Aluminum-induced stomatal closure is related to low root hydraulic conductance and high ABA accumulation

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ABSTRACT

Many studies ask how aluminum (Al) reduces the root growth, but as Al is mostly retained in the root system, the physiological explanations for the also expected Al-induced decrease in stomatal conductance (g_s) are unclear, mainly in well-watered conditions. We exposed tomato plants (*Solanum lycopersicum*) to 0, 25, 50 and 100 μM Al in nutrient solution to investigate 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 measured ABA delivery rate, xylem sap pH and the root/leaf area ratio in order to explain the low g_s in plants exposed to Al. Declines in Lp_r and g_s were proportional to the increase in Al concentration, and all Al treatments similarly decreased Ψ_{leaf}, indicating the plant's attempt to maintain leaf water status while accumulating more ABA. Despite Al-induced increases in root ABA, the root-to-shoot delivery of ABA did not enhance, but Al caused root xylem sap alkalization. Despite the stability of root/leaf area ratio across a range of Al concentrations (0, 25 and 50 μM Al), the leaf hydration and stomatal opening was not conserved. Here we provide the first evidence that decreases in Lp_r and increases in ABA might explain Al-induced stomatal closure.

Key words: abscisic acid, aluminum, stomatal conductance, water transport, xylem sap pH

1. 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 & 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 the rapid inhibition of root growth (Delhaize & Ryan 1995; Kopittke et al, 2008; Horst et al, 2010), resulting in low root surface area and biomass, limiting water and nutrient uptake (Kochian et al. 2004). Thus, a linear and simple cause-and-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 relative leaf water content (RWC) and leaf water potential (Ψ_{leaf}) (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 frequently 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 decreases stomatal conductance (g_s) in *Solanum lycopersicum* (Simon et al., 1994b), *Coffea arabica* L. (Konrad et al., 2005), *Secale cereale* (Silva et al., 2012), *Theobroma cacao* (Ribeiro et al., 2013), *Zea mays* L. (Anjum et al., 2016) and *Citrus limonia* (Banhos et al., 2016; Silva et al., 2018). However, the mechanisms explaining how Al leads to stomatal closure remain largely unknown.

Most studies that reported reduced root and shoot growth and low g_s were performed using plants growing directly in nutrient solution where water is constantly available (Simon et al., 1994b; Konrad et al., 2005; Silva et al., 2012; Ribeiro et al., 2013; Banhos et al., 2016; Silva et al., 2018). Besides root growth inhibition, plants exposed to Al may have impaired water uptake and transport to the shoots. For instance, fibrous xylem vessels were observed in *C. limonia* grown in nutrient solution with Al and showing low Ψ_{leaf} and g_s (Banhos et al., 2016). Al causes more 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 & Maurel,

2002). Actually, 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 g_s, nor associated both variables.

Besides plant hydraulics, root-to-shoot chemical signaling could also explain the low g_s 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 decreasing g_s (Schachtman & 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 (OAs), forming non-toxic Al-OA complexes in the rhizosphere thereby avoiding excessive Al uptake (Shen et al., 2004). Al increased ABA accumulation in both roots and leaves of soybean and accelerated ABA transport from the roots, suggesting ABA may regulate Al resistance in soybean plants, even though g_s was not measured (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 and Davies 1997). However, no studies have assessed whether Al-induced ABA accumulation can decrease g_s, either due to root-to-shoot signaling (xylem ABA or pH) or local ABA synthesis in the leaf.

The present study evaluated whether low Lp_r and Ψ_{leaf} (hydraulic mechanisms) and high ABA biosynthesis (chemical mechanisms) regulates stomatal conductance and leaf growth of tomato plants (*Solanum lycopersicum* Mill.) exposed to increasing Al concentrations in nutrient solution for 10 days.

2. Material and methods

2.1. Plant material and experimental conditions

Forty 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 a nutrient solution at ½ strength and pH 5.5 ± 0.2. After three weeks growing in a glasshouse 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 x 26 x 16 cm; 15 L) containing the nutrient solution with the Al treatments.

The nutrient solution was based on Clark's solution (Clark, 1975), which was previously used to test Al toxicity (Villa et al., 2009; Silva et al. 2018; 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 $CuSO_4$ 5H_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 its 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., 1994a,b; Zhou et al., 2009; He et al., 2019).

The lids of the boxes containing the nutrient solution had 5 holes of 2.5 cm in diameter, and the plants growing on the rockwool cubes were fixed in these holes. Two boxes were used for each treatment. The boxes were maintained on benches, inside the glasshouse, with the same conditions as previously described.

2.2. 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 parameters. Non-destructive traits such as leaf length, main root length, whole-plant transpiration (E_{plant}), CO_2 assimilation rate (A) and stomatal conductance (g_s) were measured in ten 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), five plants were used to measure leaf water potential, biometric parameters in leaves (number, area and biomass) and roots (total length, surface area, diameter and biomass), and leaf and root Al concentrations. Another five plants were used to measure pressure-induced sap flow rates, root hydraulic conductance (Lp_r), xylem sap pH, abscisic acid (ABA) concentration in roots, xylem sap and leaves. The values presented are a mean of two repeated experiments.

2.3. Analysis

2.3.1. 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), 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). E_{plant} was obtained as the ratio between water uptake and time (mg H_2O s^{-1}) (Puértolas et al., 2015).

2.3.2. Stomatal conductance (g_s) and CO_2 assimilation rate (A)

Stomatal conductance and CO_2 assimilation rate were measured between 9:00h and 11:30h 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). Conditions in the leaf cuvette (2 cm^2) were set to approximately match the environmental conditions in the glasshouse: CO_2 at ambient concentration (400 µmol mol^{-1}) using the 6400-01 CO_2 mixer (LI-COR, USA), 500 µmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) using the 6400-02B LED light source, which provides 90% red and 10% blue spectra (LI-COR, USA). The air temperature of leaf cuvette was of 25°C, and relative humidity maintained at 50–60%.

2.3.3. Leaf water potential (Ψ_{leaf})

Leaf water potential was measured between 11:00h and 14:00h on the same leaf 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 a 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.

2.3.4. 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 & Diatloff, 2016). After the plant was inserted into the pressure chamber with their roots in 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 seconds 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.

2.3.5. 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) before measuring root xylem sap ABA concentration ($[X\text{-}ABA]_{root}$).

2.3.6. ABA quantification by radioimmunoassay

One leaflet (from the same leaf used for measuring g_s) and root (four root tips with 10 mm in length) samples (≈ 5-10 mg DW) were frozen in liquid nitrogen and stored at –18 °C. Leaf samples were collected before shoot removal to measure Lp_r, while root samples were collected after Lp_r assessment to avoid damaging the root apices prior to Lp_r analyses. 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; dry sample(g):water(g)) 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 ($[ABA]_{leaf}$), root ($[ABA]_{root}$), and root xylem sap ($[X\text{-}ABA]_{root}$) was measured by radioimmunoassay method, using the monoclonal antibody AFRC MAC 252 (Quarrie et al. 1988). While $[ABA]_{leaf}$ and $[ABA]_{root}$ were measured in the aqueous extract, the $[X\text{-}ABA]_{root}$ was measured directly in sap samples. $[X\text{-}ABA]_{root}$ was determined in the sample with the closest sap flow rate to E_{plant}.

