UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal

FELIPE DELLA TORRE

ECOPHYSIOLOGY OF AL-HYPERACCUMULATOR VOCHYSIACEAE SPECIES FROM THE BRAZILIAN CERRADO

BELO HORIZONTE – MG
2022

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal

Área de concentração: Fisiologia Vegetal e Ecologia

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RESUMO GERAL

O alumínio é o metal mais abundante na crosta terrestre e é considerado tóxico para a maioria das culturas em baixo pH. Mesmo sendo bastante tóxico, algumas espécies nativas de solos ácidos desenvolveram diferentes estratégias para lidar com o metal. Alguns são exclusoras de Al, liberando ácidos orgânicos para evitar que o metal entre no sistema radicular e outras são tolerantes ao Al, e permitem que o metal entre no sistema radicular sendo neutralizado e consequentemente acumulado em diferentes órgãos. Àquelas capazes de acumular Al recebem o nome acumuladoras ou hiperacumuladoras de Al. No Cerrado as Vochysiaceae contemplam uma grande diversidade de espécies acumuladoras de Al. Algumas delas vêm sendo estudadas há bastante tempo, mas os avanços no entendimento dessa relação tão especial entre as plantas e alumínio são insuficientes. Espécies de Vochysiaceae do Cerrado lidam diariamente com condições edafoclimáticas extremas e podem nos ajudar a entender melhor a ecofisiologia de plantas submetidas a múltiplos estresses, incluindo o caráter acumuladores de Al. O objetivo da tese foi explorar a ecofisiologia das Vochysiaceae acumuladoras de Al, com foco na fotossíntese e sua relação com o meio ambiente. O primeiro capítulo foi intitulado “Long-term Aluminum exposure improved growth in *Qualea cordata*, a native Al-accumulator species from Cerrado” descreve o cultivo das plantas em hidroponia e caracteriza alguns dos efeitos benéficos do Al para esta espécie e também orienta os próximos capítulos. O segundo capítulo intitulado “Can the Al-accumulator trait reduce the capacity of dealing with multiple environment stressors?” descreve os efeitos do Al no processo fotossintético, principalmente parâmetros de curvas de luz fornecidos por um sistema de trocas gasosas, também descreve tolerâncias térmicas e luminosas. O último capítulo intitulado “Photosynthesis and energy partitioning, different Al-accumulator species respond equally to the metal availability?” traz uma análise mais madura e robusta de como a fotossíntese e partição de energia em três espécies acumuladoras de Al são afetadas pela disponibilidade de Al. Com mais espécies e as informações capítulos 1 e 2, o terceiro capítulo é capaz de trazer algumas discussões ecológicas e novas percepções.

Palavras-chaves: Fotossíntese, termotolerância, fotoinibição, estresse oxidativo e nutrição mineral.

GENERAL ABSTRACT

Aluminum is the most abundant metal in Earth's crust and is considered toxic for most crops under low pH. Even being very toxic, some acid soil native species have developed different strategies to deal with the metal. Some are Al-excluders, releasing organic acids to avoid the metal entering the root system and others are Al-tolerant, and allow the metal to enter the root system and are neutralized in different internal organs, being consequently accumulated in different organs. To those capable of accumulating Al are given the name Al-accumulator or hyperaccumulator species. With high abundance and diversity in different types of Cerrado vegetation, Vochysiaceae contemplates a wide diversity of Al-accumulator species. Some of which have being studied from quite some time, but the advances in understanding this special connection between plant and aluminum are insufficient. Vochysiaceae species from Cerrado deal daily with extreme edaphoclimatic conditions and they can help us to better understand the ecophysiology of plants subjected to multiple stresses including the Al-accumulators trait. The objective of the thesis was to further explore the ecophysiology of the Vochysiaceae Al-accumulator species, focused mostly on photosynthesis and its relation with the environment. The first chapter was entitled "Long-term Aluminum exposure improved growth in *Qualea cordata*, a native Al-accumulator species from Cerrado" describes how we cultivated the plants in hydroponics and characterize some of the beneficial effects of Al for this species and also orientate the next chapter. The second chapter entitled "Can the Al-accumulator trait reduce the capacity of dealing with multiple environment stressors?" describes the effects of Al in the photosynthetic process, mostly light curves parameters provided by a gas exchange system, it also describes thermic and light tolerances. The last chapter entitled "Photosynthesis and energy partitioning, different Al-accumulator species respond equally to the metal availability?" brings a more mature and robust analyses of how the photosynthesis and energy partitioning of three Al-accumulator species are affected by Al availability. With more species and the previous information from the chapters 1 and 2 the third chapter is able to bring some ecological discussions and new insights.

Keywords: Photosynthesis, thermotolerance, photoinhibition, oxidative stress and mineral nutrition.

SUMÁRIO

GENERAL INTRODUCTION

The relationship of aluminum (Al) with plants was always a very intriguing subject to plant scientists (McLean and Gilbert 1928). The contrast of being extremely toxic for some sensitive plants, while beneficial for those that accumulate the metal, reassures this intriguing physiological dichotomy. When soil pH drops below 5, the most rhizotoxic Al species (Al^{3+}) is solubilized, and sensitive plants suffer with root growth inhibition, reduced and damaged root system and limitations on water and nutrient uptake (Kochian et al. 2004). Those primary rhizotoxic effects are the main reason for reducing crop yield in acid soils, which compromised about 40-50% of all world's potentially arable land (Kochian et al. 2015). Most of those areas are found in the tropical regions, where agriculture is the basic source of income and food safety is a concern (Kochian et al. 2015).

Under harsh environment conditions, likewise soil acidity, high exchangeable Al, poor in nutrient and water availability and high temperatures and light, native plants usually shows a wide range of physiological adaptations to survive and cope with those very harmful aspects (Haridasan 2008; de Carvalho et al. 2014). When looking specifically to Al toxicity its well established that plants can respond in two major ways, they can be Al-excluders or Al-resistant (Bojórquez-Quintal et al. 2017). Aluminum excluders avoid Al toxicity avoiding the entrance of Al to the cell through the exudation of high Al affinity organic acid (mostly malate and citrate; Ma et al. 2001). On the other hand, Al resistant plants have the capacity to tolerate high amounts of internal Al and utilize mechanisms to compartmentalize in vacuoles and/or stabilize the metal in order to reduce its toxicity (Bojórquez-Quintal et al. 2017). When plants store high amounts of Al in the chloroplast or other tissues (more than 1,000 mg Al Kg^{-1} DW) those are considered Al-accumulator or hyperaccumulator species (Jansen et al. 2002).

Acid, rich in exchangeable Al and poor in nutrients are some of the typical characteristics of a *sensu stricto cerrado* that contributes to the high diversity of A-accumulator plants there. (Haridasan 1982, 2008). Such an amazing trait, the Al-accumulation was already found in Euphorbiaceae, Myrtaceae, Rubiaceae, Melastomataceae and Vochysiaceae families (Jansen et al. 2002). But only the Vochysiaceae have the trait expressed in all species, which makes the family even more especial in order to study the metabolism (Jansen et al. 2002). The plant-metal relationship in those specific groups are so unique that they not only tolerate and accumulates, but also benefits from the metal (Cury et al.; Osaki et al. 1997; Bojórquez-Quintal et al. 2017). Among most well established beneficial effects of Al are stimulating growth, nutrient uptake, pathogen protection and increase in metabolism (Bojórquez-Quintal et al. 2017). Most studies for Al-accumulator species are from the cultivated species *Camellia sinensis* (Mukhopadyay et al. 2012; Hajiboland et al. 2013, 2014; Xu et al. 2016; Zhao et al. 2018; Sun et al. 2020). And besides our rich biodiversity very little is known about the physiology of the Cerrado native species. Most studies are either the recognition of the Al-Accumulator trait, or the comparison of the influence of different types of soils on those organisms, which compromises the accuracy of interpretation of the effects of isolated Al, with those associated with pH (Gonçalves Malta et al.; de Andrade et al. 2011; Bressan et al. 2016; Alvim et al. 2017; de Souza et al. 2020). More recently some advance was made in order to elucidate the effects of Al in the metabolism of *Qualea grandiflora* a Cerrado Al-accumulator species (Cury et al. 2019). Knowing that studies focused on the physiology of Al-accumulator species, especially those from Cerrado, are very insipient we propose here to deepen in that subject to gather enough knowledge for that, in the future, we get a little closer to understand the biological functions of Al on those very special plants.

Hereafter we presented three chapter focused mostly on the ecophysiology of four Al-accumulator Vochysiaceae species. The first chapter describes the beneficial effects of Al on growth, nutrition and water relations using *Qualea cordata* as the study object. The second chapter also with *Qualea cordata* was focused on understanding the implications of Al on photosynthetic process, water relations, oxidative stress and tolerance to high temperature and light. The last chapter dive deeper in the photosynthetic process, a more complex carboxylation and energy partitioning methodologies allowed us to better understand the possible effects of Al on the photosynthesis and photoprotection in Al-accumulator species.

CHAPTER 1 - Long-term Aluminum exposure improved growth in *Qualea cordata,* a native Al-accumulator species from Cerrado.

Felipe Della Torre[1]; Ana Kelly Gomes de Oliveira[1]; Tiago Vilas Boas da Silva[1]; Igor Vasconcelos[1]; Maurílio Assis Figueiredo[2], Joni Esrom Lima[1]; Marcel Giovanni Costa França[1]

[1] Department of Botany, Universidade Federal de Minas Gerais, Av Antonio Carlos 6627 CP 486. Belo Horizonte, Minas Gerais, 31270-901, Brazil.

[2] Department of Geology, Universidade Federal de Ouro Preto, Campus Morro Do Cruzeiro, 35400-000, Ouro Preto, Brazil.

Abstract

Aims It is unknown why aluminum (Al) accumulator plants growth better when cultivated in the presence of Al and neither why, several times, the absence of such metal is associated with severely limitations in plant growth. Aiming at a greater understanding of this behavior, in this study we evaluated metabolic features induced by differences in Al accumulation in *Qualea cordata* plants, an Vochysiaceae Al-accumulator.

Methods We evaluated plant biomass, allometric relationships in roots, stem and leaves, mineral leaf content, relative water content (RWC) and specific leaf area (SLA) of plants growing in hydroponics subjected to 0, 800 and 1600 μM of $AlCl_3$.

Results Aluminum significantly increased plant growth, leaf area expansion, RWC, SLA and as expected, leaf Al content. Contrastingly, the absence of Al resulted in significantly increases in Cu, Ca, P and S leaf content, root elongation and the number of branches.

Conclusions Aluminum availability significantly improved growth, strengthening the hypothesis of the beneficial effects of Al for accumulator species. The lack of aluminum clearly impaired biomass accumulation, affecting directly root growth and resulting in imbalances in the soil-water-plant relationship (diminished relative water content). Also, the higher levels of Ca and P detected in the treatment with no aluminum supplementation, might indicate possible toxicity of those elements, once both elements are usually found in low availability in native Cerrado soils.

Keywords: Calcium, phosphorous, specific leaf area, relative water content.

Introduction

Aluminum (Al) is the most abundant metal on Earth crust and a primary factor of impairments in crop yield production under acid soil conditions (Exley 2003; Kochian et al. 2015). The acid soils pH < 5.5 are estimated in almost 25% of topsoil and 75% of subsoils of all world ice free lands (FAO 2015) and represent almost 50% of all potentially arable lands (von Uexküll and Mutert 1995; Liu et al. 2014). Sixty per cent of these soils are found in the tropical and subtropical regions, where agriculture is a basic source of income and food safety is a concern (Kochian et al. 2015). Like most tropical regions, *Cerrado* soils are mostly acid, rich in exchangeable Al and poor in nutrient availability (Haridasan 2008).

The *Cerrado* edaphic properties are determinant to species plant diversity, distribution and the development of different survival strategies to cope with different nutrient availability (de Carvalho et al. 2014). From this range of strategies, the ability of some *Cerrado* native species to tolerate Al stands out. Aluminum tolerance mechanisms are basically divided into Al excluders or resistant (Bojórquez-Quintal et al. 2017). Aluminum excluders avoid Al toxicity avoiding the entrance of Al to the cell through the exudation of high Al affinity organic acid (mostly malate and citrate) (Ma et al. 2001). On the other hand, Al resistant plants have the capacity to tolerate high amounts of internal Al and utilize mechanisms to compartmentalize in vacuoles and/or stabilize the metal in order to reduce its toxicity (Bojórquez-Quintal et al. 2017). Some plants have such intimate relationship with the metal, that not only tolerate it, but also accumulates high amounts of Al inside their leaf tissues, and those which accumulate more than 1,000 mg Al Kg^{-1} DW are called Al-accumulators or Al-hyperaccumulators (Jansen et al. 2002a).

Hyperaccumulator species are very representative in a typical *sensu stricto cerrado* (Haridasan 1982). The Al-hyperaccumulator trait was already found to be present in Euphorbiaceae, Myrtaceae, Rubiaceae, Melastomataceae and Vochysiaceae families (Jansen et al. 2002a, b) and apparently present in all species of Vochysiaceae (Jansen et al. 2002b). Not only it is tolerated, but its metal-plant relationship seems to promote possible benefits such as stimulating growth, nutrient uptake, pathogens protection, increased metabolism and others (Bojórquez-Quintal et al. 2017). In order to better understand the Al relationship with Vochysiaceae species, some interesting findings were made in the last few years, for instance, the presence of calcicole and calcifuge behavior (de Souza et al. 2017; 2020), the Al accumulation inside the chloroplast without any apparent damage (de Andrade et al. 2011) and the benefits of soil acidification for growth stimulation (Alvim et al. 2017), but none of that characterized the influence of the aluminum itself, free from pH and soil influences. We compared the seedlings performance of the Vochysiaceae species *Qualea cordata* on three levels of aluminum supplementation in hydroponics, during their early development. Based on the hypothesis that Al-accumulation is beneficial to *Q. cordata* species, we expected that the performance of plants grown on low Al availability would be impaired by nutrient imbalance, particularly Al, P and Ca. We also expect to observe a better development on those plants subjected to higher aluminum availability confirming the beneficial effects of Al for *Q. cordata*.

Materials and methods

Plant material and experimental condition

Qualea cordata (Vochysiaceae) seeds were collected in the Cerrado region, in the city of Santana do Riacho-MG, and germinated in plastic trays containing a filter paper. After germinated the seedlings were grown in hydroponic standard solution (Modified from Watanabe and Osaki 2001) under low light intensity, 200 µmol photons m^{-2} s^{-1} in 4.5 pH for one month. The standard nutrient solution was composed of 1.07 mM N (NH_4NO_3), 48µM P ($NaH_2PO_4 \cdot 2H_2O$), 0.385 mM K (K_2SO_4:KCl = 1:1), 0.625 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.41 mM Mg ($MgSO_4 \cdot 7H_2O$), 17.9 µM Fe ($FeSO_4 \cdot 7H_2O$), 9.1 µM Mn ($MnSO_4 \cdot 4H_2O$), 46.3 µM B (H_3BO_3), 3.1 µM Zn ($ZnSO_4 \cdot 7H_2O$), 0.16µM Cu ($CuSO_4 \cdot 5H_2O$), and 0.05 µM Mo ($(NH_4)6Mo_7O_{24} \cdot 4H_2O$). After one month, plants were transferred to 3L pots – two plants per pot, 12 plants per treatment - constantly aerated, containing the standard nutrient solution with pH adjusted to 4.5 and supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. The plants were grown for 7 months under greenhouse conditions, when they were harvested for physiological and morphological evaluations.

