University of São Paulo
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Experimental approaches to study the responses to Al stress in cultivated crops

Berenice Kussumoto de Alcântara

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

Piracicaba
2014

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I dedicate this thesis to my
grandparents Massayashi Kussumoto
and Nobuco Kussumoto (in memoriam)

With all my love, I offer this work to André
Gracioso, to my mother Lioko, to my father
Benedito and to my sister Brígida

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“Life is like riding a bicycle – in order to keep your balance, you must keep moving.”

Albert Einstein

Source: In a letter to his son Eduard (5 February 1930). As quoted in "Einstein: His Life and Universe" (2007), Walter Isaacson, p. 367

CONTENTS

RESUMO

Abordagens experimentais para o estudo das respostas ao estresse por Al em plantas cultivadas

Esta tese foi realizada no âmbito do Programa Internacional de Pós-Graduação em que a Universidade de São Paulo e *The Ohio State University* estão inseridos. No primeiro ano do trabalho de doutorado, o objetivo foi de compreender o efeito do alumínio (Al) no crescimento de raízes de *Eucalyptus spp.* e a relação com parâmetros bioquímicos e respostas de transcrição já que há pouca informação sobre estas respostas para espécies arbóreas. O alumínio é encontrado naturalmente na crosta terrestre, porém sob condições ácidas o íon Al^{3+} torna-se disponível e tóxico para as plantas. Os Estados Unidos e o Brasil possuem grandes áreas com Al tóxico e grandes áreas de florestas crescem sob estas condições. A fim de executar a análise transcricional, o RNA dos materiais de eucalipto foi transportado para os Estados Unidos, entretanto, o método de transporte não foi eficiente para garantir a integridade do RNA. Assim sendo, mudamos nossos objetivos e realizamos novos experimentos utilizando milho como sistema biológico, porque esta espécie tem importância econômica para os EUA e também para o Brasil. Ademais, substanciais áreas de cultivo do milho se encontram em solos sob estas condições. Nos novos experimentos realizados, o objetivo foi de verificar se o pré-tratamento de sementes de milho antes da germinação poderia ser eficiente para induzir tolerância ao alumínio em genótipos Al-sensíveis. Esta tese foi organizada a fim de construir uma história com base nas sequencias dos experimentos. Em geral, resultados interessantes foram obtidos para a linhagem de milho sensível ao Al que apresentou redução no teor de lignina e na sua expressão gênica em parte aérea, também para a variedade tolerante ao Al que apresentou aumento no crescimento, produtividade e melhoria da qualidade nutricional dos grãos.

Palavras-chave: Solo ácido; Alumínio trocável; Estresse oxidativo; Lignina; Produtividade

ABSTRACT

Experimental approaches to study the responses to Al stress in cultivated crops

This thesis was performed in the scope of International Graduation Program in which *Universidade de São Paulo* and The Ohio State University are inserted. In the first year of Ph.D. work we aimed to understand the effect of aluminum (Al) in root growth of *Eucalyptus spp.* and the relationship with biochemical parameters and transcriptional responses since there is little information about these responses for tree species. Aluminum is found naturally in the Earth crust, but under acidic conditions Al^{3+} ion becomes available and toxic to the plants. United States and Brazil possess large areas with Al toxicity and great area of forests growing under these conditions. In order to perform the transcriptional analysis, *Eucalyptus* RNA was transported to United States, however, the method of transportation was not efficient to guarantee RNA integrity. Therefore we changed our objectives and we performed new experiments using maize as biological system because this species has economical importance to U.S. and Brazil. Furthermore, substantial area of this crop is growing in soils under these conditions. In these experiments, we aimed to verify if pre-treating maize seeds before germination could be efficient to induce Al-tolerance in Al-sensitive genotypes. This thesis was organized in order to build a story based on the sequences of the experiments. In general, we obtained interesting results for Al-sensitive maize that presented reduction in lignin content and gene expression in shoots, also for Al-tolerant variety that present increase in growth, productivity and improvement of nutritional quality of the kernels.

Keywords: Acidic soil; Exchangeable aluminum; Oxidative stress; Lignin; Productivity

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1 INTRODUCTION

The present thesis is the result of the first International Graduation Program in Brazil that consisted in the partnership of three recognized Universities: Universidade de São Paulo (Brazil), The Ohio State University and Rutgers (United States). Since this doctorate work is inserted into an International Tripartite Program, besides the importance of the study for the country of student's origin (Brazil), it is also essential to emphasize the justification of the work for the associate country in which this Graduate Program is inserted.

The aluminum toxicity in acidic soils is a concern for both countries since this problem causes inhibition of root growth and restriction in agricultural productivity (MA, 2007; KOCHIAN et al., 2009). Furthermore, it occurs in almost 50% of U.S. potential arable lands and almost 97% of potential arable lands of Brazil (FAO, 2011). Aluminum (Al) is the third most abundant element in the Earth's crust and an excess level in acidic soils (pH < 5.5) is toxic to plants (KOCHIAN et al., 2009). Farmers have limed the acidic soils in order to enable agricultural production. However, the effect of lime is observed only in few centimeters of soil depth (CASSIOLATO et al., 2000) and also it is a non-renewable natural resource that can eventually be exhausted. To understand how plants deal with Al stress is important to develop more tolerant and productive plant varieties.

Why did we choose eucalyptus and maize as systems to study the effects of Al stress? In the beginning of the thesis, we aimed to utilize *Eucalyptus* species because studies that correlate biochemical parameters and transcriptional responses with the effect of Al in root growth are lacking in the literature for tree species. The first year of experiments using *Eucalyptus* (Section 2) occurred in Brazil, however because of the difficulties to transport the experimental materials to the United States, new goal was designed and we choose maize as biological system because both countries are the main producers of corn in world, also a considerable part of maize is growing in soils with Al toxicity in U.S. and Brazil, a problem that can cause yield losses up to 80% (BARINAGA, 1997). Since then, the novel objective of the study was to test the hypothesis that certain seed treatments can induce tolerance in Al-sensitive maize genotypes.

The experiments performed in U.S are found in the sections 3 and 4, in which we investigated if Al-tolerance could be induced by seed priming with acidic water or aluminum solution and seed treatment using ascorbic acid or boric acid powder. We utilized these treatments based on the observations obtained in literature. After the results analysis, we

raised more questions and when the Ph.D. student arrived to Brazil, other experiments were performed in field (Section 5) in order to try to answer these questions.

The experiments performed in the sections 3, 4 and 5 show interesting observations as example, the reduction of lignin content and gene expression for Al-sensitive maize shoots, also the improvement of productivity and kernels quality for Al-tolerant maize, which give directions for further studies regarding economical applicability of the treatments in maize, also new tests for other species and type of stresses could be interesting to be evaluated.