2.3.7. 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 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 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).

2.3.8. Aluminum quantification

Al quantification was performed according to Havlin & Soltanpour (1980). 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 an inductively coupled plasma optical emission spectrometry (ICP-OES).

2.3.9. 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 LSD (least significant difference) at 0.05 confidence level using Tukey's test ($P < 0.05$). In addition, a Pearson correlation analysis was performed between individual values of g_s and $[ABA]_{leaf}$, Ψ_{leaf}, xylem sap pH, Lp_r and A obtained from plants exposed to Al.

3. **Results**

3.1 Biometric parameters

Aluminum decreased leaf length (Fig. 1a) and terminal leaflet length (Fig. 1c) from 5 DAT in a concentration-dependent manner. All treatments had significantly 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 by 55 and 48% respectively. Thus, Al treatment decreased both petiole and leaflet expansion similarly.

At 10 DAT, leaf number, leaf area and leaf biomass decreased with increasing Al concentration (Table 1; Fig. 1b). 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 (Table 1). Compared to control plants, the 100 μM Al treatment decreased leaf number (-33%), leaf area (-82%) and leaf biomass (-64%) (Table 1). Thus, Al decreased leaf initiation, expansion and biomass accumulation.

Within 1 day, all Al treatments limited the main root length (Fig. 1e). Thereafter, roots exposed to 100 μM Al almost ceased growing (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. 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. 1e). Thus, increasing nutrient solution Al concentrations proportionally decreased root elongation (Fig. 1d).

At 10 DAT, all Al concentrations significantly increased root diameter by 36% compared to control plants, with no differences between Al concentrations (Table 1). In addition, increasing Al concentration significantly decreased root surface area and root biomass in a concentration-dependent manner (Table 1). 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 (Table 1). Moreover, all Al concentrations significantly increased root diameter by 36% compared to control plants, with no differences between Al concentrations (Table 1). Thus, Al rapidly inhibited root growth, but caused root thickening.

As Al concentrations in the root environment increased, the leaf area and the root surface area decreased proportionally (Table 1), 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 lower root/leaf area ratio (Fig. 1f). Therefore, inhibition of leaf area expansion compensated for the decrease in root area only up to 50 μM Al.

3.2 Water relations

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 (Fig. 2a). Compared to the control plants, at 10 DAT, the 25, 50 and 100 μM treatments decreased g_s by 30, 53 and 62%,

284 respectively (Fig. 2a). Thus, stomatal closure was detected immediately after root growth
285 inhibition (compare Fig. 1e x Fig. 2a) and earlier (by two days) than leaf growth inhibition
286 (compare Fig. 1a and 1c x 2a). At 10 DAT, CO_2 assimilation rate (*A*) decreased with increasing Al
287 concentration (Fig. 2b). Compared to the control plants, at 10 DAT, the 25, 50 and 100 μM
288 treatments reduced *A* by 27, 40 and 53% respectively (Fig. 2b). Thus, the decrease in g_s might
289 explain the reductions observed in *A*. As expected, g_s showed inversely proportional correlation
290 with $[ABA]_{leaf}$, Ψ_{leaf} and xylem sap pH, while exhibiting a direct proportional correlation with Lp_r
291 and *A* (Table 2).
292 Increasing the pneumatic pressure applied to de-topped root systems linearly increased sap
293 flow rates in all Al treatments, but the slopes of the curves were lower as Al concentration was
294 raised in the nutrient solution (Fig. 3a). Al reduced Lp_r of plants exposed to 25 (-25%), 50 (-60%)
295 and 100 (-70%) μM Al, when compared to 0 μM Al (Fig. 3b). Thus Al-induced decreases in
296 whole plant transpiration were correlated with the decrease in Lp_r.
297 All Al treatments reduced Ψ_{leaf} by 0.3 MPa (-40%) compared to control plants, with no
298 differences between the Al treatments (Fig. 4a). Root xylem sap pH increased 0.5, 0.6 and 0.7
299 units in plants treated with 25, 50 and 100 μM Al, respectively, when compared to the control
300 plants (Fig. 4b).
301
302 *3.3 ABA and plant signaling*
303 In general, Al treatments increased tissue ABA concentrations in a concentration-
304 dependent manner (Fig. 5a, b). Leaf ABA concentrations (Fig. 5a) were more than 10 times higher
305 than root ABA concentrations (Fig. 5b), with significant differences between Al treatments for
306 both organs.
307 Increasing the Al concentration in the nutrient solution significantly decreased E_{plant}
308 measured at 10 DAT (Fig. 6a), being 13, 42 and 68% lower in plants treated with 25, 50 and 100
309 μM Al respectively, in relation to control plants. Root xylem sap ABA concentrations were
310 indistinguishable between control (0) and 25 μM Al treatments, and between the 50 and 100 μM
311 Al treatments (Fig. 6b). This parameter was 35% higher in the latter two treatments when
312 compared to the former. ABA delivery rate ([ABA] x transpirational flow rate), however, was the
313 same for control, 25 and 50 μM Al treatments, and it decreased by 47% in 100 μM Al treatment
314 (Fig. 6c). Thus, despite being present in xylem sap, ABA transport from roots to shoots does not
315 seem to explain the high ABA concentration in leaves. However, ABA concentrations increased

throughout the plant in response to Al, especially in leaves, evidencing the existence of chemical signaling between Al in the roots and shoot responses, possibly explaining the low g_s (Fig. 7).

3.4 Aluminum concentration in plant organs

As expected, Al concentration in the roots was approximately 100 times higher than that in the leaves, and it increased as Al concentration in the nutrient solution was raised. Root Al concentration was 13-, 25- and 46-fold higher in plants treated with 25, 50 and 100 μM Al, respectively, when compared to the control plants (Supplementary material; Fig. S1).

4. Discussion

Even though Al reduced 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 g_s (Banhos et al., 2016; Cavalheiro et al., 2020). In the present study, lower leaf water status and g_s of plants exposed to high Al concentration may be associated with low Lp_r (hydraulic mechanism) (Fig. 3a, b) and ABA accumulation in leaves (Fig. 5 a) (chemical mechanism), respectively.

