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Metabolic and physiological adjustments of maize leaves in response to aluminum stress

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Abstract Acidic soils with elevated aluminum (Al) saturations are worldwide distributed and harm the crop production in most of the tropical and subtropical regions. Under these conditions, root elongation may be impaired and thus disturbs water and nutrient uptake. Consequently, physiological responses of plants challenged with excess Al may resemble those of drought stresses. Here, we hypothesized that drought tolerant plants are also Al tolerant due to changes in growth, metabolic and physiological adjustments in leaves. Two maize genotypes, BRS1010 and BRS1055, sensitive and tolerant to drought, respectively, were hydroponically grown under controlled conditions and challenged with two Al concentrations (0 and 100 μM $AlCl_3$) for 5 days. After treatment with Al, BRS1055 plants displayed increased leaf and stem elongation whereas the relative root growth rate remained unchanged. This was accompanied by unaltered root structure, photosynthetic efficiency and leaf primary metabolism. In sharp contrast, the BRS1010 plants were sensitive to Al, exhibiting a reduction in leaf and stem elongation and biomass accumulation in shoot and root, as well as greater structural damages in root tips. Additionally, in response to Al, lipid peroxidation increased in BRS1010 leaves in parallel to inhibition of photosynthetic performance and dark respiration. Moreover, compared to control treatment, the genotype BRS1010 displayed a large accumulation of sugars, amino acid, proteins and organic acids in leaves under Al stress. Therefore, the leaf physiology and metabolism are pivotal players in modulating Al tolerance in maize.

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1 Introduction

Aluminum (Al) is a major constraint for plant growth and development in acidic soils (Kochian et al. 2015), affecting largely food production in developing countries. Initially, root elongation is inhibited under Al toxic conditions, triggering water and nutrient deficits, which lead to drastic losses on crop yield (Kochian et al. 2004). However, Al-tolerant plants may sustain root elongation rate under Al stress and simultaneously increasing nutrient and water uptake (Giannakoula et al. 2008).

Al-tolerant plants apparently display a range of responses that are common to water deficit and Al excess, and thus genotypes with improved drought tolerance may be also tolerant to excess Al. Moreover, drought tolerance involves the regulation of shoot growth, inducing changes in leaf-to-root interactions (Moles et al. 2018; Tardieu et al. 2018). Drought sensitive genotypes are unable to control the features mentioned above and display reduced photosynthetic performance under water limitation (Moles et al. 2018). In maize (*Zea mays*), sorghum (*Sorghum bicolor*) and in most Al sensitive plants, even low Al concentrations inhibits root and leaf elongation and impairs photosynthetic performance (Peixoto et al. 2002; Wang et al. 2015). Additionally, leaf expansion reduction, stomatal closure and reduced photosynthesis performance are common responses observed under drought and Al stresses (Yang et al. 2013). In addition to the direct damage caused by Al to the root system, this metal can decrease water transport due to low stomatal conductance (g_s) (Ahmed et al. 2016; Silva et al. 2018). Therefore, plants exposed to Al develop unknown mechanisms that can decrease water uptake, resulting in low leaf hydration which in turn alters leaf metabolism.

The mechanisms that confer resistance to Al can be divided into those that facilitate the exclusion of Al from root cells (exclusion mechanism) and those that enable plants to tolerate Al once it has entered the root and shoot symplast (internal tolerance mechanism) (Brunner and Sperisen's, 2013). Key metabolic components related to Al tolerance have been associated with mitochondrial activity as well as to mitochondrial metabolism and organic acid transport (Nunes-Nesi et al. 2014). In the last years, enormous advances have been achieved demonstrating how and to which extent organic acids neutralize Al inside root cells and around rhizosphere (Kochian et al. 2015). Thus, understanding the physiological basis that mediates Al tolerance in plants is essential to identify metabolic alterations disrupting photosynthesis and respiration, which are the major processes producing and consuming photoassimilates.

Mitochondrial metabolism plays an essential role on carbohydrate consumption allowing the biosynthesis of cellular ATP through oxidative phosphorylation in heterotrophic tissues. In fact, plant mitochondria appear to be involved in many other aspects of plant growth and performance (Zhan and Fernie 2018) as well as in response to environmental stresses (Araújo et al. 2014). Accordingly, the tricarboxylic acid (TCA) cycle intermediates, mainly citrate and malate, can neutralize Al inside root cells as well as around rhizosphere, evidencing the mitochondrial role in Al tolerance (Nunes-Nesi et al. 2014; Kochian et al. 2015). Notably, the organic acids malate, citrate and oxalate may be exudated to prevent Al uptake, whereas in the intracellular environment these organic acids promote Al transport into the vacuole (Nunes-Nesi et al. 2014; Kochian et al. 2015). Thus, photosynthesis-related products may be used to produce organic acids during Al stress (Wang et al. 2010), suggesting that photosynthesis improvement could increase plant Al tolerance. Likewise, the leaf metabolism may represent the key trait to mediate plant growth and development under exposure to Al toxic concentrations, and thus the identification of the major alterations would contribute to improve Al-tolerance.

Studies seeking to elucidate differential responses involved in Al tolerance have been mostly performed using root samples (Kochian et al. 2015). Nevertheless, it has been demonstrated that transcriptional changes also occur in leaves of maize plants cultivated under high concentrations of Al (Mattiello et al. 2014). Interestingly, this study revealed that genes encoding TCA cycle enzymes were upregulated whereas no specific organic acid transporters were differentially expressed in leaves. This fact suggests the shoot metabolism involvement in Al responses, but it

remains unclear if these changes resemble those of drought stress.