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SECTION 2 - RESPONSES OF *EUCALYPTUS SPP.* TO AL STRESS – EXPERIMENTAL APPROACHES

The Section 2 presents three different experimental approaches to study Al stress in the roots of *Eucalyptus* species. These experiments were performed in Brazil. In our first approach, we tried to use tissue culture. For the second and third approaches, we used a hydroponic system. We show the results obtained for all of these approaches and discuss only the interesting results that were observed using the third, most refined experimental approach. This Ph.D. work was performed in the context of the International Tripartite Program; therefore, one part of the study was executed in the United States. For that reason, RNA samples obtained from the third approach were transported to the U.S. to investigate the expression of lignin-related genes. However, the RNA was degraded during transportation, which led to further new experiments with maize (described in Sections 3, 4 and 5). The objective of this section is to present the experiments that were conducted in the first year of the student's Ph.D. study in Brazil and to show which approaches are interesting for the study of the effects of Al in *Eucalyptus* roots.

2 TEMPORAL DYNAMICS OF THE RESPONSE TO AL STRESS IN *EUCALYPTUS SPP.*

Abstract

This section describes three different approaches to study the effect of Al in *Eucalyptus* roots. The two initial approaches suffered from methodological problems, so only the results of the third approach are discussed. For the last approach, the 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. The present study demonstrated that the hybrid exhibited root growth reduction and increased the levels of lipid peroxidation after 24 h of treatment with 100 μM of Al, which was followed by a reduction in lipid peroxidation levels and the recovery of root elongation after 48 h 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 designing new experiments with predetermined periods that are chosen to identify rapid-response genes for bioengineering applications.

Keywords: Aluminum; Lipid peroxidation; Root growth

2.1 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, molding, 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 area of planted forest in the world, currently estimated at 6.66 million ha, 76.6% of which is composed of *Eucalyptus* trees (Figure 1), which create 4.4 million direct and indirect jobs (ASSOCIAÇÃO BRASILEIRA DE PRODUTORES DE FLORESTAS PLANTADAS - ABRAF, 2013). The socioeconomic importance of forestry in Brazil is undeniable, and the *Eucalyptus* crop has expanded in Brazil due to its fast growth rate and high productivity, particularly in the Brazilian "Cerrado" region (SILVA; MENDES, 2009), an area known to have acidic soils and high levels of exchangeable aluminum (Al^{3+}), which are associated with low fertility and are the primary factors that limit agricultural productivity (ABREU et al., 2003; KOCHIAN et al., 2004). However, unlike annual crops, some species of *Eucalyptus* have demonstrated tolerance for 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.

In the United States, wood production is concentrated in the Southeast, Northeast, Northwest and Rocky Mountains (Figure 2). Approximately 29.3% of total wood production is destined for the pulp industry, of which 55% is utilized for printing/writing paper production, 15% for towels/tissues, 13% for packaging and 9% for sanitary goods (JOHNSON et al., 2009).

Fiber sources used to produce paper include wood, waste paper, non-wood agricultural fibers, and inorganic fibers. In the United States, the most economical and practical fibers are those derived from wood. Approximately 63% of total fiber consumption in domestic paper and paperboard mills is derived from wood pulp (KOPLAN et al., 2002).

Waste paper supplies most of the rest of the consumption; non-wood fibers supply less than 1% of total consumption. In the United States, both softwood (conifers) and hardwood (broad-leaves) trees are important sources of fiber for the manufacture of wood pulp. Softwoods, which generally have longer fibers than hardwoods, are used when strength is a desirable quality in the finished product (e.g., paperboard). The short fibers from hardwoods are used for pulping when smoothness is a desirable quality (e.g., printing and writing papers) (KOPLAN et al., 2002).

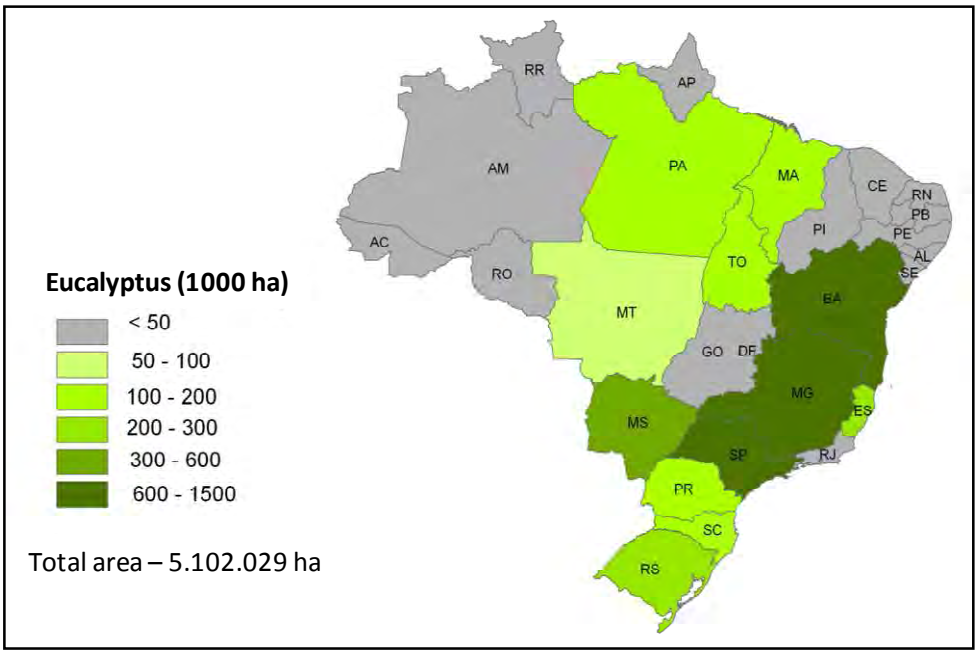


Figure 1 - Areas with eucalyptus-planted forests in Brazil (ABRAF, 2013)

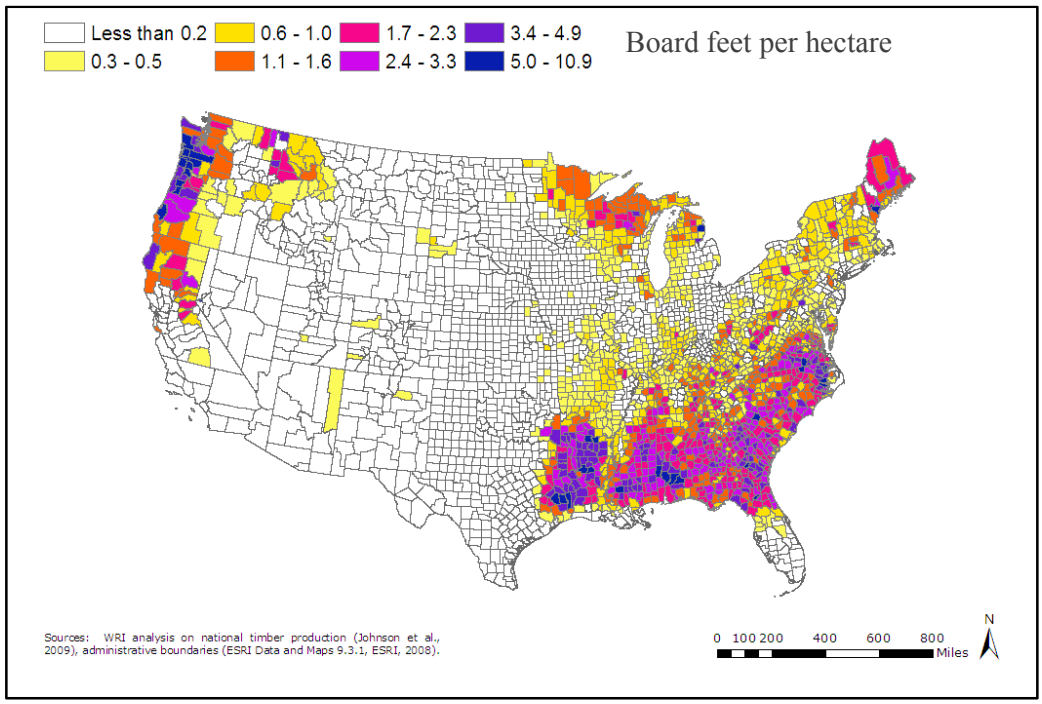


Figure 2 - Timber production in United States by county (JOHNSON et al., 2009)

