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Temporal dynamics of the response to Al stress in *Eucalyptus grandis* × *Eucalyptus camaldulensis*

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ABSTRACT

Lipid peroxidation and root elongation of *Eucalyptus grandis* × *Eucalyptus camaldulensis* were studied under stress conditions in response to aluminum (Al), a metal known to limit agricultural productivity in acidic soils primarily due to reduced root elongation. In Brazil, the Grancam 1277 hybrid (*E. grandis* × *E. camaldulensis*) has been planted in the "Cerrado", a region of the country with a wide occurrence of acidic soils. The present study demonstrated that the hybrid exhibited root growth reduction and increased levels of lipid peroxidation after 24h of treatment with 100 μM of Al, which was followed by a reduction in lipid peroxidation levels and the recovery of root elongation after 48h of Al exposure, suggesting a rapid response to the early stressful conditions induced by Al. The understanding of the temporal dynamics of Al tolerance may be useful for selecting more tolerant genotypes and for identifying genes of interest for applications in bioengineering.

Key words: aluminum, eucalypt, lipid peroxidation, oxidative stress, root growth.

INTRODUCTION

The *Eucalyptus* genus is considered to be the most commonly planted, fast-growing hardwood in the world, with an estimated total planted area of 20 million ha (Trabado 2009). The greater part of this resource has been managed for the production of pulpwood, fuelwood, lumber, moulding, millwork, sliced and rotary peeled veneer, plywood, composite panels, flooring, furniture, and engineered wood products (Donnelly et al. 2003).

Brazil is one of the countries with the largest areas of planted forest in the world, currently estimated at 6.5 million ha, 74.8% of which is composed of *Eucalyptus* trees, creating 4.73 million direct and indirect jobs (ABRAF 2012). The socioeconomic importance of the forestry activity in Brazil is undeniable, and due to its fast growth rate and high productivity, the *Eucalyptus* crop has expanded in Brazil, particularly in the Brazilian "Cerrado" region (Silva et al. 2009, Soares and Nunes 2013) an area known to have acidic soils and a high occurrence of exchangeable aluminum (Al^{3+}) that leads to low fertility and may also compromise symbiotic efficiency and plant growth, which are the primary factors that limit agricultural productivity (Abreu et al. 2003, Kochian et al. 2004, Vendrame et

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al. 2010, Soares et al. 2014). However, unlike annual crops, some species of *Eucalyptus* have demonstrated tolerance to acidic soils (Tahara et al. 2005, 2008), which demonstrates the potential for this crop to be an economic alternative for producers whose annual crop productivity is adversely affected by Al.

The link between the reduction of growth and lipid peroxidation, induced by Al and associated with a low pH, is still controversial (Basu et al. 2001, Yamamoto et al. 2001, Ribeiro et al. 2012, Silva 2012) and the correlation between lipid peroxidation and growth varies depending on the species. In contrast, it is well known that Al induces oxidative stress and increases the activity of a number of antioxidant enzymes related to the detoxification of reactive oxygen species (ROS) and lipid peroxidation (Boscolo et al. 2003, Giannakoula et al. 2010), due to an imbalance in the cellular redox status that leads to oxidative stress (Gratão et al. 2005, Boaretto et al. 2014), a response that is quite similar to those produced by other types of stresses, such as exposure to ozone (Azevedo et al. 1998, Bulbovas et al. 2014), heavy metals (Gratão et al. 2012, Monteiro et al. 2012), nutritional deficiencies (Lidon and Barreiro 1999, Muneer et al. 2013), drought (Cia et al. 2012, Boaretto et al. 2014) and diseases (Lamb and Dixon 1997) and even the use of nanoparticles (Arruda et al. 2015). Curiously, various aluminium-containing salts have also been shown to provide an alternative to the use of synthetic fungicides to control pathogens (Kolaei et al. 2013).

Previous studies have elucidated some of the mechanisms that lead to Al tolerance, such as the production of organic acids (Marschner 1991, Nguyen et al. 2003, Tahara et al. 2008, Ezaki et al. 2013), the deposition of lignin on the cell wall (Ezaki et al. 2005, Moura et al. 2010), and the efflux of Al by proteins (Ezaki et al. 2005). However, studies regarding the temporal dynamics of the Al stress response for a large number of important crop species, including *Eucalyptus*, are not available. In addition, there is a lack of studies that correlate the biochemical parameters with root growth. Therefore, the objective of the present research was to analyze the effects of Al on the root elongation of a *Eucalyptus* hybrid (*E. grandis* × *E. camaldulensis*) and to examine the plant stress responses through lipid peroxidation measurements following 0, 24 and 48h of Al exposure.

