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BASIC AREAS
Article

Malate and cell wall aluminum immobilization act as resistance mechanisms in soybean roots

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ABSTRACT: Toxic levels of aluminum (Al) in the soil can reduce the growth of different grain crops. To understand the effects of Al in soybean (*Glycine max* L.) seedlings Al accumulation and its effect on anatomy, morphology, and metabolism in roots of two soybean genotypes were evaluated: Suprema and A7002. For this, soybean plants were grown in nutrient Clark solution, pH 4.0, without Al (control) and with 100 µM of Al for 72 hours. Both genotypes accumulated Al in the roots, however, Suprema showed a higher Al accumulation than A7002. The latter genotype showed Al accumulated only in the cell walls of the epidermis and root cap, which did not affect root growth. On the other hand, in Suprema, Al accumulated in the cells of the root cap, epidermis, and the nucleus of the ground meristem cells, which resulted in inhibited root growth and structural damage in the root epidermis cells. Al modulated the primary metabolism with increases in the levels of glucose, fructose, sucrose, and malate in the roots of the genotype A7002 and reduced the starch levels in the Suprema genotype. The findings of this study suggest the A7002 genotype seems to be more resistant to Al than Suprema, mainly by the use of two important mechanisms: an increase in malate content and Al immobilization in the external cells of the root.

Key words: accumulation sites, *Glycine max*, histochemistry, organic acids, primary metabolism.

INTRODUCTION

Cultivated plants are exposed to several stress conditions that can impact growth and cause yield loss (Kochian et al. 2015). Among them stands out aluminum (Al), which is found in tropical and subtropical soils with higher bioavailability in soils with low pH (Chauhan et al. 2021; Ofoe et al. 2023). Al-induced phytotoxicity negatively affects major grain crops (Kochian et al. 2004; Ryan et al. 2011), such as soybean (*Glycine max*) which is a very sensitive species to acidic soil in the presence of Al (Reis et al. 2018; Yang et al. 2011b). This sensitivity is mainly due to the direct effect of this metal on the root system (Kopittke et al. 2015).

The root apex is the first site of Al phytotoxicity (Kochian et al. 2015; Kopittke et al. 2015), consequently reducing water and nutrient uptake (Gupta et al. 2013; Ofoe et al. 2023). Al can accumulate in the root apoplastic region (Frantzios et al. 2005; Yoshida et al. 2023; Zhu et al. 2017), binding to chemical components of the cell wall and plasma membrane, and thus induce its toxicity at the cellular level (Yang et al. 2011a). On the other hand, the secretion of organic acid (AOs) anions into the apoplast space by membrane proteins can reduce Al toxicity through the exclusion mechanism (Chauhan et al. 2021; Shavrukov et al. 2016) by preventing the binding of this metal to important cellular sites (Ikegawa et al. 2000; Nunes-Nesi et al. 2014).

Under Al stress, the exudation of AOs, such as malate, citrate, and oxalate is an important mechanism of plant resistance to Al toxicity (Chauhan et al. 2021; Kochian et al. 2015;Ofoe et al. 2023). AOs are exuded in the rhizosphere and can chelate with trivalent Al ions (Al3+). Thus, there is a neutralization of toxic Al due formation of a stabilized non-phytotoxic complex with Al (Kochian et al. 2005). In some plant species, more than one OA exudate has already been identified in response

to Al. In soybean the main OAs involved in Al-resistance are citrate and malate through exudation by the respective transporters GmMATE and GmALMT1 (Liang et al. 2013; Liu et al. 2016).

In addition to the quantitative analysis of Al in plant tissues (mg ·Kg-1 dry mass), the evaluation of the Al accumulation in cellular sites is important to the identification of Al resistance mechanisms (Ribeiro et al. 2022; Silva et al. 2020; Yoshida et al. 2023). The accumulation of this metal can be observed in situ using specific dyes, such as hematoxylin (Brito et al. 2020; Ribeiro et al. 2022; Siqueira et al. 2020; Souza et al. 2016; Yoshida et al. 2023), and at the anatomical level the Chrome Azurol S staining (Silva et al. 2020). Additionally, the use of scanning electron microscopy (SEM) and X-ray spectroscopy (EDS) allows the detailed observation of the accumulation sites and the morphological and structural changes induced by Al (Brito et al. 2020; Ribeiro et al. 2022; Souza et al. 2016; Yoshida et al. 2023). Therefore, the use of microscopic techniques can provide important insights into Al sensitivity or tolerance (Ribeiro et al. 2022; Yoshida et al. 2023).