From the above, we selected two maize genotypes with contrasting tolerance to drought: BRS1010 and BRS1055, respectively sensitive and tolerant (Lavinsky et al. 2015). These genotypes have been shown to present varying leaf growth and photosynthesis performance under drought stress, as denoted by the reduced photosynthesis rates and maximum rate of carboxylation of phosphoenolpyruvate in BRS1010, whereas these changes were not observed in BRS1055 (Lavinsky et al. 2015). Here we hypothesized that genotypes with superior drought tolerance are also better able to cope with Al excess due to changes in growth coupled with metabolic and physiological adjustments in leaves. We aimed, therefore, to demonstrate whether differential growth and biochemical adjustments can mediate Al tolerance in these genotypes. To reach these goals, we evaluated growth, root tip damages, leaf gas exchanges, antioxidant system and leaf primary metabolism in both genotypes challenged with toxic Al concentrations.

2 Material and methods

2.1 Plant growth conditions and experimental design

Seeds of two genotypes of maize (*Zea mays* L.) were obtained from the National Maize and Sorghum Research Center (Embrapa, Brazil). BRS1010 (Al-sensitive) and BRS1055 (Al-tolerant) genotypes with distinct physiological responses to drought (Lavinsky et al. 2015) were used. After germination, 4-day-old seedlings were transferred to 2 L pots with ½ Clark solution (pH 4.0) (Clark 1975) under continuous aeration for 5 days. Then, plants were grown in full-strength Clark solution, pH 4.0, with 100 µM $AlCl_3$ (stressful condition) and 0 µM (control condition) for 5 days, with pH adjusted daily to 4.0. Plants were grown in a temperature-controlled (25 ± 1°C) chamber under 200 µmol photons m^{-2} s^{-1} light intensity, under continuous aeration, 80% relative humidity and photoperiod of 16/8 h day/night. The experimental unit consisted of four plants per pot and the experiments were conducted in a completely randomized design with five replicates, totalling 20 plants of each genotype.

2.2 Aluminum concentrations

The Al concentrations were quantified in root tips (0.5 cm) and shoots (Silva et al. 2020). The samples were analyzed in an inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 3000XL, Maryland, USA).

2.3 Growth parameters

The relative elongation rates of leaf, stem and root were calculated as described by Matonyei et al. (2014): Relative elongation rate (%) = (L_{Al} / L_c) * 100; where L_{Al} and L_c represent length of plants exposed to Al and length of plants exposed to control treatment, respectively. The relative growth rate (RGR) of roots and shoots was determined according to Hunt (1978): RGR = (ln w_1 − ln w_0) × 1000/(t_1 − t_0), where ln w_1 and ln w_0 represent neperian logarithm of the mass at the end and beginning of the experiment, respectively; and t_1 − t_0 represents the duration of the experiment (days).

2.4 Detection of aluminum in root tips by hematoxylin staining

Root tips (0.5 cm) were dipped in 0.2% iron hematoxylin and 0.02% KIO_3 solution for 15 min (Polle et al. 1978). After, the samples were washed in deionized water by 15 min to remove the dye excess, and then photographed under a stereomicroscope (Zeiss, Stemi DV4, Germany). The positive reaction of hematoxylin with Al is represented by turquoise-blue color (Baker 1962). The intensity of this staining is related to the intensity of Al accumulation in the tissue, thus higher Al accumulation refers to more intense staining.

2.5 Micromorphology and microanalyses of root tips by energy dispersive X-ray spectrometer (EDS) coupled to scanning electron microscopy (SEM)

Root tips (0.5 cm) were fixed in 2.5% glutaraldehyde (v/v) in 0.05 M cacodylate buffer (pH 7.2) for 2 h at room temperature. Next, the samples were dehydrated in an ethylic series, dried with CO_2 at critical point dryer (Baltec model CPD 030, Liechtenstein) and fixed on a metallic stub. The samples were covered

with gold in sputter (FDU 010, Balzers, Liechtenstein) for scanning electron microscopy (SEM) while samples for X-ray dispersive energy spectrometry (EDS) were covered with carbon in the carbon evaporator (Q150T, Quorum Technologies, Ashford, Kent, UK). In root tips the relative content of Al (%) (proportion of Al relative to N, P and S) was estimated at 250 µm (Root Cap Zone) and 1500 µm (Distal Transition Zone - DTZ) from the root tip. The relative content of Al (%) was determined by EDS and expressed as a mean value of five points in each replicate. EDS microanalyses and SEM imagens were performed using scanning electron microscope Leo 1430VP (Cambridge, England) with an accelerating voltage of 20 kV coupled to the IXRF Iridium Ultra EDS.

2.6 Gas exchange measurement

Gas-exchange measurements were performed using a two cross-calibrated portable open-flow infrared gas exchange analyzer (IRGA) systems (LI-6400XT, Li-Cor Inc. Lincoln, NE, USA). Stomatal aperture was maximized using irradiance of 200 µmol photons m^{-2} s^{-1} with 10% blue and 90% red light. The analysis was performed on the third leaf from the apex, with leaf temperature controlled at 25°C, vapor pressure deficit of approximately 1.0 kPa, chamber CO_2 concentration of 400 µmol CO_2 mol^{-1} air and a flow rate of 500 µmol s^{-1}. Before data recording, the CO_2 concentration and the water vapor between the leaf and the reference chamber were automatically matched. Intrinsic water use efficiency (iWUE) was determined according to the protocol described by Sanglard et al. (2014); dark respiration rate was determined at night (dark-acclimation for at least 4 h) using the same leaf used to determinate photosynthesis parameters.

