 8A–D, Fig. 9).

3.4. Aluminum concentration in plant organs

After 168 h of exposure, Al concentration was increased in more than twofold in leaves (Fig. 10A) and fifteenfold in the roots (Fig. 10B) of Al-treated plants compared with the CT.

4. Discussion

4.1. Stomatal conductance and early hydraulic responses

The rapid decline in g_s observed within 24 h of Al exposure (Fig. 2) indicates that stomatal closure is among the earliest physiological responses to Al toxicity. Similar reductions have been reported in tomato (Simon et al., 1994; Gavassi et al., 2020), maize (Lidon et al., 1999), rye (Silva et al., 2012), and 'Rangpur' lime (Banhos et al., 2016, Gavassi et al., 2021), often ranging from 30 to 80 %. While attributed to reduced water uptake from root growth inhibition (Kochian et al., 2015) and "*consequently*" lesser surface area to absorb water, our data, combined with decreases in Lp_r and E_{plant} (Fig. 3A and B), support the theory that Al can also prompt impaired water transport efficiency, even in hydroponic conditions, as previously shown (Batista et al., 2013; Banhos et al., 2016; Silva et al., 2018a; Cavalheiro et al., 2020; Gavassi et al., 2020, 2021). These results suggest that Al may suppress parameters that rapidly affect *Lpr*, such as AQP expression and activity (Fig. 11).

4.2. Leaf water status and dehydration onset

The alkalinization of xylem sap from 48 h onward (Fig. 4) suggests a chemical signal contributing to stomatal closure. Increased sap pH is a well-known mechanism for enhancing ABA sensitivity in guard cells during drought (Hartung and Radin, 1989; Wilkinson and Davies, 1997) and has been documented in Al-stressed tomato plants (Gavassi et al., 2020). Studies using ABA-deficient or ABA-insensitive mutants would be highly informative for further clarifying the role of ABA in modulating aquaporin expression under Al toxicity. Such approaches would allow differentiation between direct aluminum effects and those mediated by ABA signaling, helping to determine whether AQP downregulation

results primarily from hormonal control, hydraulic constraints, or their interaction.

The earlier decline in Ψ_{leaf} compared to RWC (Fig. 5A and B) may indicate a reduction in whole-plant hydraulic conductance, as demonstrated in 'Rangpur' lime under Al stress in nutrient solution (Cavalheiro et al., 2020), leading to a drop in leaf water potential before detectable dehydration symptoms (Trueba et al., 2019). However, after 120 h, significant RWC reduction confirmed the onset of tissue-level water deficit. This pattern matches reports in other Al-sensitive species (Yang et al., 2011; Silva et al., 2012; Cavalheiro et al., 2020) and underscores the progressive nature of Al-induced hydraulic stress.

4.3. PIP expression and water transport regulation

PIPs play a pivotal role in facilitating water transport across plant cell membranes and are key determinants of root hydraulic conductivity (Maggio and Joly, 1995; Sutka et al., 2011). Despite their importance, information on how Al toxicity affects PIP expression and activity is surprisingly scarce. Downregulation of *SlPIP1;1* and *SlPIP2;6* coincided with the early drop in g_s (Figs. 2 and 6A, L). Downregulation of *SlPIP1;3*, *SlPIP1;5*, *SlPIP1;7*, and *SlPIP2;8* aligned with reduced Lp_r, E_{plant} and increased sap pH (Fig. 3A–B, 4, and 6C–E, J), linking transcriptional regulation of AQPs to both hydraulic and chemical signaling. Although *SlPIP2;11* showed significant and persistent upregulation (Fig. 6M), this increase was insufficient to maintain water status (Fig. 5A and B), indicating that compensatory expression of individual *PIP* genes may not counterbalance the systemic impact of Al on root hydraulics. The upregulation of *SlPIP2;11*, contrasting with the downregulation of seven other members of the *PIP2* subfamily (*SlPIP2;4*, *SlPIP2;5*, *SlPIP2;6*, *SlPIP2;8*, *SlPIP2;9*, *SlPIP2;10*, and *SlPIP2;12*; Fig. 6G–N), may reflect an indirect role in maintaining water transport. Considering recent findings in tomato under high vapor pressure deficit (VPD), where *SlPIP2;11* overexpression improved root morphology, stomatal performance, antioxidant capacity, and photosynthetic stability (Zhao et al., 2025), it is plausible that a similar mechanism could be triggered under Al stress. In Al-sensitive plants such as 'Rangpur' lime, Al toxicity considerably reduces g_s responsiveness to increasing VPD, leading to decreased leaf hydration Silva et al. (2018b). In this context, *SlPIP2;11* upregulation might contribute to sustaining hydraulic function and/or mitigating oxidative damage. Although speculative, this hypothesis suggests that *SlPIP2;11* could participate in a broader signaling or protective pathways under abiotic stress conditions.

