MARCELLE FERREIRA SILVA

METABOLIC AND PHYSIOLOGICAL ASPECTS ASSOCIATED WITH DIFFERENTIAL ALUMINUM TOLERANCE IN MAIZE

Dissertation presented to the Universidade Federal de Viçosa, as part of the requirements of the Plant Physiology Graduate Program to obtain the degree of *Magister Scientiae.*

Advisor: Wagner L. Araújo

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Assent:

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ABSTRACT

FERREIRA-SILVA, Marcelle, M.Sc., Universidade Federal de Viçosa, February, 2020. **Metabolic and physiological aspects associated with differential aluminum tolerance in maize**. Adviser: Wagner Luiz Araújo.

Maize (*Zea mays*) is a major crop cultivated worldwide with several uses including animal feeding human consumption and alcohol production. Notably, it is mostly cultivated in tropical and subtropical regions, where acid soils are prevalent. In those acidic soils, the toxicity triggered by aluminum (Al), in special Al^{3+}, is the main factor limiting agricultural production. In this context, strategies aiming at developing stress-resistant crops could increase productive capacity and reduce yield penalty. Al tolerance in maize has been associated with organic acid (OA) exudation, mediated mainly by the membrane transporter family MATE (MULTIDRUG AND TOXIC COMPOUND EXTRUSION). Which are responsible for citrate exudation to rizosphere in an OA/H^+ antiport in root cells in response to Al toxicity. In this study, we used five genotypes derived from Al-intermediate tolerant (L3) and Al-sensitive (L53) genotypes with differential expression of the gene *MATE* that culminated with differential Al tolerance. Given that OA is intimately related with tricarboxilic acid cycle the metabolic consequences of this differential Al-tolerance were investigated. Higher Al content was observed in Al treated samples in all genotypes comparing with its respective controls. Interestingly, Al treated seedlings of tolerant genotypes showed higher increase in Al content than seedling of sensitive ones. This fact aside, higher accumulation of Al was observed in roots of genotypes with lower OA exudation. Moreover, this change in Al uptake and transport also lead to significant changes in mineral elements content including calcium and magnesium. Histochemical evaluation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in roots indicate that accumulation of those reactive oxygen species was actually higher in absence of Al and that it was similar in presence of Al for tolerant genotypes, suggesting that cell division was less affected in those genotypes. Al tolerant genotypes were characterized by minor disturbances in primary metabolism (i.e. photosynthesis and respiration) while the sensitive genotypes, with little if any OA exudation, were characterized by Al-damage effects (i.e. root and shoot growth) since the first hours of Al exposure. Although our findings indicate that different organs of the same species can present distinct Al resistance and/or tolerance mechanisms they were collectively able to provide a

better understanding of the mechanisms used by maize genotypes to avoid or to minimize Al toxicity.

Keywords: Citrate exudation. Abiotic stress. Root growth. *ZmMATE1*

RESUMO

FERREIRA-SILVA, Marcelle, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Aspectos metabólicos e fisiológicos associados com a tolerância diferencial em milho.** Orientador: Wagner Luiz Araújo.

O milho (*Zea mays*) é uma das principais culturas cultivadas no mundo, com vários usos, incluindo alimentação animal, consumo humano e produção de etanol, sendo cultivado principalmente em regiões tropicais e subtropicais, onde os solos ácidos são predominantes. Nesses solos ácidos, a toxicidade provocada pelo alumínio (Al), em especial o Al^{3+}, é o principal fator que limita a produção agrícola. Nesse contexto, estratégias visando o desenvolvimento de culturas resistentes ao estresse podem aumentar a capacidade produtiva e reduzir a perda na produção. A tolerância ao Al em milho tem sido associada à exsudação de ácidos orgânicos (AO), mediada principalmente pela família de transportadores MATE (MULTIDRUG AND TOXIC COMPOUND EXTRUSION). Sendo essa responsável pelo antiporte de AO/H^+ na membrana celular das raízes, exsudando citrato em resposta ao estresse por Al. Neste estudo, foram utilizados cinco genótipos derivados dos genótipos com tolerância intermediária ao Al (L3) e sensível (L53) com expressão diferencial do gene *MATE* que culminou com tolerância diferencial ao Al nesses genótipos. Dado que AO estão intimamente relacionados ao ciclo do ácido tricarboxílico, as consequências metabólicas dessa tolerância diferencial ao Al foram investigadas. Um maior teor de Al foi observado em amostras tratadas com Al em todos os genótipos comparado com seus respetivos controles. Vale ressaltar que plântulas tratadas com Al de genótipos tolerantes apresentaram um maior aumento nos níveis de Al do que as plântulas sensíveis. Este fato à parte, foi observado maior acúmulo de Al nas raízes dos genótipos com menor exsudação de AO. Além disso, essa mudança na absorção e transporte de Al também leva a mudanças significativas nos conteúdos de elementos minerais, incluindo cálcio e magnésio. A avaliação histoquímica do peróxido de hidrogênio (H_2O_2) e superóxido (O_2^-) nas raízes indica que o acúmulo dessas espécies reativas de oxigênio foi mais alto na ausência de Al. Resultado que foi semelhante em presença de Al para genótipos tolerantes, sugerindo que a divisão celular foi menos afetada nesses genótipos. Além disso, os genótipos tolerantes foram caracterizados por pequenos distúrbios no metabolismo primário (i.e. fotossíntese e respiração), enquanto os genótipos sensíveis, com pouca ou nenhuma

exsudação de AO, foram caracterizados por danos (afetando crescimento radicular) desde a primeiras horas de exposição ao Al. Embora nossos resultados indiquem que diferentes órgãos da mesma espécie podem apresentar mecanismos distintos de resistência e/ou tolerância ao Al, eles coletivamente foram capazes de fornecer um melhor entendimento dos mecanismos utilizados pelos genótipos de milho para evitar ou minimizar a toxicidade do Al.

Palavras-chave: Exudação de citrato. Estresse abiótico. Crescimento radicular. *ZmMATE1*

SUMMARY

INTRODUCTION

Given that Al is the most abundant metallic element in Earth's crust (Singh et al., 2017), the toxicity triggered by Al is considered the main factor limiting world agricultural production in acidic soils (Kochian et al., 2015). In tropical and subtropical regions, soil acidity is an important constrains that hinder the increase of food production (Gupta et al., 2013), required to cope with an expected growth in global population. Not only usual farming practices, such as application of ammonium-based fertilizers, but also industrial pollution unceasingly increase the acidification of soils (Kochian et al., 2004; Kochian et al., 2015). It is important to mention that most of the potentially arable land worldwide is located in acid soils (Singh et al., 2017), exhibiting a pH equal or lower than 5.5 (Bojórquez-Quintal et al., 2017). In Brazil, more than 2 million km^2 of the territory, mostly in savannah areas, are covered by acidic soils (Vitorello et al., 2005).

In highly acid soils (pH <5.0), rhizotoxic aluminum (Al) species, Al^{3+}, is solubilized inhibiting root growth and function (Kochian et al., 2015). The formation of toxic Al species occurs from aluminum silicates which are solubilized in the forms of aluminum hydroxide $Al(OH)^{2+}$ and the trivalent cationic form Al^{3+} (Delhaize and Ryan, 1995). Of these, the last one has a greater capacity to strongly and rapidly interfere with root growth and development of several plants. Even at micromolar concentrations (Kochian et al., 2004) and within 30 min to 2h of exposure, Al^{3+} may promote rhizotoxic effects in higher plants (Vitorello et al., 2005) which results in significant losses in crop productivity.

The abundance of Al in acid soils contributes to its contact with the roots, resulting not only in changes in the growth pattern of the roots but also in their physiological functions, such as water and nutrient absorption (Gupta et al., 2013). In this context, strategies aiming at developing stress-resistant crops could increase productive capacity in acid soils and in the presence of Al^{3+} (Setotaw et al., 2015).

The inhibition of root elongation in response to Al is a consequence of alterations in both division and cellular elongation (Kochian et al., 2002). Furthermore, Al toxicity can result in complex interactions between this metal and the plant cell wall (apoplast), plasma membrane and / or cytosol (simplast), favoring the generation of reactive oxygen species (ROS) (Kochian et al., 2004; Illés et al., 2006). Such ROS can directly cause irreversible damage to the structure of DNA, ultimately halting the cell cycle progression and stopping root growth through changes

in differentiation process of stem cells (Eekhout et al., 2017; Horvath et al., 2017). Accordingly, the root distal transition zone (DTZ) is considered the root region most susceptible to Al (Kopittke et al., 2015). Not only growth related issues are evident in response to Al but a misbalance in nutrient absorption (e.g. magnesium and iron) might occur in acidy soils (Vitorello et al., 2005).

As sessile organisms, plants cannot escape from adverse conditions, and thus, throughout their evolution, they have developed strategies capable of preventing or mitigating Al toxicity effects. These include: (i) mechanisms of tolerance – that are based in sequestration and internal detoxification of Al, and (ii) exclusion mechanism – that prevents the entrance of toxic Al species into the root apex (Ryan et al., 2007; Kochian et al., 2015). Both mechanisms involve different transporters and the formation of Al complexes with organic acid (OA) anions, more specifically malate, citrate and, in some species, oxalate (Nunes-Nesi et al., 2014). In the tolerance mechanism, Al enters the cytoplasm and, once transported into the cell, it can be complexed with OAs and/or phenolic compounds, thus mitigating its toxic effects (Kochian et al., 2015). Various compounds, including OAs and proteins, can form stable complexes with Al within the cell (Simões et al., 2012). Moreover, phenolic compounds, such as catechol, catechin, and quercetin, have also been related to Al resistance in some species, including maize (Kidd et al., 2001; Kochian et al., 2015). On the other hand, the Al exclusion mechanism is associated with the transport of organic compounds from the interior of the cell to the external environment (rhizosphere), mainly at the root apex, in the presence of solubilized Al. In these last one mentioned, once complexed with OAs, Al^{3+} form non-toxic compounds and are unable to be absorbed by the roots (Gupta et al., 2013; Nunes-Nesi et al., 2014). The OA exudation mechanism via Al-dependent anion channels, present in the plasma membrane of plant cells (Kochian et al., 2015) is the most known mechanism.