Growth and biomass allocation

For morphological evaluations, plants of each treatment (n = 7) were segmented in stem, leaf, and root. Each part was photographed with a scale to determine, using ImageJ software, the following parameters: root length, main stem length, number of branches, mean branch length, total leaf area, mean leaf area and number of leaves. Then the plant material was taken to a forced-air circulation oven at 60ºC until the material dried completely. The parts were weighted separately to obtain stem dry mass (SDM), leaf dry mass (LDM), root dry mass (RDM) and total dry mass (TDM). The total leaf area and LDM were used to estimate specific leaf area (SLA).

Mineral leaf content

Leaves of *Qualea cordata* plants (n = 3) were completely dried in a forced air circulation oven at 60ºC, and the dried material was then grounded in a knife mill (Wiley TE-648, Tecnal, Brazil). Samples of 0.250g were digested in nitric acid and oxygen peroxide and quantified in inductively coupled plasma atomic emission spectrometer (ICP – OES, Agilent 725), according to Barros et al. (2016) to determinate the concentration of Cu, Zn, Mn, Al, Fe, Ca, K, Mg, P, and S.

Relative water content

To evaluate the plant water status, leaf discs of 0.5 cm diameter (n = 5) subjected to different Al concentrations were weighted and the fresh weight (FW) obtained. The same discs were then let on the Petri plate with distilled water for 24 h to reach a full turgid status and weighed again to obtain turgid weight (TW). The dry weight (DW) was obtained weighting the same leaf discs dried in a forced air circulation oven at 60ºC. The relative water content (RWC) was calculated as RWC = [(FW-DW)/(TW-DW)] *100.

Statistical analyses

A completely randomized design was used for all the experiments, and each experimental unit was composed of a *Qualea cordata* plant. The data was previously evaluated using the Brown-Forsythe Test for variance homogeneity and the comparison between means was performed using ANOVA followed by Tukey test at 5% of significance. Statistical analyses and figures were done using GraphPad Prism software, version 7.00 (San Diego, CA; www.graphpad.com).

Results

Biomass accumulation, growth pattern and specific leaf area

The presence of Al positively influenced biomass accumulation in root, leaves, and stem (Fig. 1). The total dry weight increased ($P<0.05$) about 134 and 138% in treatments supplemented with Al, when compared with plants not subjected to Al. The increase in biomass accumulation was more evident in roots, resulting in increases as high as 355% in plants subjected to 800 μM of Al, compared to plants subjected to no Al in the same organ. Leaf and stem biomass had the same pattern of biomass increase in response to Al presence as total and root biomass (Fig. 1). Despite the differences in biomass accumulation detected between plants in all organs, there were no differences in biomass accumulation between plants subjected to 800 and 1600 μM of Al (Fig. 1).

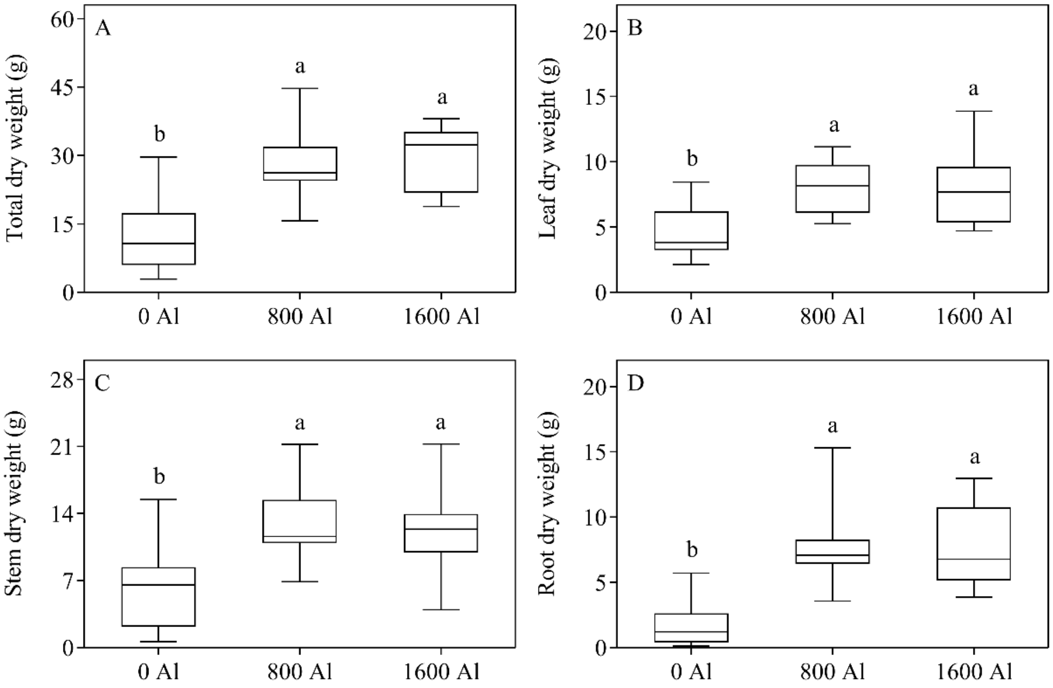

Fig. 1. Total (A), leaf (B), stem (C) and root (D) dry weight (g) of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. (n = 7).

The Al presence affected not only the leaf biomass accumulation but also the leaf number and leaf area parameters (Fig. 2 A, B, and C). The total and mean leaf area showed a well-defined pattern of increase in response to the aluminum presence (88% increase in total leaf area and a 121% increase in mean leaf area). A different response in leaf number was detected, leaf number was significantly reduced in response to the presence of Al (Fig. 2 C). Plants growing in absence of Al showed a higher number of leaves (44) followed by an intermediate number of leaves for 800 Al (37) and a lower number for high Al concentration (22).

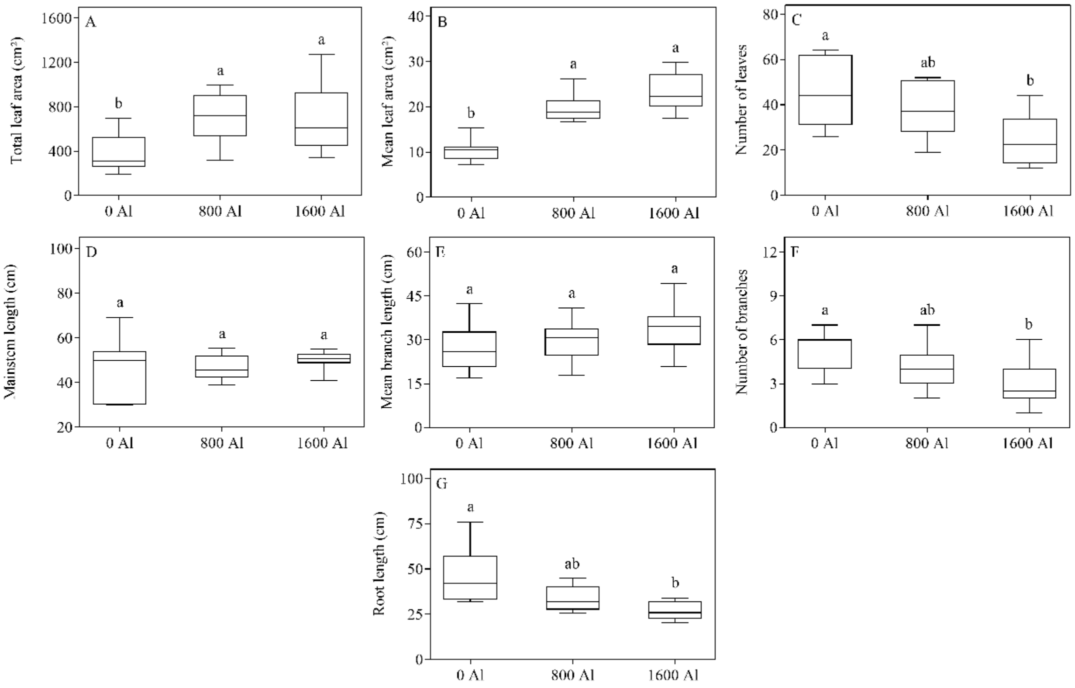

Fig. 2. Total leaf area (A), mean leaf area (B), number of leaves (C), main stem length (D), mean branch length (E), number of branches (F) and root length (G) of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. (n = 7).

The main stem branch and the mean branch size were not affected by Al presence (Fig. 2 D and E). On the other hand, the branching process was affected in the same way as the number of leaves, having the plants subjected to no Al showing a higher branching, 71% higher, when compared to plants subjected to 1600 Al (Fig. 2 F). Plants growing in the absence of Al showed root length values 72% higher ($P<0.05$) than plants growing in high Al concentration. Besides the absence of Al, the different concentrations also affected the root size (Fig. 2 G).

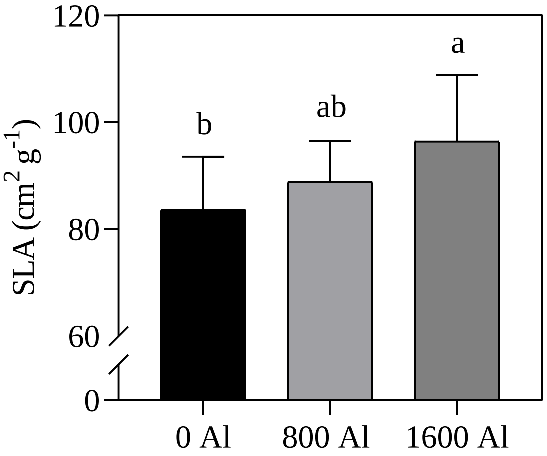

Fig. 3 Specific leaf area of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. Bars with different letters differ significantly between treatments by Tukey's test at $P \leq 0.05$. Bars indicate means values ± standard deviation (SD) with n = 7.

The results obtained for specific leaf area (SLA) were significantly different between the treatments ($P<0.05$). The mean values obtained were 83.6 for the lowest Al treatment 96.41 for the higher Al treatment as indicated in Figure 3.

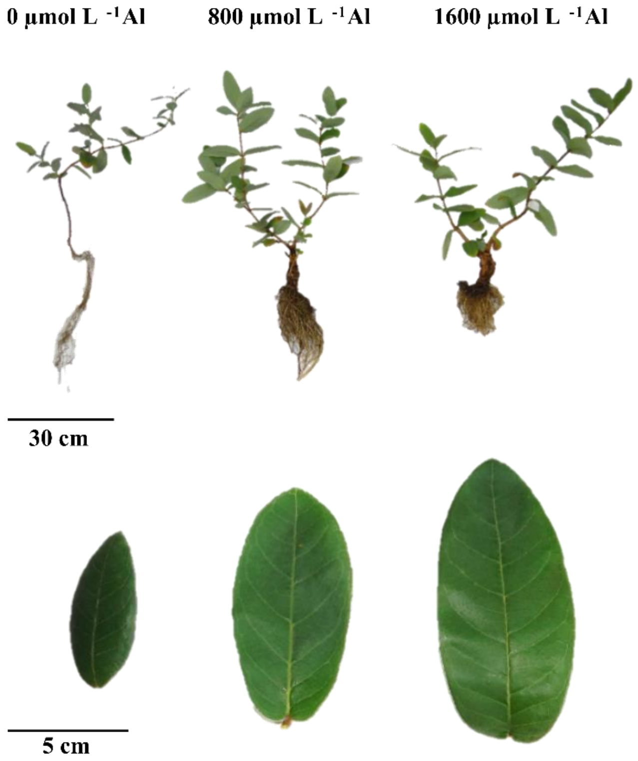

Fig. 4 Morphological comparison of full plants A and leaves B of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$.

Leaf mineral content

The evaluation of leaf mineral content showed no significant differences ($P<0.05$) for Fe, K and Mg content between treatments. Small concentrations of Al were detected (162.47 mg Kg^{-1}) even in plants not subjected to Al. The Al treatment was effective, once the 800 Al concentration resulted in an Al accumulation of 17 times higher than the plants without Al (2757 and 162.47 mg Kg^{-1}, respectively). Different from what was expected, 1600 Al treatment resulted in a lower Al concentration when compared to 800 Al growing condition (Table 1). For Cu, P, S and Ca the absence of Al resulted in higher values of leaf mineral content (the concentrations were 3 times higher for Cu, 6 times higher for P, 2 times higher for S and 47% more Ca in plants without Al). Also, the presence of Zn and Mn was significantly higher in 800 Al treatment when compared to plants not subjected to Al and those subjected to 1600 Al.

Table 1. The Al effect on the mineral concentration in leaves of *Qualea cordata*. Mineral concentrations are expressed as mg Kg^{-1} of dry weight. Values are the mean of three replicates ± SD. Different letters in each element indicate differences among treatments (*P* <0.05).

Leaf mineral content (mg/Kg)	Al (µmol L^{-1} of $AlCl_3$)		
	0	800	1600
Cu	4.9±0.6 a	1.6±0.3 b	1.5±0.5 b
Zn	18.8±2.8 a	14.2±1.7 b	20.3±4.7 a
Mn	44.6±10.5 ab	49.4±16.8 a	32.6±4.9 b
Al	162.5±71.2 c	2757.5±419.1 a	1933.8±191.9 b
Fe	180.8±40.34 a	185.0±40.6 a	208.3±25.1 a
Ca	5843.7±1567.6 a	4763.8±1261.8 ab	3321.6±543.0 b
K	9909.4±1281.2 a	9302.7±1678.5 a	9598.3±538.7 a
Mg	3243.4±463.4 a	3293.9±829.0 a	2233.4±525.9 b
P	3634.1±2621.5 a	592.7±71.3 b	504.8±112.5 b
S	2106.7±790.0 a	1005.9±146.9 b	849.3±78.7 b

Relative water content

Related to water status, there was significant differences (*P*<0.05) among treatments in relative water content (RWC) (Fig. 5). The mean values of RWC were 80.3 % for 0 Al, 84.29 % for 800 Al and 85.72% for 1600 Al treatment plants.

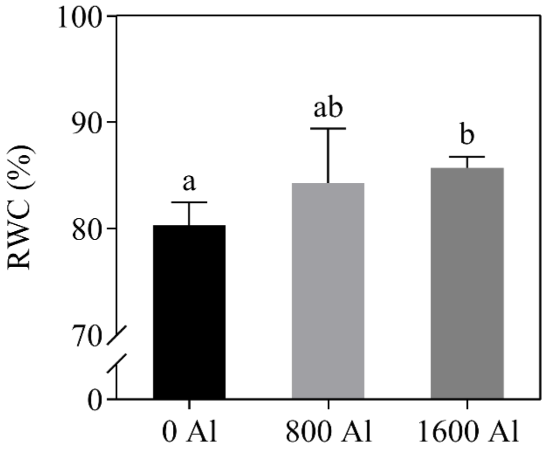

Fig 5. Relative water content (RWC) of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. Bars represented with different letters differ significantly among treatments by Tukey's test at $P \leq 0.05$. Bars indicate mean values ± standard deviation with n=5.