2.7 Measurement of sugars, starch, protein, amino acids, malate and citrate

The intermediary portions of third and fourth leaves from the apex 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 until use. The metabolite extraction was performed with an ethanol gradient, 98%, 80% and 50% and cycles of 80°C. Subsequently, glucose, sucrose, starch, total protein and total amino acid concentrations were measured as described by Fernie et al. (2001); Cross et al. (2006). Malate and fumarate contents were determined according to Nunes-Nesi et al. (2007).

2.8 Enzyme assays

Fresh leaf samples (0.3 g) were ground in liquid nitrogen and homogenized in 0.1 M potassium phosphate buffer (pH 6.8), 0.1 m M EDTA, 1 mM PMSF and 1% (w/v) PVPP solution for analysis of the enzymes superoxide dismutase (SOD-EC 1.15.1.1), ascorbate peroxidase (APX-EC 1.11.1.11) and catalase (CAT-EC 1.11.1.6) (Ribeiro et al. 2012). After filtration through cheesecloth, the homogenates were centrifuged at 12,000 *g* for 15 min at 4 °C, and the supernatants were used as the crude extract of enzyme.

The SOD activity was determined by adding 30 µL of enzyme extract to 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM *p*-NBT, 0.1 m M EDTA and 2 µM riboflavin. The reaction was conducted in a chamber under illumination of a 15 W fluorescent lamp at 25 °C. After 5 min, the illumination was interrupted and the blue formazan intensity produced by photoreduction of NBT was measured at 560 nm (Giannopolitis and Ries 1977). One unit of SOD was defined as the amount of enzyme required to inhibit 50% NBT photoreduction. The protein content of the enzyme extracts was determined by the method described by Lowry et al. (1951) using BSA as the standard.

We added 0.1 mL of enzyme extract to 2.9 mL of reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0) and 12.5 mM H_2O_2 for assessing CAT activity, and 50 mM potassium phosphate buffer (pH 6.0), 0.8 mM ascorbate and 1 mM H_2O_2 for APX activity (Ribeiro et al. 2012). In all cases, the enzyme activities were estimated by absorbance change during the first minute of the reaction at 30°C. Molar extinction coefficients: CAT (240 nm, $\varepsilon = 36$ M^{-1} cm^{-1}) and APX (290 nm, $\varepsilon = 2.8$ mM^{-1} cm^{-1}) were used for expressing enzyme activities.

2.9 Lipid peroxidation

Lipid peroxidation in whole roots and leaves was estimated via analysis of malondialdehyde accumulation (Cakmak and Horst 1991). The samples were homogenized in 2 mL of 1% (m/v) trichloroacetic acid

and centrifuged at 12,000 *g* for 15 min at 4 °C. Aliquots (0.5 mL) of the supernatant were added to 1.5 mL of 0.5% thiobarbituric acid (TBA) (m/v) in 20% trichloroacetic acid (m/v) and incubated in a water bath at 95 °C. After 30 min, the reaction was stopped, the tubes were centrifuged at 10,000 *g* for 10 min, and the absorbance of the supernatant was determined at 532 and 600 nm. The concentration of malondialdehyde-TBA complex was estimated using a molar absorptivity coefficient of 155 mM^{-1} cm^{-1}.

2.10 Data analyses

Data were statistically examined using analysis of variance and tested for significant ($P < 0.05$) differences using Student's *t* tests for comparison of Al effects.

3 Results

In Al-treated plants, Al concentrations in shoots did not differ significantly relative to their respective controls (Fig. 1a). In contrast, Al concentrations in roots were 80% higher upon Al exposure irrespective of genotypes, which did not differ significantly between them (Fig. 1b). Al reduced the shoot relative growth rate (RGR) in both genotypes in comparison with their respective untreated controls (Fig. 1c) whereas in roots Al reduced RGR only in BRS1010 plants (Fig. 1d). Root elongation did not differ after Al exposure regardless of genotypes (Fig. 1e). Comparing the two genotypes after Al exposure, leaf and stem elongation was higher in BRS1055 (Fig. 1e).

After 5 days of Al stress, the root apex of BRS1055 genotype exhibited hematoxylin staining more intense than the BRS1010 genotype (Fig. 2a). This result was confirmed by SEM-EDS (Fig. 2d). The relative content of Al (%) in distal transition zone (DTZ)