In maize (*Zea mays*) and sorghum (*Sorghum bicolor*), the family of transporters MATE (Multidrug And Toxic Compound Extrusion) have been shown to contribute to Al tolerance (Kochian et al., 2015). ZmMATE1 and SbMATE proteins perform an OA: H^+ antiport and governs the transport of citrate in maize (Maron et al., 2010) and sorghum (Magalhaes et al., 2007). On the other hand, in species such as wheat and arabidopsis the main OA exuded by the roots and used to chelate Al in the rhizosphere is malate. This process of transporting the OA out of the cell involves a transporter belonging to the multigenic family of ion channels ALMT

(*Al-Activated Malate Transporter*), TaALMT1 and AtALMT1, in wheat and arabidopsis respectively (Sasaki et al., 2004; Kobayashi et al., 2007). Although the genetic control of Al tolerance is mostly shared between two main gene families (*ALMT* and *MATE*), other membrane transporters also appears to play an important role in Al tolerance, such as the ABC (*Atp-Binding Cassette)*, as well as the NRAMP *(Natural Resistence-Associated Macrophage Protein)* gene families (Kochian et al., 2015).

The *MATE* family encode membrane transporters and participate in the citrate exudation by the roots in the presence of Al (Kochian et al., 2015). This gene family was initially identified through map-based cloning of the loci of greatest effect for resistance to Al in sorghum (Magalhaes et al., 2007) and barley (Furukawa et al., 2007). Notably, their counterparts were found, *a posteriori*, in Arabidopsis (*AtMATE1*) (Liu et al., 2009), maize (*ZmMATE1*) (Maron et al., 2010), rice beans (*Vigna umbellata*) (*VuMATE1*) (Yang et al., 2011) and rice (*Oryza sativa*) (*OsFRD1*) (Yokosho et al., 2011).

Much more is required to elucidated the real impact of Al resistance mechanisms in consuming respiratory commodities. Mitochondrial metabolism plays an essential role on carbohydrate consumption allowing the biosynthesis of cellular ATP through oxidative phosphorylation in heterotrophic tissues (Nunes-Nesi et al., 2013). In fact, plant mitochondria appear to be involved in many other aspects of plant growth and performance (Zhang and Fernie, 2018) as well as in response to environmental stresses (Araújo et al., 2014). Accordingly, the tricarboxylic acid (TCA) cycle intermediaries 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 complex with Al before it be transport into vacuole (Nunes-Nesi et al., 2014; Kochian et al., 2015). Thus, photosynthesis-related products may be used to produce organic acids during Al stress (Nunes-Nesi et al., 2014), suggesting that photosynthetic carbon metabolism should also investigated in response to Al. Studies seeking to elucidate differential responses involved in Al tolerance have been performed in several species and thus significant advances have been obtained on the mechanisms modulating Al resistance in crops (Kochian et al., 2015).

Maize is a major crop cultivated worldwide, it is used for animal feeding, followed by human consumption and alcohol production (Ranum et al., 2014). However, it is mostly

cultivated in tropical and subtropical regions, where acid soils are prevalent (Gabur et al., 2019), and thus maize breeding in those areas focused on Al tolerance (Gabur et al., 2019). The genetic control of resistance to Al in maize is usually considered polygenic (Magnavaca et al., 1987; Sibov et al., 1999; Ninamango-Cárdenas et al., 2003; Conceição et al., 2009; Guimaraes et al., 2014). It have been associated with quantitative trait loci (QTLs) (Guimaraes et al., 2014), and studies involving Al tolerance usually used mapping populations of inbred lines, using crosses between Al-tolerant (Cateto Al237) and Al-sensitive (L53) genotypes (Gabur et al., 2019). In this study, we used genotypes derived from these lines with differential expression of the gene MATE related with OA exudation.

The physiological and metabolic responses associated with differential Al responses and whether and to which extent these responses are associated to the expression of the *MATE1* remains rather unclear. Once the identification of genetic factors associated with Al resistance in crops is of crucial importance to allowing breeding of more resistant and yet productive materials with lower yield penalties in crops, here, we investigated such responses in maize genotypes. This genotypes were previously demonstrated to be sensitive (L 53 and NIL 5) and tolerant (L3, L3MATE and L3TT) to Al stress (Guimaraes et al., 2014). In addition, the negative effects caused by Al^{3+} were evaluated both in autotrophic organs (leaves) and in heterotrophic organs (roots), and the contribution of central metabolism to the differential tolerance to Al^{3+} in maize was analyzed. Our results demonstrated that the maintenance of root growth in tolerant genotypes was correlated with an intense accumulation of Al in whole plant tissues, as well as with an increase in ROS production in root apexes despite changes in both photosynthesis and respiration in leaves and in primary metabolism of either roots or shoots under Al conditions. It also suggest that plant tolerance to Al are not restricted to roots and that the changes in growth as well as photosynthetic and respiratory metabolism are of crucial significance to modulate Al tolerance in maize and that the combination of the known mechanisms of Al tolerance are clearly required.

MATERIAL AND METHODS

Plant material and experimental conditions

Maize (*Zea mays*) genotypes were used in all experiments and are described in Figure 1. Briefly, we analyzed genotypes with differential Al tolerance due to an increased expression of MATE1 from maize (via backcrossing) or sorghum (via transgenic approach). The genotypes were (i) L53, as the standard Al-susceptible line (Maron et al., 2008; Guimaraes et al., 2014; Matonyei et al., 2017) and (ii) NIL 5, which is a near-isogenic line (NIL) and presented the L53 genetic background and the qALT6 region, QTL that contains the ZmMATE1 gene, and confers Al-tolerance (Guimaraes et al., 2014). NIL5 was bred through assisted backcrossing with genetic marks (Tinoco et al., 2010); (iii) L3, an elite maize line (Guimaraes et al., 2014) with intermediate tolerance to Al; (iv) L3MATE line, which is the L3 introgressed with the gene ZmMATE1 derived from the tolerant line Cateto Al237 (Guimaraes et al., 2014) line through assisted backcrossing and (v) L3TT has the genetic backgound of L3, displaying an over expression of SbMATE gene that confers Al tolerance in sorghum (Magalhaes et al., 2007); notably, the L3TT has low yield performance when compared to its genetic background (half grain production of L3). All materials were kindly provided by Embrapa Milho e Sorgo Research Center (Sete Lagoas, Brazil).

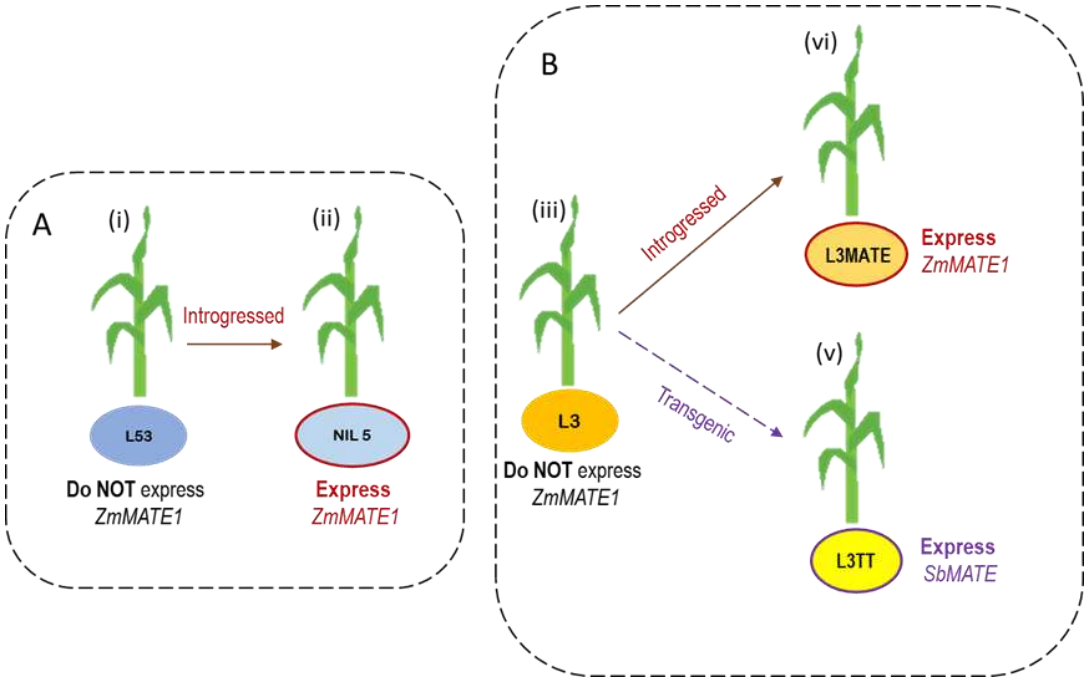


Figure 1. Schematic representation of all maize (*Zea mays*) genotypes with differential aluminum (Al) tolerance used. (A) Al-susceptible lines namely (i) L 53 used as genetic background to assisted backcrossing to introgress the *ZmMATE1* gene in NIL 5 line (ii). (B) elite maize lines displaying (iii) intermediate tolerance to Al (L3), and it was used as genetic background in both, (iv) assisted backcrossing to introgress *ZmMATE1* in L3, here called L3MATE and (v) to transgenic insertion of *Sb*MATE from sorghum in maize line (L3TT).

Seeds were surface-sterilized with 0.5% (w/v) sodium hypochlorite for 5 minutes, thoroughly rinsed in deionized water and germinated on moistened germination paper rolls. Four day-old seedlings with a 3,0cm long radicle were transferred to plastic pots filled with nutrient solution, as previously described (Magnavaca et al., 1987; Piñeros et al., 2002) under constant aeration and for an adaptation period of 24 h in full nutrient solution, pH 4.0. Afterwards, Al treatments were imposed by replacing the nutrient solution with solution of identical composition but without (-Al - control) or with Al (+Al) containing {39} µM Al^{3+} (brackets denote free Al^{3+} activity) supplied as $KAl(SO4)_2$ and pH 4.0. Free Al^{3+} activities were calculated using the GEOCHEM-EZ speciation software (Shaff et al., 2010). Plants were grown in a temperature-controlled chambers (27°C/21℃ throughout the day/night cycle) under 330 µmol photons m^{-2} s^{-1} light intensity, 80% relative humidity and photoperiod of 12h/12 h day/night

Biometric parameters

Plant height was determined as the distance from the edge of the pot until the apex of the highest leaf, and was monitored at three time points (24, 48 and 120h after Al treatment). In addition, dry weight of shoots (leaves and stem) and roots was determined at the end of the experiment (120 hours of Al stress). To this end, maize seedlings were harvested at the end of the photoperiod and thoroughly rinsed with deionized water. Shoots and roots were placed in labelled paper bags and brought to a forced air circulation oven at 70 °C for 5 days; after which, the dry weight of shoots and roots was determined, and the shoot/root dry mass ratio was further calculated.