4.1 Plant growth

As expected, the root size (Fig. 1d), main root length (Fig. e), root surface area and root biomass (Table 1) decreased as Al concentration in the nutrient solution was raised. The Al concentration in root tissue also followed this response pattern (Supplementary material, Fig. S1b). The reasons why root growth is inhibited under Al presence have been investigated (Zheng & Yang 2005; Kopittke et al., 2008; 2015; Horst et al., 2010; 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, Al may limit leaf growth by decreasing nutrient uptake (Silva et al., 2010), the biosynthesis and transport of cytokinins (Mossor-Pietraszewska, 2001) and causing low turgor (Barceló et al., 1996). While these mechanisms seem important in water-limiting environments, here Al toxicity was imposed hydroponically, yet leaf growth was still inhibited in response to increasing Al concentrations (Fig. 1a, c and Table 1). Leaf growth of Al-exposed plants was likely regulated by low leaf water status limiting leaf expansion (Fig. 1a, c) and inhibition of leaf initiation, as evidenced by the decreased leaf number as Al was raised in the nutrient solution (Table 1). Reduced leaf area and

biomass was also noted in tomato plants exposed to 50 µM Al (Simon et al., 1994a). Irrespective of the mechanisms (hydraulic or chemical), 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. 1f). Therefore, 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 are unclear. Although leaf length was reduced from 5 DAT (Fig. 1a, c) and g_s decreased from 3 DAT (Fig. 2a), such compensation may not be sufficient to maintain leaf hydration to keep stomata open.

4.2 Hydraulic mechanism

All Al treatments reduced Ψ_{leaf} in comparison with control plants (Fig. 4a), suggesting that Al exposure impaired root-to-shoot water transport, lowering shoot water status. Increasing Al concentration in the nutrient solution decreased Lp_r (Fig. 3a, b) and E_{plant} (Fig. 6a), perhaps due to less developed and smaller protoxylem vessels or even structural damage in the vascular cylinder as observed in maize plants exposed to 300 µM Al (Batista et al., 2013). Low gene expression of aquaporins (partially responsible for water transport) was also observed in *Secale cereale* (Milla et al. 2002), *Arabidopsis* (Shen et al., 2008) and *Citrus limonia* (Cavalheiro et al. 2020) exposed to Al. As far as we are aware, the Al-induced decrease in Lp_r was only measured in maize (Gunsé et al., 1997), although this study did not assess g_s, nor associated both parameters. However, the difficulty about Lp_r measurement can be related to the expectation of normalized data per unit root area (m^2) or root biomass (g), as usually calculated in studies of plant water deficit (Rodríguez-Gamir et al., 2015; Ding et al., 2019). But unlike plants exposed to Al, in which the root system does not grow (Delhaize & Ryan 1995; Kopittke et al, 2008; Horst et al, 2010; Fig. 1d, e) and the roots are anatomically damaged (Batista et al., 2013; Banhos et al., 2016; Silva et al., 2019), roots of plants under water deficiency grow significantly *more*, including the involvement of ABA (Saab et al., 1990) and are *not* anatomically damaged. In addition, in water deficiency studies, water availability is limited in the substrate/soil, whereas plants tested in Al toxicity studies are, usually, grown directly in nutrient solutions, where water availability is unlimited, like in the present study. Thus, in studies with Al toxicity, when Lp_r is normalized by any root parameter, which is significantly lower in relation to plants not exposed to Al, Lp_r will result in higher and not lower values for plants exposed to Al (Supplementary material, Fig. S2), which does not make any physiological sense because higher Lp_r in plants exposed to high Al concentration would have to directly correlate with increased leaf water status, what did not happen in the present study. For

instance, g_s values of plants exposed to Al showed inversely proportional correlation with Ψ_{leaf}, while exhibiting a direct proportional correlation with Lp_r (non-normalized data) and A (Table 2), corroborating, indeed, that A is controlled by g_s in plants exposed to Al, as observed by other studies (Ribeiro et al., 2013; Banhos et al., 2016; Cavalheiro et al., 2020). Furthermore, absolute Lp_r (non-normalized data) is valid, and is an important tool to understand the root capacity to transport water (Dodd & Diatloff, 2016), especially under non-limiting conditions (Jackson et al., 1996).

4.3 Chemical mechanisms

Whether rapid root ABA accumulation in response to Al (within 3 h in rice bean – Fan et al. 2019) changes shoot physiology is of interest, since 50 µM Al increased [ABA] in both roots and leaves of soybean plants (Hou et al. 2019). Moreover, these plant species showed 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, in the present study, was the same between plants exposed to 0, 25 and 50 µM Al (the increase in $[X\text{-}ABA]_{root}$ at 50 µM Al (Fig. 6b) was offset by decreased sap flow rate (Fig. 6a)), it is difficult to argue that foliar ABA accumulation (Fig. 5a) was due to root-to-shoot ABA signaling. That is, even though $[ABA]_{root}$ was increased with the raise of Al in the nutrient solution (Fig. 5b), the decrease in sap flow rate seemed to be more important. While studies investigating leaf ABA accumulation in plants exposed to Al are rare, 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 likely determined by foliar ABA biosynthesis, and seemed sufficient to induce stomatal closure due to inversely proportional correlation between g_s and $[ABA]_{leaf}$ in plants exposed to Al (Table 2).

However, increased ABA concentration in roots reduced proton pumping (from symplast to apoplast) of the plasma membrane of squash (Ahn et al., 2002) and *Arabdopsis* (Brault et al., 2004) exposed to Al. This may be related to Al increasing root xylem sap pH from 6.5 to 7.2 (Fig. 4b). Similar pH values (6.3 to 7.2) were found in root xylem sap from water-stressed *Phaseolus vulgaris* plants (Hartung & Radin, 1989). Increased xylem sap pH decreases stomatal aperture in an ABA-dependent manner, most probably by increasing ABA concentration in the apoplast (Wilkinson & 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 sequestration by mesophyll cells.

5. Conclusion

In conclusion, even when plants are grown in nutrient solution, where water is constantly available, Al toxicity decreases water transport from roots to the leaves as evidenced by low values of g_s, 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. Al appears not to enhance root-to-shoot ABA signaling but leaf ABA is likely the major cause of Al-induced stomatal closure.

Author contributions

MAG and GH raised the hypothesis; MAG, ICD and GH developed the experimental design, MAG proceeded the experiment and collected the data, GSS gave assistance to all data analysis; JP measured ABA and helped to interpret these data; MAG, GH and ICD wrote the manuscript; all the authors made significant contributions to the manuscript revision.

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Tables

Table 1. Biometric parameters of tomato plants (*Solanum lycopersicum*) cultivated for 10 days in nutrient solution containing 0, 25, 50 and 100 µM of aluminum.

Variable/ Treatment (µM Al)	Leaf number	Leaf area (cm^2)	Leaf biomass (g)	Root diameter (mm)	Root surface area (cm^2)	Root biomass (g)
0	7.5 ± 0.3 a	464.5 ± 17.4 a	1.75 ± 0.09 a	0.35 ± 0.02 b	580.4 ± 11.2 a	0.21 ± 0.01 a
25	6.0 ± 0.1 b	287.1 ± 16.4 b	1.11 ± 0.05 b	0.45 ± 0.01 a	358.8 ± 5.7 b	0.15 ± 0.01 b
50	5.5 ± 0.3 bc	139.9 ± 8.0 c	0.73 ± 0.03 c	0.49 ± 0.01 a	135.2 ± 3.3 c	0.10 ± 0.02 b
100	5.0 ± 0.1 c	82.2 ± 4.1 c	0.64 ± 0.04 c	0.45 ± 0.01 a	44.8 ± 2.5 d	0.04 ± 0.01 c

For each variable (column), distinct letters indicate significant differences ($P < 0.05$) between Al treatments.