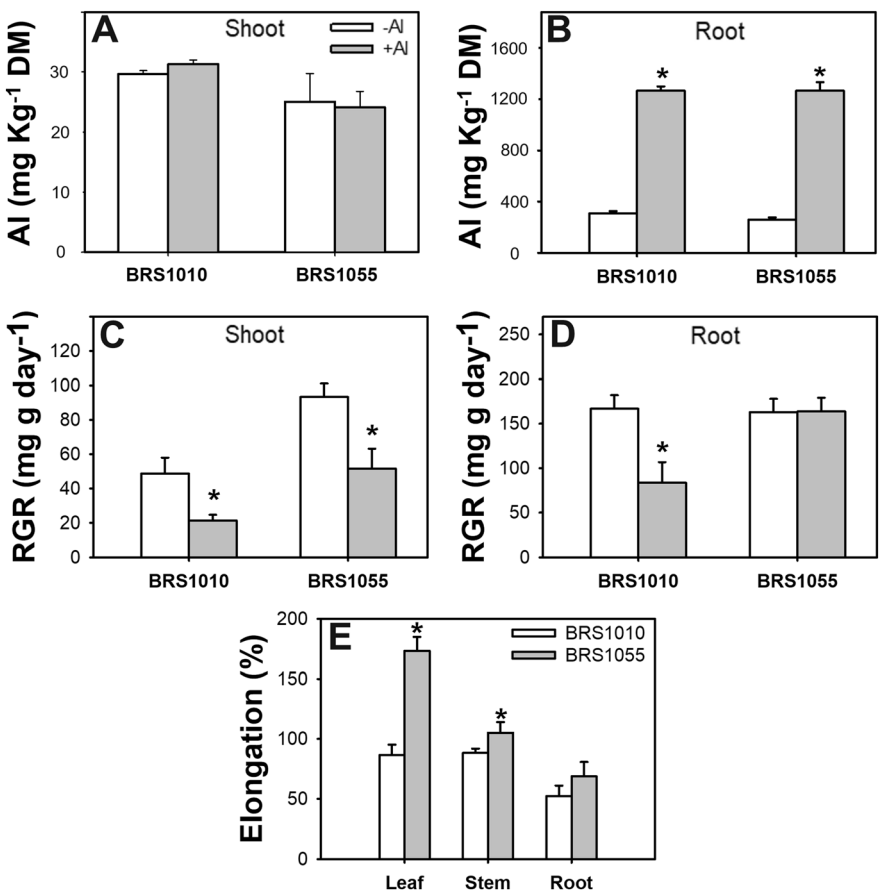
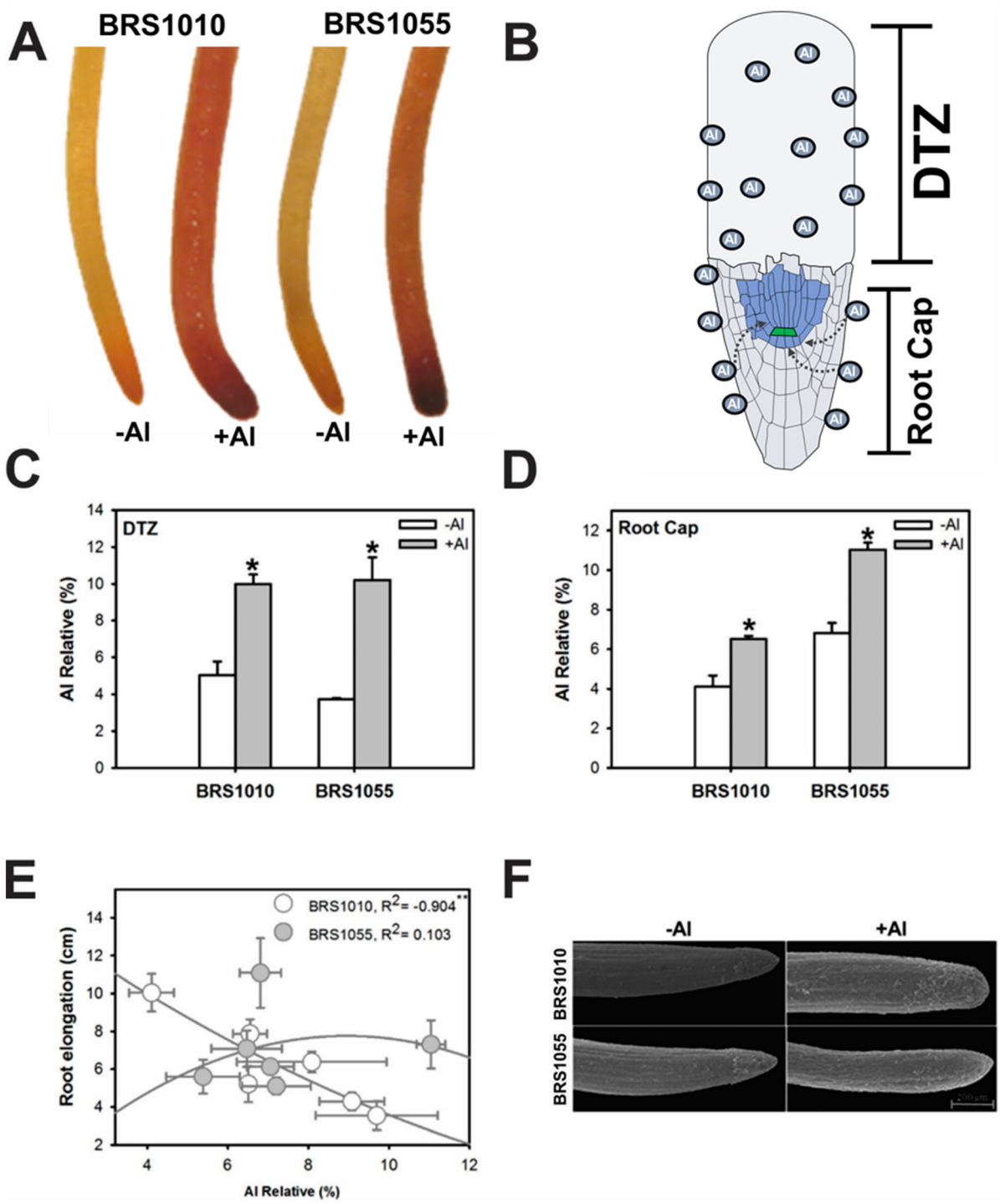