Determination of mineral content

The mineral content of whole plants was determinate in samples harvested at the end of the Al stress (120 h). For that, plants were harvested, and their roots were washed with deionized water. Samples (~0.2g) were then oven-dried at 70 °C for five days, reduced to powder (using a mill CIENLAB CE-430; 8 blades, 1,725 r.p.m., 20 mesh size), and submitted to a nitric-perchloric digestion (3:1) (nitric acid – HNO_3 70% and then percloric acid – $HClO_4$ 70%). The content of calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P) and aluminum (Al) were determinate by atomic absorption spectrophotometry coupled to flame emission graphite furnace system (Varian Spectra AA, 220FS, added with graphite oven GTA 110).

Histochemical hematoxylin assay

At the end of the experiment, root apexes (5 cm) were dipped in iron hematoxylin 0.2% (mass/volume) solution with sodium iodide ($NaIO_3$) for 15 min (Souza et al., 2016). After, the roots were washed in deionized water with constant aeration by 15 min to remove the dye excess, and then samples were observed and photographed under a stereomicroscope (Zeiss model Stemi 2000-C).

Photosynthetic parameters

Leaf gas-exchange parameters were determined in the first fully expanded leaf from the apex of 5 days of Al exposure using a portable open-flow infrared gas analyzer (Li 6400XT, Li-Cor, Inc., Lincoln, NE, USA) were measured after 1 h illumination during the light period. Instantaneous gas exchanges including the net CO_2 assimilation rate (A_n), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and transpiration (E) were measured after 1 h illumination during the light period under 330 µmol photons m^{-2} s^{-1} light intensity at leaf level. The reference CO_2 concentration was set at 400µmol CO_2 mol^{-1} air. All measurements were performed using the $2cm^{-2}$ leaf chamber at 25°C, as well as a 0.5 stomatal ratio (amphistomatic leaves), with a flow rate of 300 µmol s^{-1}, and the leaf-to-air vapour pressure deficit was kept at 1.2 kPa, while the amount of blue light was set to 10% PPFD to optimize stomatal aperture.

Dark respiration (R_d) was determined using the same gas exchange system described above after at least 2h in the dark period (at night) using the same leaf previously used to determine A_n.

Metabolites analyses

Samples were harvested in four different time points at 6, 24, 72 and 120 hours of Al^{3+} exposure and were flash-frozen in liquid nitrogen and stored at -80 ºC until further analyses. Methanolic extraction was performed by rapid grinding in liquid nitrogen and immediate addition of the appropriate extraction buffer. The levels of glucose, fructose and sucrose were determined as described previously (Fernie et al., 2001), while malate was measured as described by Nunes-Nesi et al., 2007. Moreover, free amino acids were determined as previously described by Yemm and Cocking (1955) and total proteins as described by Bradford, (1976).

The metabolite profiling was carried out in samples for both shoot and roots by using an established gas chromatography coupled to mass spectrometry (GC-MS), as previously described (Lisec et al., 2006). Briefly, the extraction was performed using 1ml of methanol and shaking (35 g) at 70°C during 15min; 60µl of Ribitol (0.2 mg ml^{-1}) was added as an internal standard. After that, the derivatization procedure was performed exactly as described in Roessner et al., (2001). Chromatograms were manually evaluated using the TagFinder software

(Luedemann et al., 2012), using the reference library available in the Golm Metabolome Database (Kopka et al., 2005) and following the recommended reporting format (Fernie et al., 2011).

Histochemical ROS assays

Qualitative evaluation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) was performed by histochemical staining in maize roots as previously described by Kong et al., (2011) with modifications in time exposure and reagents concentration. Briefly, for the identification of H_2O_2 species, the 3,3'-Diaminobenzidine (DAB) at 1.0 mg ml^{-1} was used whereas for the O_2^- species it was used Nitrobluetetrazolium (NBT) at 0.1 mg mL^{-1}, with an exposure time of 7 and 5h respectively. Afterwards, the staining solution (i.e. DAB or NBT) was removed and a distaining solution (ethanol: acetic acid: glycerol 3:1:1) was added until all samples had been completely covered allowing storage until samples were observed and photographed under a stereomicroscope (Zeiss model Stemi 2000-C).

Statistical analyses

The experiment was performed in a completely randomized blocks design with seven plants for replicate and four replicates per treatment. The data was expressed as means ± standard error (SE). All data passed the normality and equal variance Kolmogorov-Smirnov tests and then the means were further analyzed by a one-way analysis of variance. In the absence of restrictions, the data was submitted to analysis of variance ($P < 0.05$) and comparison of means by Student-*t* test ($P < 0.05$) using the SISVAR software (Ferreira, 2011).The means ± *SE* presented in the tables and figures were obtained from at least four independent replicates per genotype and when was the case, over time.

RESULTS

Changes in plant growth and biomass partitioning under Al stress

We investigate the physiological and metabolic impacts of Al stress conditions on previously characterized maize genotypes with differential Al tolerance due to an increased expression of *MATE1*. Clear reductions of root growth were observed in seedlings after120 h of Al exposure and grown side by side with compared without Al treatment (Fig. 2), with the only exception being the transgenic genotype overexpressing the *MATE1* from sorghum (L3TT), that was virtually unaffected by Al (Fig. 2). In agreement with the previous studies that root elongation inhibit is the most sensitive response among various Al toxicity symptoms (Kochian et al., 2004; Singh et al., 2017). L53 was the most affected genotype, while its isogenic line (NIL 5) is seemingly less affected by Al in terms of root length in comparison with L53. Conversely, these genotypes used here did not show changes in biomass presenting similar values of dry weight in seedlings grown with (+) or without (–) Al (Fig. 3). Although dry weight of both shoot (leaf and stem) and root is not significantly affected by Al, it is possibly to observe that seedlings of L3 MATE and L3TT and of their corresponding counterpart L3 invested more in root biomass than seedlings of NIL5 and its background L53, regardless of the treatment (Fig. 3D). During stress establishment, seedlings of L53 line presented the highest values of height in the first 24 hours in both conditions (with or without Al) (Fig. 3E). Moreover, NIL 5 and L3 showed height reductions only after 48 hours of Al exposure, whereas L3 MATE exhibited those reductions later (at 120 h). On the other hand, the height of L3TT did not display reductions in the presence of Al, yet it seems to present smaller plants than its genetic counterparts at the same time point (120 h) under control conditions (without Al) (Fig. 3E).

Figure 2. Differential tolerance to aluminum (Al) in maize (*Zea Mays*) lines. Representative images of 9 days seedlings after 120 h in presence of Al Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. The plants were previously cultivated in hydroponic system with nutritive solution in the absence (-Al) or presence (+Al) for 120h.

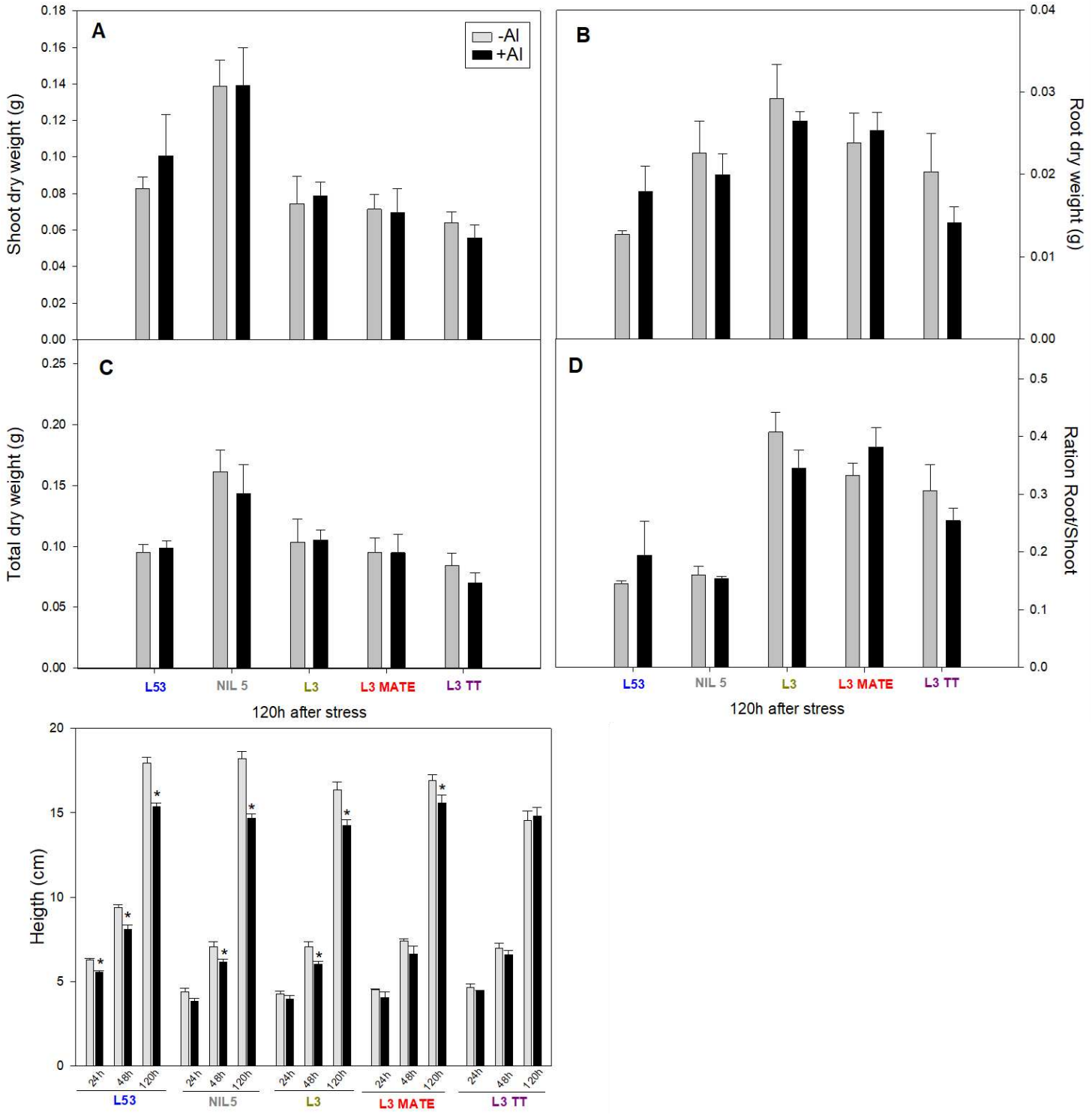


Figure 3. Differential growth response of maize (*Zea Mays*) lines with differential Al tolerance. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. (A) Shoot dry weight; (B) Root dry weight; (C) Root/Shoot Ratio; (D) Total dry weight determined after 120 h of Al treatment; (E) Height after 24h, 48h and 120 h of Al treatment. Values or means ±SE (n=5). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$).