The United States is the world's leading producer of cellulosic market pulp, with 52.6 million tons of production (U.S. CENSUS BUREAU, 2012). In 2011, Brazil was third in the production of cellulose, with 14.0 million tons, most of it coming from hardwoods (Figure 3) (ABRAF, 2012).

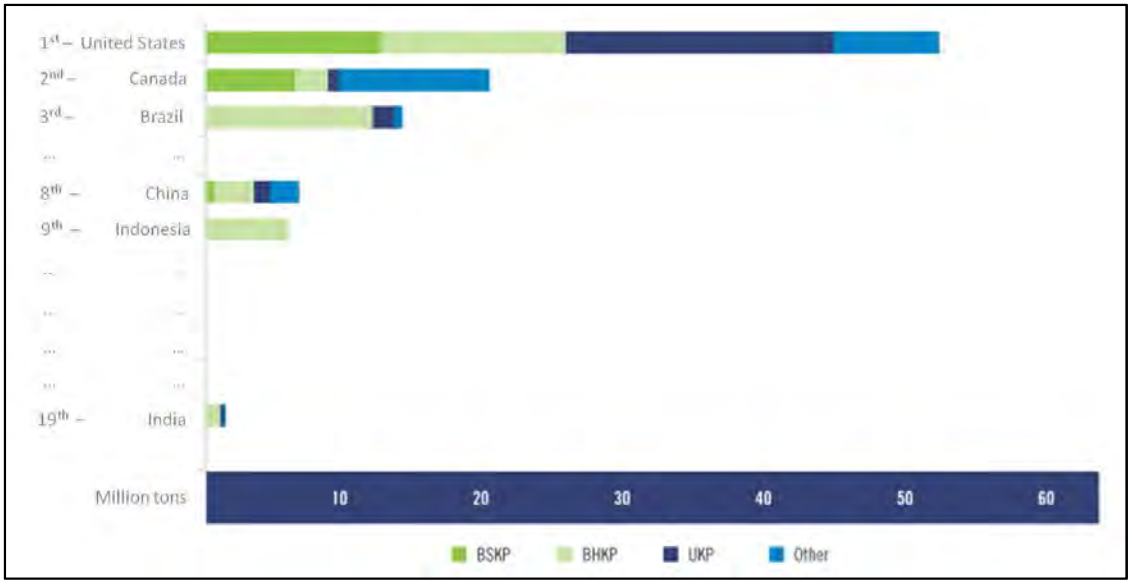


Figure 3 - Major producers of cellulosic pulp in the world (reference year 2011). Labels of pulp types: BSKP, Bleached Softwood Kraft Pulp; BHKP, Bleached Hardwood Kraft Pulp; UKP, Unbleached Kraft Pulp (ABRAF, 2012)

Forests and woodlands grow in approximately 66.3% of soils exhibiting Al stress (HEDE et al., 2001); however, there is a lack of studies regarding the effects of Al on the growth of tree roots and its correlation with biochemical parameters. Furthermore, the link between the reduction of growth and lipid peroxidation induced by Al in crop plants is still controversial (BASU et al., 2001; YAMAMOTO et al., 2001); the correlation between lipid peroxidation and growth most likely depends on the species.

The major components of wood are cellulose (41-51%), hemicellulose (23-38%) and lignin (19-33%) that binds cellulose fibers together (RAGAUSKAS et al., 2005). The process of chemical pulping actually separates usable cellulose fibers from the lignin (KOPLAN *et al.*, 2002).

In addition to the effect of Al on growth, there is also a need to investigate the consequences of Al stress in lignin synthesis, especially for pulpwood plants. For example, Grisel et al. (2010) analyzed the transcriptome response caused by aluminum stress in the roots of *Populus tremula* using an Affymetrix GeneChip poplar genome microarray, which was designed based on the sequence information of different poplar species. They observed an increase in the callose content in Al-treated poplar and changes in nine genes related to pectin formation. However, genes for lignin biosynthesis, such as the key genes *COMT* and *F5H*, were not found among the genes studied. For *Pinus* and *Eucalyptus,* there were no transcriptome data under aluminum stress until this moment.

Previous studies have elucidated some mechanisms that lead to Al tolerance, especially for agricultural crops, such as the deposition of lignin on the cell wall (EZAKI et al., 2005; MOURA et al., 2010), the production of organic acids (MARSCHNER, 1991; NGUYEN et al., 2003; SILVA et al., 2004; TAHARA et al., 2008), and the efflux of Al by proteins (KOCHIAN et al., 2004 and references therein).

In this section, we will describe three experimental approaches for *Eucalyptus spp.* to demonstrate the effect of Al on eucalyptus roots. The analysis of gene expression related to lignin biosynthesis was initially planned to be performed in the U.S., but we encountered problems with the transportation of materials. At the end of this section, we will discuss the results of the third experiment obtained in Brazil. For the subsequent sections (Section 3, 4 and 5), we changed our objectives, as different, new experiments were performed.

2.1.1 Objectives

The general objectives of this present section are 1) to describe several experimental approaches to study the stresses caused by Al on the roots of a *Eucalyptus spp.* and 2) to verify how Al affects eucalyptus roots during that time.

2.1.2 Hypothesis

Based on prior information about the responses to Al stress in *Eucalyptus*, we can expect that:

1) *Eucalyptus* can avoid oxidative damage in roots caused by Al;
2) Lignin-gene expression could be modulated during Al exposure;
3) We can expect the highest expression of lignin-related genes in roots under Al stress.

2.2 Materials and Methods

2.2.1 Experimental approach 1 – root medium culture

We defined a protocol for seed sterilization and germination *in vitro* (ALCÂNTARA *et al.*, 2011) and we observed that the amount of plant material produced, especially root material, was insufficient for all intended analyses. Thus, an attempt was made to optimize the

production of roots *in vitro* because we required at least 1 g of fresh material for metabolic analysis, 0.2 g of fresh material for RNA extraction, 0.2 g of fresh material for lipid peroxidation analysis and 0.3 grams of dry matter for aluminum quantification.