Fig. 1 Aluminum concentrations and growth parameters of BRS1010 and BRS1055 maize genotypes under Al treatment. Plants were grown in control (0 µM Al, -Al) or Al stressful condition (100 µM Al, +Al) for 5 days. Assessment of aluminum concentrations in the shoot (**a**) and root (**b**). Relative Growth Rate (RGR) of shoot (**c**) and root (**d**). Relative elongation rate (%) of leaf, stem and root after exposition to Al (**e**). Values are means ± SE of 5 replicates, asterisks (★) are associated with differences in the same genotype with absence (-Al) or presence (+ Al) of Al according with *t-student* test in a 95% of confidence interval



increased in both genotypes after stress when compared to the Al-untreated plants (Fig. 2c). The relative content of Al in the root cap increased after Al exposure in both genotypes and the highest increase was observed in BRS1055 (Fig. 2d). Interestingly, the relative content of Al in BRS1010 root cap showed a

◀**Fig. 2** Micromorphology and location of Al in BRS1010 and BRS1055 maize genotypes roots. Plants were grown in control (0 μM Al, -Al) or Al stressful condition (100 μM Al, +Al) for 5 days. Hematoxylin stain for Al localization in root sections with 1 cm, $n = 05$ (**a**). Root representative model for analyzes performed in different root sections for the relative content of Al (%) detected by X-ray probe: Root cap and distal transition zone (DTZ) (**b**). The relative content of Al (%) in the DTZ (**c**) and root cap (**d**) detected by EDS coupled to scanning electron microscopy (SEM-EDS). Values are means ± SE of 5 replicates, asterisks (★) are associated with differences in the same genotype with absence (-Al) or presence (+ Al) of Al according with *t-student* test in a 95% of confidence interval. Pearson correlation among root elongations with relative content of Al in root cap of BRS1010 and BRS1055, ** represent significant correlation to $p \leq 0.01$ (**e**). Root apices evaluated by scanning electron microscopy (**f**)

negative correlation with root elongation rates ($r = -0.904$, $p = 0.013$), while in BRS1055 this relationship was not significant ($r = 0.103$, $p = 0.845$) (Fig. 2e).

Thickening of BRS1010 roots was greater than that of BRS1055 upon Al treatment (Fig. 2f and Fig. S1). Roots of Al-treated BRS1010 plants displayed more damages on the fourth and fifth day of stress, which were less pronounced in the BRS1055 roots (Fig. S1). We observed transverse ruptures in the protoderm and outer cell layers of cortex only in BRS1010 roots (Fig. S1 arrows).

Whereas in BRS1055 Al toxicity did not alter the photosynthetic performance (Fig. 3a-e), Al stress led to decreases (25%) in net photosynthesis rates in Al-treated BRS1010 plants (Fig. 3a) in parallel to increases in the intercellular CO_2 concentration (Fig. 3b), while g_s and intrinsic water use efficiency (iWUE) did not respond to treatments (Fig. 3c, e). Transpiration rate did not differ among treatments and genotypes (Fig. 3d). Interestingly, Al stress led to increases in dark respiration rate in BRS1055 in contrast to decreases in BRS1010 (Fig. 3f).

Overall, Al excess triggered metabolite accumulation in BRS1010 leaves when compared with Al-untreated BRS1010 plants and Al-treated BRS1055 plants (Fig. 4). Glucose, fructose, sucrose and starch concentrations increased by 60% 45%, 35% and 55%, respectively, in Al-treated BRS1010 leaves in comparison with their untreated plants (Fig. 4a, c, e, g). Total amino acids and proteins also accumulated in Al-treated BRS1010 in comparison with control BRS1010 and Al-treated BRS1055 (Fig. 4b, d). There was no accumulation of fumarate in Al-treated BRS1010 leaves while fumarate content of Al-treated BRS1055 leaves was reduced by 40% when compared to their respective control leaves (Fig. 4f). In contrast, Al exposure triggered malate accumulation in both genotypes (which did not differ between them) compared to their respective control plants (Fig. 4h).

Lipid peroxidation in both leaves and roots of BRS1055 was unaffected upon exposure to Al (Table 1), whereas it increased by 33% in roots and 43% in leaves after challenging BRS1010 plants with Al stress. Indeed, lipid peroxidation of Al-treated BRS1010 plants was higher in both roots (21%) and leaves (44%) than in their BRS1055 counterparts. Regardless of Al treatment or genotype, the lipid peroxidation rate was higher in leaves than in roots. Superoxide dismutase (SOD) and catalase (CAT) activities increased in BRS1010 leaves after exposure to Al, contrasting with reduced activity of ascorbate peroxidase (APX). CAT activity was 1.4 times higher in Al-treated BRS1010 plants than in their control counterparts.