Mineral composition is modified after Al stress

To further investigate to what extent the impacts of Al on plant growth could be associated with changes in the levels of mineral nutrient concentration. As expect, higher Al content was observed in all genotypes cultivated under Al stress than without Al (Fig. 4A). Remarkably, Interestingly, the seedlings with L3 background treated with Al showed ~2.5-fold more Al concentration than untreated seedlings, whereas the seedling of L53 background presented ~1.6-fold increase in Al concentration due to Al treatment. Seedlings of L3 and L3MATE exhibited lower phosphorous levels, whereas L3TT presented higher phosphorous level in the presence of Al compared with untreated seedlings (Fig. 4B). Lines with L53 background showed similar phosphorous and calcium concentration independently on the presence or absence of Al (Fig. 4B-C), and presented lower levels of both minerals than those observed in seedlings with L3 background (L3, L3MATE and L3TT). Seedlings with L3 background showed lesser calcium and magnesium concentrations under Al treatment compared to the controlled condition, without Al (Fig. 4C and D). L53 also presented lower magnesium in presence of Al exposure (Fig. 4D). Potassium concentration in the seedlings was not significantly changed by Al treatment (Fig. 4E).

Al location in roots was evaluated by hematoxylin staining and we observed that seedlings of L53 and L3 which display low expression of *ZmMATE1*, the gene responsible for citrate exudation in maize (Guimaraes et al., 2014), showed more intense staining with iron hematoxylin than those lines that carry the introgression or transgenic *MATE* and thus have organic acids exudation. On the other hand, roots of the lines carrying ZmMATE1 (NIL5 and L3MATE) or overexpressing SbMATE were much less stained with hematoxylin (Fig. 5), confirming the Al exclusion mechanism conferred by these genes (Magalhaes et al., 2007; Maron et al. 2010). (L3TT). NIL5, L3MATE and L3TT (Fig. 5). These findings therefore indicate a higher accumulation of Al in roots of maize genotypes with lower organic acid exudation.

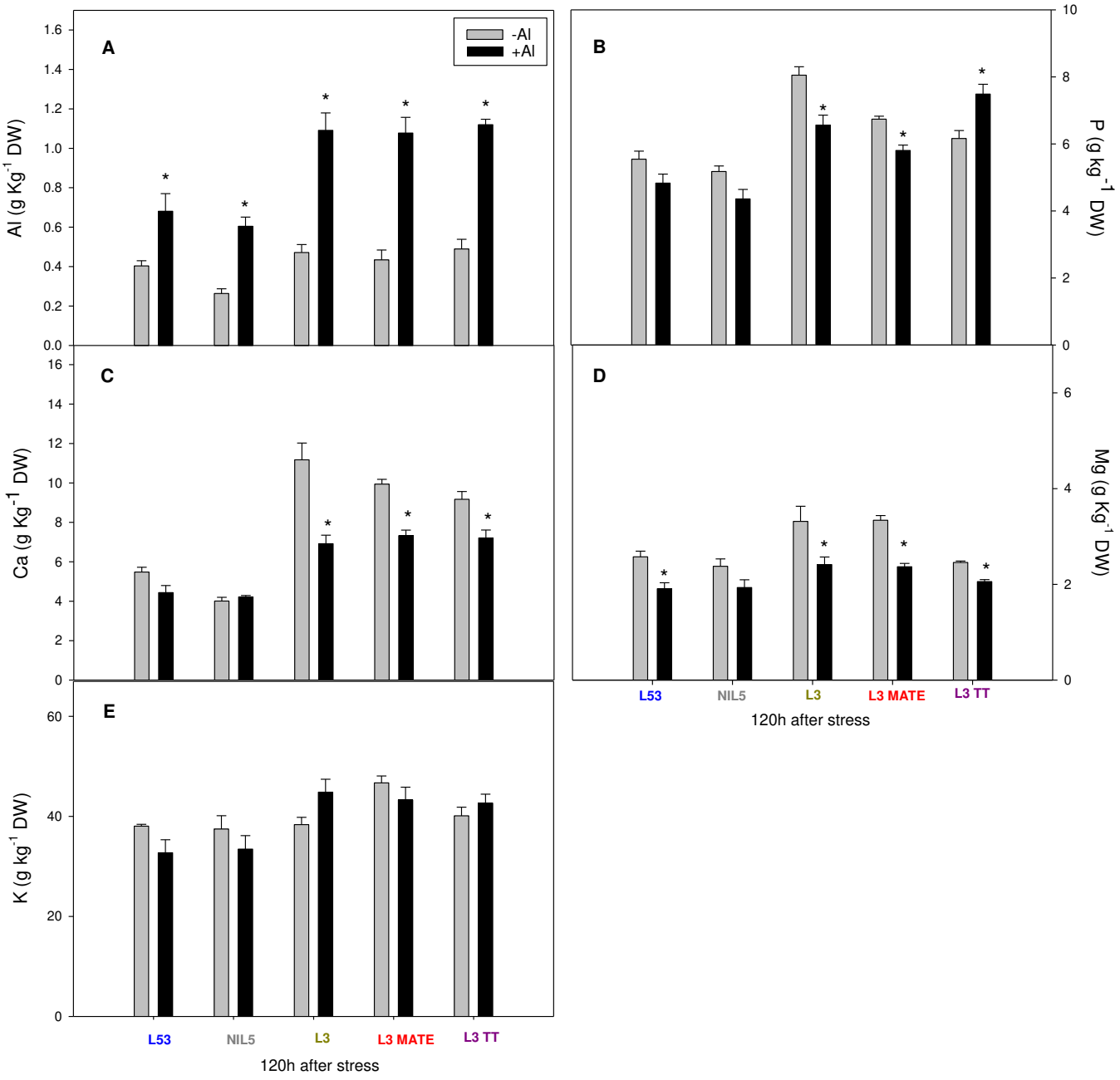


Figure 4. Mineral elements are altered in whole plants of maize (*Zea Mays*) lines with differential tolerance to aluminum. Line L53,Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. (A) Phosphorus; (B) Potassium; (C) Calcium; (D) Magnesium; (E) Aluminum. The determination was made in 9 days seedlings after 120 h of Al treatment or control (-Al). Values or means ±SE (n=4). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$).

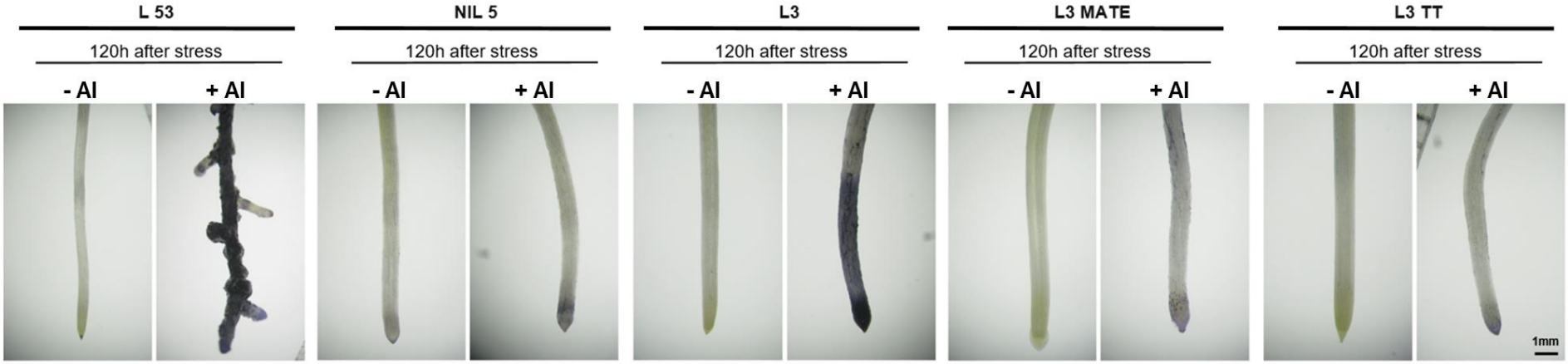


Figure 5. Differential Al location in seminal roots apex evaluated by iron hematoxylin staining. Representative images were taken 120 h after Al treatment in 9 days seedlings of maize (*Zea Mays*) lines with differential Al tolerance. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line.

Photosynthetic responses are affected following Al stress

Given the aforementioned alterations in morphology and growth, we next performed a characterization of the photosynthetic capacity in response of Al on maize genotypes with differential Al tolerance. After 120h of Al exposure, all genotypes displayed significant alterations in net CO_2 assimilation rate (A_n) (Table 1). L53 seedlings increased A_n after Al treatment, whereas the other lines reduced A_n in the presence of Al in comparison with their respective seedlings without Al. L3MATE and L3TT seedlings displayed lower dark respiration rate (R_d) under Al compared with their counterparts without Al treatment. However, stomatal conductance (g_s), transpiration (E), water use efficiency (WUE), electron transport rate (ETR), and internal CO_2 concentration (C_i) remained unchanged in Al-treated seedlings compared to the control condition (Table 1). Notably, seedlings of L53 exhibited lower C_i compared with the other genotypes under both conditions (-Al or +Al).

Table 1. Al modifies gas exchange parameters of 9 days seedlings maize (*Zea Mays*) lines with differential Al tolerance. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. Values or means ±SE (n=5). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$). Abbreviations: $A_{n,}$ Net CO_2 assimilation rate; g_s, stomatal conductance; E, transpiration; WUE, water use efficiency; R_d, dark respiration rate; ETR, electron transport rate and C_i, internal CO_2 concentration.