Discussion

Lack of Al impairs biomass accumulation and affect water relations

As expected for Al-accumulator species, *Qualea cordata* showed greater biomass accumulation when subjected to Al (Fig. 1). A positive correlation between Al accumulation and biomass accumulation was already identified for the same species by Alvim et al. (2017). The authors used soil deacidification to reduce the availability of Al, resulting in lower Al-accumulation and consequent nearly half of the total dry biomass. In our study changing the Al availability induced a similar response with ~138% increase in total dry biomass in plants growing under high Al concentrations. This positive correlation with Al and biomass accumulation seems to be typical response for the most well studied Al-accumulator species like *Camellia sinensis* L. (Hajiboland et al. 2013) and *Melastoma malabathricum L.*

(Watanabe and Osaki 2001a). For most plants Al is toxic and causes great damage to plant growth and development, especially for roots (Bojórquez-Quintal et al. 2017a) conversely, for Al-accumulator species the presence of Al seems to be essential for root growth and development (Sun et al. 2020a). In our study the lack of aluminum, and consequently lower stimulus for root growth, seems to result in prejudice to plant-water relations, being expressed by lower RWC. Lower RWC, a water drought like response might also be the responsible for the low values of SLA observed for plants under 0Al treatment (Kalapos et al. 1996). Even exhibiting increases in root biomass under high levels of Al, this data should be interpretated as a stimulus to growth and development but we should not disregard the possible toxic effects imposed by Al in other metabolic pathways, once we also observed reductions in root length, a toxicity indicative and a known strategy for root stress avoidance (Poschenrieder et al. 2008; Hodge et al. 2009; Kochian et al. 2015).

Allometric changes implications

Allometric changes that took place through ontogeny in the different Al concentrations might be an important indicative to better understand how this element affected the growth of these accumulator plants (Farnsworth 2004; Weiner 2004). Auxins are among the most studied class of phytohormone and some of the functions attributed to them are apical dominance and cell expansion (Kebrom 2017; Du et al. 2020). It is a fact that auxin produced in young leaves and shoot apex are transported basipetally and results in inhibition of auxiliary bud outgrowth (Ljung et al. 2001). The lower number of branches in plants cultivated under high Al concentrations, when compared to plants without Al exposure, might indicate a possibility of Al inducing an increase in auxin levels in buds and young leaves. Different from Al-sensitive plants, Al-accumulator species which can tolerate high Al levels are able to benefit from those higher auxin levels under long-term exposure. Increases in auxin levels in response to Al toxicity were already identified and usually result in cell wall modification and/or regulation of organic acid release (Kopittke 2016).

Leaf mineral content, benefits of Al vs nutrient toxicity

Like most Vochysiaceae from Brazilian Cerrado, the native species *Qualea cordata* shows Al accumulator behavior (Haridasan 1982; de Andrade et al. 2011; de Souza et al. 2020). Therefore, it is expected in *Qualea cordata* that the presence of Al in hydroponic solution resulted in significant biomass and growth improvement. Our results were similar to those obtained by Alvim et al. (2017) with the same species, but with soil deacidification instead of Al addition. The mechanisms behind the enhanced Al induced plant growth are not yet fully understood (Bojórquez-Quintal et al. 2017), but are sometimes attributed to H^+ toxicity amelioration (Kinraide 1993; Llugany et al. 1995) and improvement in P nutrition (Osaki et al. 1997; Watanabe and Osaki 2001b; Bojórquez-Quintal et al. 2017). Both explanations for Al growth improvement do not seem to be suitable for *Q. cordata*, once the H^+ amelioration does not appear to be related to native acid soils species (Osaki et al. 1997) and results did not show any improvement in P leaf uptake, and contrastingly, there was a significant reduction in P uptake in the presence of Al (Table 1). The combination of low leaf P and Ca concentration in the presence of Al seems to be beneficial for improving growth. Species of some Proteaceae from South-western Australia P-impoverished soils showed high levels of P-toxicity even when grown under low P concentrations. This toxicity is attributed to lack of a P downregulation mechanism (Hawkins et al. 2008; De Campos et al. 2013; Pereira et al. 2018; Hayes et al. 2019). In general, Cerrado soils are not as P-impoverished as those of South-western Australia, but the high iron and Al-saturation usually cause significant reductions in the P available in these soils (Furley and Ratter 1988). Evaluating leaf mineral content from diverse native Cerrado Al-accumulator species, Haridasan (1982) found the highest value of leaf P accumulation of 1,750 mg Kg^{-1} (*Vochysia thyrsoidea*) and lowest values of 650 mg Kg^{-1} (*Vochysia elliptica*). Even the highest value found for native Vochysiaceae is half of the mean values found for *Q. cordata* growing without Al. This difference between native plants and those cultivated without Al might indicate a possible P-toxicity.

Besides P, Ca also plays an important role in Vochysiaceae distribution. Calcifuge behavior is attributed to some native plants from acid soils with Ca sensitivity. For example, the native Cerrado species *Vochysia tucanorum* is not able to survive in calcareous soils due to its extreme Ca sensitivity (de Souza et al. 2017). This means that the presence of Al plays a major role in the contrasting biomass accumulation pattern and growth improvement among treatments. Despite that, we cannot disregard that the high P and Ca concentrations in leaf tissue might also be an indicator of another stress source. Those relations need to be further investigated, especially because there is no well-defined pattern threshold of how much Ca or P those species can endure.

Conclusion

Aluminum availability significantly improved growth, strengthening the hypothesis of the beneficial effects of Al for accumulator species. The lack of aluminum clearly impaired biomass accumulation, affecting directly root growth and resulting in imbalances in the soil-water-plant relationship (diminished relative water content). Also, the higher levels of Ca and P detected in 0AL treatment might indicate possible toxicity of those elements, once both elements are usually found in low availability in native Cerrado soils.

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CHAPTER 2 - Can the Al-accumulator trait reduce the capacity of dealing with multiple environment stressors?

Felipe Della Torre[1]; Ana Kelly Gomes de Oliveira[1]; Tiago Vilas Boas da Silva[1]; Alexandre Aparecido Duarte[1]; Cristiane Jovelina Da-Silva[2]; Joni Esrom Lima[1]; Marcel Giovanni Costa França[1]

[1] Department of Botany, Federal University of Minas Gerais, Av Antonio Carlos 6627 CP 486. Belo Horizonte, Minas Gerais, 31270-901, Brazil

[2] Department of Botany, Federal University of Pelotas, Capão do Leão 96160-000, Brazil

E-mail: felipedtorre@gmail.com, marcel@icb.ufmg.br

Abstract

High temperatures and irradiances, low relative air humidity, soil acidity highly Al saturated and poor in soil nutrient availability, are among the roughest hindrances imposed by the Brazilian Cerrado environment to plants. Al-accumulator species are capable to survive with all those abiotic stressors while accumulates Al inside its tissues without apparent damage. Classic studies with Cerrado Al-accumulator species highlighted the importance of studying these plants and its intriguing capacity to deal with the metal. We focused on understanding the possible effects of the accumulation of Al on the photosynthesis and the capacity of those plants to resist to high temperatures and light while dealing with Al toxicity. In our work high levels of Al was associated with higher photosynthetic rates and high level of ROS, antioxidant enzymes and MDA. Although Al have affected assimilation rates, no differences in thermotolerance or photoinhibition were detected, meaning that this Al-accumulator species is able to manage multiple stressors without losing its competitive fitness.

Keywords: Photoinhibition, thermotolerance, photosynthesis, chlorophyll fluorescence and metal accumulation.

Introduction

Amazingly fitted to its natural environment, the Cerrado plants deal with a wide range of edaphoclimatic extremes (Oliveira and Marquis 2002). High temperatures and irradiances, low relative air humidity, especially during the dry season, concomitant with the acid and nutrient-poor soils with high exchangeable aluminum levels, are among the roughest hindrances imposed by this environment (Haridasan 2008). The Al toxicity of Cerrado soils impose to those organisms a mandatory need of having an Al exclusion or tolerance mechanism (Kochian et al. 2015). In nature the balance between stress and adaptation results in a rich flora with a variety of strategies to deal with all these abiotic stressors (Laliberté et al. 2014). The Al-accumulator trait seems the most fitted, and is very representative in Cerrado phytophysiognomy, especially in Vochysiaceae, Rubiaceae and Melastomataceae species (Haridasan 1982; Jansen et al. 2002b, a). Classic studies with Cerrado Al-accumulator species highlighted the importance of studying these plants and it's intriguing capacity to deal with the metal (Haridasan 1982; Haridasan and De Araújo 1988) without apparent damage (de Andrade et al. 2011). More recently some effort has been done to unravel the mysteries of the Al-tolerance and beneficial effects of the metal to Al-accumulator species (Alvim et al. 2017; de Souza et al. 2017; Cury et al. 2019; de Castro et al. 2022). But facing the highly Al-saturating soils is not the only abiotic stressor that challenge the Al-accumulator species.

The same energy that drives photosynthesis can also cause great harm to the photosynthetic apparatus depending on its amount and one's capacity to dissipate this excess energy (Barber and Andersson 1992). The limits of light intensity an organism can handle are a combination of its internal mechanisms and the environmental conditions in which it grew in (Oquist et al. 1992). When the excess light causes more damage to the photosynthetic apparatus than it is capable of repairing (photosystem II D1 protein) it is considered a state of photoinhibition (Nishiyama et al. 2006; Takahashi and Murata 2008). Depending on the duration and the intensity of the photoinhibition it can be referred to as dynamic (fast recovery; Teixeira et al. 2015) or chronic (long-lasting; Long and Humphries 1994; Kitao et al. 2018). The techniques used to measure the occurrence of photoinhibition are based on measurements of quantum yield using chlorophyl fluorescence and it can be associated with light curves as a great tool to help understanding the limits of plants optimal and excess light (Lobo et al. 2013). When under excess light, plants can also experience heat stress due to the conversion of excess energy on leaves (Mittler 2006).

High temperatures can have an immense effect on plant physiology, causing irreversible damages (Allakhverdiev et al. 2008), mainly on the photosynthetic activity, one of the most heat sensitive cell function (Berry and Bjorkman 1980; Yordanov 1986). Heat can harm the thylakoid membrane integrity and the photosynthetic machinery, including CO_2 assimilation system, PSII and oxygen evolution complex (Berry and Bjorkman 1980; Gounaris et al. 1984; Mamedov et al. 1993; Havaux and Tardy 1996; Semenova 2004; Sharkey 2005; Allakhverdiev et al. 2008). The tolerance of the PSII to thermal stress can be assessed by chlorophyl fluorescence analyses of the maximum quantum yield (Fv/Fm) subjected to different temperatures reveling one’s fragility or resistance to heat (Godoy et al. 2011; Chaves et al. 2015).

The capacity to induce the production of reactive oxygen species (ROS) is a common ground between all abiotic stressors. When under extreme conditions, plants usually produce an excessive amount of ROS (superoxide radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, peroxy radical and alkoxyl radicals) (Hasanuzzaman 2019), which are capable of causing oxidation in biomolecules likewise lipids, carbohydrates, proteins and others. These damages can be so intense and result in plant death (Hasanuzzaman et al. 2018). In order to avoid death, plants have enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, peroxidase and others) a non-enzymatic antioxidants (vitamins, flavonoids, stilbenes and carotenoids) (Hasanuzzaman et al. 2020b). Quantifying both the products and the antioxidants can help us to understand the intensity of stress plants are going through (da-Silva et al. 2018).

For this work the Al-accumulator species *Q. cordata* was chosen because it was already characterized as being favored by the presence of Al (Alvim *et al.* 2017; Della Torre et al. 2022, Unpublished Manuscript) and it is a very frequent species in the Cerrado phytophysiognomy. In order to contribute to the understanding of the ecophysiology of those plants, especially under harsh environmental conditions two main questions motivated this work: 1) How photosynthesis and the antioxidant system is affected by absence of Al in Al-accumulator plants and 2) how the accumulation of Al affects the resistance of the PSII to high light and heat stress. Our objective was to evaluate which are the impacts of differential long-term aluminum exposure on ecophysiological traits, focused on photosynthesis, thermotolerance, oxidative stress and photoinhibition in the Al-accumulator species *Qualea cordata*. We expected that, even having an intrinsic relationship with aluminum, *Q. cordata* plants can show some physiological disadvantages due to the energetic coasts of dealing with Al.

Materials and methods

Plant material and experimental condition

Qualea cordata (Vochysiaceae) seeds, collected in august 2018 in Santana do Riacho-MG, were germinated in plastic trays containing a filter paper. After germinated the seedlings grown in hydroponic standard solution (Modified from Watanabe and Osaki 2001) under low light intensity (200 µmol photons m^{-2} . s^{-1}) and pH of 4.5 for one month until the Al treatments begin. The standard nutrient solution were composed of: 1.07 mM N (NH4NO3), 48µM P (NaH2PO4·2H2O), 0.385 mM K (K2SO4:KCl = 1:1), 0.625 mM Ca (CaCl2·2H2O), 0.41 mM Mg (MgSO4·7H2O), 17.9 µM Fe (FeSO4·7H2O), 9.1 µM Mn (MnSO4·4H2O), 46.3 µM B (H3BO3), 3.1 µM Zn (ZnSO4·7H2O), 0.16µM Cu (CuSO4·5H2O), and 0.05 µM Mo ((NH4)6Mo7O24·4H2O). In September 2018 the plants were transferred to 3L vessels (two plants per vessel), constantly aerated, containing the standard nutrient solution with pH adjusted to 4.5 and supplemented with 0, 800 and 1600 µmol. L^{-1} of $AlCl_3$. The plants were grown for 7 months under greenhouse conditions and the physiological and morphological evaluations were conducted between the fifth and seventh months.

Leaf gas exchange and pigment content

Leaf gas exchanges were evaluated using an infrared gas analyzer LI6400XT Li-Cor, with a LED 6400-02B chamber. All measurements were performed in a fully expanded leaves, with no signs of damage, in plants of all treatments (n = 4 per treatment), leaf temperature was fixed to 28 ºC and the CO_2 reference kept in 400 µmol CO_2 µmol air^{-1}. The light responses curves of net CO_2 assimilation (A, µmol CO_2 m^{-2} s^{-1}) to photosynthetically active radiation (PAR, µmol photon m^{-2} s^{-1}) were performed from high to low light level intensities (2000, 1500, 1250, 1000, 800, 500, 250, 100, 50, 25, 0 µmol photon m^{-2} s^{-1}). To estimate dark respiration (R_D), light compensation point (I_{comp}), maximum net A (A_{max}), light saturation point beyond which there is no significant change in A (I_{max}), and the maximum quantum yield (Φ) the light curve response were adjusted to the best-fitted model according to Lobo et al. (2013). In order to determinate leaf and air temperature the measurements were performed between 9 to 11 am in the same leaves described above.

Leaf pigments content were obtained from three leaf discs of 0.5 cm diameter of each leaf, one leaf per plants, 6 leaves per treatment, submerged into 4 mL of dimethyl sulfoxide (DMSO) for 72 hours until complete extraction. After this, the absorbance was read in UV-spectrophotometer in wave-lengths of 480, 649 and 665nn to determine the chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoid (Car) contents, according to Wellburn (1994).

Thermotolerance

Before the effective heat tolerance evaluation, the plants were adapted to dark for 30 min at 25 ℃ and the maximal photochemical efficiency (Fv/Fm) accessed using a chlorophyll fluorometer (PAM-2500, Walz, Effeltrich, Germany). One leaf segments of each plant (approximately 2 cm^2) of each treatment (n = 6 per treatment) were subjected to increases in temperature from 28 to 60 ℃ using a thermostatic bath. At each 2 ℃ of temperature variation, the fragments were acclimated for 3 minutes and the Fv/Fm was measured. The decrease in Fv/Fm related to heat tolerance was fitted in a non-linear regression (Sigmoidal) and the values of 15% (T_{15}) and 50% (T_{50}) of Fv/Fm reduction were calculated. This evaluation was performed as described by Chaves et al. (2015).