Since soybean is an acid soil-sensitive plant in the presence of Al than other crops, studies are necessary to understand the responses of this species to Al bioavailability. Besides, studies on the modulation of metabolism and morphoanatomical alterations in soybean roots against Al are still scarce. Thus, the objective of this work was to evaluate the sites of Al accumulation in the roots and the structural and metabolic alterations induced in this organ by this metal in two soybean genotypes.

METHODS

Plant material and experimental conditions

Seeds of soybean (*Glycine max* L.) genotypes Suprema and A7002 were obtained from the Soybean Breeding Program for Quality Traits of the Agricultural Biotechnology Research Institute (Bioagro) at the Federal University of Viçosa, Brazil. Seeds were sterilized with 5% (v/v) sodium hypochlorite for 5 min and then washed with deionized water. The seeds were germinated in rolls of paper dipped in 100 μM $CaCl_2$ (pH 7.0) and maintained under continuous aeration in the dark at 25 °C. After three days in the dark, the seedlings were exposed to light and remained for another three days in the growth room. Seven-day-old seedlings were selected and transferred to a polypropylene pot with 2 L of half-strength Clark solution (pH 4.0) Clark (1975) with the following composition (mM): 1.3 Ca, 0.9 K, 0.5 -NH_4^+, 3.5 -NO_3^-, 0.3 Mg, 0.3 S, 0.0345 P, 0.0035 Mn, 0.0095 B, 0.001 Zn, 0.0003 Mo, 0.00025 Cu, and 0.0225 Fe, for 48 h under continuous aeration for acclimatization. Next, nine-day-old seedlings were grown in total-strength Clark (1975), pH 4.0, with $AlCl_3$ at the final concentration of 100 μM (stressful condition) and 0 μM (control condition) during 72 h. Plants were kept in a temperature-controlled (25 ± 1 °C) chamber under 200 μmol photons·m^{-2}·s^{-1} light intensity, 80% relative humidity, and photoperiod (8h light and 16h dark). The pH of the nutrient solution was adjusted daily to 4.0.

Determination of Al content

Root tips (0.5 cm) were oven-dried at 70°C until constant weight and were powdered within a knife mill. Samples of 0.2g dried root tips were digested with nitro-perchloric solution (3:1) (Silva et al. 2020). The Al concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 3000XL, Maryland, USA).

Histochemical staining by hematoxylin

Intact roots were dipped in 0.2% iron hematoxylin (w/v) containing 0.02% KIO_3 (w/v) for 15 min (Polle et al. 1978). Subsequently, the samples were washed in deionized water for 15 min to remove excess dye. The root tips (1.0 cm) were sectioned and photographed under a stereomicroscope (Zeiss, Stemi DV4, Germany).

Al localization in root tips by Chrome Azurol S staining

Root tips (1.0 cm) were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) (Karnovsky 1965). Next, the samples were dehydrated through an ethanol series and included in methacrylate (Historesin, Leica Instruments,

Heidelberg, Germany). Longitudinal sections with 5 μm thickness were obtained with an automatic rotary microtome (RM2155, Leica Microsystems Inc., Deerfield, EUA) and stained with 0.5% Chrome Azurol S (Kukachka and Miller 1980) for 1 h. A positive reaction was identified by bluish staining. After the sections were washed in distilled water and mounted in Permount. Photographs were taken using a light microscope (AX70TRF, Olympus Optical, Tokyo, Japan) equipped with an image capture system (AxioVision Release 4.8.1, Carl Zeiss Vision, Germany).

Plant growth and development

The roots were digitized using the WinRHIZO® Pro software (Regent Instruments, Québec, Canada) coupled to an EPSON Perfection 10000XL (Epson, Nagano, Japan). The following traits were measured: root length, average root diameter, number of root bifurcations, and total root volume.