We tested methods for growing roots in liquid culture medium as an attempt to obtain enough roots for setting up an *in vitro* experiment. For this propose we utilized JADS medium (CORREIA et al., 1995) supplemented with 0.5 $mg.L^{-1}$ indole-3-butyric acid (IBA) in 4 treatments and 5 replications:

1) Liquid culture medium under agitation in the light;
2) Liquid culture medium under agitation in the dark;
3) Liquid culture medium under no agitation in the light; and
4) Liquid culture medium under no agitation in the dark.

2.2.2 Experimental approach 2 – first experiment in a hydroponic system

a) Plant material and a hydroponic system

Seeds of *Eucalyptus urophylla* S. T. Blake and hybrid *E. grandis* x *E. camaldulensis* and *E. urophylla* x *E. grandis*, the last two known as "E. grancam" and "E. urograndis", respectively, were collected from a seed orchard, located in Anhembi-SP, Brazil, belonging to the Institute of Forestry Research and Studies (IPEF). The cultivars were identified, respectively, as LCF A009, AEC 0601 and RIP 01. The seeds were germinated in phenolic foam *Green-up* as shown in Figure 4.

We used 4 plants per vase and three repetitions per treatment, totalizing 216 *Eucalyptus* plants in this experiment.

The application of Al in the experiment was performed 36 days after plant growth in modified Hoagland nutrient medium (HOAGLAND; ARNON, 1950). During the first two weeks, the seedlings were grown in nutrient medium at 25% strength, and after this adaptation period, the nutrient medium was raised to 100% strength (Table 1). Aluminum was added to the nutrient medium in the form of $AlCl_3 \cdot 6H_2O$ (BASSO *et al.*, 2007), with the following concentrations: 0 mM of $AlCl_3 \cdot 6H_2O$ (control) and 1 mM of $AlCl_3 \cdot 6H_2O$. During the growth of plants, the nutrient medium was prepared at pH 5.8. Upon application of the experimental conditions, the pH of the nutrient medium was changed to 4.2 using HCl. The samples were collected at 0 hours (before the addition of $AlCl_3 \cdot 6H_2O$), 24 hours and 48 hours for

cultivations with and without aluminum. After collecting leaves and roots, we analyzed lipid peroxidation and the amount of hydrogen peroxide.

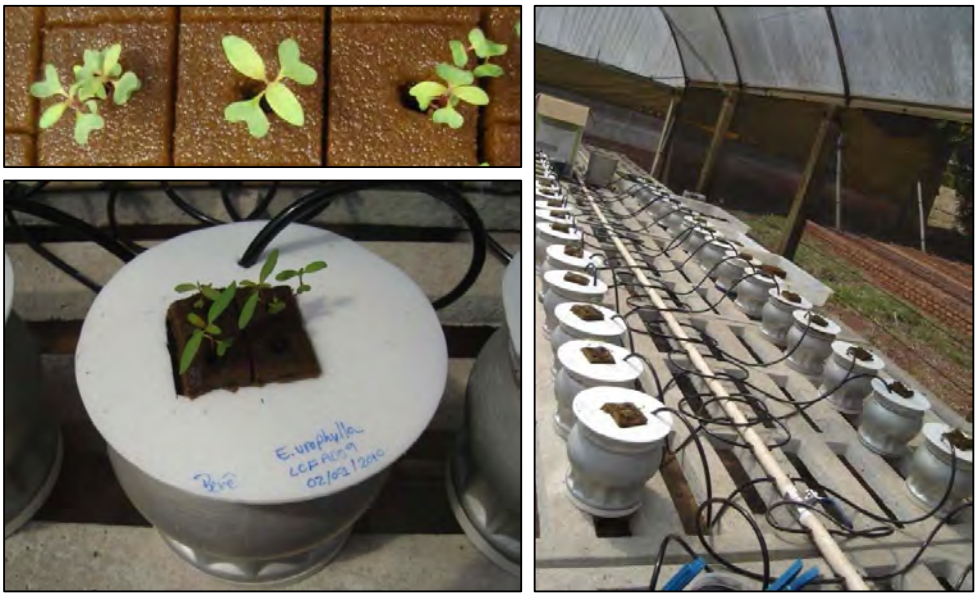


Figure 4 - Structure of first hydroponic experiment in a phenolic foam *Green-up*

Table 1 - Nutrient solution (pH 4.2) utilized in the second approach performed in a hydroponic system

Macronutrients	**Stock ($g \cdot L^{-1}$)**	**Volume (ml)**	**Final [ ] ($g \cdot L^{-1}$)**
KNO_3	50.5	10	0.505
KH_2PO_4	13.6	10	0.136
$MgSO_4$	49.2	10	0.492
$Ca(NO_3)_2 \cdot 4H_2O$	118	10	1.18
Micronutrients			
$MnSO_4$	4.52	1	0.00452
$ZnSO_4$	0.55	1	0.00055
H_3BO_3	7.2	1	0.0072
Na_2MoO_4	0.225	1	0.000225
$CuSO_4$	0.02	1	0.00002
Na_2EDTA	3.72	1	0.00372
$FeSO_4 \cdot 7H_2O$	2.78	1	0.00278

b) Lipid peroxidation and hydrogen peroxide

Oxidative damage was biochemically quantified based on Cakmak and Horst (1991). Metabolites reactive with 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 (1):

$$C = [ABS(535 - 600) \div 155] \times 10^6 \quad (1)$$

The results were expressed as nmol MDA·g^{-1} fresh matter.

We determined the levels of hydrogen peroxide according to methods described by Alexieva et al. (2001). Leaves and roots of *Eucalyptus* were homogenized in 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 *g* for 15 min at 4 °C. Following centrifugation, 200 µL of the supernatant was added to 200 µL of 100 mM potassium phosphate buffer (pH 7.0) and 800 µL of 1 M KI. Readings were taken at a wavelength of 390 nm.

2.2.3 Experimental approach 3

In the second approach, the nutritive solution was not suitable for the Al-stress experiment because of the presence of Na_2EDTA and $FeSO_4 \cdot 7H_2O$ that complex and precipitate Al in the solution. Additionally, the amount of KNO_3 was not ideal for this type of experiment because this nutrient increased the pH of the solution, making Al less available to the plants. Therefore, in addition to KNO_3, nitrogen was also supplemented in the form of $(NH_4)_2SO_4$. In this new approach, we modified the nutritive solution for use in the Al-stress experiments that are detailed as follows:

a) 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). We used 12 *Eucalyptus* plants per container and four plants per three repetitions totalizing 108 plants in this experiment. After six months, the seedlings were transferred to a hydroponic system (Figure 5) containing the Hoagland and Arnon (1950) nutrient solutions 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 pH 4.0. The nutrient solution was supplied with $AlCl_3 \cdot 6H_2O$ at concentrations of 0, 10, or 100 µM. The roots were collected for analysis 0, 24 and 48 h after Al exposure.

Figure 5 - Structure of the third approach in a hydroponic system

b) 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% (v/v) in deionized water and was immediately used in inductively coupled plasma - optical emission spectroscopy (ICP-OES).

c) Relative root growth

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

d) Lipid peroxidation

In addition to biochemical quantification (see item 2.2.2 – method b), in approach 3, the oxidative damage was also 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 deionized 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 color.