MATERIALS AND METHODS

Plant Material and Al Stress Exposure

Hybrid clones of *E. grandis* × *E. camaldulensis*, cultivar Grancam 1277, were donated by the Estação Experimental de Itatinga, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo. The seedlings were grown in tubes, with a substrate composed of Basaplant® and vermiculite in equal proportions (1:1). After six months, the seedlings were transferred to a hydroponic system containing a Hoagland and Arnon (1950) nutrient solution with modifications. The components of the nutritive solution were as follows: 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.4 mM KNO_3, 0.3 mM $(NH_4)_2SO_4$, 0.2 mM KH_2PO_4, 0.82 mM $MgSO_4 \cdot 7H_2O$, 3.3 µM $MnSO_4$, 1 µM $ZnSO_4 \cdot 7H_2O$, 3.3 µM H_3BO_3, 0.034 µM Na_2MoO_4, 0.003 µM $CuSO_4 \cdot 5H_2O$, and 2.7 µM Fe-EDTA, at a pH of 5.8. The *Eucalyptus* seedlings were transferred to the hydroponic system, where they were maintained for 30 days to acclimatize the plants. After this period, the nutrient solution was removed and replaced with a new solution that lacked Fe-EDTA, at a pH of 4.0. The nutrient solution was supplied with $AlCl_3 \cdot 6H_2O$ at concentrations of 0, 10, or 100 µM. We used 12 *Eucalyptus* plants per container and four plants per three repetitions totalizing 108 plants in this experiment. The roots were collected for analysis 0, 24 and 48h after Al exposure.

Aluminum Content Determination

The roots were dried at 60 °C, macerated until a fine powder was obtained, and digested in pure

nitric acid. After the acid digestion, the extract was diluted to 10% in deionised water and immediately used in inductively coupled plasma - optical emission spectroscopy (ICP-OES).

Relative Root Growth

The root length was measured 24h before treatment with Al and during the treatment periods (0, 24, and 48h). The root elongation was determined by calculating the differences in root length for the following periods: 24h before Al application; 0 and 24h after Al application; and 24 and 48h after Al application. These differences were used as the basis for growth comparisons among the treatments.

Lipid Peroxidation

Oxidative damage was visually evaluated by histochemical analysis according to Yamamoto et al. (2001) with modifications. The root tips were stained with Schiff's reagent for 20 min. After the reaction, the roots were washed with deionised water and immediately analyzed with a stereoscopic microscope (24× lens). Schiff's reagent associates with the aldehydes that are derived from lipid peroxidation (Yamamoto et al. 2001), resulting in different degrees of a purple colour. Oxidative damage was biochemically quantified based on Cakmak and Horst (1991). Metabolites reactive to 2-thiobarbituric acid (TBA) were used to estimate the levels of malondialdehyde (MDA), which is an indicator of lipid peroxidation. The readings were performed using a spectrophotometer at 535 and 600 nm, and the concentration of MDA was determined using the following formula: $C = [ABS (535 - 600) / 155] \times 10^6$. The results were expressed as nmol MDA·g^{-1} fresh matter.

Experimental Design and Statistical Analyses

The experiment was completely randomised, using three repetitions per treatment. Analysis of variance (ANOVA) was performed using Statistica Software (version 7.0 StatSoft, Tulsa, OK, USA). Significant differences between the averages of the treatments were determined by performing the Duncan test with a confidence level of 95%.

RESULTS

In the present manuscript, we aimed to understand the aspects related to Al toxicity in *Eucalyptus* because information regarding the dynamics of toxicity and the recovery potential following metal exposure is largely lacking in the literature.

To estimate oxidative stress, we quantified the concentration of malondialdehyde (MDA), a product of lipid peroxidation. An interesting result was observed when the roots treated with 100 µM $AlCl_3$ for 24h exhibited higher levels of MDA than the roots exposed to the same concentration of $AlCl_3$ for 48h (Fig. 1). To compare quantitative and qualitative data, we performed a histochemical analysis and similar results were observed. *E. grandis* × *E. camaldulensis* plants that were submitted to 24h treatment with 100 µM $AlCl_3$ exhibited the highest levels of lipid peroxidation in the transitional region of the roots (Fig. 2).