Micromorphology by scanning electron microscopy (SEM) and microanalyses by energy-dispersive X-ray spectrometer (EDS) coupled with SEM

Root tips (0.5 cm) were harvested and fixed in 2.5% (v/v) glutaraldehyde. Next, the root tips were dehydrated in ethylic series, dried with CO2 in a critical point dryer (CPD 030, Bal-Tec, Balzers, Liechtenstein), and fixed on the metallic stub. Samples were covered with carbon in the carbon evaporator (Quorum Q150 T, East Grinstead, West Sussex, UK) for Al localization using energy-dispersive X-ray spectrometry. The photographic documentation was conducted using an IXRF Iridium Ultra EDS (X-EDS, IXRF systems, Houston, TX, USA) coupled to the scanning electron microscope (LEO 1430 VP, Zeiss, Cambridge, UK) operating at an accelerating voltage of 20 kV. Al microanalysis (positive red color) was performed by SEM-EDS by the distribution map of this metal at the root tip.

Determination of primary metabolites

Samples of 200 mg of fresh roots were harvested in the middle of the photoperiod (8 h after the start of the light period), immediately frozen in liquid nitrogen, and stored at -80°C. The metabolite extraction was performed with an ethanol gradient, of 98%, 80%, and 50% and cycles of 80°C. Subsequently, glucose, fructose, sucrose, total amino acid, total protein, and starch were measured as described by Fernie et al. (2001) and Cross et al. (2006). Malate and fumarate contents were determined according to Nunes-Nesi et al. (2007).

Determination of lipid peroxidation

Lipid peroxidation was estimated by MDA content produced after a reaction with thiobarbituric acid (TBA) (Cakmak and Horst 1991). Fresh roots (0.3 g) were homogenized in 2 mL of 1% TCA (w/v) and centrifuged at 12,000g at 4°C for 15 min. For the reaction, an aliquot of the supernatant was added to a reaction mixture containing 0.5% TBA (w/v) in 20% TCA (w/v), then incubated at 95°C for 30 min and centrifuged at 10,000 g at 4°C for 10 min. The absorbance of the supernatant was determined at two wavelengths (535 nm and 600 nm) in a microplate reader. The MDA–TBA content complex was estimated using a molar extinction coefficient of $155\times 10^{3}\cdot M^{-1}\cdot cm^{-1}$.

Statistical analysis

Data were statistically examined using analysis of variance (ANOVA) and their means tested for normality by Shapiro-Wik test and Bartlet's homogeneity of variance compared by Tukey's test at ($p < 0.05$), in software R (R Core Team 2021). The experimental design was completely randomized with six replications, composed of four treatments: (1) absence of Al + Suprema, (2) presence of Al (100 μM) + Suprema, (3) absence of Al + A7002, and (4) presence of Al (100 μM) + A7002. The experimental plot consisted of a pot with four seedlings.

RESULTS AND DISCUSSION

Regardless of the crop, it is known that the root is the organ most affected by Al toxicity (Chauhan et al. 2021; Kochian et al. 2015; Ofoe et al. 2023). In cultivated plants this problem is intensified, because besides compromising the development and growth of the plant, the plant yield can also be reduced. Sites of Al accumulation in roots have been well studied in crop plants such as *Zea mays* (maize), *Oryza sativa* (rice), and *Sorghum bicolor* (sorghum) (Kollmeier et al. 2000; Too et al. 2014; Yang et al. 2008). However, in soybean, one of the most important crops in the world, it is not clear where this metal accumulates in the root (Silva et al. 2020) and, consequently, what would be the changes induced by this metal.

In the present work, Al accumulation (Figs. 1 and 3) and its primary toxic effects were observed only in the root system of the Suprema genotype (Fig. 2). Despite the six-fold increase in Al content in the roots of genotype A7002 (Fig. 1a), most of this metal was accumulated in the root outer cells, specifically in the cell walls of the epidermis and the root cap (Fig. 1e and 1f). This accumulation did not alter root development (Fig. 2a and 2e). Al accumulated in root cells may be bound mainly to the pectin matrix of the cell wall, more specifically to the negatively charged carboxylic groups (Ofoe et al. 2023). Thus, the strong and rapid binding of Al to these chemical components of the cells allows lower mobility of this metal, preventing its arrival at the symplastic region. Consequently, there is a reduction in the damage to important cellular regions, such as apical meristems and, mainly, the cell nucleus, as well as organelles, such as mitochondria and chloroplasts (Kochian et al. 2005). Thus, the immobilization of Al in the root outer cells in A7002 allows root growth under Al conditions. The maintenance of root growth in the presence of phytotoxic Al is generally associated with Al-tolerant plants (Kochian et al. 2015; Ofoe et al. 2023). Therefore, under the experimental conditions in this work, the results indicate that genotype A7002 is Al-tolerant.