Table 2. Pearson correlations between individual values of parameters obtained from plants exposed to aluminum treatments.

	$[ABA]_{leaf}$	Ψ_{leaf}	Xylem sap pH	Lp_r	A
g_s	-0.817	-0.700	-0.838	0.932	0.855
	0.00116	0.0112	0.000668	0.000009997	0.000392

For each variable, the first line represents the correlation coefficient (R^2) and the second line, the P-value. For abbreviations of parameters (g_s, $[ABA]_{leaf}$, Ψ_{leaf}, xylem sap pH, Lp_r and A) see 'Material and methods'.

694 **Figure legends**
695
696 **Fig 1.** Accumulated leaf length (A), terminal leaflet length (C), main root length (E) of tomato
697 plants (*Solanum lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100
698 μM of aluminum. Morphological details of shoots and leaves (B) and roots (D) of the plants.
699 Relationship between leaf area and root area (F). Distinct lowercase letters indicate significant
700 differences ($P < 0.05$) between Al treatments on each evaluation date. Dots are mean values (n =
701 10 plants for A, C, E and 5 plants for F). Bars are standard errors. Ellipses indicate statistically
702 similar treatments.
703
704 **Fig 2.** Stomatal conductance (g_s) and CO_2 assimilation rate (A) of tomato plants (*Solanum*
705 *lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100 μM of
706 aluminum. Distinct letters indicate significant differences ($P < 0.05$) between Al treatments on
707 each evaluation date. Dots and columns are mean values (n = 10 plants) and bars are standard
708 errors. Ellipses indicate statistically similar treatments.
709
710 **Fig 3.** Relationship between xylem sap flow rate (J) and applied pressure (MPa) (A) of tomato
711 roots (*Solanum lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100
712 μM of aluminum. The slopes of the linear regression lines indicate the root hydraulic conductance
713 (Lp_r) (B). Distinct letters indicate significant differences ($P < 0.05$) between Al treatments. Dots
714 and columns are mean values (n = 5 plants) and bars are standard errors.
715
716 **Fig 4.** Leaf water potential (Ψ_{leaf}) (A) and root xylem sap pH (B) of tomato plants (*Solanum*
717 *lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100 μM of
718 aluminum. Distinct letters indicate significant differences ($P < 0.05$) between Al treatments.
719 Columns are mean values (n = 5 plants) and bars are standard errors.
720
721 **Fig 5.** Abscisic acid (ABA) concentration in leaves ($[ABA]_{leaf}$) (A) and roots ($[ABA]_{root}$) (B) of
722 tomato plants (*Solanum lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50
723 and 100 μM of aluminum. Distinct letters indicate significant differences ($P < 0.05$) between Al
724 treatments. Columns are mean values (n = 5 plants) and bars are standard errors.
725
726 **Fig 6.** Whole-plant transpiration (E_{plant}) (A), root xylem sap ABA concentration ($[X\text{-}ABA]_{root}$) (B)
727 and ABA delivery rate from root-to-shoot (C) of tomato plants (*Solanum lycopersicum*) grown for

10 days in nutrient solution containing 0, 25, 50 and 100 μM of aluminum. Distinct letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n = 5 plants) and bars are standard errors.

Figure. 7 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 suggested effect.

Appendix A. Supplementary data

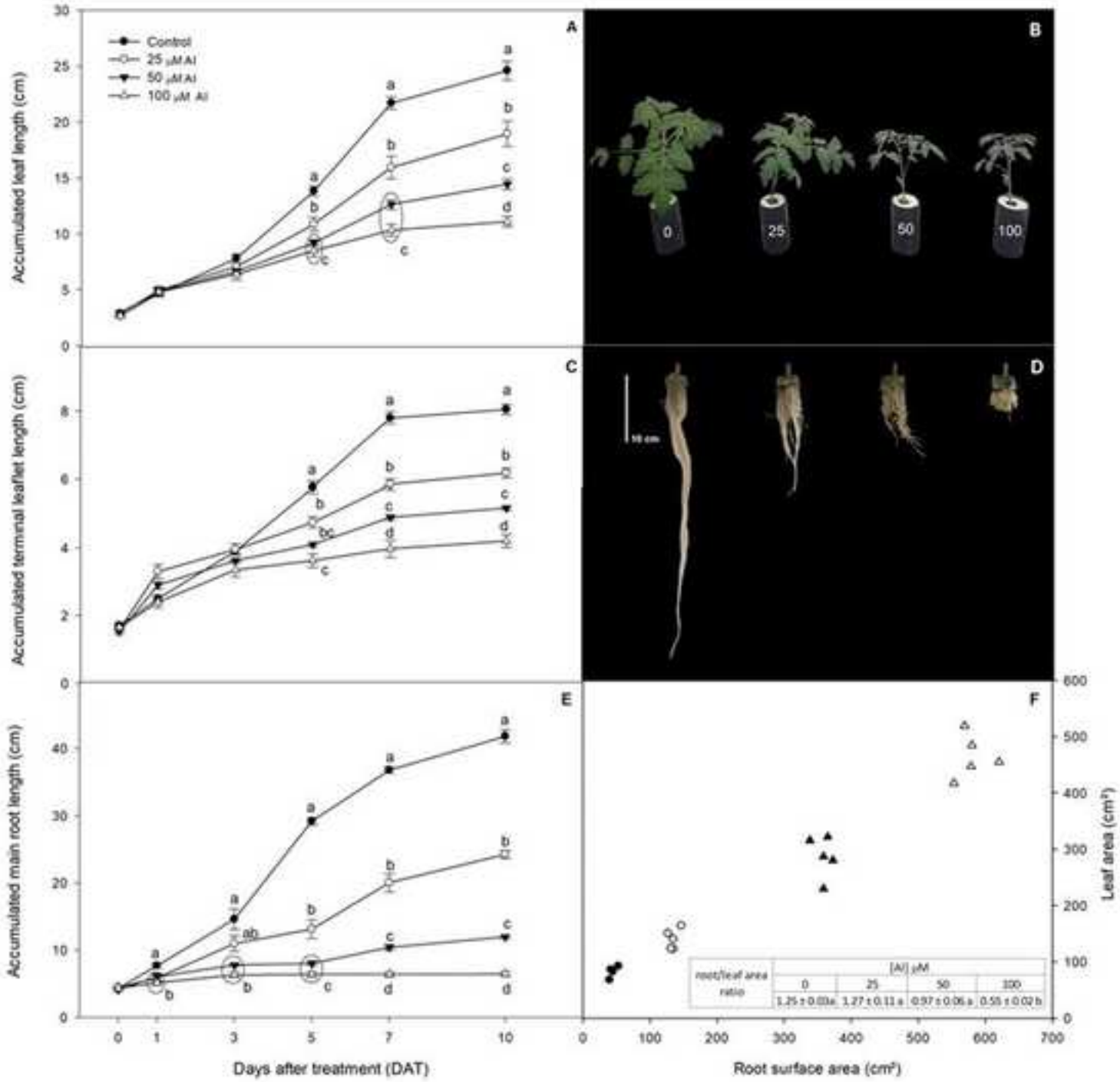
Additional supporting information may be found in the online version of this article.