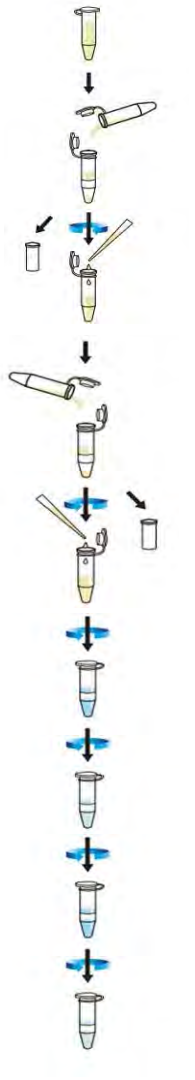
e) Statistical analysis

The experiment was completely randomized, using three repetitions per treatment. Analysis of variance (ANOVA) was performed using the Statistica software package (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%.

f) RNA extraction and transport to U.S.

Total RNA extraction was performed using a kit (#74904, RNeasy Plant Mini Kit, Qiagen). The details are shown in Figure 6.

Following RNA extraction, we quantified and evaluated total RNA in a NanoDrop (NanoDrop® ND-1000 UV/VIS, von NanoDrop Technologies) and in 2% agarose gel. RNA samples were stored in glycogen to permit their transport at room temperature. This procedure was based on testimony from colleagues who have transported RNA from Piracicaba to Rio de Janeiro and obtained good results using this method of transportation.



1) Lyse the cell wall and membranes in buffer;

2) Filter the lysate;

3) Discard the filter and add the ligand solution;

4) Transfer the lysate with RNA ligand solution into a new tube and filter this mixture;

5) Keep the filter (with RNA) and discard the filtrate;

6) Treat the filter with DNase on column to eliminate genomic DNA;

7) First wash;

8) Second wash; and

9) Elute RNA.

Figure 6 - Steps of total RNA extraction

2.3 Results

2.3.1 Experimental approach 1 – root medium culture

After setting a protocol for seed sterilization and germination *in vitro*, we observed that the amount of plant material, especially the roots, was insufficient for all of the intended analyses. Therefore, an effort was made to optimize an *in vitro* culture for root production. Table 2 shows the results of four different methods of root growth in liquid medium culture JADS (CORREIA et al., 1995) supplemented with 0.5 $mg \cdot L^{-1}$ indole-3-butyric acid. We

observed that there is no significant difference between the growth in ambient light and dark, but there is a difference regarding the presence/absence of agitation.

Table 2 - Mean values of the Fresh Weight (FW) and Water Content (WC). Evaluation after 15 days of growth

Treatment	FW (g)	WC (g)
Agitation/light	0.826±0.352 A	0.786±0.320 A
Agitation/dark	0.830±0.290 A	0.788±0.273 A
No agitation/light	0.372±0.115 B	0.352±0.112 B
No agitation/dark	0.252±0.038 B	0.238±0.041 B

In the columns, the means followed by the same letter do not differ significantly ($p < 0.05$)

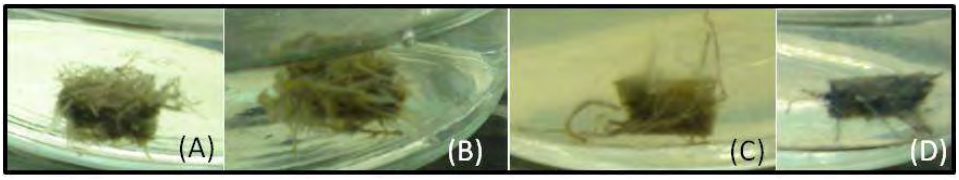


Figure 7 - Root culture in liquid medium JADS: (A) under agitation/light; (B) under agitation/dark; (C) no agitation/dark; (D) no agitation/light

Although the roots in the liquid medium culture under agitation grew better, the amount of roots produced *in vitro* after 15 days was still not enough to carry out all the analysis required for the proposed experiment; moreover, the flasks should be placed under agitation, and insufficient equipment to agitate the calculated amount of flasks was available. Thus, the experimental system was redesigned and modified for a hydroponic system.

2.2.4 Experimental approach 2

In this second approach, we aimed to test a hydroponic system for *Eucalyptus* to study Al stress in the roots of this tree species. Although this approach was not corrected for the effects of the nutritive solution utilized for the experiment, we show some results regarding lipid peroxidation and hydrogen peroxide. As we note in Tables 3 and 4, we did not observe any significant increase of MDA production within the species; however, in *Eucalyptus*

grandis x E. camaldulensis (E. grancam), the content of H_2O_2 increased in the first 24 hours of Al stress, although after 48 hours it was reduced.

Table 3 - Contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the roots of *Eucalyptus* species

Roots	**Species**					
	E. urophylla		***E. grancam***		***E. urograndis***	
	MDA	**H_2O_2**	**MDA**	**H_2O_2**	**MDA**	**H_2O_2**
Time 0 (solution pH 5.8)	0,4 a	0,39 A	0 mM Al 0,48 a	0,14 B	0,38 a	1,05 B*
Time 24 hours (solution pH 4.2)	0,63 a	0,97 B	0 mM Al 0,45 a	0,63 A	0,34 a*	1,02 B
	0,65 a	0,45 A	1 mM Al 0,54 a	0,53 A	0,51 a	0,43 A
Time 48 hours (solution pH 4.2)	0,48 a	0,62 A	0 mM Al 0,62 a	0,48 A	0,49 a	0,69 A
	0,61 a	0,96 B	1 mM 0,44 a	0,25 B	0,42 a	0,48 A

In the columns, the means followed by the same capital letter do not differ in H_2O_2 content, while the means followed by the same lower-case letter do not differ in MDA content within the species. In the rows, the asterisk symbolizes different averages between the species ($p < 0.05$)

Table 4 - Contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the leaves of *Eucalyptus* species

Leaves	Species					
	E. urophylla		***E. grancam***		***E. urograndis***	
	MDA	**H_2O_2**	**MDA**	**H_2O_2**	**MDA**	**H_2O_2**
Time 0 (solution pH 5.8)	6,607 b*	6,46 A	21,637 a	5,17 A	17,91 a	7,13 A
Time 24 hours (solution pH 4.2)	21,72 a	6,08 A	23,28 a	7,56 A	34,32 b*	6,75 A
	27,90 a	11,41 B*	24,41 a	5,74 A	19,97 a	4,3 A
Time 48 hours (solution pH 4.2)	20,35 a	9,84 A	21,84 a	9,95 A	32,29 b*	6,55 A
	32,51 c	7,16 A	27,01 a	8,5 A	20,61 a*	4,68 A*

In the columns, the means followed by the same capital letter do not differ in H_2O_2 content, while the means followed by the same lower-case letter do not differ in MDA content within the species. In the rows, the asterisk symbolizes different averages between the species ($p < 0.05$)

2.2.5 Experimental approach 3

In this experiment, we aimed to understand Al toxicity in *Eucalyptus* because information regarding the dynamics of toxicity and the recovery potential following metal exposure was largely lacking from the literature.