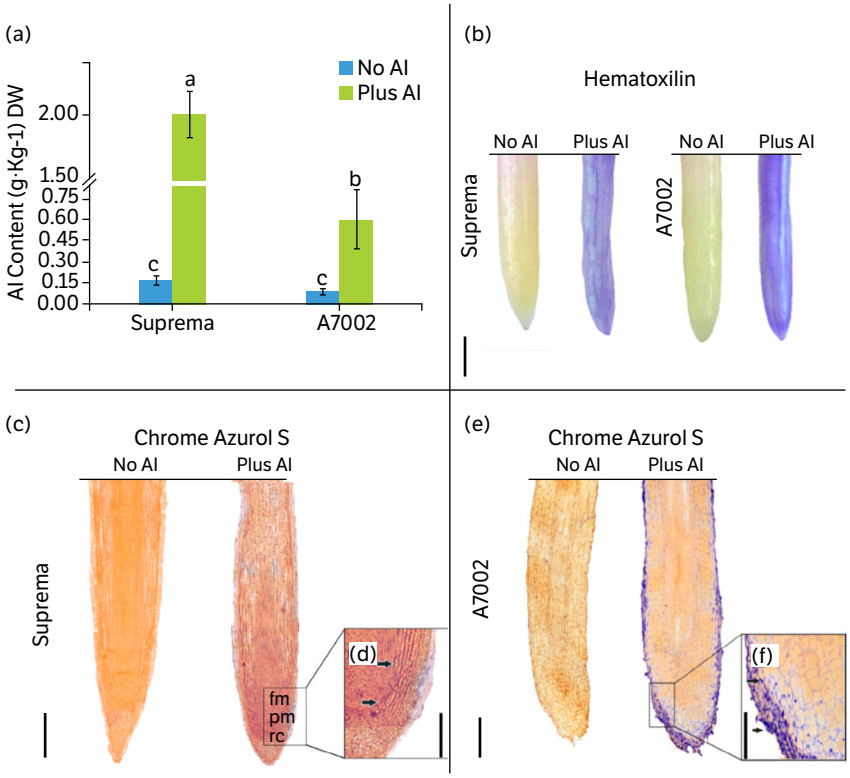

Figure 1. Aluminum content and histolocalization (Al) in root tips of soybean seedlings Suprema and A7002 grown in Clark solution in the absence: No Al (0 µM) or presence: Plus Al (100 µM Al) for 72h. a: Aluminum content in the root. Vertical bars represent the standard deviation (n = 5) and the same letters do not differ statistically by the Tukey's test ($p < 0.05$). b: Histolocalization of Al by hematoxylin dye in root tips. c, d, e and f: Morfoanatomic and histochemical of Al by Chrome Azurol S dye in longitudinal cut of root tip. Purple-blue staining indicates positive reaction with hematoxylin and Chrome Azurol S dye. c and d: genotype Suprema. e and f: genotype A7002. d: Black arrows indicate the presence of Al in the cell nuclei. f: Black arrows indicate presence of Al between the cells. Bars: b: 2 mm; c and e: 500 µm; d and f: 1000 µm. rc: root cap, pm: promeristem e fm: fundamental meristem.

Source: Elaborated by the authors.

In the Suprema genotype, besides the 11-fold increase in Al concentration in the roots (Fig. 1a), there was Al labeling in the innermost cells of the root apex and in the nucleus of the fundamental meristem cells (Fig. 1d), which may have resulted in a 25% reduction in root length (Fig. 2a and 2e). In the root apex, the meristematic and elongation zones are highly sensitive to Al, with a high rate of accumulation of this element due to its ability to bind to numerous sites in the cells, potential targets of its injury, such as the cytoskeleton and the nucleus (Kochian et al. 2004; Ofoe et al. 2023). Thus, one of the most damaging effects of Al in cells is its binding to the cell nucleus (Ofoe et al. 2023). Al can bind to the phosphate groups of DNA, forming the Al-DNA complex, damaging the double helix, and hindering the replication of the genetic material, which can compromise cell division, and consequently organ development, especially in sensitive genotypes.