To assess whether lipid peroxidation was occurring simultaneously with root growth inhibition,

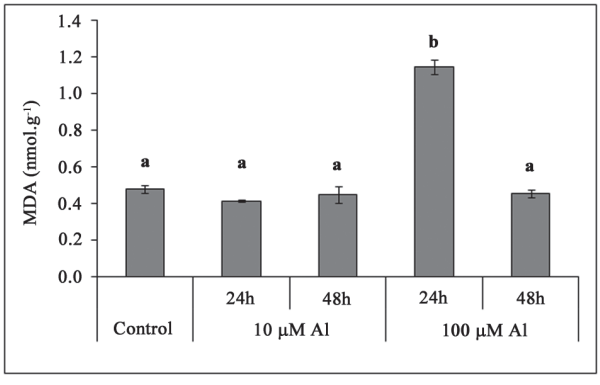

Figure 1 - Malondialdehyde (MDA) quantification to estimate lipid peroxidation in the roots of *E. grandis* × *E. camaldulensis* under Al stress. Bars with the same letters indicate no significant difference between the means at the 95% confidence level using the Duncan test. The error bars represent the standard error (n = 3).

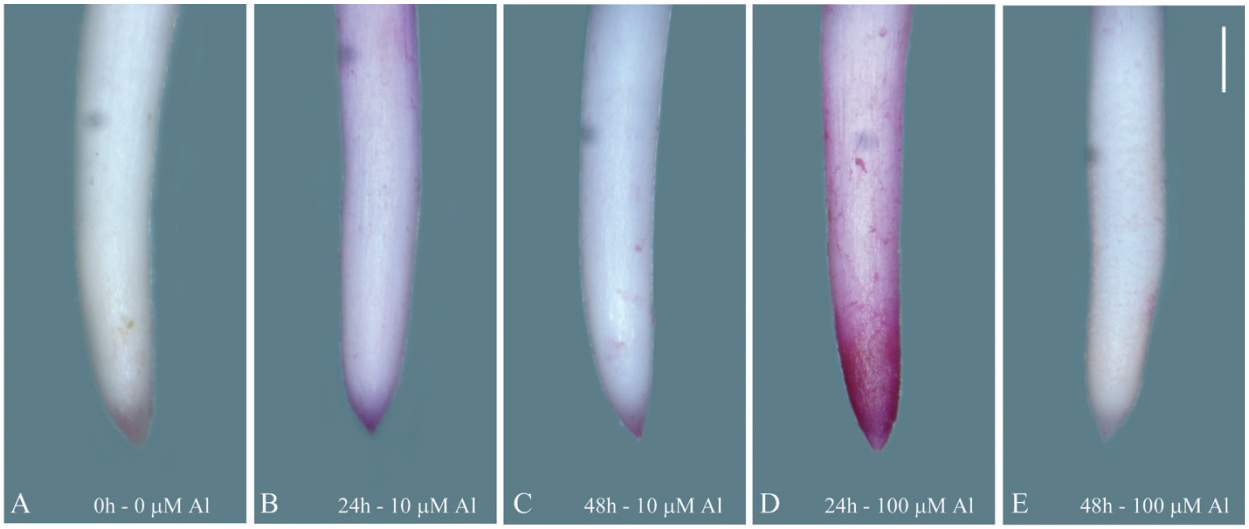

Figure 2 - Histochemical analysis of lipid peroxidation in the roots of *E. grandis* × *E. camaldulensis*. **(A)** Control, before the application of $AlCl_3$; **(B)** 24h after the application of 10 µM $AlCl_3$; **(C)** 48h after the application of 10 µM $AlCl_3$; **(D)** 24h after the application of 100 µM $AlCl_3$; and **(E)** 48h after the application of 100 µM $AlCl_3$. The scale bar represents 1 mm.

we measured the roots during the treatments and 24h prior to the beginning of the experiment. A reduction in root elongation of approximately 32% was observed following 100 µM $AlCl_3$ exposure for 24h; however, there was no inhibition after 48h (Fig. 3).

Al concentration in the roots was measured for all treatments. The results revealed that the reduction in Al uptake by the roots exposed to 100 µM $AlCl_3$ for 48h was sufficient to increase root growth and reduce the lipid peroxidation levels (Fig. 4).