	L53		NIL 5		L3		L3 MATE		L3 TT	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
A_n (µmol CO_2 m^{-2} s^{-1})	4.6711 ± 0.2100	**5.6925 ± 0.2342**	6.4567 ± 0.3266	**5.2810 ± 0.3606**	7.3685 ± 0.1911	**6.1787 ± 0.4080**	7.5127 ± 0.2451	**6.4906 ± 0.2662**	6.8070 ± 0.4053	**6.4353 ± 0.1776**
g_s (mol CO_2 m^{-2} s^{-1} Pa^{-1})	0.0244 ± 0.0015	0.0257 ± 0.0019	0.0348 ± 0.0025	0.0256 ± 0.0042	0.0517 ± 0.0048	0.0440 ± 0.0056	0.0553 ± 0.0057	0.0418 ± 0.0037	0.0512 ± 0.0025	0.0406 ± 0.0053
E (mmol m^{-2} s^{-1})	0.3511 ± 0.0313	0.3970 ± 0.0225	0.5138 ± 0.0499	0.4111 ± 0.0807	0.7515 ± 0.0863	0.6850 ± 0.1067	0.8077 ± 0.0892	0.6489 ± 0.0765	0.7574 ± 0.0428	0.6275 ± 0.0895
WUE (A_n g_s^{-1})	194.2064 ± 13.3672	165.6676 ± 41.6366	187.8433 ± 11.9450	220.8933 ± 26.3682	107.2920 ± 27.2203	128.5781 ± 37.1836	140.7339 ± 12.4116	160.1218 ± 15.3144	132.8873 ± 5.0047	169.5014 ± 21.4215
R_d (µmol CO_2 m^{-2} s^{-1})	0.8395 ± 0.0816	0.9723 ± 0.0307	0.9500 ± 0.0711	0.9709 ± 0.0402	1.0569 ± 0.0779	1.0254 ± 0.1031	0.9905 ± 0.0809	**0.7921 ± 0.0687**	0.9585 ± 0.0974	**0.7231 ± 0.0430**
ETR (µmol e^- m^{-2} s^{-1})	41.9159 ± 1.0541	43.5985 ± 0.2189	38.9520 ± 1.0971	39.9783 ± 1.1230	41.3415 ± 0.6805	40.1132 ± 0.7933	42.9036 ± 0.5267	41.6347 ± 0.7609	42.9526 ± 0.1941	42.1904 ± 0.3027
F_v'/F_m'	0.6798 ± 0.0090	0.6912 ± 0.0009	0.6440 ± 0.0174	0.6684 ± 0.0089	0.6825 ± 0.0061	0.6629 ± 0.0078	0.7009 ± 0.0018	0.6910 ± 0.0082	0.7029 ± 0.0022	0.6990 ± 0.0034
C_i (µmol CO_2 mol^{-1})	46.5485 ± 3.8055	58.7488 ± 7.5209	101.0195 ± 16.0039	74.2966 ± 19.8966	168.6941 ± 8.3510	221.5062 ± 29.1318	161.9428 ± 19.6675	152.7488 ± 13.4901	174.7811 ± 7.8493	167.9527 ± 8.0375

Metabolite levels in shoot and root of maize plants under aluminum stress

Given the recognized differential roots and shoots responses to Al and that roots are the foremost site for Al toxicity (Kopittke et al., 2015; Singh et al., 2017), we next decided to investigate whether such a differential response also extends to metabolic features in these organs. Neither L53 nor L3MATE exhibited significant differences in total proteins, while NIL5 and L3TT showed a reduction in the first 6 h that did not proceed in the last analyzed time points (Fig. 6A). Reduced levels of amino acids were observed in +Al shoots for L53 after 24 h, whereas shoots of both L3 and L3 MATE exhibited higher levels of amino acids after 6 h in comparison to their respective -Al controls (Fig. 6C). Moreover, +Al shoots of L3 displayed an increase in amino acids content in the first 6 h followed by reduction after 24 and 72 h in comparison to -Al shoots. Lower alterations were observed in the protein content of roots than of shoots (Fig. 6B). In fact, the only observed difference was the reduced levels of protein found in roots of +Al L3 seedlings after 24 h of exposure (Fig. 6B). No differences were observed in amino acids levels in +Al roots after 6 h when comparing with their respective -Al controls (Fig. 6D). Moreover, higher levels of amino acids were observed in roots at 24 h (for L3 MATE), 72 h (for L53 and L3) and at 120 h (for L3 and L3 MATE) (Fig. 6D). For amino acids in shoots, L53 line was characterized by reduction of amino acids in shoots at 24 h, while L3 and L3MATE showed an increase at 6 h following Al treatment. No differences were found in the content of amino acids in shoots for either NIL 5 or L3TT seedlings after Al exposure (Fig. 6C).

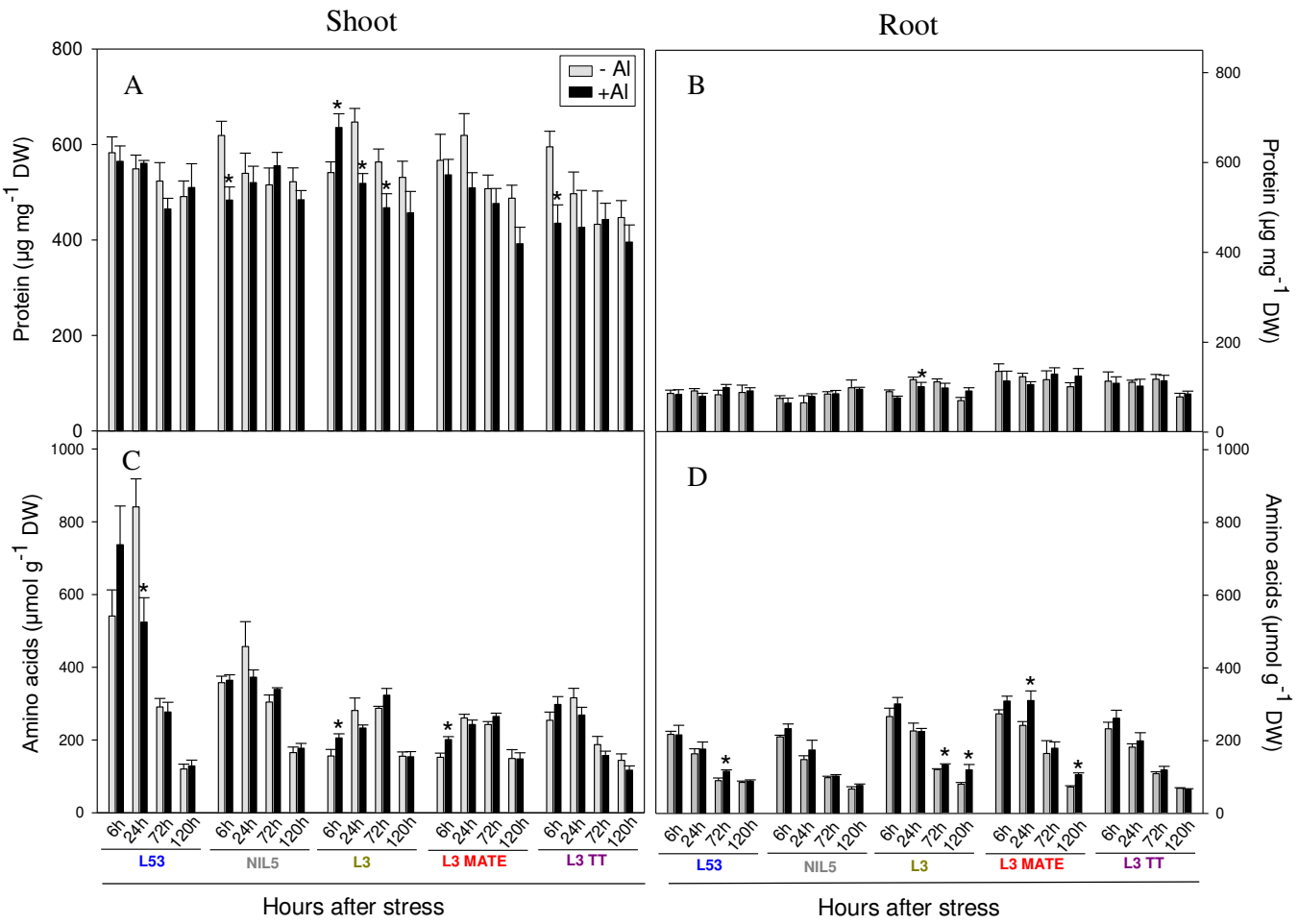


Figure 6. Changes in the levels of nitrogen metabolism related metabolites in maize (*Zea Mays*) lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. (A) Shoot amino acids; (B) Root amino acid; (C) Shoot protein ; (D)Root protein; after - 6h, 24h, 72 and 120h of Al treatment. Values or means ±SE (n=4). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$).

It was also possible to observe an overall decrease in glucose and fructose over time in both shoot and root of seedlings submitted to both Al treatments (Fig. 7A-D). Notably, reductions were significant –without Al, most likely due to higher growth, and thus higher levels of glucose and fructose were generally found in organs of seedlings grown with Al in comparison with the controlled condition, without Al (Fig. 7A-D). L53 and NIL5 exhibited a similar response with an increase in the levels of glucose and fructose in shoots after 72 h in presence of Al. Moreover, +Al seedlings of both L3 and L3MATE exhibited higher levels of glucose at 72 h in shoots, while higher levels were found in roots for NIL 5 and L53 (at 120 h) and L3 MATE at 6 h (Fig. 7B). By contrast, lower levels of glucose were found in roots of L3TT +Al seedlings after 72 h. We did not notice significant chances between -Al and +Al seedlings of the L3TT. L53 did not presented significant changes in roots, whereas for both NIL5 and L3 higher glucose levels were only observed after 120 h in +Al.

Neither L3MATE nor L3TT exhibited differences in the levels of fructose in shoots between -Al and + Al seedlings (Fig. 7C-D). Elevated levels of fructose were found in shoots of L53 (also at 120 h), as well as in NIL5 and L3 treated seedlings at 72 h of +Al (Fig. 7C). No differences were observed in the fructose levels in roots of L53 comparing -Al and +Al seedlings, while elevated levels of fructose were found after 120 h in +Al for NIL5 and L3 and at 24 h for L3 MATE (Fig. 7D). On the other hand, L3 TT exhibited significantly lower levels of fructose in roots at both 72 and 120 h at +Al.

L53, NIL5 and L3 behave similarly, with an accumulation of sucrose in shoots of +Al seedlings after 72 h and 120 h (Fig. 7). Moreover, higher levels of sucrose were also observed in shoots at 24 h, 72 h in +Al L3MATE seedlings, whereas L3TT did not show any changes in sucrose levels in both evaluated organs. Furthermore, L53 and L3 exhibited a higher sucrose content in roots at 72 and 120 h in +Al seedlings. NIL5 generally presented higher levels of sucrose than the others genotypes and an increased level of sucrose was also observed in +Al NIL5 roots in comparison to -Al roots (Fig. 7F). Neither L3MATE nor L3TT presented changes of sucrose in roots.