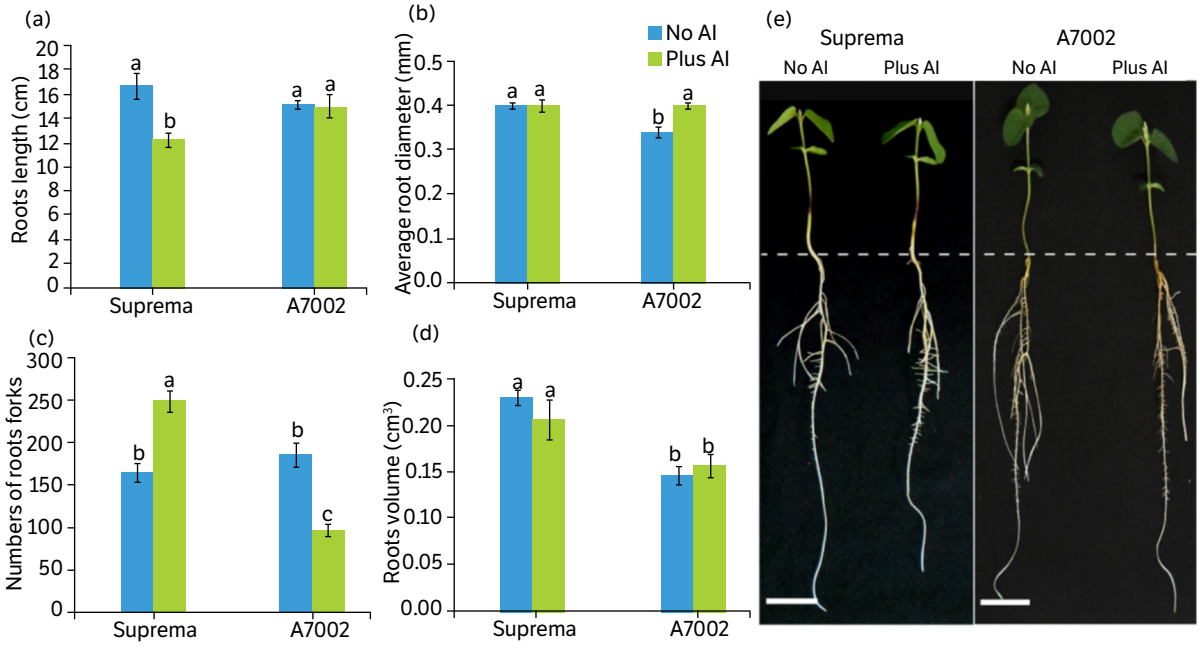

Figure 2. Biometrics and phenotypics parameters the soybean genotypes Suprema and A7002 grown in Clark solution in the absence: No Al (0 µM) or presence: Plus Al (100 µM Al) for 72h. a: root length; b: average root diameter; c: numbers of roots forks; d: root volume. Vertical bars represent the standard deviation (n = 5) and the same letters do not differ statistically by the Tukey's test ($p < 0.05$). e: Phenotype of the genotypes Suprema and A7002 after of exposition to the nutrient solution without aluminum (-Al) and presence of 100 µM of aluminum (+Al). Bars = 2 cm.

Source: Elaborated by the authors.

Tests with hematoxylin staining (Fig. 1b) and EDS (Fig. 3d and 3h), were used to detect the presence of Al in the outer root cells. For these evaluations, intact roots were used. Thus the positive reaction with Al is a result of the presence of this metal adsorbed in the outer cells. Consequently, this Al accumulation resulted in damage to the root apex cells in both soybean genotypes (Fig. 3c and 3g). The outer root cells, especially the epidermis, are primary targets of Al injury since they are in direct contact with Al (Ciamporova 2002; Delhaize and Ryan 1995). The binding of Al with the components of the cell walls can result in an unequal expansion of the cells, generating mechanical stress that can induce damage to the peripheral tissues of the root, such as in the cortex and epidermis (Ciamporova 2002). Depending on the intensity of this damage, the uptake of water and nutrients by the roots may be affected, compromising the growth and development of the plant (Chauhan et al. 2021).