Fig. S1 Aluminum concentration in leaves (Leaf [Al]) (A) and roots (Root [Al]) (B) of tomato plants (*Solanum lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100 μM of aluminum. Distinct letters indicate significant differences ($P < 0.05$) between Al treatments. Columns are mean values (n = 5 plants) and bars are standard errors.

Fig. S2 Relationship between xylem sap flow rate (*J)* and applied pressures (MPa) normalized by root surface area (cm^2) (A) of tomato roots (*Solanum lycopersicum*) grown for 10 days in nutrient solution containing 0, 25, 50 and 100 μM of aluminum. The slopes of the linear regression lines when normalized by root surface area (cm^2) indicate the root hydraulic conductivity (K_r) (B). Distinct letters indicate significant differences ($P < 0.05$) between Al treatments. Dots and columns are mean values (n = 5 plants) and bars are standard errors.

Figure

Click here to access/download;Figure;Figure 1 - growth.jpg



Figure

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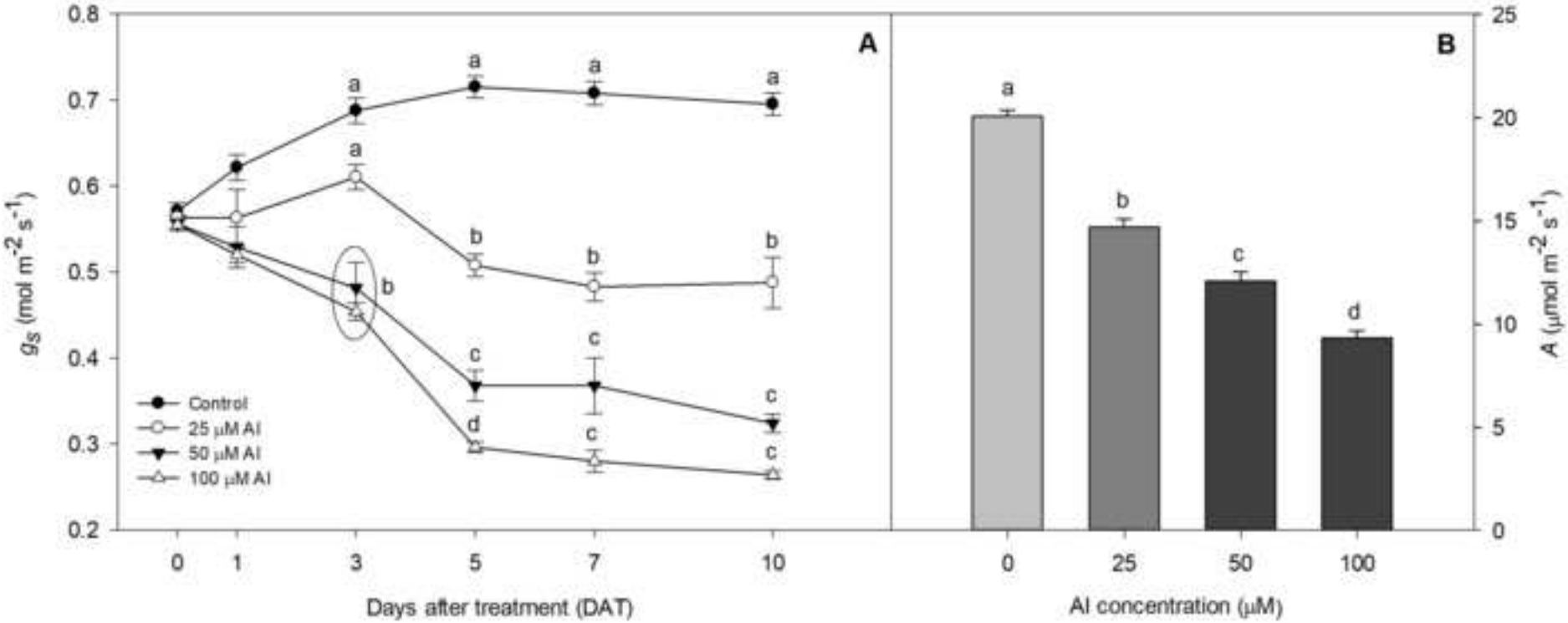

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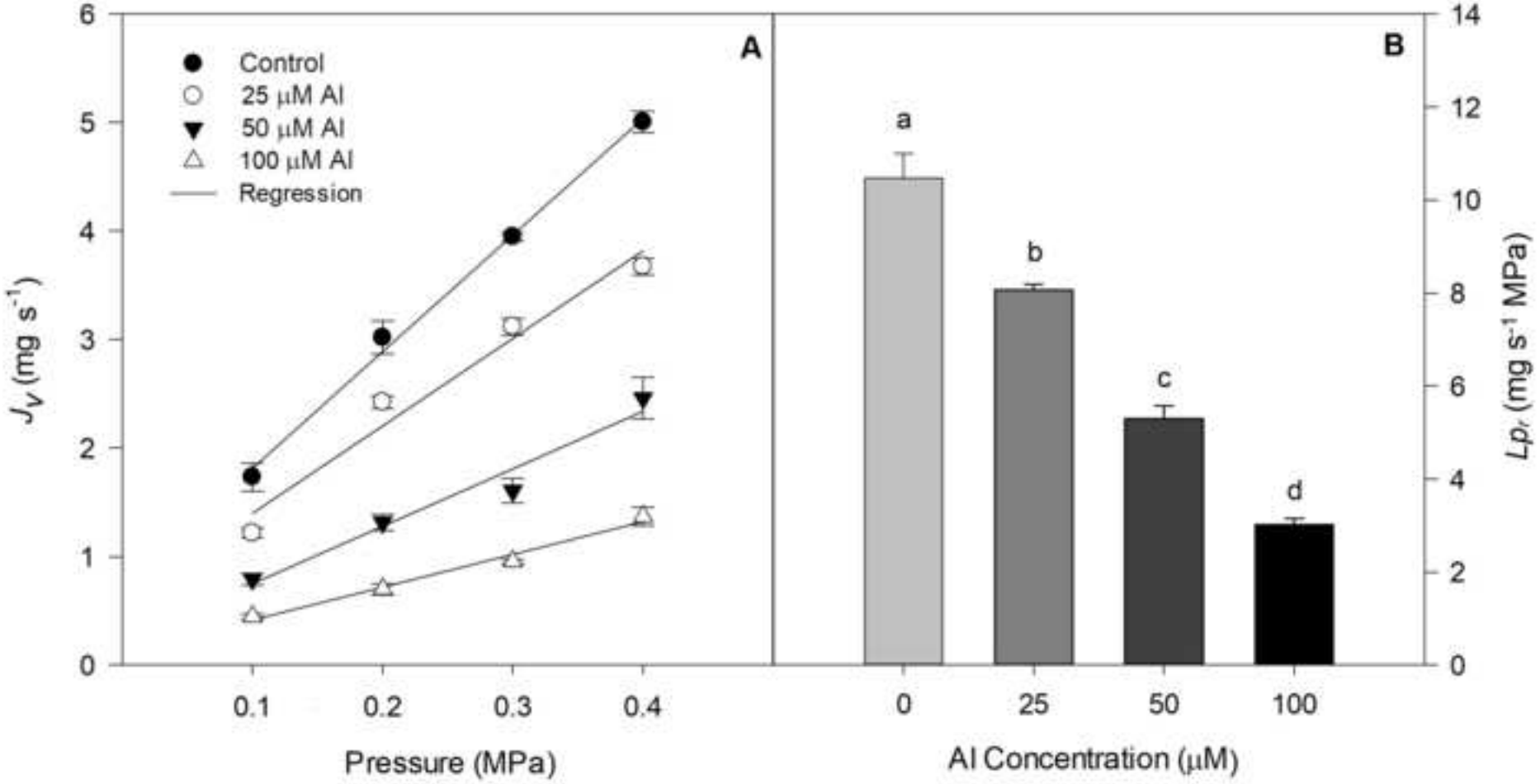