Levels of reactive oxygen species, lipid peroxidation and antioxidant enzymes activity

Approximately 0.2 g of leaf material (from 3 different plant for each treatment) were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 13,000 × *g* at 4ºC for 20 min. The oxygen peroxide (H_2O_2) amount was determined in leaves as described by Velikova et al. (2000). Aliquots of 300 µL of supernatant were added to the reaction medium containing 10 mM phosphate buffer (pH 7.0) and 1 M potassium iodide. Samples were incubated at 30ºC for 30 min, and the absorbance was determined at 390 nm. The H_2O_2 concentrations were estimated based on a calibration curve, and the concentration of malondialdehyde (MDA) was used as a parameter to evaluate lipid peroxidation. Subsequently, the supernatant was incubated in a reaction medium containing 0.5% thiobarbituric acid (w/v) and 10% TCA (w/v) at 90ºC. After 20 min, the reaction was stopped in an ice bath for 10 min. The absorbance was measured at 535 and 600 nm, and the MDA concentration was calculated as described by Cakmak and Horst (1991).

To determine reactive oxygen species ($^{\bullet}O_2^-$) levels, leaves (0.1 g) were completely homogenized in 1.8 mL of 65 mM phosphate buffer (pH 7.8). The homogenates were centrifuged at 5,000 × *g* for 10 min at 4ºC and the supernatants (0.05 mL) were incubated with 2.75 mL of 65 mM phosphate buffer containing 10 mM hydroxylamine at 25 ºC for 20 min. Then, 0.1 mL of 17 mM sulfanilamide and 0.1 mL of 7 mM α- naphthylamine were added to the mixture. After 20 min, samples were analyzed at 530 nm and $^{\bullet}O_2^-$ levels were determined from a calibration curve prepared using sodium nitrite ($NaNO_2$; Li et al. 2010).

The crude enzyme extract was prepared by homogenizing leaves (0.2 g) with 100 mM phosphate buffer (pH 7.8) containing 100 µM EDTA, 10 mM ascorbic acid and 10% (w:w) polyvinylpolypyrrolidone. Homogenates were centrifuged at 13,000 × *g* at 4ºC for 20 min, and the supernatant was collected for the analysis of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The SOD (EC 1.15.1.1) activity was measured by adopting the method of nitroblue tetrazolium (NBT) photoreduction, according to Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. The CAT (EC 1.11.1.6) activity was determined by incubating leaf extracts with a 100 mM phosphate buffer (pH 7.0) and 12.5 mM H_2O_2 (Azevedo et al. 1998). The CAT activity was estimated using the molar extinction coefficient (ε) of 36 M^{-1} cm^{-1} from measurements of H_2O_2 degradation at 240 nm. APX (EC 1.11.1.11) activity was assayed in a medium with 37.5 mM phosphate buffer (pH 7.0), 0.25 mM ascorbic acid and 5 mM H_2O_2. The rate of ascorbic acid oxidation was monitored at 290 nm and the quantification carried out using ε equal to 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981).

Resistance of the Photosystem II to high light

To evaluate if the differential Al supplementation influences the resistance of the photosystem II to high light intensities, we used two different experimental setups. The first setup was performed evaluating diurnal fluctuation of the maximal photochemical efficiency (Fv/Fm) using a chlorophyll fluorometer PAM-2500, Walz, Effeltrich, Germany, during a typical sunny day. Evaluations were performed from predawn to 5 pm, every 2 h, according to He et al. (1996). For the second setup, leaf fragments samples of approximately 4 cm^2, from each treatment (n = 7) were subjected to 2000 $\mu mol\ m^{-2}\ s^{-1}$ of high intensity LED artificial light. At every 5 min of high light exposure, the fragments were acclimated to dark for 20 min and the Fv/Fm measured. After 20 min of exposure, time exposure was increased, according to the light response until the Fv/Fm reached values around 0.55.

Statistical analyses

A completely randomized design was used for all the experiments, and each experimental unit was composed of a *Qualea cordata* plant. The data was previously evaluated using the Brown-Forsythe Test for variance homogeneity and the comparison between means was performed using ANOVA followed by Tukey test at 5% of significance. Statistical analyses and figures were done using GraphPad Prism software, version 7.00 (San Diego, CA; www.graphpad.com).

Results

Temperature and humidity

During a typical sunny week, the plants of *Qualea cordata* growing in hydroponics were subjected to low and high mean temperatures of 21.6 °C and 41.6 °C respectively. The lowest and highest mean humidity values obtained in the same week were 34.29% and 75.22% respectively (Figure 1).

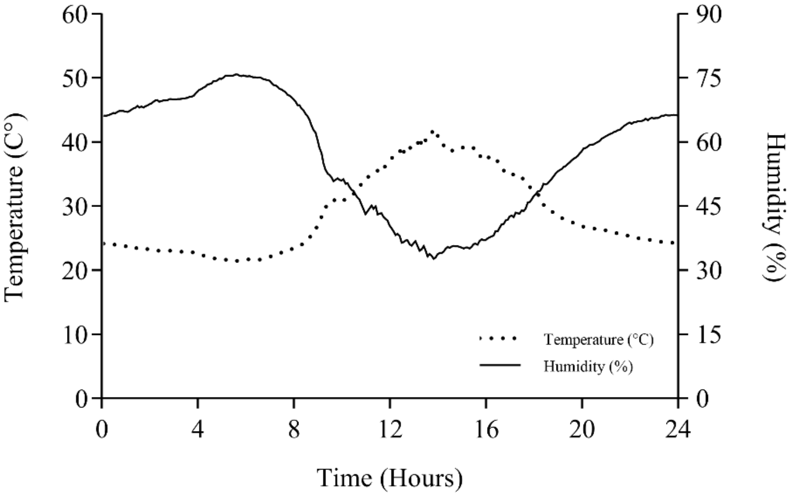

Figure 1. Daily mean temperature and humidity during a typical sunny week in the greenhouse where plants of *Qualea cordata* grown.

The light curve response in Figure 2 A showed a clear positive relation between CO_2 assimilation and the presence of aluminum. Figure 2 B shows the relationship between stomatal conductance at 1000 µmol (photons) m^{-2} s^{-1} (g_{s1000}) and the aluminum for these plants. Stomatal conductance (g_{s1000})was 0.046 in plants grown without aluminum, 0.080 for 800 Al and 0.117 for 1600 Al plants. Resulting in a significant increase ($P < 0.05$) of about 156% from 0 Al to 1600 Al plants. The same pattern of increase observed in g_{s1000} was observed in Maximum assimilation rates (A_{max}) (Figure 2 C) and light saturation point (I_{max}) (Figure 2 D) with significant increases of 73% and 88% from 0 AL to 1600 Al treatments, respectively. Light compensation point (I_{comp}) and dark respiration (R_D) were not affected by the presence of aluminum.

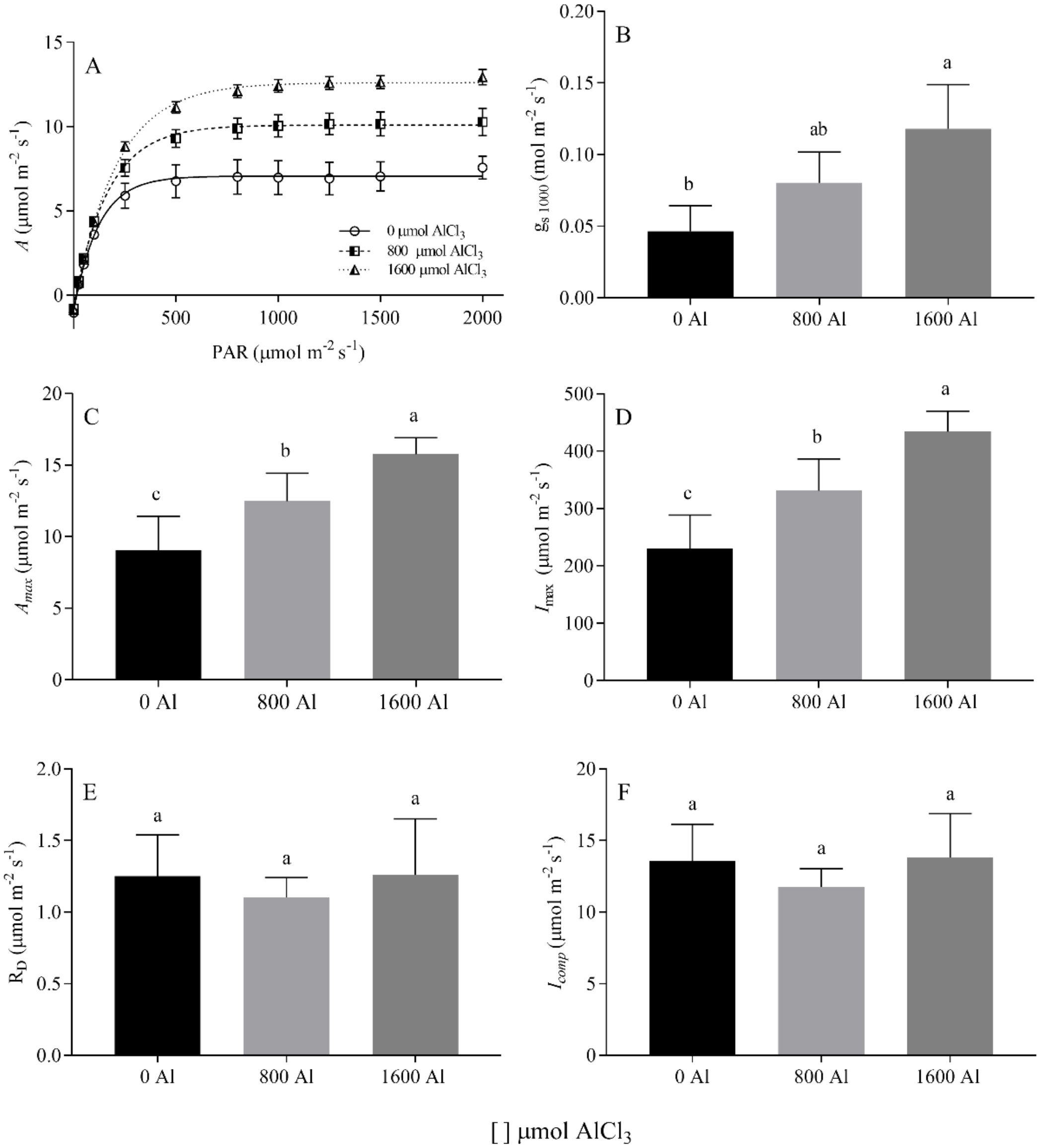

Figure 2. Light response curves variables of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 μmol. L^{-1} of $AlCl_3$. **(A)** - Light curves, **(B)** – g_{s1000} = stomatal conductance at 1000 μmol (photons) m^{-2} s^{-1}, **(C)** - A_{max} = maximum assimilation rate, **(D)** - I_{max} = light saturation

point beyond which there is no significant change in net photosynthesis, **(E)** – R_D = dark respiration, **(F)** - I_{comp} – light compensation point. Bars represented with different letters differ significantly among treatments by Tukey's test at $P \leq 0.05$. Bars indicate means values ± standard deviation with n=4.

Under grown condition plants showed significant differences in Δ temperature leaf/air as a result of lower transpiration, leaf temperature of plants growing under no aluminum condition was 0.6℃ hotter than the air temperature, while plants from high aluminum treatment (1600 Al) showed only 0.3℃ of difference (Figure 3).

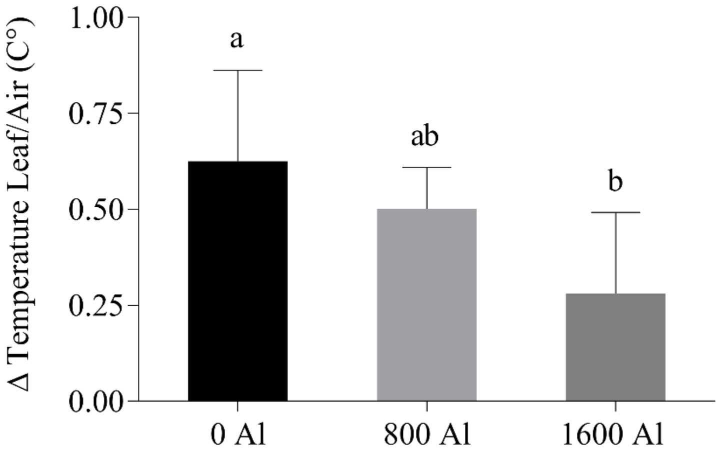

Figure 3. Delta leaf temperature, determined in situ, of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol. L^{-1} of $AlCl_3$. Bars Represented with different letters differ significantly among treatments by Tukey's test at $P \leq 0.05$. Bars indicate means values ± standard deviation with n=5.

Pigment content was affected by aluminum supplementation as expressed in Figure 4. Chlorophyll a and total chlorophyll did not show significant differences between treatments, reaching the highest values of 175.44 µg.g^{-1} FW and 241.148 µg.g^{-1} FW respectively, both from 0 Al treatment plants. Chlorophyll b and carotenoids were detected in higher content for plants cultivated without aluminum. Values of chlorophyll b were 37% higher in plants of 0 Al treatment when compared to 1600 Al plants (Figure 4) B. Carotenoids showed a similar response, 24% higher values in 0 Al treatment plants.

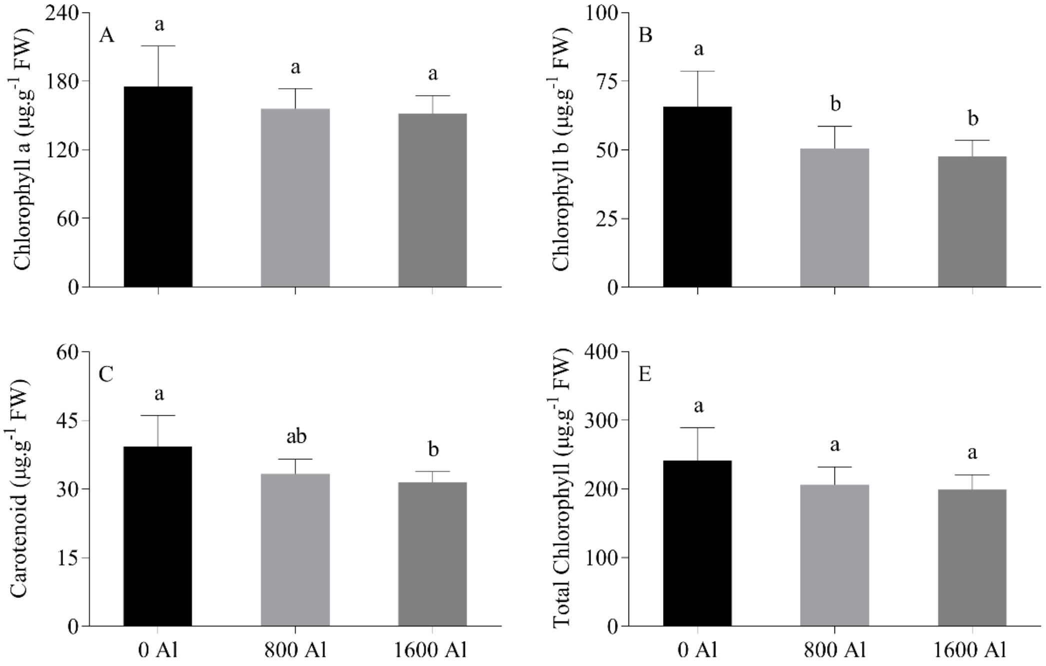

Figure 4. Pigment content of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol. L^{-1} of $AlCl_3$. (A) – chlorophyll a, (B) – chlorophyll b, (C) carotenoids, (D) – Total chlorophyll. Bars Represented with different letters differ significantly among treatments by Tukey's test at $P \leq 0.05$. Bars indicate means values ± standard deviation with n=6.