To estimate oxidative stress, we quantified the concentration of malondialdehyde (MDA), a product of lipid peroxidation. Interestingly, roots treated with 100 µM $AlCl_3$ for 24 h exhibited higher levels of MDA than the roots exposed to the same concentration of $AlCl_3$ for 48 h (Figure 8). 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 24-h treatments with 100 µM $AlCl_3$ exhibited the highest levels of lipid peroxidation in the transitional region of the roots (Figure 9).

To assess whether lipid peroxidation was occurring simultaneously with root growth inhibition, we measured the roots during the treatments and 24 h prior to the beginning of the

experiment. A reduction in root elongation of approximately 32% was observed following 100 µM $AlCl_3$ exposure for 24 h; however, there was no inhibition after 48 h (Figure 10).

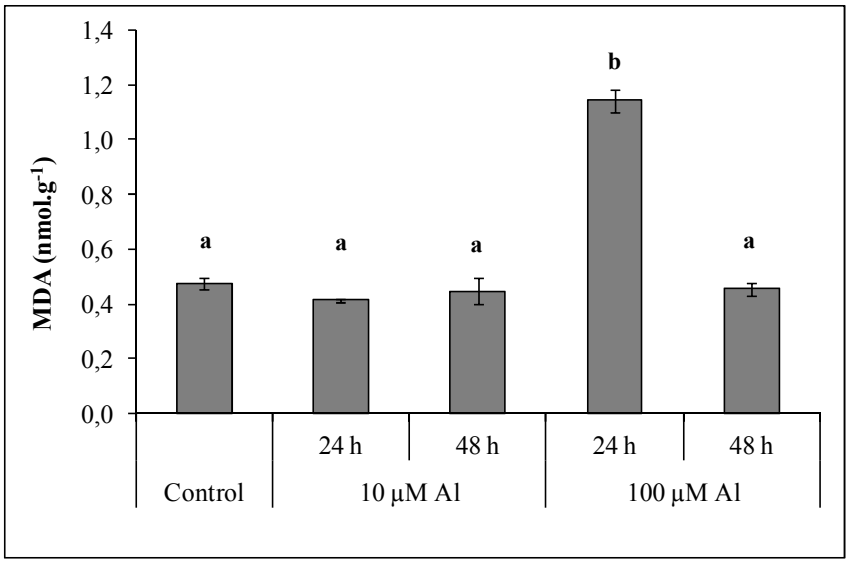


Figure 8 - Malondialdehyde (MDA) quantification to estimate lipid peroxidation in the roots of *E. grandis* × *E. camaldulensis* under Al stress. Bars with the same letters indicate no significant difference between the means at the 95% confidence level using Duncan's test. The error bars represent the standard error (n = 3)

Figure 9 - Histochemical analysis of lipid peroxidation in the roots of *E. grandis* × *E. camaldulensis*. (A) Control, before the application of $AlCl_3$; (B) 24 h after the application of 10 µM $AlCl_3$; (C) 48 h after the application of 10 µM $AlCl_3$; (D) 24 h after the application of 100 µM $AlCl_3$; and (E) 48 h after the application of 100 µM $AlCl_3$. The scale bar represents 1 mm

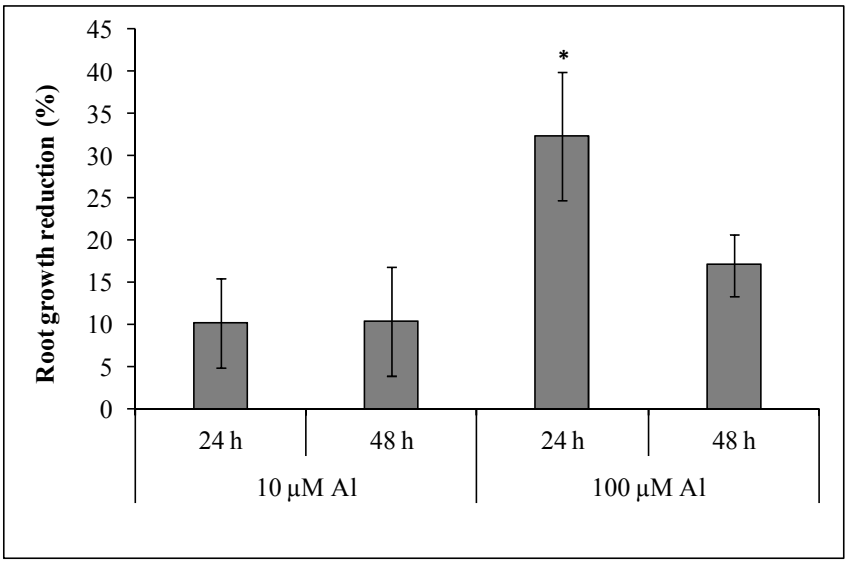


Figure 10 - Reduction in the root growth of *E. grandis* × *E. camaldulensis* exposed to 10 µM and 100 µM Al compared to the control 24 h before Al application. The asterisk indicates a significant difference at the 95% confidence level. The error bars represent the standard error (n = 12 plants)

The reduction in cell growth has been shown to be related directly to Al uptake and accumulation inside the cell (VITORELLO; HAUG, 1999). Therefore, 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 48 h was sufficient to increase root growth and reduce the lipid peroxidation levels (Figure 11).

In an attempt to analyze gene expression of *Eucalyptus grandis* × *E. camaldulensis*, total RNA extracts were kept in Brazil and then sent to the United States (Figure 12). These materials were stored in glycogen to allow for the transport of RNA at room temperature.

However, after the student's arrival in the United States and analysis of the RNA quality using the Bioanalyzer platform, we observed that the RNA was degraded and unable to be used in expression analysis. We do not recommend RNA transport using glycogen; instead, the shipping should be performed with the use of RNAlater® to avoid degradation during transportation.

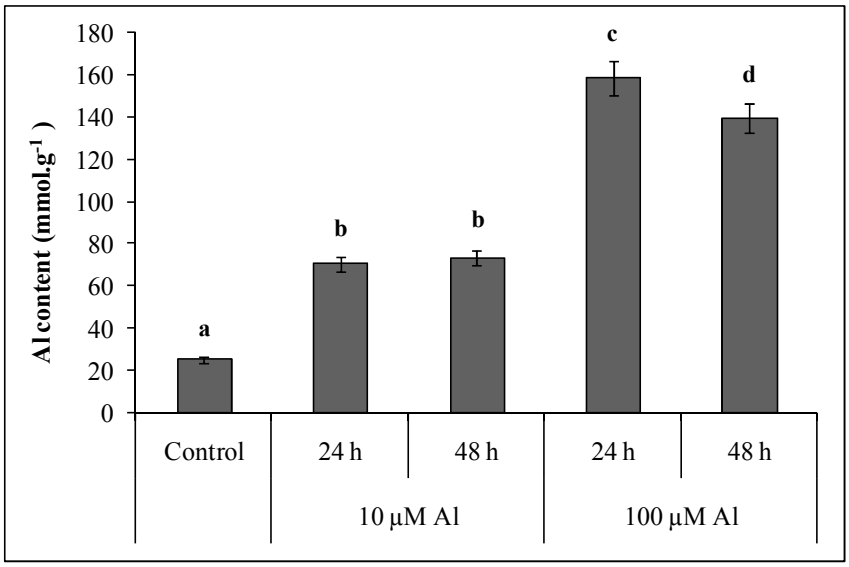


Figure 11 - Aluminum quantification in *E. grandis* × *E. camaldulensis* roots. Bars with the same letters indicate no significant difference between the means at the 95% confidence level. The error bars represent the standard error (n = 3)