4 Discussion

Acidic soils constrain agriculture expansion in developing countries because of their high Al saturations, limiting plant cultivation. Plants exposed to excess Al are characterized by limitations in water and nutrient uptake due mainly to root growth impairment (Kochian et al. 2004). Here, we demonstrated that although both genotypes accumulated high concentrations of Al in roots (Fig. 1b), this was not associated with increased root damages (Fig. S1) or inhibition on root relative growth rate (Fig. 1d) in the drought tolerant BRS1055 genotype. Thus, the maintenance of root growth under Al toxic concentrations, which is often displayed by Al tolerant plants (Kochian et al. 2015), indicates that the drought tolerant genotype is likely to be also tolerant to Al toxicity, as judged from its unchanging photosynthetic performance and

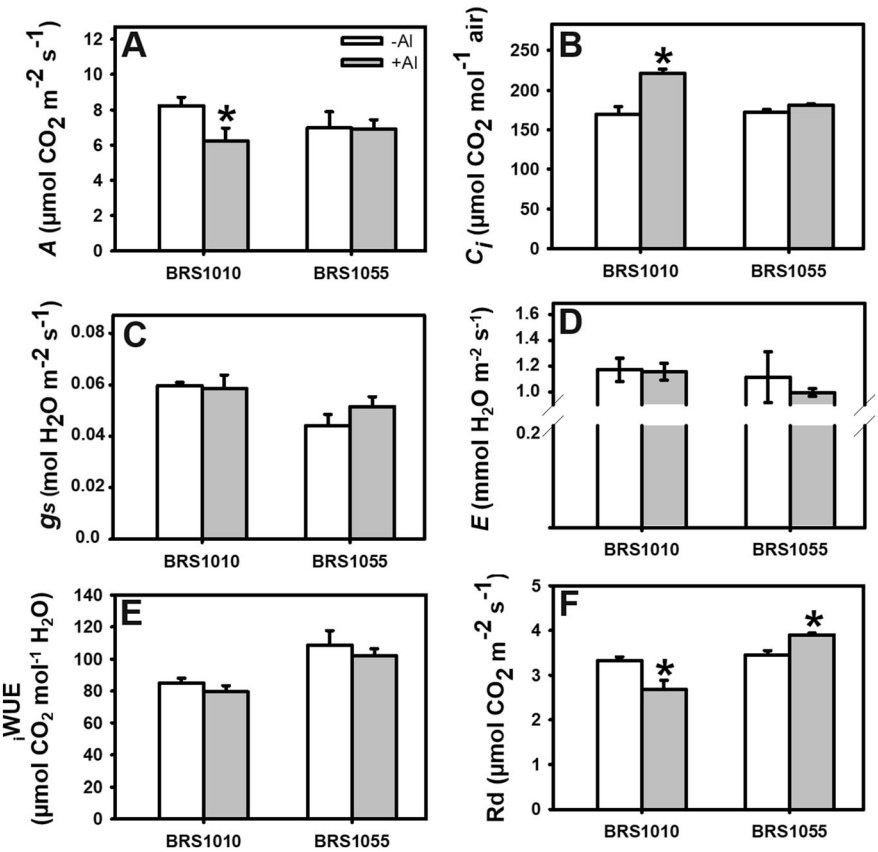


Fig. 3 Gas exchange measurements in BRS1010 and BRS1055 maize genotypes under aluminum (Al) exposure. Plants were grown in control (0 µM Al, -Al) or Al stressful condition (100 µM Al, +Al) for 5 days. Photosynthesis rate (**a**). Intercellular CO_2 concentration (**b**). Stomatal conductance (**c**). Transpiration rate (**d**). Intrinsic water use efficiency (iWUE) (**e**). Dark respiration rate (**f**). Values are means ± SE of 5 replicates, asterisks (★) are associated with differences in the same genotype with absence (-Al) or presence (+ Al) of Al according with *t-student* test in a 95% of confidence interval

remarkable metabolic homeostasis under Al toxic conditions. In sharp contrast, BRS1010 showed reduced root RGR (Fig. 1d) and displayed transverse rupture in root tips after Al stressful conditions (Fig. S1), in addition to exhibiting higher root thickening (Fig. 2f). Therefore, BRS1010 was proven to show higher sensitivity to Al as previously reported for other sensitive maize genotypes (Souza et al. 2016). Although Al induced damage to the root system, the short time of Al exposure (5 days) was apparently not enough to trigger nutritional disturbances in roots, except in the content of Mg (both genotypes) and Ca (BRS1055), which reduced (Fig. S2). However, it seems reasonable to suppose that the damage generated by Al in the roots of the BRS1010 genotype may, over longer time of Al exposure, compromise nutrient and water uptake and, consequently, generate nutritional or drought stress.

Despite both genotypes displaying similar concentrations of total Al at the root apex when challenged with Al (Fig. 1b), results obtained by hematoxylin staining (Fig. 2a) and by SEM-EDS (Fig. 2d) revealed more Al in the BRS1055 genotype. There are several reports evidencing the histolocalization of Al in tissues by the hematoxylin technique (Chowra et al. 2017; Bera et al. 2019; Rahim et al. 2019). These two techniques can detect the Al that is immobilized on the root surface, or immediately below it (approximately 1 micrometer deep), which means that Al is present in the apoplast or in border cells around the tip of the root (Ownby 1993; Támas et al. 2006). Therefore, the higher Al content in root tips of BRS1055 genotype suggests an external immobilization of Al in the apoplast. This would prevent Al entrance into the symplast, allowing it to be retained in the apoplast, thus ultimately contributing to the Al tolerance observed in the BRS1055 genotype.