The resistance of photosystem II to temperature increasing was evaluated and is expressed in figure 5. No significant differences ($P < 0.05$), in photosystem II resistance, were detected between aluminum treatments. Reductions of 15% in Fv/Fm (T_{15}) was observed at temperatures as high as 51.5 °C for 800 Al treatment plants. To reduce 50% of Fv/Fm (T_{50}) was necessary 54.9 °C (Figure 11 B) for the same treatment.

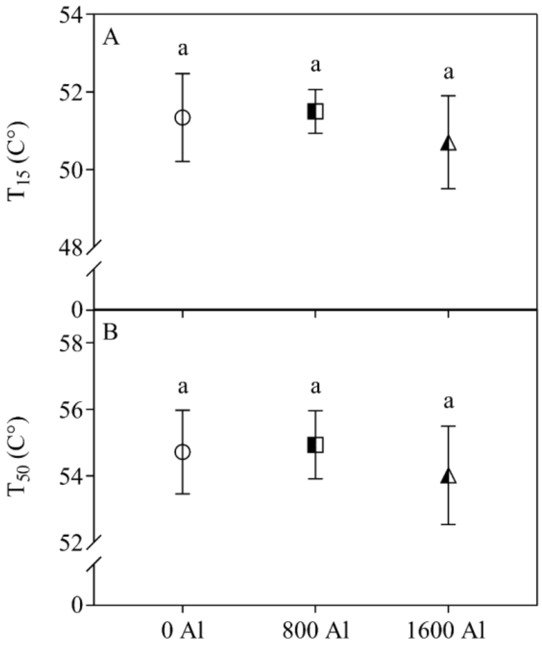

Figure 5. Thermotolerance of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 μmol. L^{-1} of AlCl3. (A) – T_{15} = temperature resulting in 15% reduction of Fv/Fm, (B) – T_{50} = temperature resulting in 50% reduction of Fv/Fm. Bars Represented with different letters differ significantly among treatments by Tukey's test at $P \leq 0.05$. Bars indicate means values ± standard deviation with n=5.

The level of reactive oxygen species (ROS), lipid peroxidation and antioxidant enzymes activity were significantly increased in response to the increase in aluminum concentration. Considered the first dangerous ROS compound superoxide anion (O_2^-) are formed in any organelle where an electron transport chain is present, for example, mitochondria and chloroplast. The concentration of O_2^- in plants subjected to higher level of aluminum was two times the amount found in plants subjected to 0 Al (2.42 for 0 Al and 4.84 µmol g^{-1} FW for 1600 Al plants). The superoxide dismutase (SOD) activity is the first line of defense to detoxify higher levels of superoxide and was also significantly higher in plants subjected to higher levels of aluminum (5.11 for 0 Al, 7.86 for 800 Al and 8.77 U min^{-1} mg^{-1} prot. for 1600 Al). The levels of hydrogen peroxide and catalase activity obtained for plants subjected to 0, 800 and 1600 were 13.26, 17.85 19.36 µmol g^{-1} FW and 101.98, 171.46, 245.83

μmol min^{-1} mg^{-1} prot, respectively. Ascorbate peroxidase (APX) is an important antioxidant to reduce hydrogen peroxide (H_2O_2) to water detoxifying cell from toxic levels of this ROS. APX was also increased in response to increase in aluminum reaching levels of 0.55 for 0 Al and 2.28 for 1600 Al. The level of peroxidized lipids (MDA) also increased in response to aluminum with values of 24.33, 42.29 and 58.52 nmol g^{-1} FW respectively for 0, 800 and 1600 Al.

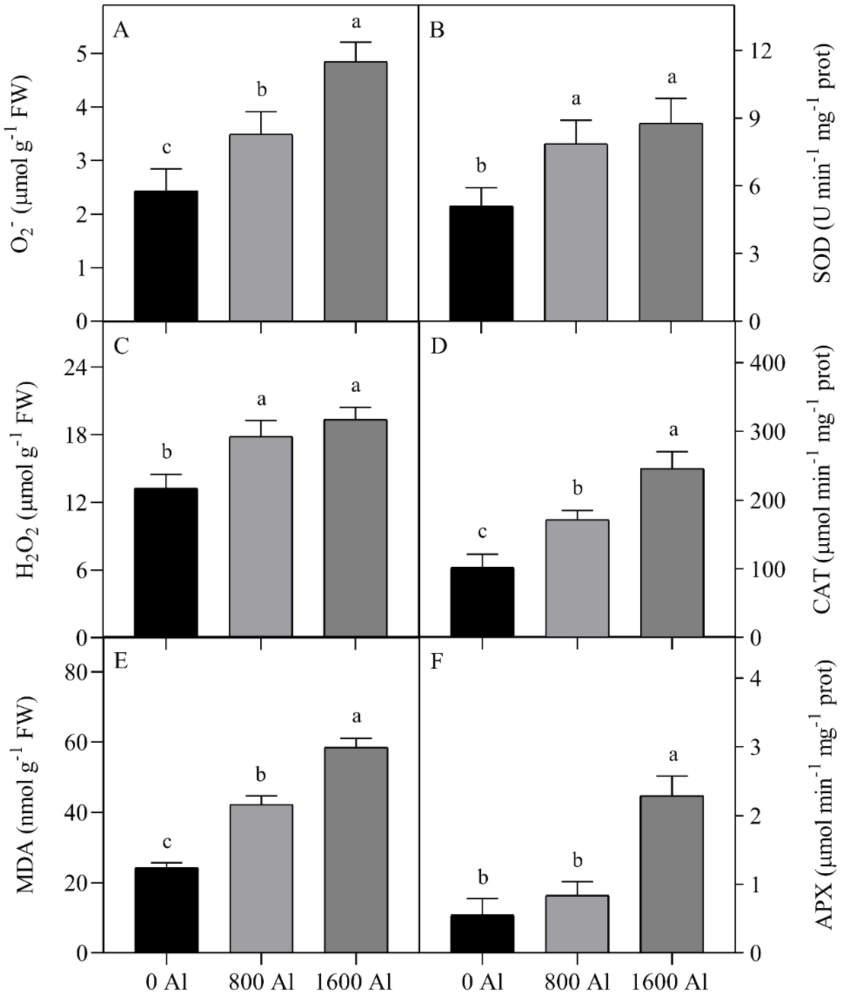

Figure 6. Reactive oxygen species (ROS), lipid peroxidation and antioxidant enzymes activity of *Qualea cordata* plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol L^{-1} of $AlCl_3$. (A) – O^{2-} = superoxide anion, (B) – SOD = superoxide dismutase activity, (C) - H_2O_2 = hydrogen peroxide, (D) – CAT = catalase activity, (E) – MDA = peroxidized lipids, and (F) – APX = ascorbate peroxidase activity. Bars represented with

different letters differ significantly among treatments by Tukey's test at P ≤ 0.05. Bars indicate mean values ± standard deviation with n=5.

Photoinhibitory responses were measured in all treatments (0, 800 and 1600 Al) in both experimental setups (Natural and artificial lights). No significant differences ($P<0.05$) were observed between treatments in neither of the experimental setups (Figure 7 A). The lower value of Fv/Fm observed under natural condition was 0,683 in plants from 1600 Al treatment at 13:00 hours (Figure 7 A). Using artificial high-intensity lights, all treatments reduced their Fv/Fm values to about 0,6 after 90 min of exposure (Figure 7 B).

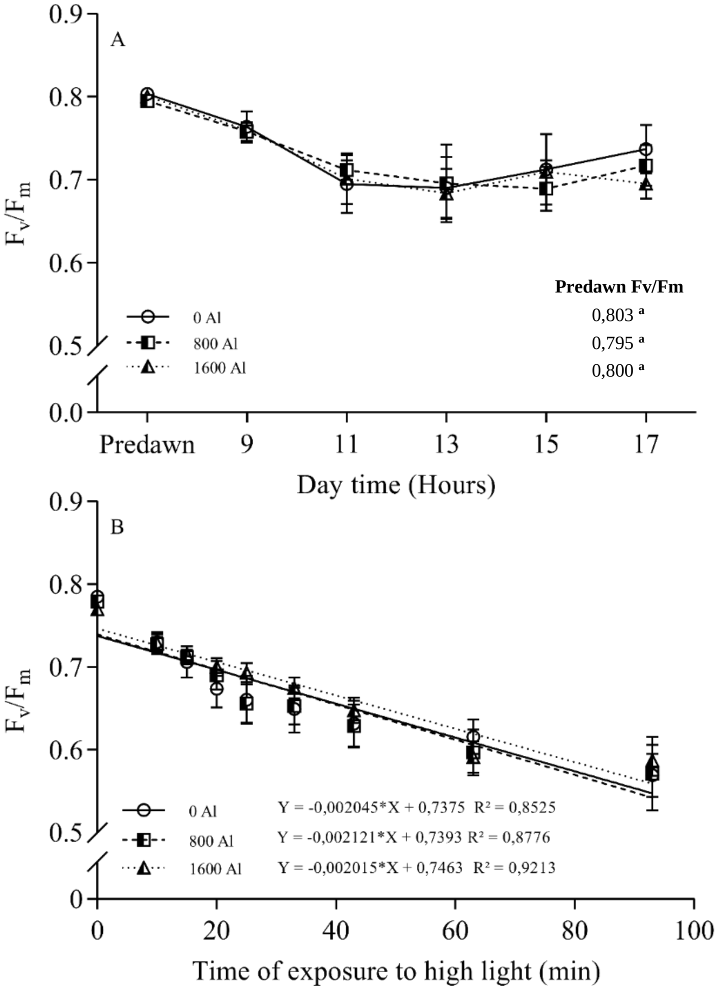

Figure 7. Photoinhibition of photosystem II in Qualea cordata plants after 7 months growing in hydroponic solution supplemented with 0, 800 and 1600 µmol. L^{-1} of AlCl3. (A) – Photoinhibition of the photosystem II by natural environmental conditions, (B) – Photoinhibition of the photosystem II by high-intensity artificial light induction. Bars Represented with different letters differ significantly among treatments by Tukey’s test at P ≤ 0.05. Bars indicate means values ± standard error with n = 5 (A) and n = 7 (B).

Discussion

The positive effects in growth and biomass accumulation in response to Al were already observed for *Q. cordata* both in soil and hydroponics experimentation (Alvim *et al.* 2017; Della Torre et al. 2022, Unpublished Manuscript). Those increases in growth and biomass accumulation were mostly assigned to the presence of the element itself mostly due to its effects in root stimulation in Al-accumulator species (Sun et al. 2020b), which consequently affect plant-water relations. The present work was able to identify that Al-root stimulation influenced positively the rates of stomatal conductance (g_s) and assimilation rates (A). Very little is known about the effects of Al in photosynthesis of Brazilian native Cerrado Al-accumulator species (de Castro et al. 2022). According to de Andrade et al. (2011) the accumulation of Al in leaf tissues of some Vochysiaceae did not cause any apparent damage to the chloroplast. Besides not causing damage to the chloroplast we were not able to detect any deleterious effects of the presence of Al in gas exchange evaluations. Although we did not locate the Al into the chloroplast, these results point to a beneficial effect of Al accumulation in the photosynthetic process, and more specifically to a stomatal conductance stimulus. An opposite response to which is usually observed for Al-sensitive plants. On those sensitive plants stomatal conductance have a reduced capacity to respond to vapor pressure deficit when exposed to high Al concentrations (Silva et al. 2012; 2018; Banhos et al. 2016). This possible positive effect of Al in stomatal conductance needs to be further investigated once the roles of beneficial Al effects are not yet clear for this species. We also highlight that the effects of Al on photosynthesis goes way beyond gas exchange evaluations and all photosynthetic pathways should be carefully and widely explored in order to fully understand its effects on photosynthesis.

When plants are under stressful condition reactive oxygen species (ROS) are a usual byproduct of oxygenic organisms, but when the amount of produced ROS is not well balanced, it can be harmful for plants (Hsu et al. 2007). In order to keep ROS levels in a not harmful range, plants can either avoid the formation of ROS or increase its scavenging system, through the increase of antioxidant enzymes, such as SOD, CAT and APX (Gill and Tuteja 2010). Together with others, already known, stressors like heavy metals, water stress, high and low temperatures, Al can increase the production of ROS and cause damages to plants (Yamamoto et al. 2003; da-Silva et al. 2018; Berni et al. 2019). For *Qualea cordata* plants the increase in Al content resulted in expressive increases in all ROS and antioxidant enzymes activity, but surprisingly no signs of damage were detected; rather the opposite, once plants subjected to higher level of Al showed growth (Della Torre et al. 2022, Unpublished Manuscript) and photosynthetic (Figure 2) improvements. As discussed by some authors, ROS production should not only be seen as a dangerous product, but should be considered a “double-edge sword” (Das and Roychoudhury 2014; Sachdev et al. 2021). For *Qualea cordata* considering the both sides of the blade, one can be the beneficial effect of increased ROS as a secondary messenger, playing a possible role in cell wall expansion, promoting leaf area increase (Kärkönen and Kuchitsu 2015). The other side of the blade are the deleterious effects of ROS seem by increases in MDA, a byproduct of membrane peroxidation (Hasanuzzaman et al. 2020a). Some have suggested that Al-accumulator species, especially those from Cerrado, are exempt from the deleterious effects of Al toxicity (de Castro et al. 2022). But our data suggests caution when making generalization. Once, even in the same plant family, species could respond very broadly, even when the overall responses seem positive. But we should also be aware that all ROS scavenging system has an energetic cost, which under limiting conditions can be detrimental for survival. Also any additional stress overlaid to that already imposed by Al, can result in very intense damages to those plants reducing greatly its competitive capacity (Das and Roychoudhury 2014).

High temperatures and excess of light are common stressful factors in natural environments and can cause great harm to plants. Especially when overlaid to other edaphic stressors like the presence of Al in the acid soils, a usual condition for Cerrado plants. Al toxicity are known to affect cell wall and plasma membrane, these modifications can result in changes in fluidity and permeability (Wagatsuma et al. 2005). Both, heat and Al toxicity can affect membrane integrity, so it was expected that plants subjected to high levels of Al were more susceptible to PSII damages under high temperature trials. But no PSII thermal tolerance differences were observed for *Q. cordata* plants.

Observing the higher levels of H_2O_2 we expected that the treatments of high aluminum would be the most susceptible to photoinhibition, mostly due to the effects of H_2O_2 in inhibiting the PSII D1 repair process (Murata et al. 2007; Takahashi and Murata 2008). But we did not detect a higher susceptibility of 1600 Al treatment to photoinhibition, in fact there was no difference between treatments (Fig. 7). Although it seems odd the photoinhibition should not be studied from only one perspective because it is the result of two separated processes, the rate of photodamage and the rate of the D1 protein repair (Nishiyama et al. 2006; Takahashi and Murata 2008). Even knowing that H2O2 inhibits PSII D1 repair rates we should also consider the dependence of ATP as a driving force in repairing the PSII, the differences in the amount of excess light between the treatments and the biochemical adjustments likewise increases in photoprotection pigments. (Choudhury and Behera 2001; Sacharz et al. 2017; Murata and Nishiyama 2018). What we observed for *Q. cordata* was a trade-off adjustment in response to the intrinsic condition it was facing, resulting in no differences in the photoinhibitory processes. The high Al treatment had the highest concentration of H_2O_2, it also had the highest CO_2 assimilation rates, resulting in more ATP for D1 repair along with slightly lower carotenoid amount due to the lower excess light faced. In contrast the 0 Al treatment have its photosynthetic rates saturated with only 53% (Fig. 2 D) of the light amount necessary to saturates the photosynthesis of 1600 Al, meaning higher surplus of light. That was enough to result in increases in carotenoid levels in 0 Al treatment.