In addition to morphological and anatomical changes (Figs. 1-3), Al can also alter root primary metabolism (Silva et al. 2023; Siqueira et al. 2022). Al positively modulated the primary metabolism of genotype A7002 (Fig. 4). After exposure to Al the content of the malate increased 1.2-fold in the roots (Fig. 4f). Malate is an important molecule for plant resistance to Al (Kochian et al. 2015; Nunes-Nesi et al. 2014). Al can be chelated by this organic acid forming a stabilized non-phytotoxic complex or can be exuded by the roots, preventing Al influx (Kochian et al. 2015). Thus, the exudation of malate may be an important response for differentiating Al-tolerance between soybean genotypes analyzed in this work since during Al exposure the roots of sensitive genotypes may be damaged and over time stop exuding AOs (Nian et al. 2005).

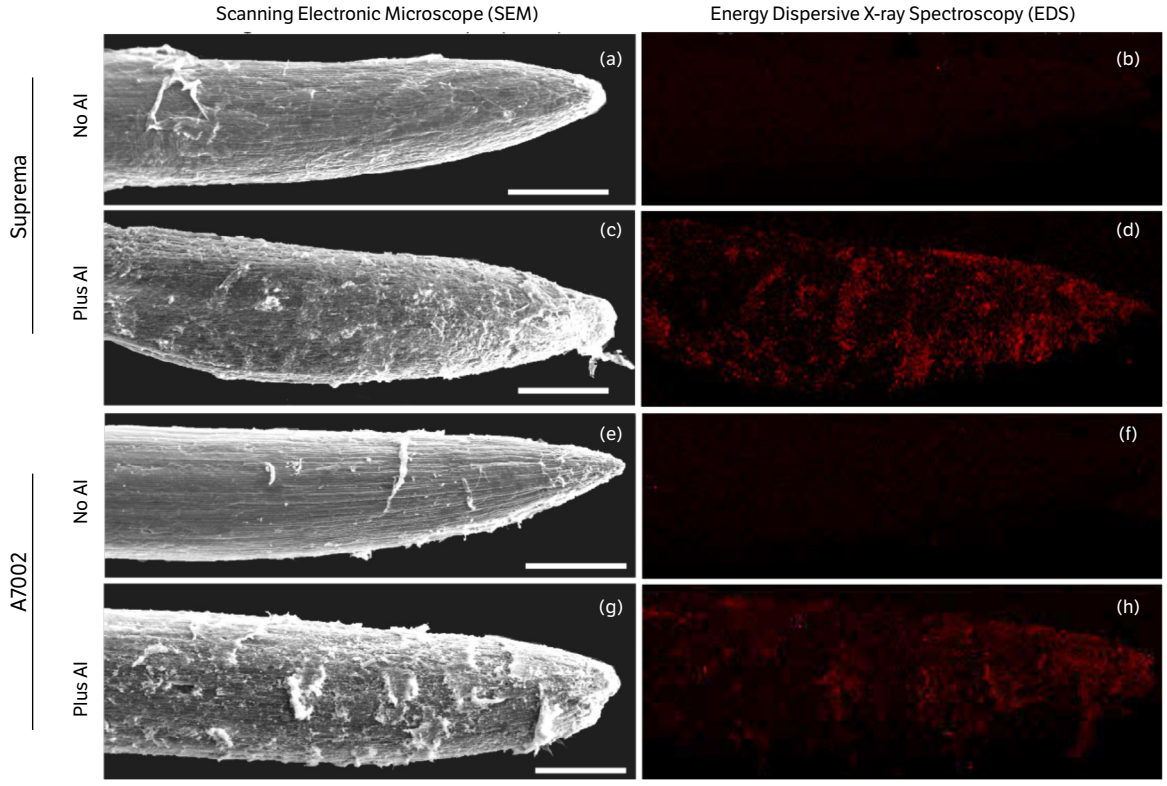

Figure 3. Micrograph in root tips of soybean seedlings Suprema and A7002 grown in Clark solution in the absence: No Al (0 µM) or presence: Plus Al (100 µM Al) for 72h. a, c, e and g: scanning electron microscopy (SEM); b, d, f and h: aluminum localization by scanning electron microscopy coupled with an X-ray probe (EDS). The red areas indicate the presence of Al. a - d: genotype Suprema. e – h: genotype A7002. a, b, e and f: no Al (0 µM). c, d, g and h: Plus Al (100 µM Al). Bar = 200 µm.

Source: Elaborated by the authors.