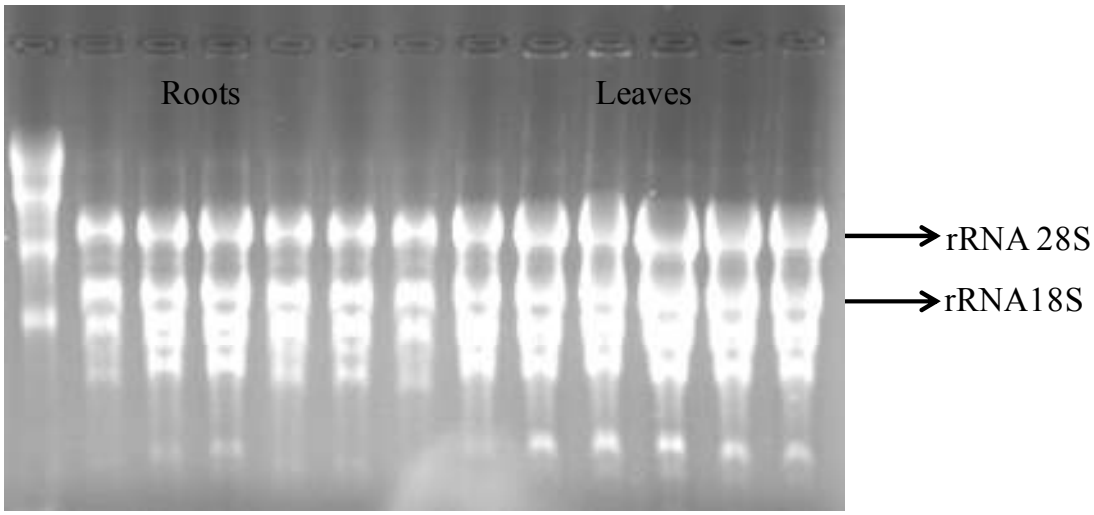


Figure 12 - Agarose gel of total RNA of *Eucalyptus grandis* x *E. camaldulensis*

2.4 Discussion – Experimental approach 3

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 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 24 h of stress). Interestingly, we observed the highest levels of lipid peroxidation in the transitional region of the roots. This result is consistent with other reports that found that the root distal transition zone is a critical site for the perception of Al toxicity (SIVAGURU; HORST, 1998; SIVAGURU et al., 2013). However, subsequently (after 48 h of stress), the roots were able to maintain rates 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 24 h of exposure to 1 mM Al (NGUYEN et al., 2003; TAHARA et al., 2005); however, growth was normal after 20 days of Al exposure (TAHARA et al., 2005). Tahara et al. (2008) found that the root growth inhibition of *E. camaldulensis* occurred after 5 days of exposure to 1 mM Al. According to these data, the growth recovery shown by *E. camaldulensis* when exposed to 1 mM Al occurred between 5 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 48 h. This result suggests that the time for recovery depends on the Al concentration, and it is possible that the rapid response observed in the hybrid was inherited from the parent, *E. camaldulensis*.

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. However, 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; some 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 the sequential citrate rinses to remove exchangeable Al in the apoplast. Considering that high levels of Al in the roots were still detected after 48 h of exposure to the metal in the present study, it is likely that avoidance mechanisms are occurring at the cell wall level, contributing to the swift recovery of *E. grandis* × *E. camaldulensis*.

Marschner (1991) proposed that the inhibition of root growth, particularly by Al, may affect the capacity for mineral nutrient acquisition, raising the risk of drought stress. This paper also suggested that plants adapted to adverse soil conditions employ two primary

strategies: tolerance and/or avoidance. According to Marschner (1991), the mechanism of avoidance is more commonly used for adaptation to acidic soils. More recent reports have suggested a third strategy that involves cell death (REIS et al., 2011). Furthermore, it has been suggested that it is possible to increase drought resistance through the inhibition of cell death signaling.

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 48 h of Al exposure, which may reflect growth recovery during this period. However, further research regarding the correlation between cell death signaling and lipid peroxidation in the *Eucalyptus* species is necessary.

It is also possible that the rapid Al 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 their leaf area and an increase in ground water absorption by their 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 allow 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 that more fully elucidates the joint action between mechanisms for tolerance to drought and Al in *E. camaldulensis* is needed.

The present work contributes information regarding the dynamics of the Al stress response in *E. grandis* × *E. camaldulensis*, demonstrating a rapid 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, 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.

2.5 Conclusions – Experimental approach 3

The data produced in this experiment raised interesting questions regarding how the duration of exposure impacts the toxicity of Al in *E. grandis* × *E. camaldulensis*. We observed that the hybrid exhibited an increased rate of lipid peroxidation after 24 h of stress; however, after 48 h, the levels of lipid peroxidation were reduced to normal levels. A similar pattern was observed for root growth inhibition, which implies a swift recovery.

We suggested new directions for additional work to correlate cell death signaling and lipid peroxidation in *Eucalyptus* as well as studies of the coupling of tolerance mechanisms to Al and drought.

Although was not possible to test the suggested hypothesis regarding lignin-genes expression, 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-stress response.

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SECTION 3 - WORK DEVELOPED IN THE UNITED STATES – MEETING THE TRIPARTITE PROGRAM REQUIREMENTS

In Section 2, when transporting samples of eucalyptus RNA, we found that the applied method of transport was not the most appropriate for long distances. Therefore, we decided to perform an experiment using maize to test the hypothesis that seed priming with acidic water or an $AlCl_3$ solution can induce tolerance to Al stress. After the first trial, we were able to propose a hypothesis that seed priming could increase hydrogen peroxide in the seeds, signal that is important to germination, and we performed further experiments to validate this hypothesis. In Section 3, we present the details of these experiments, their results and possible explanations based on the literature.