Conclusion

The higher antioxidant enzymes level observed in plants subjected to Al was enough to reduce the oxidative stress, in a way that the PSII function was kept intact, but further investigations are needed in order to better understand the benefits and risks of constant higher levels of ROS for those plants, especially when subjected to additional environmental stress. Although Al have affected assimilation rates no differences in thermotolerance or photoinhibition were detected, meaning that this Al-accumulator species is able to manage multiple stressors without losing its competitive fitness. Also, the high MDA levels detected indicates that besides not being overall affected the Al accumulation do cause some harm to those plants, being necessary more studies to identify the extent of those internal damages.

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CHAPTER 3 - Photosynthesis and energy partitioning, different Al-accumulator species respond equally to the metal availability?

Felipe Della Torre[1]; Rowan F. Sage[2] and Marcel G. C. França[1]

[1] Department of Botany, Federal University of Minas Gerais, Av Antonio Carlos 6627 CP 486. Belo Horizonte, Minas Gerais, 31270-901, Brazil

[2]Department of Ecology and Evolutionary Biology, The University of Toronto, Toronto, ON M5S 3B2, Canada

E-mail: felipedtorre@gmail.com, marcel@icb.ufmg.br

Abstract

We investigated the influence of Al supplementation in the photosynthetic and energy partitioning in *Vochysia elliptica*, *Qualea grandiflora* and *Qualea multiflora*, three Cerrado Al-accumulator species. A/Ci curves allowed us to identify important differences in the carboxylation rates of those species. *Vochysia elliptica* showed reductions in A_{max}, V_{cmax}, J and TPU which means toxicity of Al for this species. On the other hand, the same parameters were improved by the presence of Al in *Q. grandflora* and *Q. multiflora*. These results indicate that differences in photosynthetic Al sensibility might be linked to the specific Al storage strategy differences observed for different Vochysiaceae species. Also, the energy partitioning evaluation indicates an important role of Al in reducing photoinhibition (lower ΦNF) and increasing ΦNPQ in high Al treatments, but the photoprotective role of Al seems not to be related to the differences in Al-storage strategies.

Keywords: Photoprotection, energy partitioning, gas exchange, metal accumulation, photoinhibition, photosynthesis.

Introduction

Aluminum is widely present in Earth's crust, but its only considered toxic for plants when under low pH, found as Al^{3+}, its most toxic form (von Uexküll and Mutert 1995; Horst et al. 2010). A lot is known about Al impairing growth and development due to interferences in cell walls, root tips, cellular respiration, photosynthesis, genetic processing and even absorption of essential nutrients (Kochian et al. 2015; Kopittke 2016; Bojórquez-Quintal et al. 2017b; Rahman et al. 2018). To avoid and/or cope with the metal, native plants from acid soils seems have developed two different strategies. The first used by excluder species or resistant species, are based on the use of organic acids to immobilize the Al^{3+} in to the rhizosphere not letting it entering the root system (Poschenrieder et al. 2019). The other strategy is used by tolerant species, and are based on allowing the metal to access inner tissues and neutralizing it in different internal organs, and consequently accumulating the metal (Kochian et al. 2015).

In the Cerrado, the soil properties allowed Al-accumulating strategy to be widely used, with some Al-accumulator families like Vochysiaceae, presenting high importance value index in different types of vegetation (Haridasan and De Araújo 1988; Simon and Pennington 2012). Due to its ecological importance and intrinsic relation with Al, the Vochysiaceae species are being used to better understand the possible beneficial effects of Al on the metabolism of Al-accumulator species (Alvim et al. 2017; Cury 2017; de Souza et al. 2017, 2020; Cury et al. 2019; de Castro et al. 2022). In 1982, Haridasan observed large amounts of Al storage in the species *Qualea parviflora*, *Q. grandiflora*, *Q. multiflora*, *Vochysia elliptica* and *V. thyrsoidea* without any visible deleterious effects. A similar observation was made by de Andrade (2011) with the Al-accumulator species *Q. grandiflora* and *C. major* and *V. pyramidalis*. In this work the author was able to locate the Al-accumulated inside the chloroplast with no apparent damage in *Q. grandiflora* and *C. major* while *V. pyramidalis* did not allowed the Al to reach the chloroplast. Aiming to better understand the photosynthetic effects of Al accumulation inside the chloroplast Della Torre et al. (2022a and 2022b – not published) cultivated *Q. cordata* under different levels of Al supplementation and observed increases in photosynthetic rates associated with increases in stomatal conductance (gs), and root growth. The positive relation between Al and CO_2 assimilation rates were assigned to stomatal limitations disguising the true effects of Al in the photosynthesis of Al-accumulator species. To remove the restraint imposed by stomatal limitation the responses of CO_2 uptake to intercellular CO_2 mole fraction, also known as A/Ci curves, can be performed and help us to better understand the specificities of the photosynthetic metabolism of Al-accumulator species (Farquhar et al. 2001; Long and Bernacchi 2003; Sharkey et al. 2007; Sharkey 2016). As important as carboxylation, energy partitioning evaluations should be considered in the investigating of the effects of Al in the photosynthesis of Al-accumulator species (Lazár 2015). The energy partitioning method will allow us to understand the quenching of energy into PSII, NPQ, NF and f,D (Hendrickson et al. 2005). In order to better understand the physiology of Al-accumulator species, three main questions motivated this work: 1) How the accumulation of Al affects the carboxylation responses without the interference of stomatal resistance? 2) How aluminum accumulation influence energy partitioning? 3) Are the responses in carboxylation and energy partitioning to Al the same for the al-accumulator species evaluated?

We do not expected interferences of Al in carboxylation in any of the Al-accumulator species. We might expect some negative effects in energy partitioning in response to Al, especially in ΦNF, once its related to photoinhibition and previous reports have suggested increases in ROS in Al-accumulator species. Some differences in responses between species should be expected due to its differences in Al compartmentalization.

Materials and methods

Plant material and experimental condition

Vochysia elliptica, Qualea grandiflora and Qualea multiflora (Vochysiaceae) seeds, were collected in the Brazilian Cerrado region, in the city of Jaboticatubas-MG and germinated in Petri plates containing a filter paper. After germinated the seedlings were grown in hydroponic standard solution (Modified from Watanabe and Osaki 2001) suplemented with 200 µmol L^{-1} of $AlCl_3$ in 4.5 pH until they grew enough to begging the treatments. The standard nutrient solution was composed of 1.07 mM N (NH_4NO_3), 48µM P ($NaH_2PO_4 \cdot 2H_2O$), 0.385 mM K (K_2SO_4:KCl = 1:1), 0.625 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.41 mM Mg ($MgSO_4 \cdot 7H_2O$), 17.9 µM Fe ($FeSO_4 \cdot 7H_2O$), 9.1 µM Mn ($MnSO_4 \cdot 4H_2O$), 46.3 µM B (H_3BO_3), 3.1 µM Zn ($ZnSO_4 \cdot 7H_2O$), 0.16µM Cu ($CuSO_4 \cdot 5H_2O$), and 0.05 µM Mo ($(NH_4)6Mo_7O_{24} \cdot 4H_2O$). After five months, plants were transferred to 5L pots – two plants per pot - constantly aerated, containing the standard nutrient solution with pH adjusted to 4.5 and supplemented with 0, 400 and 800 µmol L^{-1} of $AlCl_3$. After 60 days under this condition, it was observed that species respond differently to Al concentrations, so the concentrations were re-adjusted for each species. The new Al concentrations was 0, 400 and 800 µmol L^{-1} of $AlCl_3$ for *V. elliptica*, 0, 100 and 200 µmol L^{-1} of $AlCl_3$ for *Q. grandiflora* and 0, 300 and 600 µmol L^{-1} of $AlCl_3$ for *Q. multiflora*. The hydroponic solution was changed every 4 days. The plants grew under glasshouse of the Earth Science building in University of Toronto, under 14h photoperiod. The light intensity of 200 ± 50 Photosynthetic active radiation (PAR) offered by a high-pressure sodium lamps in addition to natural illuminations (1500 µmol photons m^{-2} s^{-1} in sunny days) for 4 months until the experiment was evaluated.

Leaf gas exchange, chlorophyll assay and specific leaf area

Leaf gas exchange were performed on the first or second healthy fully expanded leaves. The infrared gas analyzer (model LI-6400, LICOR Biosciences) was used for gas exchange evaluations. Responses of net CO_2 assimilations rates (A) to intercellular CO_2 concentrations were measured at leaf temperature of 30°C with light intensity of 1500 µmol m^{-2} s^{-1}. First the leaves were acclimated to the desired light intensity and then the A/Ci curves were measured using a sequence of reference CO_2 concentrations of 400, 60, 75, 90, 105, 120, 250, 400, 650, 900, 1150, 1250 and 1500 µmol mol^{-1}. Data were recorded 5 times for each step in sequential order, with an equilibration time of 90 to 180 s at each step. From the A/Ci curves was possible to estimate the values of Gamma (Γ), Maximum assimilation rates (A_{max}), carboxylation efficiency (CE), Assimilation at 400 reference CO_2 (A_{400}), Stomatal conductance at 400 reference CO_2 (g_{S400}), Vapor pressure deficit at 400 reference CO_2 (VPD_{400}), The ratio of internal to atmospheric CO_2 concentration at 400 reference CO_2 (Ci/CA_{400}), Maximum velocity of Rubisco for carboxylation (V_{cmax}), Maximum rate of electron transport (J), Maximum rate of use of triose phosphates use (TPU), Day respiration (Rd*) and mesophyll conductance (gm*) according to (Sharkey et al. 2007).

Two cm^2 leaf discs of recent, fully-mature leaves of *V. elliptica, Q. grandiflora* and *Q. multiflora* were sampled and rapidly grounded using a glass tissue homogenizer and extracted in 80% acetone. The extract was read using a Hewitt-Packard 8230 spectrophotometer at wavelength of 645 and 663 to determine concentrations of chlorophyll a, b and total (Arnon 1949). Similar leaf discs were dried in forced-air circulation oven at 60ºC and weighted for specific leaf area (SLA) determination.

Leaf chlorophyll fluorescence: energy partitioning assay

Chlorophyll *a* fluorescence emission for *V. elliptica, Q. grandiflora* and *Q. multiflora* were measured in leaf discs ~4 cm^2 using a chlorophyll fluorometer PAM-2100, Walz, Effeltrich, Germany. The discs were placed in humidified Petri plate with filter paper and all evaluations were done in the laboratory under room temperature. The discs were collected at predawn and variable (F_{vM}) and maximum (F_{mM}) fluorescence measured prior to irradiation. The discs were then subject to high irradiation (2000 µmol m^{-2} s^{-1}) for 1:30h. During irradiation, the levels of steady state (F_s), maximal (F_m’) and minimal (F_0’) levels of Chl fluorescence for light acclimated samples were measured. The F_0’ values were obtained when actinic light was off and a short-term far-red irradiation was applied. Immediately after the last measurement the samples were dark adapted for 3h until the last evaluation was made. After 3h of dark incubations the values of minimal (F_{0PI}) and maximal (F_{mPI}) post-illumination dark adapted Chl fluorescence were collected. After data collected the energy partitioning was estimated according to Hendrickson *et al. (*2005):

$(\boldsymbol{\Phi}_{f,D})$ **-** Combined quantum efficiency of fluorescence and constitutive thermal dissipation*:*

$$\frac{F_s}{F_{mPI}}\left(\frac{F_{VPI}/F_{mPI}}{F_{VPI}/F_{mM}}\right)$$

$(\boldsymbol{\Phi_{NPQ}})$ - Quantum yield of light dependent and ΔpH and xanthophyll mediated regulated thermal dissipation:

$$\left(\frac{F_s}{F_m{}'} - \frac{F_s}{F_{mPI}}\right)\left(\frac{F_{VPI}/F_{mPI}}{F_{VPI}/F_{mM}}\right)$$

$(\boldsymbol{\Phi_{PSII}})$ - Quantum yield of PS II electron flow:

$$\left(1 - \frac{F_s}{F_m{}'}\right)\left(\frac{F_{VPI}/F_{mPI}}{F_{VPI}/F_{mM}}\right)$$

$(\boldsymbol{\Phi_{NF}})$ - Quantum yield of thermal dissipation in non-functional PS II:

$$1 - \left(\frac{F_{VPI}/F_{mPI}}{F_{VPI}/F_{mM}}\right)$$

Statistical analysis

A completely randomized design was used for all the experiments, and each experimental unit was composed of a V. elliptica, Q. grandiflora or Q. multiflora plant. The data was previously evaluated using the Brown-Forsythe Test for variance homogeneity and the comparison between means was performed using ANOVA followed by Tukey test at 5% of significance or a t-test for 2 means comparison. Statistical analyses and figures were done using GraphPad Prism software, version 7.00 (San Diego, CA; www.graphpad.com). The A/Ci curves were made using GraphPad Prism software, and for the parameters estimated according to Sharkey *et al.* (2007) was made using Microsoft excel software.

Results

Carbon assimilation responses

The CO_2 assimilation rates (A) in response to reference (Ca), and consequently intercellular (Ci) carbon concentrations (A/Ci curves), allowed us to better understand the influence of Al concentration in three of the Cerrado Al-accumulator species. At the moment of the evaluations, 150 days after treatment (150 DAT), the species *Q. grandiflora* and *Q. multiflora* showed higher rates of A under high Al concentrations (Fig. 1 B and C) while *V. elliptica* assimilated more amounts of CO_2 without the presence of the metal (Fig. A).

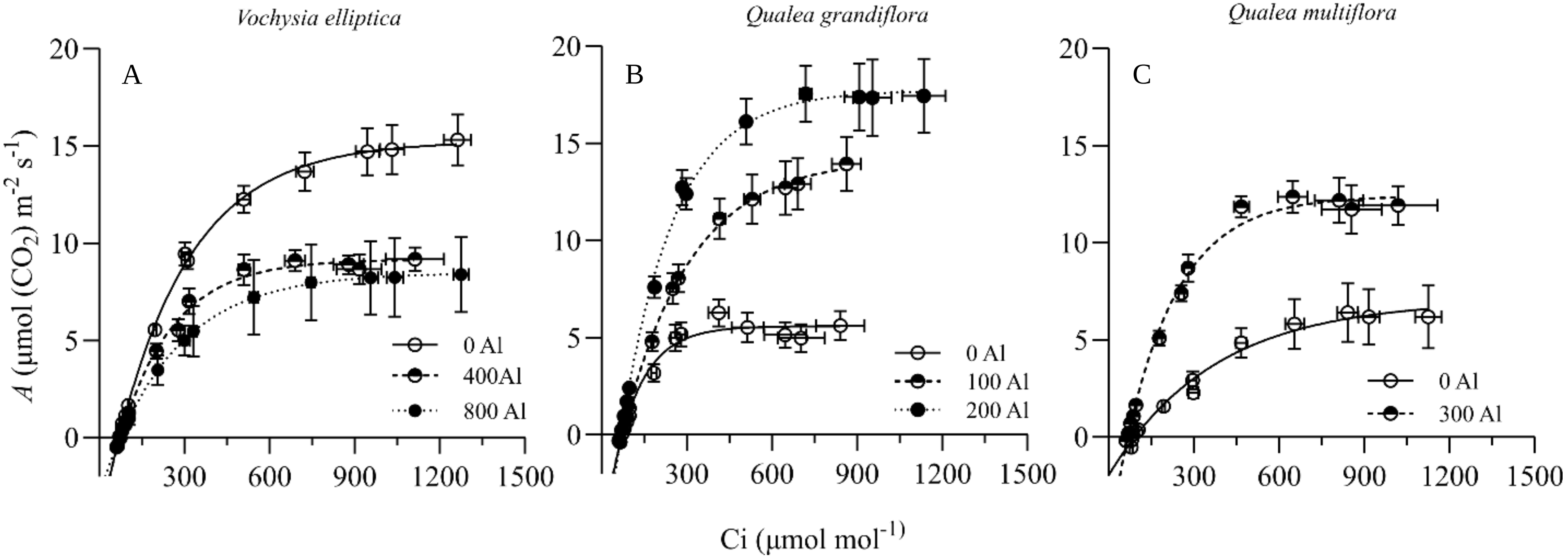

Figure 1 - A/Ci curves from leaves of *Vochysia elliptica*, *Qualea grandiflora* and *Q. multiflora* plants growing under different levels of Al concentrations. All responses in each panel were generated from the mean of 3 plants, one leaf per plant ± SE, with Ca concentrations ranging from 60 to 1500 μmol mol^{-1}. Light intensity used was 1500 μmol m^{-2} s^{-1}, and leaf temperature of 30°C.