Al tolerance in plants has been investigated much more in the context of root-rhizosphere dynamics, excluding or minimizing the effects of Al on leaves. Over the last years, however, compelling evidence has suggested that leaves can mediate Al tolerance in different species (Wang et al. 2010, 2012, 2015;

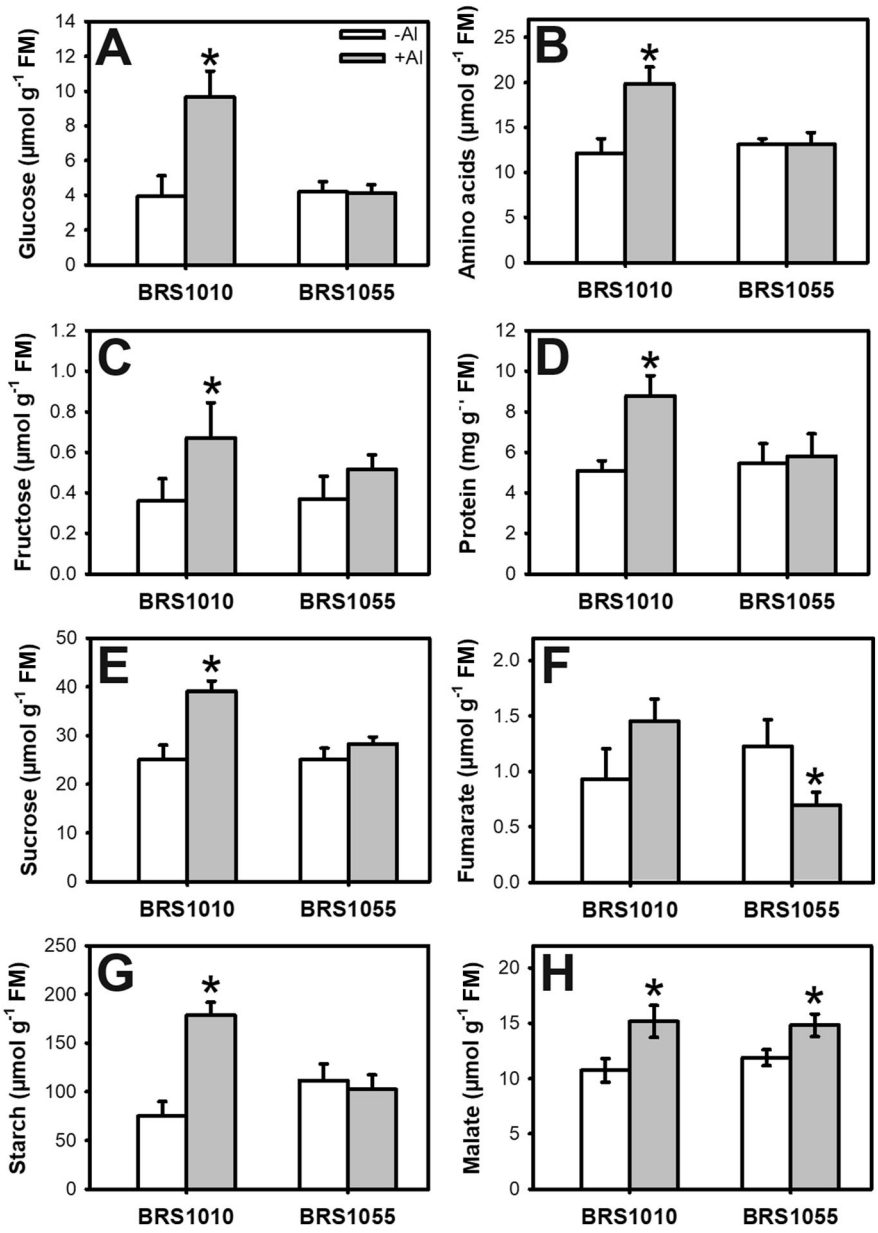


Fig. 4 Leaf primary metabolism in BRS1010 and BRS1055 maize genotypes under aluminum (Al) exposure. Glucose (**a**). Total amino acids (**b**). Fructose (**c**). Total proteins (**d**). Sucrose (**e**). Fumarate (**f**). Starch (**g**). Malate (**h**). Values are means ± SE of 5 replicates, asterisks (★) are associated with differences in the same genotype with absence (-Al) or presence (+ Al) of Al according with *t-student* test in a 95% of confidence interval

Moreno-Alvarado et al. 2017). Maize inbred parent lines with differential Al-tolerance showed root elongation inhibition in parallel to the increase of leaf growth upon Al exposure (Wang et al. 2015). Accordingly, similar reductions of root elongation in BRS1010 and BRS1055 genotypes under Al stress were unrelated to biomass accumulation since BRS1055 biomass was higher than that of BRS1010 under this condition. On the other hand, the largest difference between the genotypes upon challenged with Al resided in stem and leaf elongation in Al treatment, which were particularly noted in BRS1010 plants (Fig. 1e). The differential Al tolerance between BRS1010 and BRS1055 genotypes observed here seems therefore to be most likely based on the maintenance of leaf growth, as previously noticed in the drought tolerance of the same genotypes (Lavinsky et al. 2015).

Al can trigger damages in both proteins and lipids through oxidative stress (Boscolo et al. 2003; Yin et al.