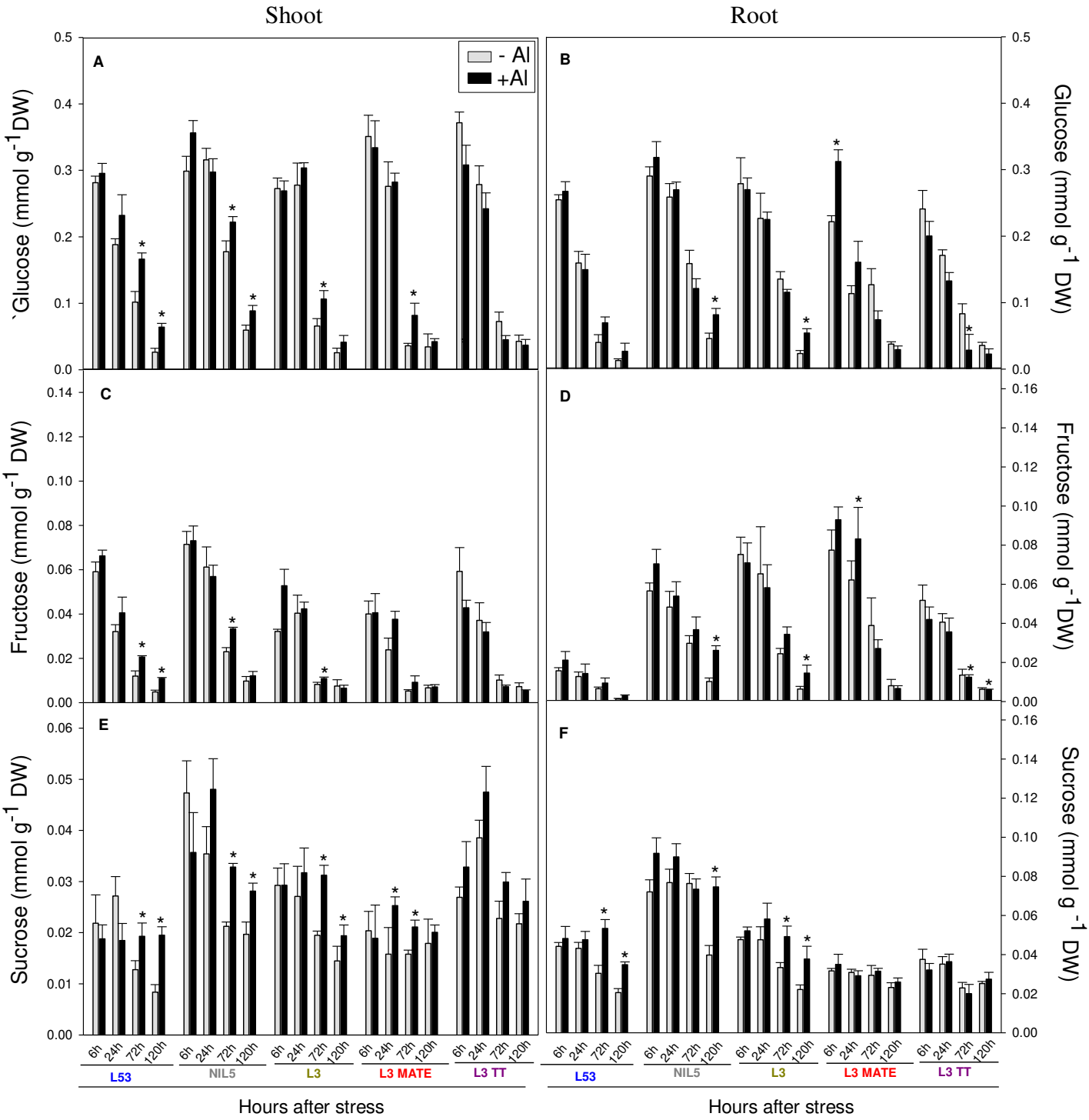


Figure 7. Changes in the levels of carbon metabolism related metabolites of maize (*Zea Mays*) lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. (A) Shoot Glucose; (B) Root Glucose; (C) Shoot Fructose; (D) Root Fructose; (E) Shoot Sucrose; (F) Root Sucrose. Values or means ±SE (n=4). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$).

Increase levels of the organic acid malate was observed in both analyzed organs and in all genotypes in +Al seedlings (Fig. 8). Only L3MATE did not present significant change in malate levels in shoots (Fig. 8A); L53 showed a significant increase only at the last time point while all the other three genotypes showed similar responses, with an enhanced malate levels at 72 and 120 h in shoots. In roots, an increased level of malate was only observed at the first 24 h for L53 (Fig. 8B), while NIL5 have an increased malate levels in all time points comparing +Al seedlings with the -Al except for the first 6 h of stress. Moreover, L3 background plants exhibited higher levels of malate in roots than the L53 background plants. L3 and its respective introgressed genotype L3MATE showed similar responses with an increase in malate levels since the first time point at 6 h in presence of Al. L3TT only change its levels of malate at the last two time points at 72 and 120 h after Al stress.

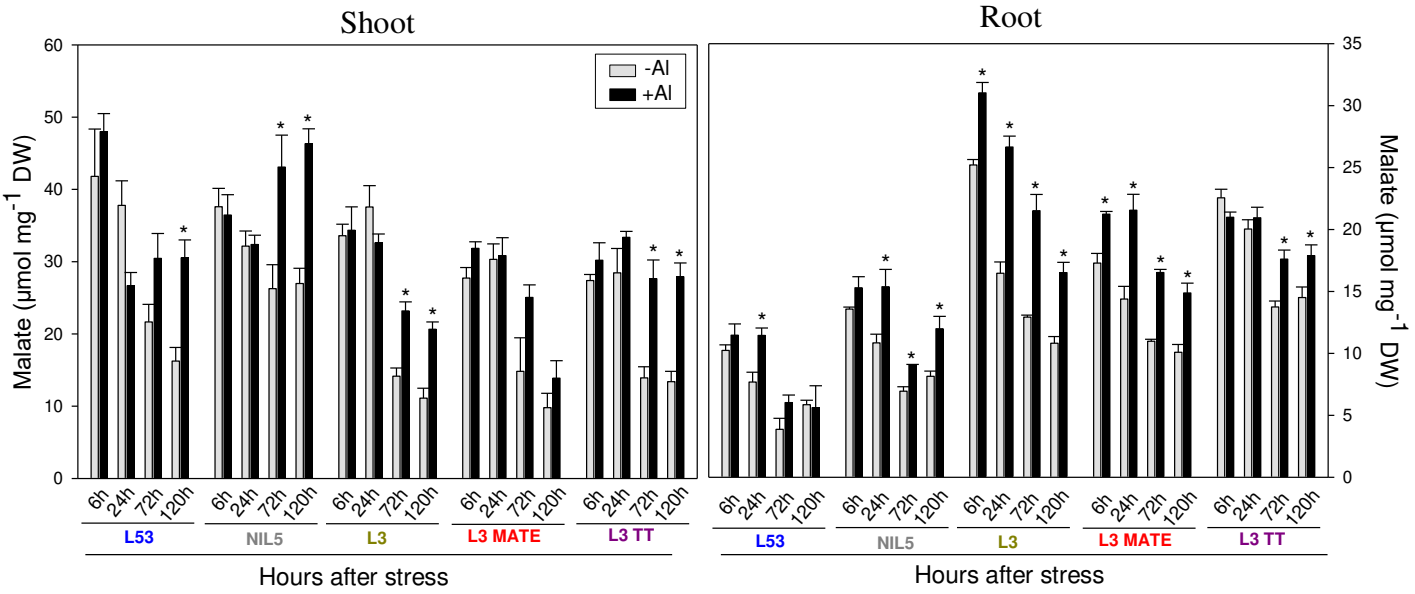


Figure 8. Changes in organic acids in seedlings of maize (*Zea Mays*) lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. (A) Shoot malate; (B) Root malate. Values or means ±SE (n=4). Asterisks (*) indicate significant difference in relation to control (-Al) as determined by Student's *t* test ($P < 0.05$).

We next decided to perform a detailed analysis of the primary metabolism of both organs using an established GC-MS approach (Lisec et. al. 2006). This analysis revealed 45 successfully compounds among amino acids, organic acids, sugars and metabolic intermediates. The number of metabolites that were modified in shoots (Table 2) in the presence of Al was smaller than in roots (Table 3). Briefly, a general reduction in the levels of amino acids was observed in shoots, specifically lysine, isoleucine, leucine, methionine and tryptophan (Table 2), reduced in almost all genotypes over the time. It is worth to mention that in L3MATE a more strong response to Al at 72h in shoot was observed, once it is possible to observe that almost half of analyzed metabolites were affected (either increased or decreased). Similar to the situation found in shoots, it was also observed that amino acids decreased over time in root as well (Table 3). Organic acids, especially malate, citrate and succinate, increased over time in all genotypes, except to L53, suggesting an involvement of TCA cycle and OA in the mechanisms of Al tolerance used by maize. Interestingly, the metabolite profile of L3TT roots was characterized by few modifications in response to Al, a feature that can be associated with the transgenic super expression of *SbMATE* even without Al exposure.

Table 2. Changes in the shoot metabolite profile in response to aluminum stress in maize (*Zea Mays)* lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. Heat map representation of the changes in the relative abundance of leaf metabolite profile in hydroponic seedlings harvested at four time points: 6, 24, 72, and 120 hours of Al treatment. Relative log_2-transformed values of signal intensities were normalized with respect to the mean response calculated for AI-susceptible line L53 at first harvest in control. Values are presented by means ± SE (n=4). Bold numbers represent values that were significantly different when comparing with the respective control at the same time point, determined by Student's *t* test ($P < 0.05$).