Figure

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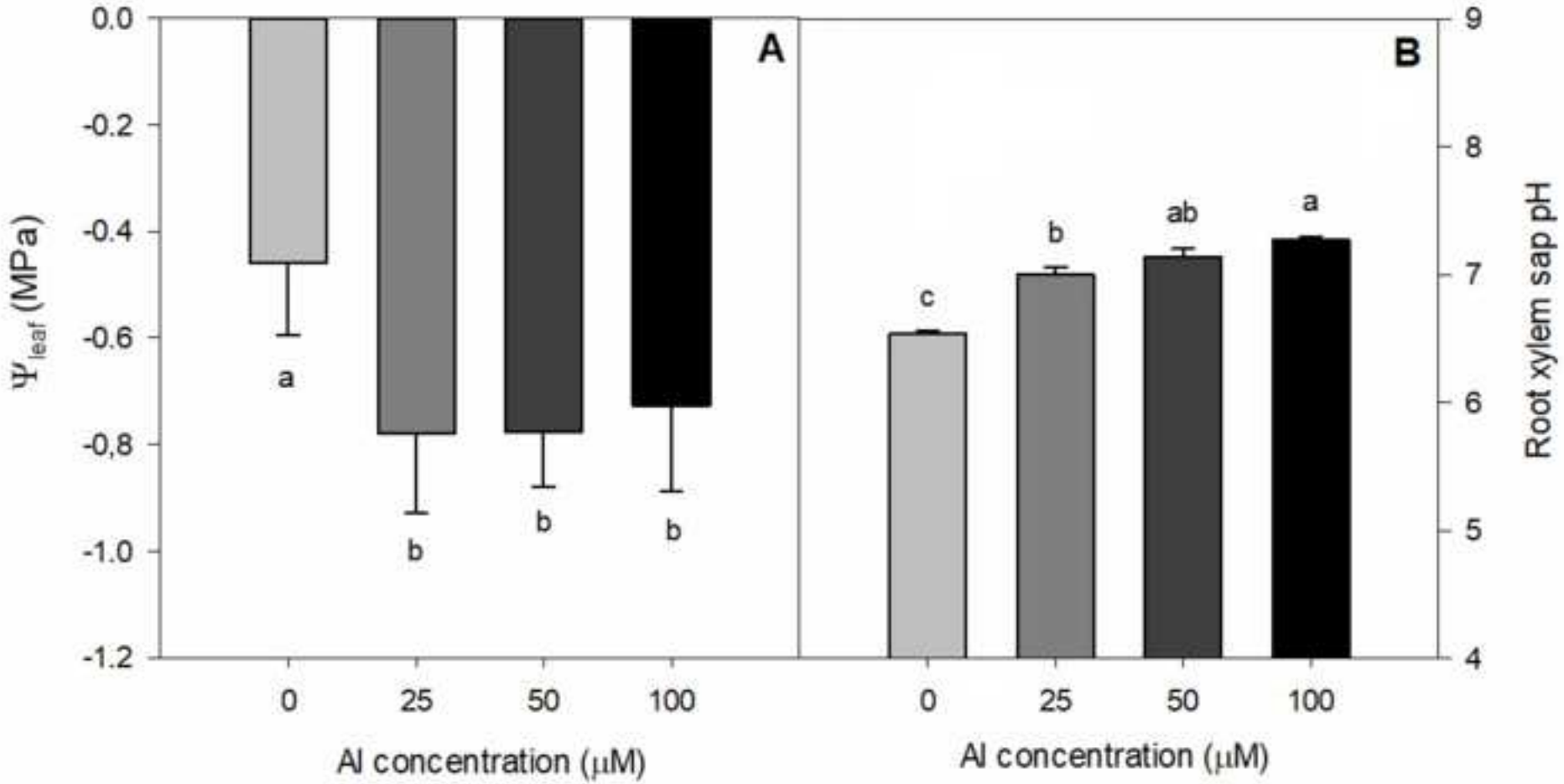