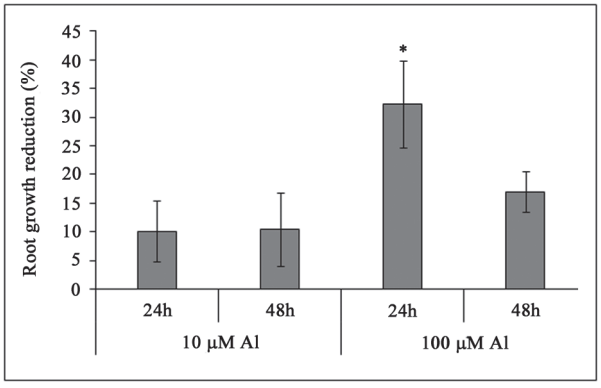

Figure 3 - Reduction in the root growth of *E. grandis* × *E. camaldulensis* exposed to 10 µM and 100 µM $AlCl_3$ compared to control 24h before Al application. The asterisk indicates a significant difference at the 95% confidence level using the Duncan test. The error bars represent standard error (n = 12 plants).

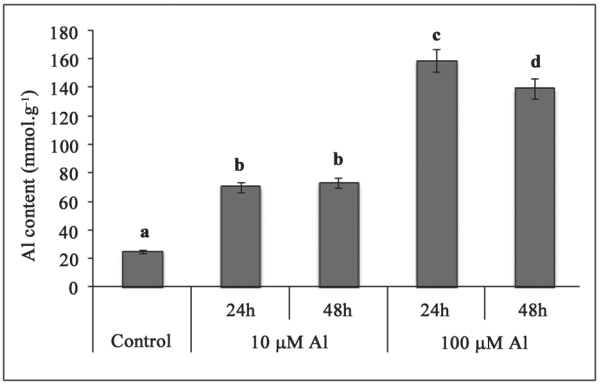

Figure 4 - Aluminum quantification in *E. grandis* × *E. camaldulensis* roots. Bars with the same letters indicate no significant difference between the means at the 95% confidence level using the Duncan test. The error bars represent standard error (n = 3).

DISCUSSION

The reduction in cell growth has been shown to be related directly to Al uptake and accumulation inside the cell (Vitorello and Haug 1999). Therefore, Yamamoto et al. (2001) demonstrated that lipid peroxidation is the first symptom triggered by Al, but growth reduction of the pea roots

was not caused by lipid peroxidation, but by Al accumulation. Working with maize, Giannakoula et al. (2008) observed that the tolerance to Al was correlated with reduced levels of lipid peroxidation and Al uptake. In the present study, it appeared that the hybrid *E. grandis* × *E. camaldulensis* exhibited an increased rate of lipid peroxidation immediately following Al exposure (after 24h stress). Moreover, we observed the highest levels of lipid peroxidation in the transitional region of the roots, a result which is consistent with other reports that found that the root distal transition zone is a critical site for the perception of Al toxicity (Marschner 1991, Sivaguru and Horst 1998). However, subsequently (after 48h stress), the roots were able to maintain the rate of lipid peroxidation within normal levels, leading to reduced root growth inhibition, which corroborates the data produced by Giannakoula et al. (2008).

Root growth reduction caused by Al exposure has been observed in one of the parental lines, *E. grandis*, when subjected to 648 μM Al^{3+} during a 10-day period of stress (Silva et al. 2004). It was also observed that among *Eucalyptus* species, *E. grandis* and *E. cloezina* were the most susceptible to Al-induced damage. For *E. camaldulensis*, a reduction in the root growth rate has been observed after only 24h of exposure to 1 mM Al (Nguyen et al. 2003, Tahara et al. 2005). However, growth was shown to be normal after 20 days of Al exposure (Tahara et al. 2005). Tahara et al. (2008) in another report observed that the root growth inhibition of *E. camaldulensis* occurred after five days of exposure to 1 mM Al and according to these same authors the growth recovery shown by *E. camaldulensis* when exposed to 1 mM Al likely occurred between five and 20 days, whereas in the present study, the hybrid *E. grandis* × *E. camaldulensis* exhibited growth recovery after treatment with 100 μM Al between 24 and 48h. This result suggests that the time for recovery depends on the Al concentration, and it is likely that the fast response observed in the hybrid was inherited from the *E. camaldulensis* parent.

Nguyen et al. (2003) and Tahara et al. (2008) suggested that *E. camaldulensis* avoids Al uptake through the production of organic acids, particularly oxalate, which can form complexes with Al^{3+}, conferring protection to the plant roots. Curiously, Silva et al. (2013) observed that when rice root apices are removed, high amounts of Al were shown to accumulate in the shoots revealing the importance of Al exclusion mechanisms in the roots of intact plants. Nevertheless, Silva et al. (2004), Nguyen et al. (2005), Jones et al. (2006) and Smith et al. (2011) proposed that there are other mechanisms that may occur in the cell wall to chelate Al and block its entrance through the cell membrane. Examples of these mechanisms include the complexation of Al by lignin, the exudation of polysaccharides that bind Al in the cell wall (i.e., pectin), and sequential citrate rinses to remove exchangeable Al in the apoplast. Considering that in the present study high levels of Al in the roots were still detected after 48h of exposure to the metal, it is likely that avoidance mechanisms were also occurring at the cell wall level, contributing to the swift recovery of *E. grandis* × *E. camaldulensis*.