In 'Rangpur' lime, Al exposure altered the expression of PIP family members in an isoform-specific manner, with *ClPIP1;1* and *ClPIP2* being downregulated and *ClPIP1;2* upregulated (Cavalheiro et al., 2020). These authors also observed that *ClPIP1;1* expression showed a positive correlation with both *A* and g_s under Al stress, suggesting that the reduced expression of *ClPIP1;1* and *ClPIP2* in roots may contribute to the low leaf hydration observed in this species.

Gene-specific patterns such as PIP2; 1 and TIP1; 1 have been reported in alfalfa *(Medicago sativa)*, and the proteins decoded by PIP2; 1 and TIP1; 1 seem to stabilize during Al exposure through interaction with the dehydrin DHN1, a process associated with increased oxalate exudation from root tips and reduced Al accumulation in root apices (Lv et al., 2021). These observations highlight that certain AQPs may perform roles beyond water transport, including the movement of metabolites or chelates that contribute to Al detoxification. Moreover, studies in hydrangea (*Hydrangea macrophylla*), buckwheat (*Fagopyrum esculentum*), tea (*Camellia sinensis*), and Arabidopsis have identified NIP and TIP aquaporins, such as *HmPALT1*, *HmVALT*, and *NIP1;2*, that mediate the transport of Al or Al–chelate complexes (Chauhan et al., 2021; Rahman et al., 2024). In these studies, increased AQP expression was associated with enhanced Al tolerance, indicating that AQP responses to Al stress are isoform- and function-specific, and that their contribution to tolerance may extend beyond the regulation of root hydraulic properties. Therefore, besides responding to the presence of Al, which attenuates water uptake and transport to the shoots in Al-sensitive species, AQPs may be also involved with mechanisms of resistance against Al in species that evidence some level of tolerance to this metal, and this warrants further investigation.

While our study highlights significant transcriptional changes in PIP aquaporins under Al toxicity, it is also important to note that aquaporin activity can be rapidly regulated through post-translational modifications. In particular, phosphorylation is a key mechanism for gating and trafficking, whereas ubiquitination controls aquaporin abundance through membrane turnover and degradation (Niño-González and Duque, 2025). Future work could therefore investigate whether post-translational regulation of PIPs contributes to the hydraulic adjustments observed under Al stress, adding another mechanistic layer beyond transcriptional control.

4.4. Growth inhibition and Al accumulation

Root thickening observed in Al-treated plants (Fig. 7C) is consistent with Al binding to cell wall components, causing stiffening and rupture of sub-apical epidermal regions (Kopittke et al., 2008, 2015). This mechanical effect explains reduced elongation and increased diameter. Reduced shoot growth (Fig. 8A–D and 9) is often considered a secondary effect of restricted water and nutrient uptake (Singh et al., 2017; Silva et al., 2018a), but the observation of Al in leaves twice as high in treated plants compared to control after 7 days (Fig. 10A) suggests potential direct effects of Al on shoot tissues during prolonged exposure.

5. Conclusion

Aquaporin genes from PIP1 and PIP2 isoforms reduce their expression in tomato roots in response to Al, with the exception of the upregulated *SlPIP2;11*. Downregulation of these AQP genes, which were associated with the decrease of hydric (Yw, g_s, and RWC) and hydraulic (E_{plant} and Lp_r) parameters highlights that Al-induced dehydration of shoots is not just a matter of inhibition of root growth. Future biotechnological initiatives should focus on AQP genes of PIPs isoforms to overcome the physiological drought imposed by Al in crop species.

CRediT authorship contribution statement

Marina Alves Gavassi: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Mariana Feitosa Cavalheiro:** Writing – review & editing, Methodology, Formal analysis. **Brenda Mistral de Oliveira Carvalho:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Giselle Schwab Silva:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Douglas Silva Domingues:** Writing – review & editing, Data curation. **Gustavo Habermann:** Writing – review & editing, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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