Figure

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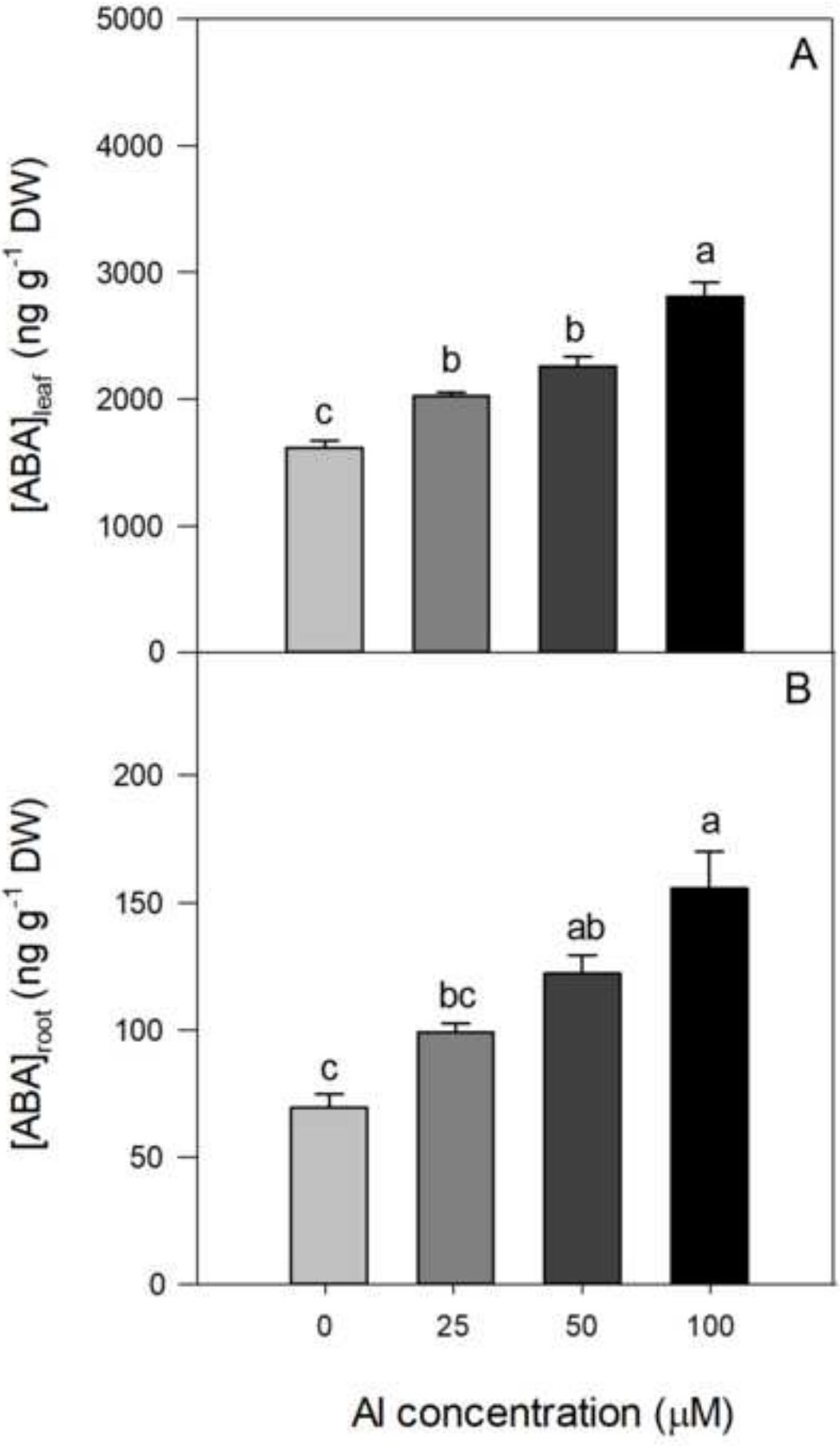
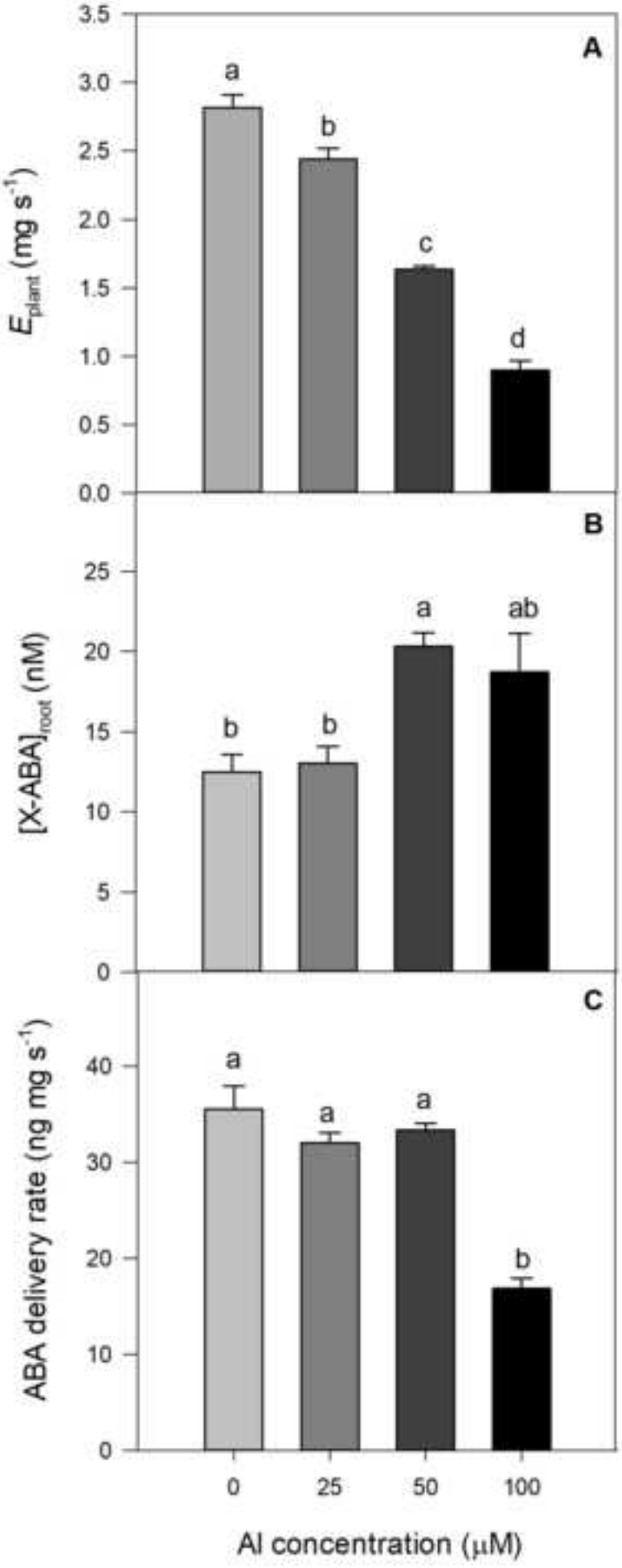