Dalal and Khanna-Chopra (1999) suggested that lipid peroxidation is a primary event required for cell death. Indeed, more recently, there have been some reports that demonstrate a correlation between lipid peroxidation and cell death (Achary et al. 2012), implying that the inhibition of root growth by Al ions is related to toxic aldehydes, such as MDA, generated downstream of ROS production. In the present research, we observed that the hybrid *E. grandis* × *E. camaldulensis* exhibited a reduction in lipid peroxidation after 48h of Al exposure, which could reflect growth recovery during this period. However, further research regarding the correlation between cell death signalling and lipid peroxidation in the *Eucalyptus* species is necessary.

It is also possible that the Al fast tolerance mechanism exhibited by *E. grandis* × *E. camaldulensis* is related to drought tolerance

because the hybrid and parent *E. camaldulensis* have both been previously characterized as being tolerant to drought stress (Reis et al. 2006; Thumma et al. 2012). Moreover, Reis et al. (2006) and Rad et al. (2011) observed that seedlings of *E. camaldulensis* cultivated in the "Cerrado" region exhibited a tolerance to dehydration due to a reduction in the leaf area and an increase in ground water absorption by the root system. As previous studies have proposed (Marschner 1991, Purcell et al. 2002), the tolerance to Al appears to be a complementary process to promote drought tolerance, which may in turn enable a faster root growth in the acidic soils of the "Cerrado" region, promoting the improved use of water by the deepening of the roots. However, further research regarding the full elucidation of the joint action between mechanisms for tolerance to drought and Al in *E. camaldulensis* is needed. As a matter of fact, water use efficiency should be on the agenda in future abiotic stress studies (Medici et al. 2014).

The present work contributes with information regarding the dynamics of the Al stress response in *E. grandis* × *E. camaldulensis*, demonstrating a fast response to Al stress that is followed by a recovery stage. Concentrations of metals in the environment may fluctuate due to natural phenomena, such as leaching and changes in metal speciation (Arruda and Azevedo 2009), or due to variable emissions from anthropogenic sources (Drost et al. 2007). Therefore, periods of low or moderate stress may be followed by periods of severe exposure and higher stress levels, highlighting the importance of the processes of adaptation and quick recovery by plants, such as those exhibited by *E. grandis* × *E. camaldulensis* in response to Al.

CONCLUSIONS

The data produced in the present study raise interesting questions regarding how the duration of exposure can impact the toxicity of Al in *E. grandis* × *E. camaldulensis*. We observed that the hybrid exhibited an increased rate of lipid peroxidation after 24h stress, however, after 48h, the levels of lipid peroxidation reduced to normal levels, as occurred to root growth inhibition, which implies a swift recovery.

We suggested new directions for additional works in order to correlate cell death signaling and lipid peroxidation in *Eucalyptus*, as well as additional studies for combined actions of tolerance mechanisms to Al and drought.

The use of *E. grandis* × *E. camaldulensis* or its parent (*E. camaldulensis*) as a genetic source appears to be promising for the investigation of which genes are involved in the rapid Al-response.

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RESUMO

A peroxidação lipídica e alongamento de raiz de *Eucalyptus grandis* × *Eucalyptus camaldulensis* foram estudados em resposta às condições de estresse com alumínio (Al), um metal conhecido por limitar a produtividade agrícola em solos ácidos, principalmente devido à redução do crescimento radicular. No Brasil, o híbrido Grancam 1277 (*E. grandis* × *E. camaldulensis*) tem sido plantado na região do "Cerrado", local com grande ocorrência de solos ácidos. O presente estudo mostra que o híbrido teve redução de crescimento das raízes e aumento dos níveis de peroxidação lipídica, após 24h de tratamento com 100 μM de Al. No entanto, um possível processo de tolerância começou após este período, uma vez que foram observadas redução na peroxidação lipídica e recuperação de alongamento

da raiz após 48h de exposição Al. A compreensão da dinâmica temporal de tolerância ao Al pode ser útil principalmente para selecionar genótipos mais tolerantes e também para identificar genes de interesse para aplicação em bioengenharia.

Palavras-chave: alumínio, eucalipto, peroxidação lipídica, estresse oxidativo, crescimento de raiz.

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