The A/Ci curves estimated values are showed in Table 1 and reveled a more detailed analysis of the photosynthetic behavior of these plants in response to Al. Gamma (Γ**)** is usually associated with photorespiration and can indicated when the competition between O_2 and CO_2 for the enzyme rubisco is pending for one or the other substrate. Q. multiflora was the only species that showed significant differences in response to Al concentration. For this species the values of Γ under 300 Al was 73% of that obtained for 0 Al treatment plants. The maximum assimilation rate ($A_{max)}$ was also affected by the Al supplementation. For *V. elliptica* high A_{max} values were obtained for plants with no Al supplementations (15.3 μmol (CO_2) m^{-2} s^{-1}) and the lowest observed for 800 Al (8.0 μmol (CO_2) m^{-2} s^{-1}). The species from *Qualea* genus showed increases in A_{max} when supplemented with Al. The higher values obtained was 17.5 for *Q. grandiflora* under 200 Al and 12.3 for *Q. multiflora* under 300 Al. The values CE, $A_{400,}$ g_{S400}, $VPDl_{400,}$ Ci/CA_{400}, Rd and gm did not differ between treatments for V. elliptica. CE increased in response to Al supplementations for Q. grandiflora (0.034 for 0 Al, 0.049 for 100 Al and 0.072 for 200 Al) and for Q. multiflora (0.015 for 0 Al and 0.041 for 300 Al). The estimated parameter of Vcmax, J and TPU was affected by aluminum presence been those for V. elliptica negatively influenced by the presence of Al and for *Q. grandiflora* and *Q. multiflora* positively influenced by the presence of Al (Table 1). The only species that showed differences for gm was *Q. grandiflora* with 1.2 for plants growing in the absence of Al, 1.6 for 100 Al and 2.9 for 200 Al.

Table 1 - Photosynthetic parameters estimated from complete A/Ci curves, for *Vochysia elliptica*, *Qualea grandiflora* and *Q. multiflora* plants growing under different levels of Al concentrations. All parameters were generated from the mean of 3 plants, one leaf per plant. The curves used to generate the parameters was measured using Ca concentrations ranging from 60 to 1500 µmol mol^{-1}. Light intensity of 1500 µmol m^{-2} s^{-1}, and temperature of 30°C. Mean ± SE

	Vochysia elliptica			***Qualea grandiflora***			***Qualea multiflora***		
	0 Al	**400 Al**	**800 Al**	**0 Al**	**100 Al**	**200 Al**	**0 Al**	**300 Al**	**600 Al**
Γ	68.76±6.08^{A}	72.54±1.24^{A}	74.55±5.73^{A}	71.72±6.33^{A}	71.95±1.52^{A}	66.29±2.23^{A}	87.43±4.14^{A}	63.9±4.3^{B}	-
A_{max}	15.27±1.43^{A}	9.23±0.37^{AB}	8.04±1.99^{B}	5.48±0.71^{B}	14.07±2.34^{A}	17.53±1.76^{A}	6.99±1.92^{B}	12.34±0.72^{A}	-
CE	0.048±0.002^{A}	0.038±0.007^{A}	0.027±0.001^{A}	0.034±0.006^{B}	0.049±0.004^{AB}	0.072±0.008^{A}	0.015±0.002^{B}	0.041±0.002^{A}	-
A_{400}	9.12±0.48^{A}	7.082±0.73^{A}	5.23±1.34^{A}	5.11±0.63^{C}	8.08±0.67^{B}	12.29±0.81^{A}	2.91±0.51^{B}	8.42±0.60^{A}	-
g_{S400}	0.24±0.02^{A}	0.19±0.01^{A}	0.19±0.02^{A}	0.08±0.01^{B}	0.12±0.01^{B}	0.28±0.01^{A}	0.052±0.003^{B}	0.163±0.029^{A}	-
VPD_{400}	1.71±0.17^{A}	1.79±0.13^{A}	1.93±0.17^{A}	2.03±0.11^{A}	2.00±0.09^{A}	1.62±0.10^{B}	1.99±0.10^{A}	1.75±0.12^{A}	-
Ci/CA_{400}	0.80±0.02^{A}	0.81±0.02^{A}	0.85±0.03^{A}	0.70±0.03^{A}	0.69±0.02^{A}	0.78±0.01^{A}	0.74±0.03^{A}	0.73±0.03^{A}	-
V_{cmax}	97.36±5.69^{A}	68.44±4.28^{B}	52.23±5.68^{B}	59.1±7.29^{B}	84.27±9.88^{AB}	100±9.76^{A}	33.00±7.86^{B}	64.38±4.41^{A}	-
J	80.84±6.85^{A}	55.62±4.76^{AB}	46.00±8.29^{B}	45.4±3.92^{B}	79.71±9.09^{A}	95.26±7.01^{A}	37.37±7.92^{B}	67.64±4.02^{A}	-
TPU	5.95±0.49^{A}	3.76±0.14^{B}	3.30±0.62^{B}	2.34±0.25^{B}	5.31±0.59^{A}	6.55±0.55^{A}	2.49±0.51^{B}	4.47±0.36^{A}	-
Rd*	2.55±0.72^{A}	2.02±0.21^{A}	1.60±0.44^{A}	1.56±0.29^{A}	2.34±0.43^{A}	2.02±0.09^{A}	1.30±0.27^{A}	1.32±0.25^{A}	-
gm*	1.09±0.13^{A}	1.95±0.99^{A}	0.92±0.39^{A}	1.20±0.36^{B}	1.58±0.25^{AB}	2.90±0.56^{A}	1.31±1.02^{A}	1.92±0.61^{A}	-

Chlorophyll a, b and total

Under different concentrations of Al none of the species evaluated showed significant differences in chlorophyll a, b and total (Fig. 2). V. elliptica was the species that showed the highest and lowest mean values for Chl a, b and total between treatments.

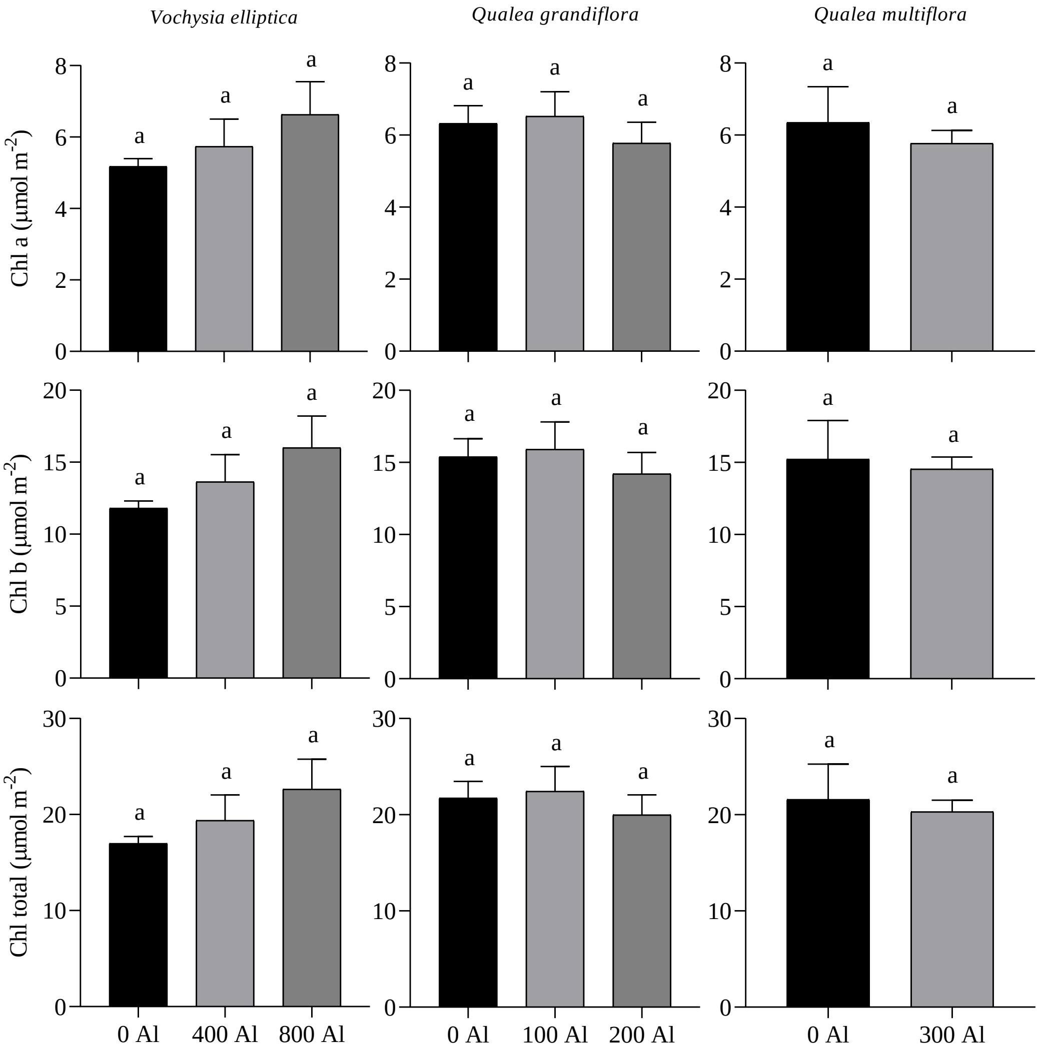

Figure 2 - Pigment content of *Vochysia elliptica, Qualea grandiflora and Q. multiflora* plants growing under different levels of Al concentrations. All parameters were generated from the

mean of 5 plants, one leaf per plant. Bars Represented with different letters differ significantly among treatments by Tukey's test at P ≤ 0.05. Bars indicate means values ± standard error.

Specific leaf area

Specific leaf area was affected by the presence of aluminum only in the species from the *Qualea* genus, both species resulted in increases in SLA when subjected to high and medium Al conditions (Fig. 3). *V. elliptica* was not affected by the presence of Al.

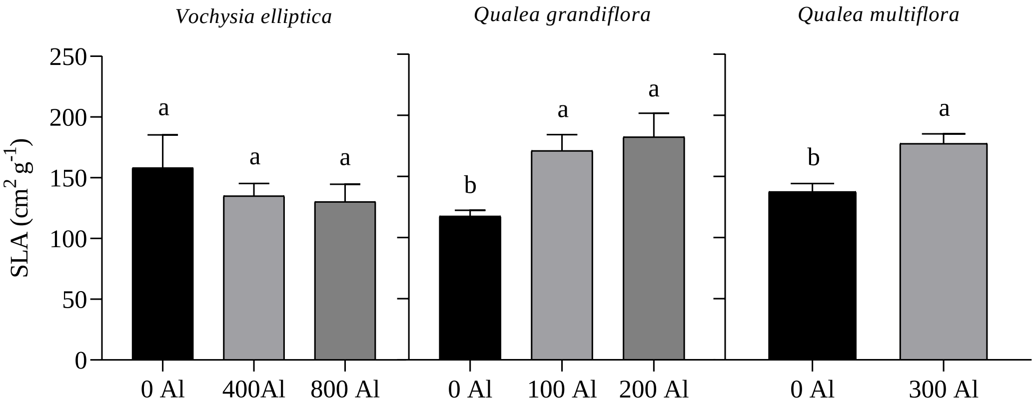

Figure 3 - Specific leaf area (SLA) of *V. elliptica*, *Q. grandiflora and Q. multiflora* plants growing under different levels of Al supplementation. All parameters were generated from the mean of 5 plants, one leaf per plant. Bars Represented with different letters differ significantly between treatments by Tukey's test at P ≤ 0.05. Bars indicate means values ± standard error.

Energy partitioning

Energy partitioning data reveal important clues in the understanding of energy destiny and influence of Al in the photosynthetic process, also in the susceptibility of those plants to photoinhibition. For all species evaluated the lack of aluminum was resulted in reductions in $\Phi_{PSII,}$ meaning lower photosynthetic capacity when in absence of the metal. Values of Φ_{NF} for *V. elliptica* (20.80 for 800 Al, 22.16 for 400 Al and 37.55 for 0Al) and *Q. grandiflora* (16.47 for 200 Al, 16.52 for 100 Al and 24.92 for 0Al) was lower in the presence of Al (Fig. 4). The higher values of Φ_{NF} in the presence of Al observed for *V. elliptica* was followed by lower values of Φ_{NPQ} meaning lower capacity of dissipate heat and consequently resulting in more excess energy, what was not observed for the other species. No differences in $\Phi_{f,D}$ was observed for any of the species.