Figure

Click here to access/download;Figure;Fig 6 - ABA delivery.tif



Figure

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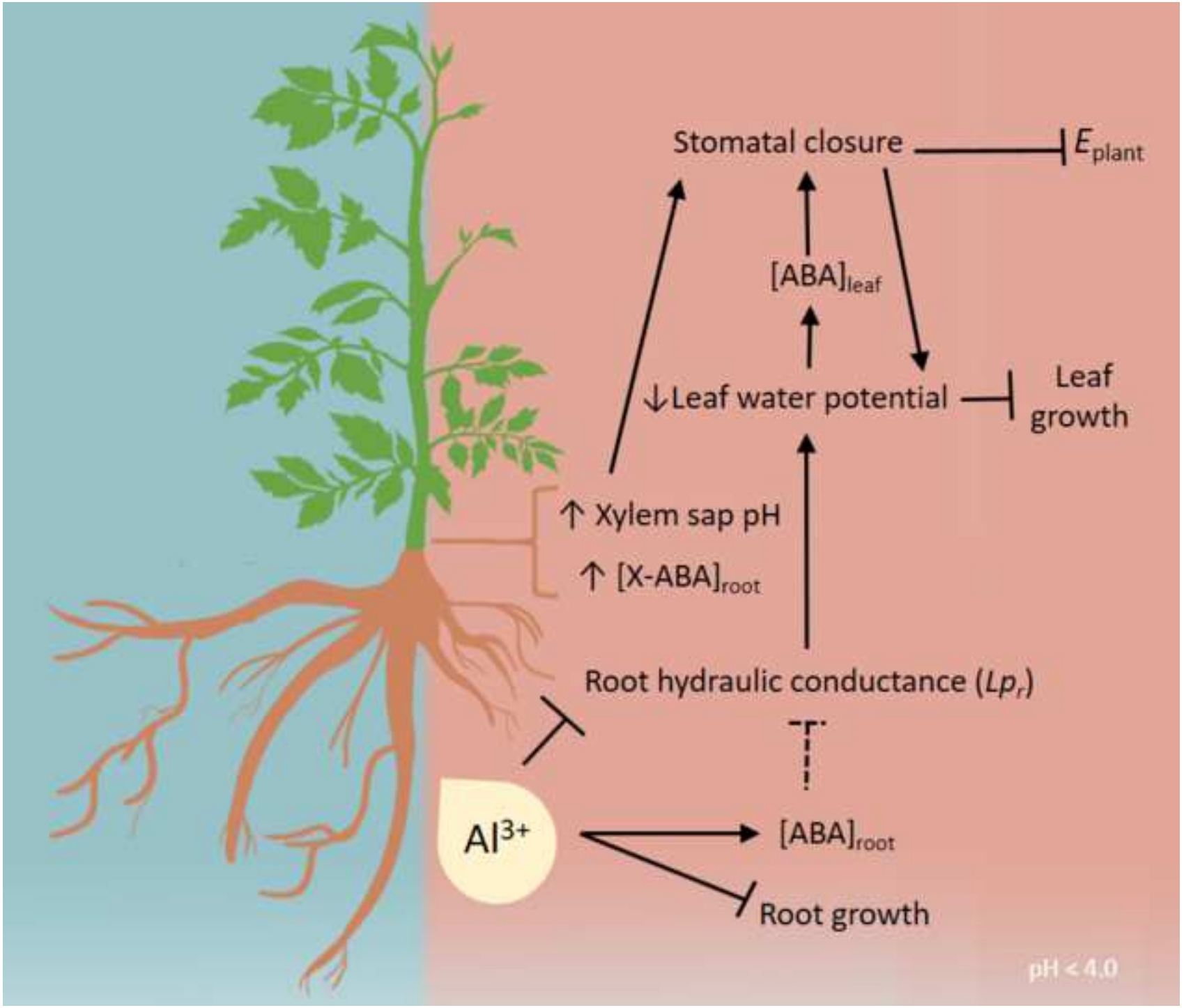
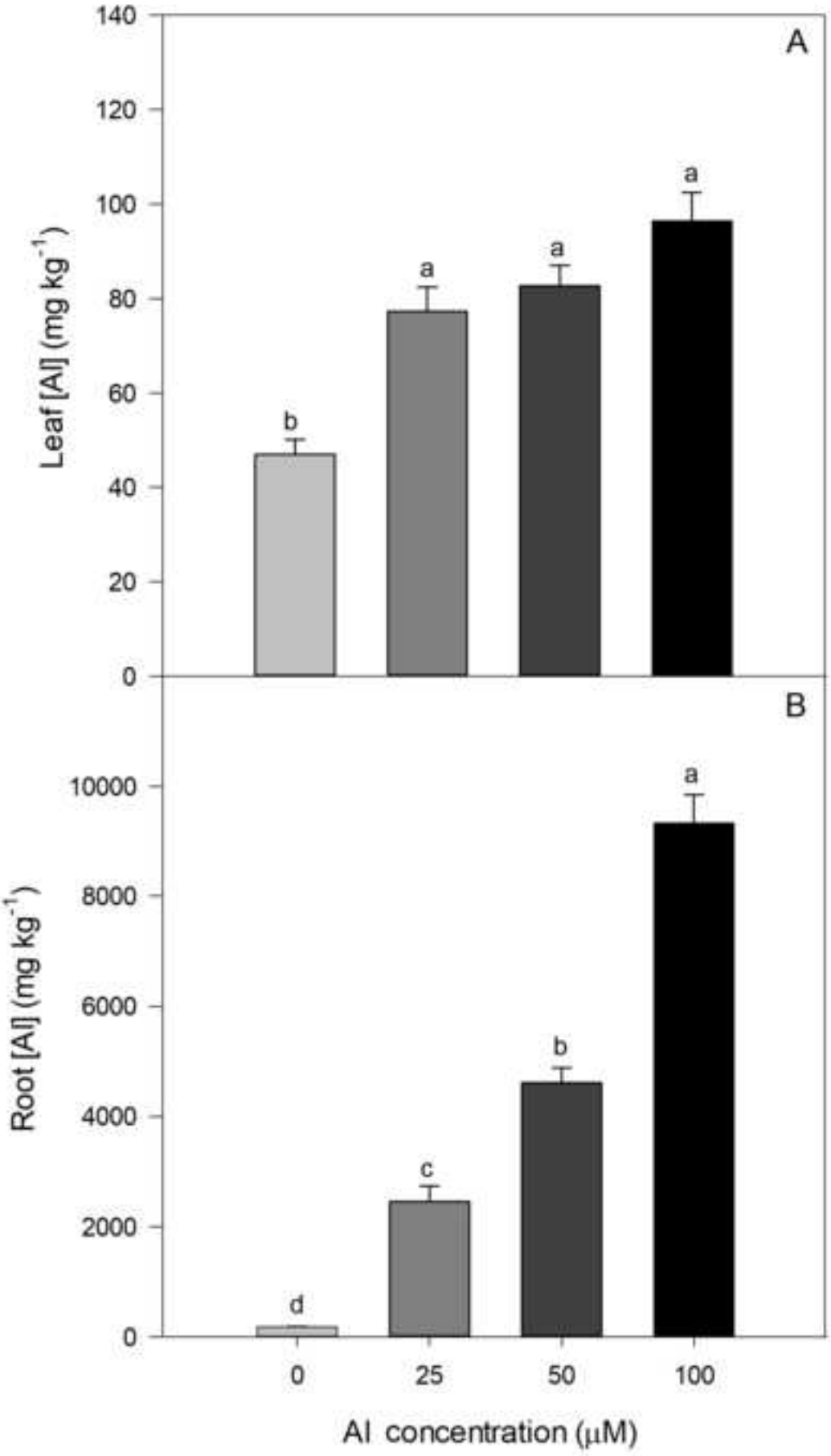


Figure

Click here to access/download;Figure;Fig S1_ [Al].tif



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