Table 1 Redox system components in BRS1010 and BRS1055 maize genotypes under Al stress

Component	BRS1010		BRS1055	
	−Al	+ Al	−Al	+ Al
MDA_R	0.9 ± 0.1	1.4 ± 0.12*	1.1 ± 0.1	1.1 ± 0.1
MDA_L	9.8 ± 1.4	17.2 ± 2.6*	8.1 ± 0.6	9.7 ± 1.14
SOD	45.3 ± 0.7	48.9 ± 0.6**	47.1 ± 0.4	48.7 ± 0.7
CAT	13.9 ± 1.5	19.1 ± 3.2*	11.7 ± 2.3	11.3 ± 0.6
APX	0.06 ± 0.01	0.03 ± 0.00*	0.04 ± 0.01	0.04 ± 0.01

Plants were grown in control (0 μM Al, -Al) or Al stressful condition (100 μM Al, +Al) for 5 days. Malondialdehyde (nmol g^{-1} FW) was analyzed in roots (MDA_R) and leaves (MDA_L) and superoxide dismutase (SOD) (U SOD min^{-1} mg^{-1} protein), catalase (CAT) (μmol min^{-1} mg^{-1} protein) and ascorbate peroxidase (APX) (μmol min^{-1} mg^{-1} protein) in leaves

The data represent means (n = 04) ± standard-errors, which are associated with differences in the same genotype with absence (-Al) or presence (+ Al) of Al according with *t-student* test to $p \leq 0.05$ (*) or $p \leq 0.01$ (**)

2010), requiring the activity of the antioxidant system, specially CAT, SOD and APX, to restore the cell redox equilibrium (Navascués et al. 2012). Al-treated BRS1010 plants were characterized by higher lipid peroxidation in both roots and leaves as well as a higher activity of CAT and SOD in leaves, suggesting the occurrence of oxidative stress in this genotype in response to Al (Table 1). Moreover, Al stress culminated not only in oxidative stress but also in changes in photosynthesis rates, as previously observed (Wang et al. 2015). Reductions in net photosynthesis rates and intercellular CO_2 concentration triggered by Al in BRS1010 (Fig. 3a, b) suggest limitations to carboxylation. In fact, it has been demonstrated that reductions in photosynthesis occurs due mainly to a limited carboxylation rate, resulting in higher intercellular CO_2 concentrations in leaves of sorghum, rye, rice and maize (Lidon et al. 1997; Peixoto et al. 2002; Silva et al. 2012; Fonseca-Júnior et al. 2013). Furthermore, the redox unbalance in single cells of pea and tobacco led to the inhibition of respiration under Al stress (Yamamoto et al. 2002; Choudhury et al. 2013). Similarly, changes in the antioxidant system coupled with respiration impairments were observed in BRS1010 plants in presence of Al (Table 1; Fig. 3f). Therefore, it seems reasonable to suggest that biochemical limitations occurred in BRS1010 leaves most likely as a result of impairment in photosynthesis and respiration processes.

The results obtained here suggest that part of the resistance of BRS1055 genotype may be associated with a mechanism that limits major disturbances to plant primary metabolism (i.e. photosynthesis and respiration) and thereby avoids a cascade of detrimental downstream effects. In good agreement, the increase in glucose, fructose, sucrose and starch concentrations under Al stress observed in BRS1010 leaves (Fig. 4) coupled with the reduced growth (Fig. 1) observed in this genotype most likely occurred regardless of reductions in photosynthesis, given the expressive accumulation of sugars. Altogether, these results clearly indicate an unbalanced metabolism in response to Al in the sensitive genotype. The accumulation of carbohydrates has already been observed in Al sensitive genotypes of different species (Giannakoula et al. 2010; Silva et al. 2012; Moreno-Alvarado et al. 2017). It has been further suggested that the reprogramming of mitochondrial metabolism may mediate Al tolerance in both microorganisms and plants (Nunes-Nesi et al. 2014).

In fact, mitochondrial malate and citrate concentrations have been demonstrated to improve Al tolerance in tobacco (*Nicotiana tabacum*), papaya (*Carica papaya*) and soybean (*Glycine max*) (De la Fuente et al. 1997; Zhou et al. 2018). Thus, the canalization of the metabolic flux to improve only malate and citrate concentrations suggests their central role in the enhancement of plant Al tolerance (Nunes-Nesi et al. 2014). Thereby, the accumulation of different leaf metabolites leading to an unbalanced metabolism may strongly compromise both growth and the tolerance for Al toxic concentrations. These findings probably reflect the different uses of carbon compounds, i.e. as fuel for energy supply and carbon

skeletons for structure build-up in Al-sensitive and Al-tolerant genotypes, respectively. Although further work is clearly required to assess the individual contribution of different metabolites to Al tolerance, our data provide insights into the strategies used by leaves when this stress occurs.

One of the interesting findings is that certain metabolic responses that were shared between genotypes showed differential behavior in response to drought or Al stress. Thus, physiological and metabolic traits appeared to play more intrinsic relationships in drought sensitive BRS1010 than in drought tolerant BRS1055 under Al exposure. Accordingly, the lower variation observed in both growth and metabolism in BRS1055 plants after Al stress indicates an improved Al tolerance. Our data demonstrated that shoot growth and metabolism play an important role in maize tolerance to Al. Accordingly, the Al-sensitive BRS1010 genotype showed lower leaf growth than the Al-tolerant BRS1055 genotype and the reduced leaf fitness in BRS1010 was associated with reductions in photosynthesis and dark respiration as well as an increased oxidative damage. Furthermore, growth limitations and accumulation of metabolites in leaves occurred in the sensitive genotype after Al exposure. We showed that not only proper root responses but also leaf metabolism reprogramming plays an essential function in response to Al stressful conditions allowing the maintenance of growth. Although our findings indicate that different organs of the same species can present distinct resistance and/or tolerance mechanisms they are collectively able to provide a better understanding of the mechanisms required to avoid Al toxicity. Taken together, our results demonstrated that Al induces physiological and metabolic changes not only in the roots but especially in the leaves, and they may be useful to increase our understanding of the Al impacts in plant fitness, helping us to breed Al tolerant genotypes.

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