The MATE (multidrug and toxic compound extrusion) family confers Al tolerance by regulating the exudation of citrate, whereas the ALMT (Al-activated malate transporter) employs malate exudation. TaALMT1 gene in wheat plants encodes transporter protein which is involved in malate secretion from the root and protects the plant against Al toxicity (Sasaki et al. 2004). In soybean, the expression of gene GmME1 increased under Al stress which encodes a cytosolic malic enzyme that contributes to increased internal malate and citrate concentrations and their external efflux to increase the resistance of the plant to this metal (Zhou et al. 2018). Based on this result, it is understood that Al induced overexpression of genes involved in the synthesis and/or transport of malate in the A7002 soybean genotype, increasing the concentration of this OA in the root tips cells, and consequently allowing greater exudation of malate into the solution of cultivation. This response demonstrates an efficient mechanism of exclusion of Al from root tips, essential to prevent the entry of significant amounts of Al into the symplasm. Thus, studies involving AO are important for the development of Al-tolerant soybean cultivars.

In addition to the increase of malate in A7002 roots, Al also increased the concentration of the soluble sugars: glucose, fructose, and sucrose (Fig. 4a-c). Similar results also were observed by Tabuchi et al. (2004) in *Triticum aestivum* (wheat) and Yang et al. (2020) in *Citrus sinensis*. In roots of a tolerant maize genotype, an increase in carbohydrate concentration under Al stress was also recorded, while in the sensitive genotype, the concentration remained unchanged (Giannakoula et al. 2010).

Metabolic energy for non-photosynthetic tissue is mainly obtained by the catabolism of carbohydrate molecules (Moriyama et al. 2016). In general, sucrose is an energy source for cells, and its cleavage products, glucose and fructose are essential for the regulation of plant growth and development (Gibson 2005, Moriyama et al. 2016). Glucose is a key compound used as an energy source and as a signaling substance to promote root growth through nitrogen (N) and carbon (C) metabolism involved with the signaling network to promote Al-induced root growth. Thus, glucose, fructose, and sucrose can be used as an energy source for maintaining root growth of soybean genotype A7002 even in the presence of Al (Fig. 2 and 2e).