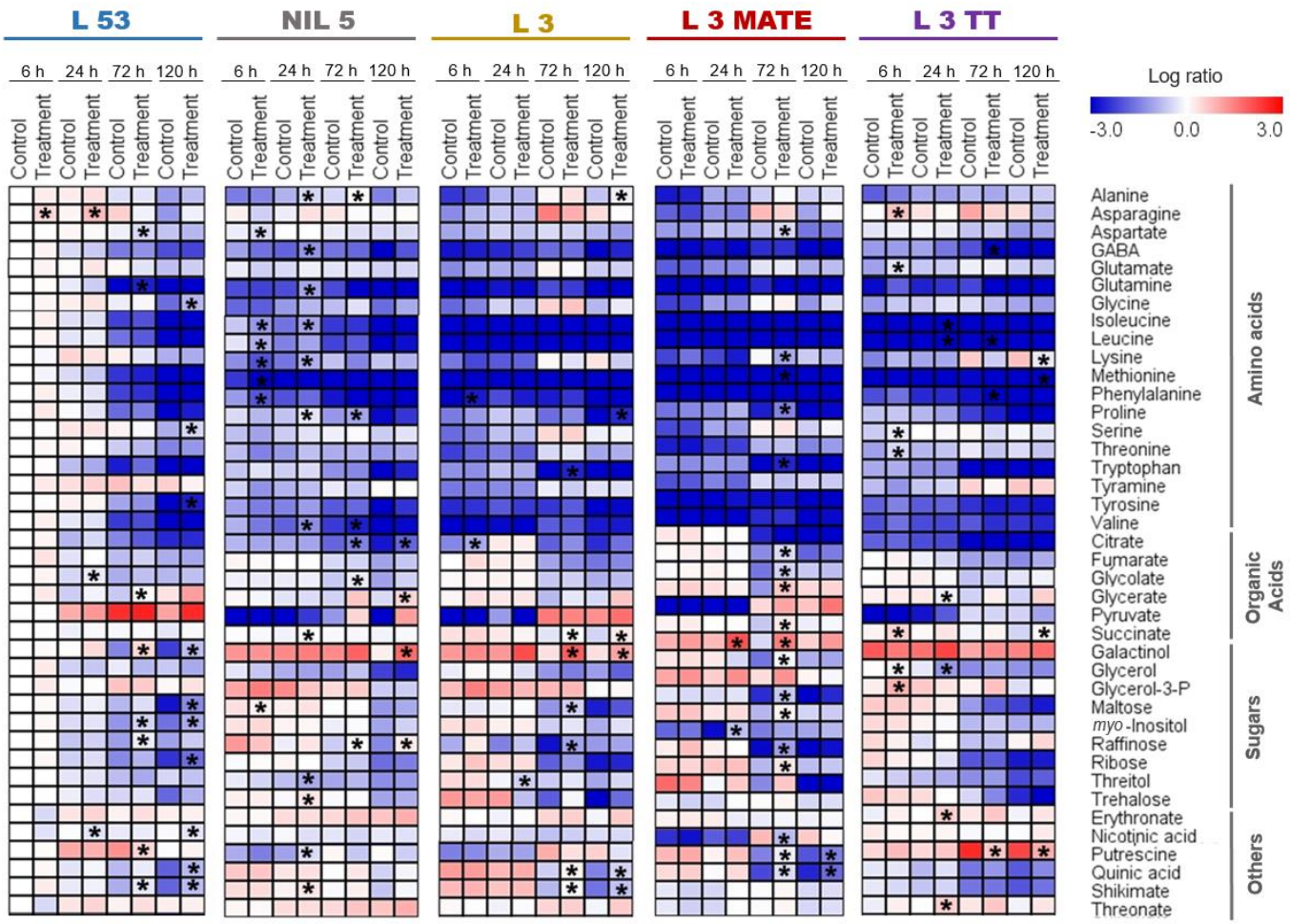
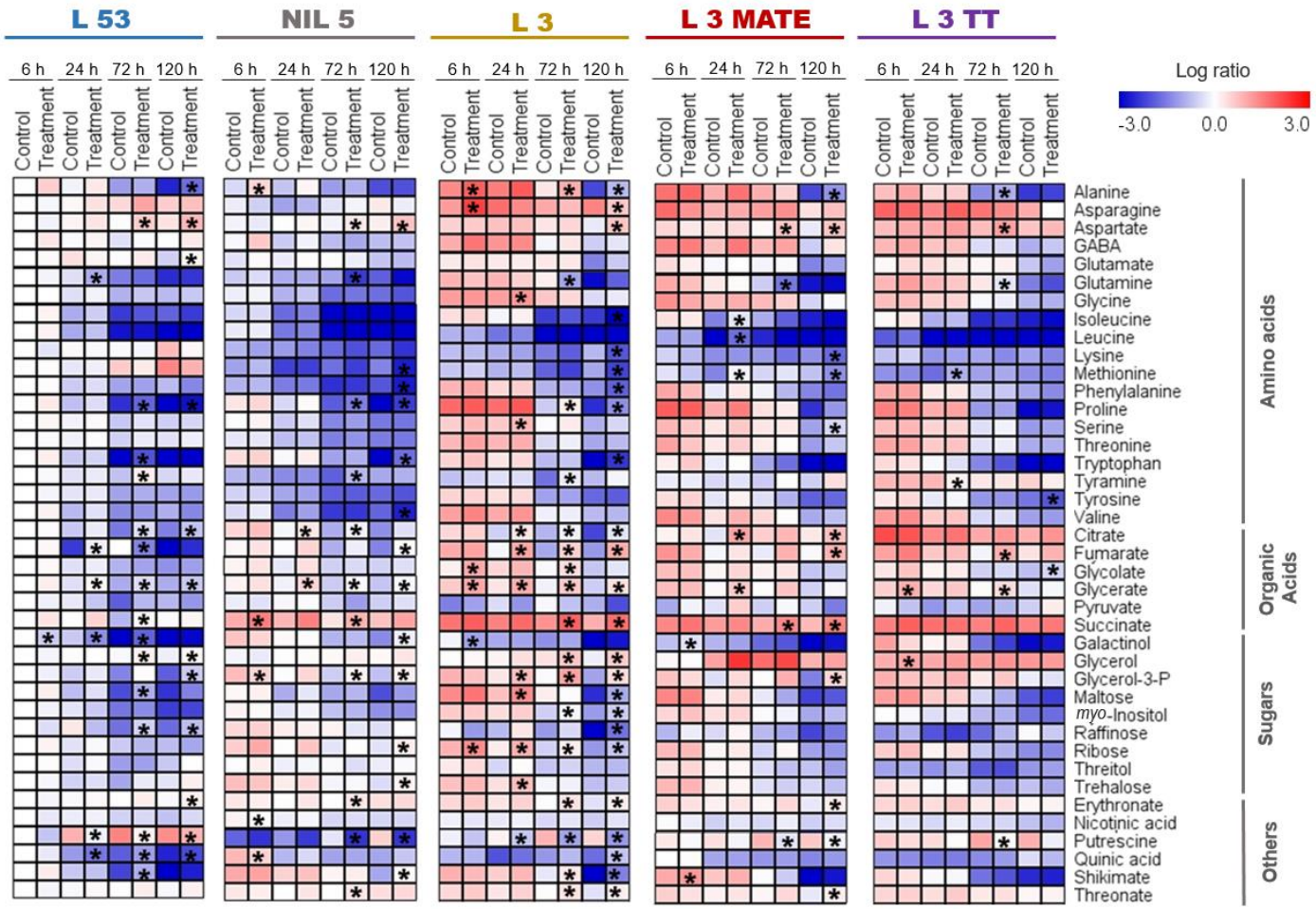


Table 3. Changes in the root metabolite profile in response to aluminum stress in maize (*Zea Mays)* lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. Heat map representation of the changes in the relative abundance of root metabolite profile in hydroponic seedlings harvested at four time points: 6, 24, 72, and 120 hours of Al treatment. Relative log_2-transformed values of signal intensities were normalized with respect to the mean response calculated for AI-susceptible line L53 at first harvest in control. Values are presented by means ± SE (n=4). Bold numbers represent values that were significantly different when comparing with the respective control at the same time point, determined by Student's *t* test ($P < 0.05$).



Alterations in histochemical ROS staining in maize roots under Al stress

Histochemical assays allowed us to observe a distinct staining with DAB (diaminobenzidine tetrahydrochloride) and NBT (nitroblue tetrazolium) as a qualitative evaluation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in roots, respectively. By exposing roots samples of cultivated with and without Al we noticed a differential coloration in the root's apex. First, the dark color was much greater along the root apex of -Al than those in of +Al (Fig. 9), suggesting that ROS (O_2^- and H_2O_2) accumulation in actually higher in -Al samples. Second, it was also clear that, after 120 h of Al-exposure, both L53 and NIL5 displayed only light stained roots, while the L3, L3MATE, and L3TT presented a much strong color that was similar to control plants, suggesting that in those last three genotypes cellular division was little, if any, affected.

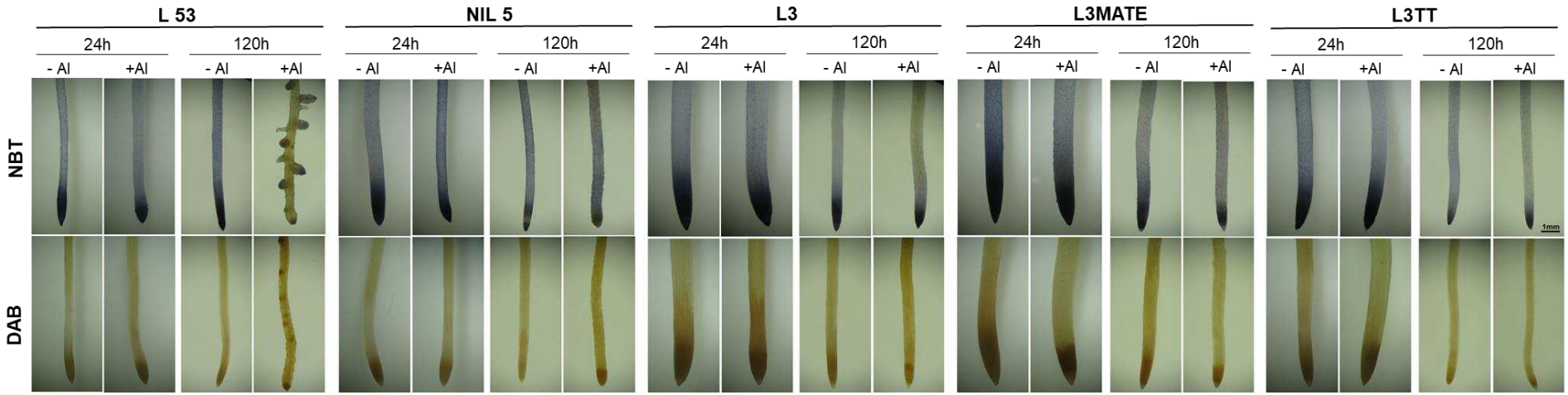


Figure 9. Reactive oxygen species (ROS) histochemical assay of root apex in maize (*Zea Mays)* lines with differential tolerance to aluminum. Line L53, Al-susceptible; NIL 5, introgressed line of *ZmMATE1* gene into L53; L3, genetic background to the introgressed line L3MATE; and L3TT, transgenic insertion of the *SbMATE* gene into the L3 line. Images of stained O_2^- (superior bar - NBT) and H_2O_2 (inferior bar - DAB) of plants exposed to control and Al stress for five days (120h). Treated plants were compared with the respective control at the same time point. Black bar = 1mm Abbreviations: DAB, Diaminobenzidine tetrahydrochloride; NBT, nitroblue tetrazolium.