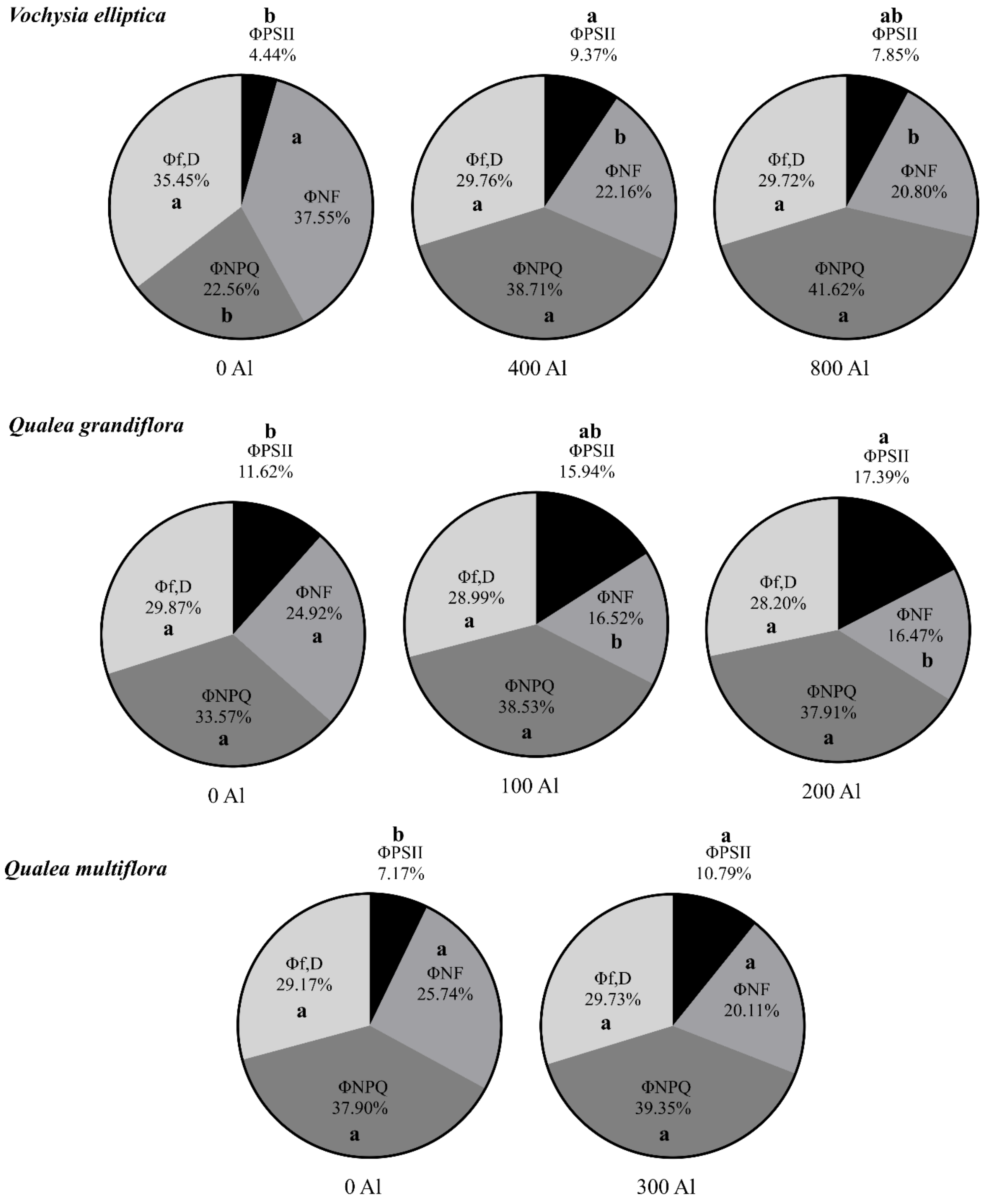

Figure 4 - Energy partitioning of *Vochysia elliptica, Qualea grandiflora and Q. multiflora* plants growing under different levels of Al concentrations. All parameters were generated from the mean of 5 plants, one leaf per plant. Percentages of the same energy partitioning

represented with different letters differ significantly among treatments by Tukey’s test at P ≤ 0.05.

Photographic records

The photographic records showed that after 60 days after treatment the plants of *V. elliptica* started showing leaf symptoms of nutrient deficiencies, likewise bronzing, under 0 Al treatment, while very little to none was observed for 400 Al and 800 treatments. After 150 days one plant from the 0Al treatment died while the other plant in the same treatment showed more signs of deficiency. The plants of 400 and 800 Al treatments kept growing normally. The species *Q. grandiflora* and *Q. multiflora* showed similar visual growth responses to treatments. Both species had an intense growth in 0Al treatment in the first 60 days of treatment, but after 150 days the 0Al plants showed clear deficiency symptoms. While the plants from medium and high Al started growing with no signs of deficiency, except for the 600 Al treatment of *Q. multiflora* that showed no growth signs in the aerial parts.

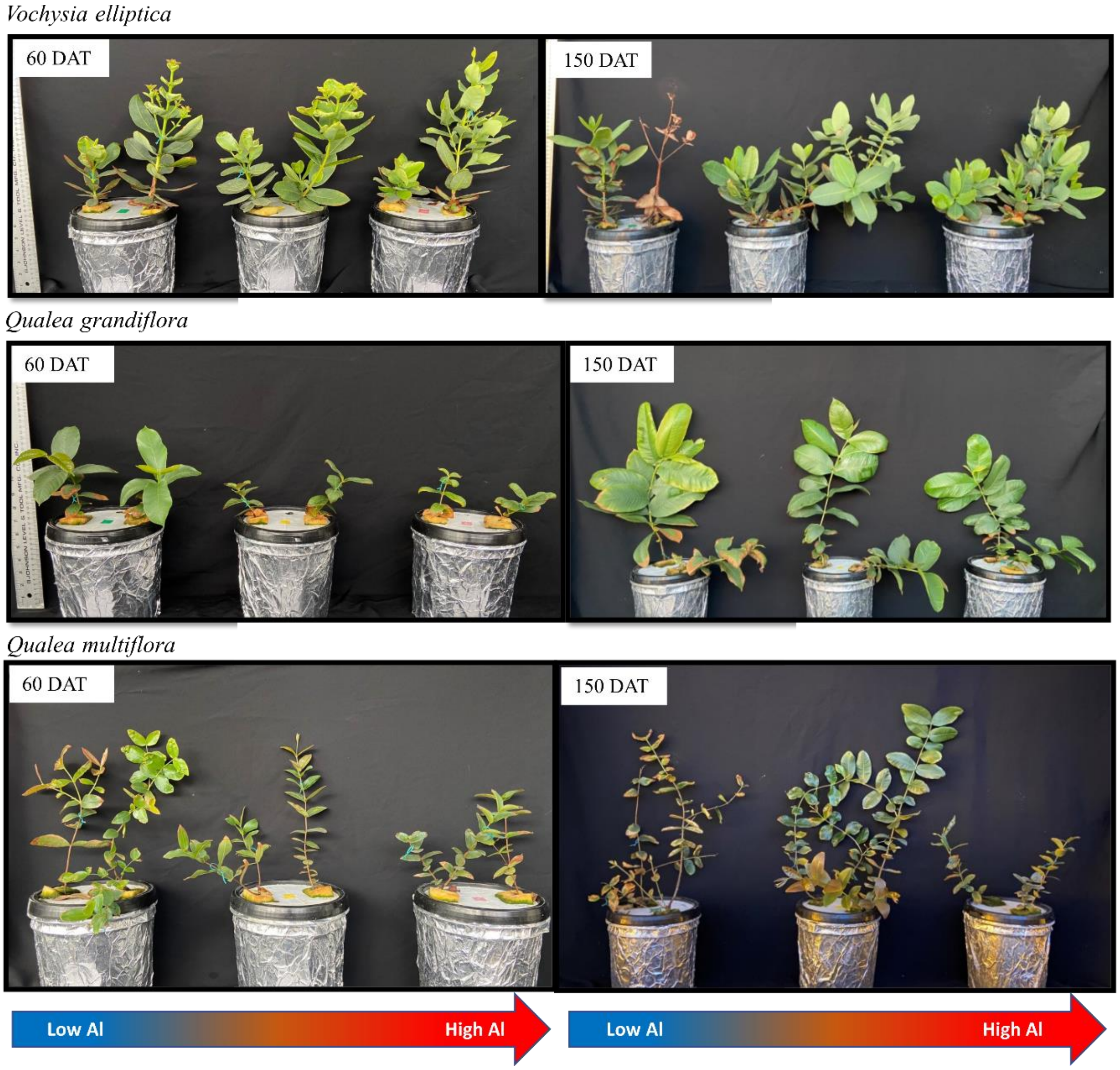

Figure 5 - *Vochysia elliptica*, *Qualea grandiflora* and *Q. multiflora* plants after growing for 60 (60 DAT) and 150 (150 DAT) under different levels of Al concentrations.

Discussion

Gas exchange and Al effects

Most of what we know about the effects of Aluminum on photosynthesis from Al-accumulator species point to stomatal limitations, mostly attributed to root inhibition under low aluminum availability (Della Torre et al., 2022a; Della Torre et al., 2022b). Our results corroborate the hypothesis of stomatal limitations in response to low Al, for *Q. grandiflora* and *Q. multiflora* (Table 1, gs400), but not for *V. elliptica*. The results point to a species-specific or even a genus-specific stomatal behavioral response. Aiming to eliminate the stomatal interferences in evaluating the influence of Al in some of the photosynthetically key biochemical limiting factors, we used the A/Ci method (based on the FvCB model) (Farquhar et al. 2001; Long and Bernacchi 2003; Sharkey et al. 2007).

The CO_2 compensation point Γ (Γ* when excluding mitochondrial respiration) is a very important component to the FvCB model, and also used characterize C3, C4 and intermediate species due to its close relation with photorespiration (Farquhar et al. 2001; Sharkey et al. 2007; Busch et al. 2013; Sage et al. 2013). Until now there is no record of Al causing changes in photorespiration, neither by toxicity or deficiency. The higher values of Γ observed for *Q. multiflora* plants under 0 Al treatments could indicate the increases in oxidative stress. Once photorespiratory pathways can be used to prevent Reactive oxygen species (ROS) accumulation (Voss et al. 2013).

The capacity of plants to growth and develop are dependent to its photosynthetic rates, the maximum assimilation rates (A_{max}) express the maximum capacity of that specific green tissue to fix carbon. Usually, lower A_{max} means reduction in photosynthetic capacity due to a stressor or adverse environmental condition (Ashraf and Harris 2013), in our study, we observed lower A_{max} in both, the presence and absence of Al in different species. In the FvCB model maximum carboxylation rates (V_{cmax}), maximum electron transport rates (J) and maximum rate of use of triose phosphates use (TPU) are used as limiting factor for CO_2 assimilation rate modeling (Farquhar et al. 2001; Sharkey et al. 2007). Aluminum is a well-studied stressor affecting photosynthesis in many sensitive plant species like citrus, *Quercus glauca*, wheat, maize and even in Al-accumulator species when cultivated under high Al concentrations (Lidon et al. 1997; Akaya and Takenaka 2001; Jiang et al. 2009; Mukhopadyay et al. 2012). For *V. elliptica* those parameters were reduced in Al supplemented plants when compared to 0Al treatments, can be a result of Al toxicity, at least for this species. The other two species from the genus *Qualea* showed increases in photosynthetic parameters when subjected to high Al. Until now, very little is known about the effects of Al in the photosynthetic processes in Al-accumulator species. De Andrade *et al.* (2011) have identified that two Al-accumulator species *C. major* and *Q. grandiflora* not only stores Al in the chloroplast but also allows the metal to enter the chloroplast without damaging it. Now we know that the Al inside the chloroplast also benefits the photosynthetic capacity of *Q. grandiflora*. De Andrade *et al.* (2011) also found that the metal only binds to cell walls epidermal and mesophyll cells, avoiding chloroplast contact in *V. pyramidalis* (same genus of *V. elliptica*). These supports the idea that there is a clear difference in chloroplast sensitivity and detoxification mechanism between the *Qualea* and *Vochysia* genus.

Between the species here analyzed only *Q. grandiflora* showed differences in mesophyll resistance (gm), Al supplementation resulted in increased gm. The difficult faced by CO_2 to move from intercellular space to the carboxylation sites inside the chloroplast is known as gm, and all the resistances faced in this path are the cell wall, plasma membrane, chloroplast membrane and stroma (Busch 2020). The Al capacity to displace calcium binds into the cell wall are well studied and can be the result we saw in gm changes (Clarkson 1967; Tabuchi and Matsumoto 2001; Silva et al. 2020).

Energy partitioning

The three species evaluated showed higher quantum yield of PSII electron flow (Φ_{PSII}) when cultivated under high Al condition, meaning that Al might be beneficial to maintain the function of the PSII after being subjected to high light levels. For both *Qualea* species these results were expected, once they already showed higher A_{max}, V_{cmax} and J. Plants with higher photosynthetic capacity are usually less prone to photoinhibition, once most of the energy from water split runs thorough the electron transport chain and are used to form NADPH and less energy is left for reactive oxygen species (ROS) production (Long and Humphries 1994; Roach and Krieger-Liszkay 2014). The excess of ROS especially H_2O_2 downregulates repair of the PSII D1 protein, usually damaged by excess light which contributes to increase photoinhibition (Takahashi and Murata 2008). Φ_{NF} is the thermal energy dissipated by non-functional PSII, and reflects the level of photoinhibition. *V. elliptica* showed lower Φ_{PSII}, but higher Φ_{NF} in plants from 0 Al treatment, indicating that lack of aluminum might reduce the resistance of these species to deal with photoinhibitory process. As a matter of fact, our data shows that Aluminum supplementation reduces the intensity of photoinhibition (Lower values of Φ_{NF}) and increases the capacity to dissipate energy as a heat thorough ΔpH dependent xanthophyll cycle (high ΦNPQ). The capacity to deal with excess light is very important feature especially in a harsh environment like the Cerrado, where added to high light intensities are high temperatures and low water availability during the dry season (Lemos-Filho 2000; Pereira et al. 2018).

Ecological considerations

Under limiting resource conditions plants are familiarized with trade-offs, like having to adjust how much CO_2 assimilation will be done at the coast of losing water in a dry environment (Manzoni et al. 2013) or maybe adjusting the chlorophyll biosynthesis under nitrogen soil limitations (Zhong et al. 2019). Our data suggests that *V. elliptica,* a Cerrado Al-accumulator species, probably deal with something similar. Giving up of some of its CO_2 assimilation capacity in order to increase its photoprotection, as a result of Al deposition in the epidermis and mesophyll cell wall, seems a good deal especially in a harsh environment like the Cerrado (Lemos-Filho 2000). No trade-off was identified for *Qualea* genus species, but it was possible to observe that when under high Al condition plants from this genus have they growth stagnant, probably a strategy to avoid toxicity, once they usually store Al inside the tissues (de Andrade et al. 2011). This differences in Al tissue allocation strategy might reflect the level soil Al tolerated by these species.

Conclusion

Based on our results the photosynthesis of the two species from *Qualea* were improved by Al supplementation, while the photosynthesis of *Vochysia* was impaired by the presence of the metal. This is probably linked to the Al-compartmentalization strategy adopted by each species or genus. We also demonstrated that Al might have some function in reducing photoinhibition susceptibility (reducing ΦNF) mostly through improving ΦNPQ.

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FINAL CONSIDERATIONS

In a very humbling way, we aimed to get closer to understanding a possible biological role of Al in Al-accumulator plants. Knowing this is too pretentious, we were able to at least walk in the right direction. The first chapter resulted in the knowledge that allowed us to cultivate the Al-accumulator species (those from chapter 1, 2 and 3) in hydroponics for a very long time (9 months). We also identified the morphological benefits resulted from the presence of Al together with discussions around the calcicole and calcifuge behavior and P toxicity in these plants. But the more relevant fact was the root growth stimulus affecting plants water relations.

The data from gas exchange measurements on the second chapter was essential to correlates the presence of Al to increases in biomass (chapter one data) due to stomatal conductance increases (consequently higher CO_2 assimilation) as a response to root growth stimulus observed in the first chapter. In chapter two we were also able to detect increases in ROS and be cautious when excluding the possibility of toxic effects of Al to Al-accumulator species. There were no differences in thermo and light tolerance but we identify the need to do a more detailed methodology. We also detected here the need to isolate the effect of stomatal limitation in order to take a closer look in to the carboxylation effects of Al on those plants.

With the new insights from chapters one and two the third chapter was more mature and allowed us to compare the photosynthesis of three species. This chapter showed us that the Al can be beneficial at carboxylation level for plants from *Qualea* genus, but can also be toxic for *V. elliptica.* We were able to inquire based on previous papers that the Al storage strategy might be directly linked to susceptibility to Al toxicity. While plants that allows Al to reach the chloroplast (*Qualea grandiflora*) are not negatively influenced by the metal. On the other hand, the species from the same genus which were only detected Al stored in cell wall are prone to impairments in the photosynthetic rates. We were also able to identify a possible positive role of Al in photoprotection, based on the observations of reductions in Φ_{NF} added to increases in Φ_{NPQ}.

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