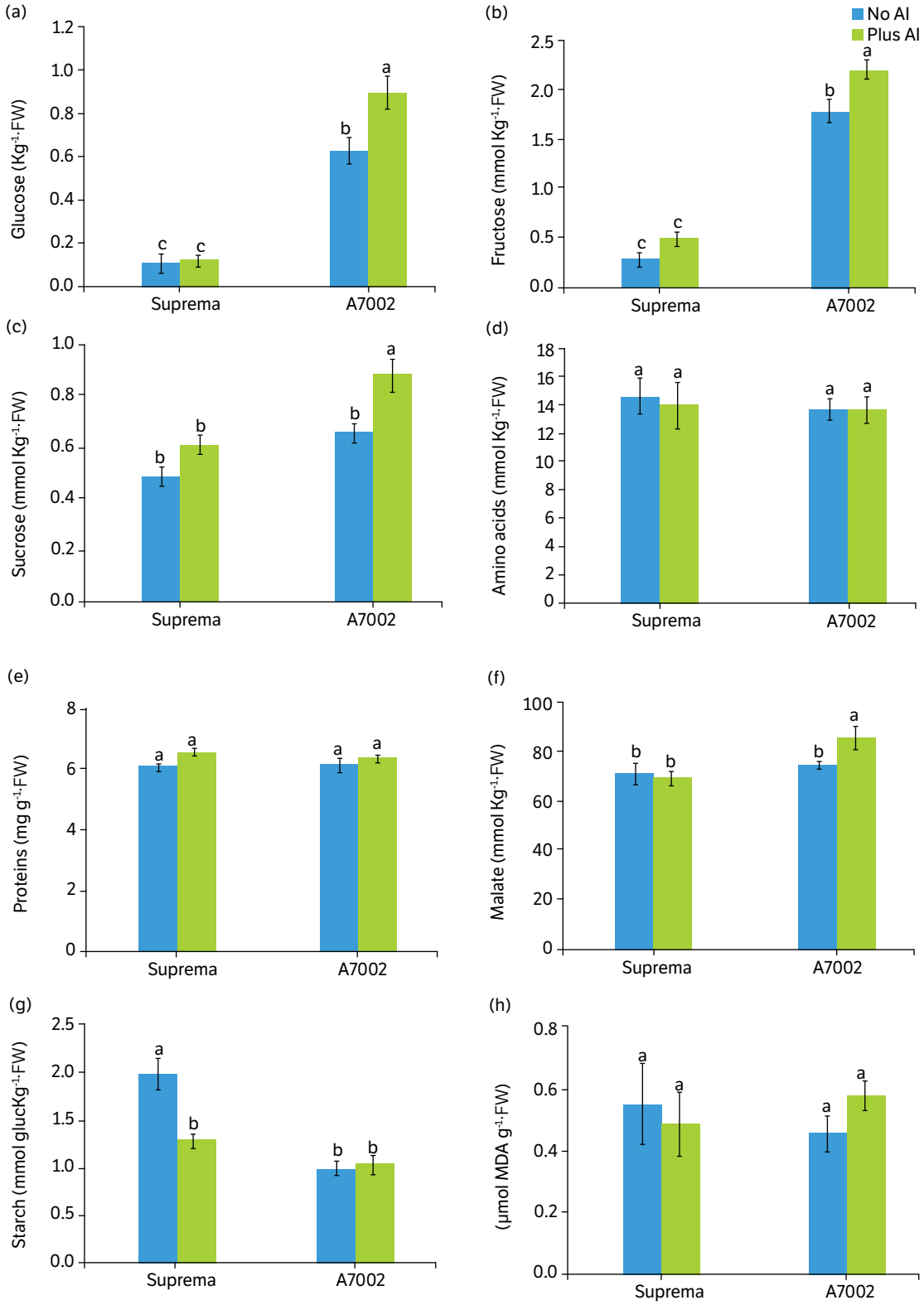

Figure 4. Content of primary metabolites and malondialdeyde (MDA) in root of soybean seedlings Suprema and A7002 grown in Clark solution in the absence: No Al (0 µM) or presence: Plus Al (100 µM Al) for 72h. a: glucose; b: fructose; c: sucrose; d: amino acids; e: proteins; f: malate; g: starch; h: malondialdeyde. Vertical bars represent the standard deviation (n = 5) and the same letters do not differ statistically by the Tukey's test ($p < 0.05$).

Source: Elaborated by the authors.

However, in the Suprema genotype, there was a reduction in starch content after exposure to Al (Fig. 4g), suggesting the cleavage of this molecule into soluble sugars such as glucose and fructose, which would result in metabolic energy for anabolic pathways. Nevertheless,this results evidenced maintenance in the levels of these carbohydrates with lower values than those found in genotype A7002 (Fig. 4a-c). Thus, even with starch cleavage, it was not possible to maintain normal root growth of the Suprema genotype in the presence of Al. Additionally, a higher concentration of soluble sugars in root tissues under Al stress may also be a tolerance response to this metal, by favoring the reduction of the osmotic potential of the roots, allowing a better water uptake, and consequent maintenance of root growth (Giannakoula et al. 2010; Tabuchi et al. 2004), as was observed for genotype A7002.

Although other works found an increase in the rate of lipid peroxidation after Al exposure (Ribeiro et al. 2022, Silva et al. 2023, Yoshida et al. 2023), in the present work no change was observed in this parameter (Fig. 4h). Increases in MDA concentration after Al exposure are related to membrane damage, which is a result of secondary damage induced by this metal, usually involving reactive oxygen species (Mahmud et al. 2019, Ofoe et al. 2023). Thus, only primary toxic effects of Al were observed on root growth of the Suprema genotype (Fig. 2a and 2e). Therefore, Al did not induce a change in the root cells' redox status in both soybean genotypes.

CONCLUSION

The A7002 genotype showed higher resistance to Al, mainly by using two important mechanisms: 1) increased malate content, and 2) Al immobilization in the outer cells of the root. In addition, this genotype showed lower Al content in the roots and increased content of soluble sugars, which may have contributed to the maintenance of root growth. However, the Suprema genotype was susceptible to Al, accumulating a greater concentration of this metal in the root apex internal cells, including the nucleus of the ground meristem cells, resulting in a decrease in root growth.

AUTHORS' CONTRIBUTION

RA and CR conceived the project and designed the experiments. RA, GAS, DSB and BGG performed the research. SVV, MDBLC and CR analyzed the data. RA, DSB and CR wrote the main manuscript. SVV, MDBLC and CR provided revisions to the manuscript. All authors have approved the fnal version.

DATA AVAILABILITY STATEMENT

All dataset were generated and analyzed in the current study.

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