DISCUSSION

Aluminum toxicity lead to an imbalanced on photosynthesis and carbon partitioning

The main symptom of Al toxicity is an inhibition of both division and cellular elongation of roots (Kochian et al., 2002; Singh et al., 2017). In good agreement, the results of this study revealed changes in seedlings growth in the presence of Al with a clear visual reduced development of root length (Fig. 2). Al can displace Ca and bind to proteins or to the lipids in the roots cell wall (Gupta et al., 2013), which will disrupt structural and mechanical features that culminate in a reduced cell wall extensibility (Kochian et al., 2004; Singh et al., 2017) leading to root shortening. Notably, Al treatment also affected shoot height (Fig. 3E), a feature also previously observed (Mattiello et al., 2014). Remarkably, shoot growth of L3TT was not affected even after 120 h and it is possibly to observe that this tolerant transgenic genotype presented short seedlings size comparing to its genetic background (L3), most likely due to the metabolic cost of this transgenic mutation that likely confer OA exudation even in the absence of stress. Indeed, the usage of OA as a resistance mechanism occur at considerable carbon cost (Kochian et al., 2015). Collectively, these results suggest that an imbalanced carbon partitioning between shoot and roots is likely occurring in response to Al conditions modulating both plant growth and Al tolerance.

Shoot height reduction in presence of Al might additionally be associated with reductions in photosynthetic rates in presence of Al (Table 1), except for L53. Al stress impacts photosynthesis in several species (Akaya and Takenaka, 2001; Zhang et al., 2007; Mattiello et al., 2014). In fact, photosynthesis is a multifactorial process affect by various elements (Zhang et al., 2007). Al interactions within the cell wall limit mineral absorption and can also interfere with water movement across the plasma membrane (Singh et al., 2017). This misbalance in nutrient acquisition and water flow can impact stomatal conductance (g_s) leading to reductions in internal carbon dioxide concentration (C_i) (Banhos et al., 2016). In any case, our results revealed that reductions the CO_2 assimilation rate cannot be explained by stomatal or photochemical limitations but rather by biochemical limitations. In agreement with this assumption, increased content of soluble sugars in shoots of +Al plants were observed (Fig. 7A and E). Higher sugar content can act as feedback inhibition for the photosynthesis (Pego et al., 2000). The higher glucose content in shoots can also be associated with lower glycolytic activity, which are in a good agreement with the reduced dark respiration in +Al plants.

Moreover, we observed a slightly increase in sucrose content in roots of Al sensitive lines after 72 h of Al exposure indicating that the osmotic potential are kept low enabling water absorption even with the disturbance in the cell wall under Al stress, as previously suggested (Giannakoula et al., 2010). In addition, sugars appears to act during signaling in response to Al stress in Al tolerant rice leaves (Moreno-Avarado et al., 2017). Although our results provided circumstantial evidence for the importance of sugar and organic metabolism in Al tolerance, we cannot rule out the presence of alternative mechanism in plants. We also are not able to ascertain whether this metabolic mechanism is independent or not of cell cycle modulating root growth. It is thus tempting to speculate the presence of combined mechanisms of Al tolerance in plants. While the exact mechanism by which this phenotype of Al tolerance seems to be unclear from this study, it remains to be tested if the blockage of the cell cycle is able to influence Al tolerance, not only in Al-related mutants but also in other mutants involved in primary metabolism.

In presence of Al reductions in Mg levels were observed (Fig. 4D) and given that Al is similar to this cation in size, this is an important feature in metal competition (Vitorello et al., 2005). We observed that increased in Al concomitantly with decreased in Mg content, an important component of chlorophyll and other chloroplasts enzymes, can explain, at least partially, the reduced photosynthetic rates. Moreover, this reduction in *A* could be triggered by Al accumulation in leaves (Akaya and Takenaka, 2001).

Aluminum interferes in cellular root division

Using histochemical assay at root apex it was observed that after 120 h of Al exposure, the samples color was much darker along the root samples in absence of Al, suggesting that the in +Al samples changes in the oxidative metabolism were evident. It is important to mention oxidative metabolism is likely involved in root elongation (Liszkay et al., 2004). It has been previously shown that promotion of root growth in maize is likely regulated by NADPH oxidases (Liszkay et al., 2004), and that active production of ROS plays a key role in root development (Causin et al., 2012). Notably, given that tip growth is related with a deposition of a new cell wall (Swanson and Gilroy, 2010), whereas Al-stress is direct related with disruption of the cell wall (Kochian et al., 2004), this could explain why control samples presented a higher ROS content, marked by a darker color (Fig. 9) than +Al- ones. In other

words, root growth in absence of Al is assumedly modulated by the constant production of ROS (Causin et al., 2012). Moreover, it was possible to observe that in response to Al exposure, the tolerant genotypes seems to be less affected (almost similar with the -Al samples), whilst the sensitive genotypes presented a much lighter color in response to Al, suggesting that Al binding process that culminate in cell wall disruption and reduced root length occurs more strongly in Al sensitive genotypes. Nevertheless, it has been also reported that Al increase ROS in maize roots (Jones et al., 2006) which might be due to the Al concentration used here. By evaluating ROS in maize roots, it was shown that in presence of {44} μM Al^{3+} the values for ROS were similar to the ones found in absence of Al (In 2010, Giannakoula et al.) and since we used {39} μM Al^{3+} it could explain, at least partially, the presence of more ROS in control samples.

Metabolites are influenced by Al-exposure and Al-resistance mechanisms

Increases in the content of OAs, specially malate, were observed in shoot tissues of +Al in all lines (Fig. 8). Malate accumulation in shoots could be explained partially by a reduction in the TCA cycle operation or biosynthetic reactions of amino acids (Araújo et al., 2010). In agreement, reduced dark respiration (Table 1) and changes in amino acid content (Fig. 6) were also observed in response to Al. Moreover, malate accumulation in roots seems to be more intense, especially in the L3 (Fig. 8). Since this line appears to not have OA exudation as part of Al-tolerance mechanism due to the lower expression of *MATE1*, it seems reasonable to assume that this increase in malate content is most likely associated with complexing Al^{3+} intracellularly (Nunes-Nesi et al., 2014). Furthermore, considering the other Al tolerant genotypes, that present OA exudation, this high malate content in roots can be connected with the occurrence of both tolerant mechanisms allowing the complexation of the Al in the rizosphere as much as intracellulary. This strategy of exclusion the metal as an insoluble chelated precipitate is present in *P. fluorescens,* and it allow to sustain it metabolic flux by enhance towards the production of chelating agents, including organic acids and lipids, that are seemingly essential for immobilization and exudation of Al (Singh et al., 2009).

Our metabolite profile revealed that differences for total amino acids content and for specific amino acids are evidence in response to Al and that is even more evident in shoots. It is worth mentioning that for adapting to hostile environments, the occurrence of noncyclic TCA cycle can lead to an accumulation of specific metabolites (Sweetlove et al., 2010; Nunes-Nesi

et al., 2014). The extensive reprogramming of metabolism has been suggested to mediate Al tolerance in both microorganisms (Mailloux et al., 2007) and plants (Nunes-Nesi et al., 2014). Accordingly, Al tolerance in tobacco (*Nicotiana tabacum*), papaya (*Carica papaya*) and soybean (*Glycine max*) (de la Fuente et al.; 1997; Zhou et al., 2018) appears to be modulated by mitochondrial malate and citrate levels. It seems therefore reasonable to suggest that the canalization of the metabolic flux to improve only malate and citrate levels might aid in the enhancement of plant Al tolerance (Nunes-Nesi et al., 2014). The results obtained here indicate thus that the accumulation of different leaf metabolites is likely leading to an unbalanced metabolism and that it may strongly compromise both plant growth and Al tolerance in maize. Although our data provides novel insights into the differential mechanism used not only by distinct genotypes but also by leaves in response to Al stress, additional work is clearly required to assess the individual and specific contribution of different metabolites to Al tolerance.

Maize could lines could present more than one Al resistance mechanism

As expected, all +Al- plants presented higher Al content than control plants (Fig. 4A), yet surprisingly the tolerant (L3, L3MATE and L3TT) genotypes were characterized by higher Al content than sensitive ones (L53 and NIL5). Taken together, the data obtained here suggest that not only OA exudation by the roots but also other mechanisms (e.g. Al-uptake), can be responsible for the higher Al-tolerance tolerance of L3, compared to L53, which was maintained in the combination with *MATE* genes, resulting in a superior Al tolerance by pyramiding different Al tolerance mechanisms. It is important to mention that such combination of tolerance mechanisms were previously proposed for other species of Poacea (Ezaki et al., 2013) and thus, further analyses concerning other mechanisms, as Al-uptake, to mitigating Al toxicity and how they can be combined to increase maize Al-tolerance are still required.

CONCLUDING REMARKS.

Here, we were able to demonstrate that the most Al tolerant line (L3TT), that present a super expression of *SbMATE* and thus display higher citrate exudation, was not characterized by the negative metabolic effects caused by Al^{3+}, yet it present a reduction at seedling development. We further observed that sensitive line (L53), without OA exudation, are characterized by Al- damage effects since the first hours of Al exposure.

In summary, the results obtained here indicate that part of the resistance of Al tolerant genotypes (and most likely Al-tolerant plants in general) can be connected with minor disturbances to plant primary metabolism (i.e. photosynthesis and respiration) and thereby avoids a cascade of detrimental downstream effects. In good agreement, the changes in sugars and organic acid levels under Al stress observed in Al tolerant genotypes of maize coupled with minor reduction in growth observed most likely occurred regardless of reductions in photosynthesis, but with reprogramming of metabolism to sustain root growth and to cope with the high cellular levels of Al. Confirmation and identification of the precise nature of this metabolic response and indeed of the exact function of cell cycle itself in the regulation of metabolism remain as important questions that should be addressed in future studies.

Finally, the results described here suggest that Al tolerance in maize is most likely not restricted to roots and OA exudation rather it might be associated with the combination of more known mechanisms of Al tolerance. Although our findings indicate that different organs of the same species can present distinct Al resistance and/or tolerance mechanisms they were collectively able to provide a better understanding of the mechanisms used by maize genotypes to avoid or to minimize Al toxicity. Further studies aiming at the elucidation of the Al-uptake mechanisms in maize, including changes in both expression and activity of enzymes related to sugars, amino acid and OA metabolism, are clearly required to maximize our understanding of the Al impacts in plant fitness, helping us to breed Al tolerant genotypes with lower to none yield penalty.

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