3 ALUMINUM TOLERANCE IN MAIZE ROOTS INDUCED BY SEED PRIMING WITH ACIDIC WATER OR $ALCL_3$ SOLUTION

Abstract

Poor fertility of acidic soils (pH < 5.5) is often caused by aluminum (Al) toxicity. Al causes oxidative damage in maize (*Zea mays* L.) roots, which can reduce productivity and growth rate. There are a number of studies published regarding the use of seed priming to increase yields under abiotic stresses, but the present study is the first to analyze the induction of Al tolerance in the Al-sensitive inbred line B73 using a seed priming technique. We tested two potential seed priming treatments, using acidic water and an Al solution. For this experiment, seeds of the inbred maize line B73 were primed with acidic water and with a 50 μM $AlCl_3$ solution (both at pH 4.0) for 6 hours. The seeds were then soaked in distilled water overnight and germinated at 25°C in the dark for 3 days on water-moistened paper towels or on paper towels wetted with 50 μM $AlCl_3$. Unprimed seeds were soaked in distilled water at pH 7.0 overnight and then germinated under the same conditions. After 3 days, we observed that under Al stress, the unprimed seeds exhibited a delay in germination. Considering root growth, the primed seeds exhibited significantly longer roots than the unprimed seeds, whereas there was no significant difference between seeds primed with acidic water and the Al solution. Quantitative real-time PCR (qRT-PCR) was used to analyze the effect of seed priming on the expression of lignin-related genes. In the roots, we observed that seed priming increased the expression of genes related to lignin biosynthesis, whereas the expression of a negative regulator of lignin was reduced in maize when germination occurred in association with the Al solution. The induction of lignin gene expression could be affecting the impact of Al on lipid peroxidation through Al chelation, as we observed a low malondialdehyde content when seeds were primed and germinated under stressed conditions. Further hypotheses and questions are raised in this section that will be answered in the subsequent sections.

Keywords: Aluminum; Seed priming; Maize; Germination; Lignin genes expression

3.1 Introduction

3.1.1 The importance of studying Al stress in maize

The United States and Brazil are among the major producers of corn in the world. In term of corn production, the U.S. is ranked first, with 313.9 million tons per year (mt/y); Brazil is third, with 55.6 mt/y; and China is second, with 192.7 mt/y (FAO, 2011). However, considering the average productivity, the U.S. is ranked eleventh in the world rank, with approximately 10 mt/ha; China is forty-first, with 5 mt/ha; and Brazil is sixty-third, producing approximately 3 mt/ha (FAO, 2011).

The presence of exchangeable aluminum (Al^{3+}) associated with acidic soils is one factor that may restrict agricultural productivity in the United States, China and especially in Brazil (Figure 13). For example, Jordan, the country with the highest average of corn yield (20 mt/ha), has no acidic soils presenting the problem of exchangeable aluminum (FAO, 2011).

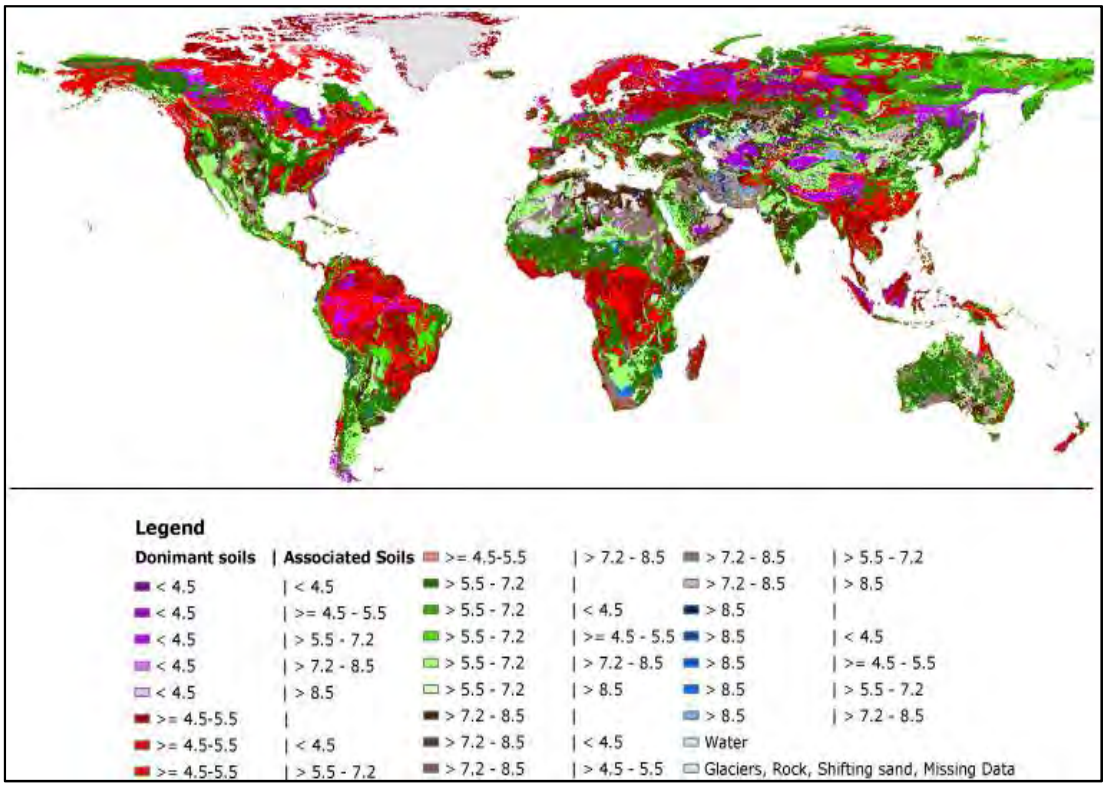

Figure 13 - Distribution of soil pH levels around the world. Source: FAO-UNESCO (2007)

After oxygen and silica, aluminum (Al) is the third most abundant element in the Earth's crust and occurs mainly in the form of alumino-silicate minerals (MA, 2007). An increase in H^+ ions in the soil water facilitates the release of aluminum ions, making it available and toxic to plants (MA, 2007). In acidic soils (pH < 5.5), an excess hydrogen level disrupts the crystal structure of soil particles, resulting in the release of Al^{3+} ions and allowing them to attach to sites of cation exchange, substituting for cationic nutrients, such as calcium (LAWRENCE et al., 1995).

Approximately 50% of the potential arable lands in the U.S. exhibit soils with Al^{3+} toxicity, and 20% of all corn planted in the U.S. is found in regions with soils showing this type of toxicity (KOCHIAN et al., 2009). In Brazil, the data is even more extreme because

approximately 97% of the potential arable area presents the problem of exchangeable aluminum in the soil (Table 5).

Table 5 - Territorial areas of the largest countries of the America Continent, their potential arable land areas (POTALAN) and Al toxicity areas (ATA)

	Total area	**POTALAN**	**ATA**	**% Al area**
Country		**'000 km^2**		**ATA/ POTALAN**
Canada	9,835	1,253	428	34.16
USA	**9,460**	**3,543**	**1,790**	**50.52**
Brazil	**8,563**	**5,493**	**5,354**	**97.47**
Argentina	2,780	905	12	1.33
Mexico	1,960	521	44	8.45
Colombia	1,139	655	636	97.10
Bolivia	1,093	619	338	54.60
Venezuela	929	550	514	93.45
Others	2,123	727	353	
Total	**37,882**	**14,266**	**9,469**	

Source: http://www.fao.org/corp/statistics/en/

Absorption of aluminum is required for the manifestation of toxicity in roots and cells in suspension (VITORELLO; HAUG, 1999; VITORELLO et al., 2005). Once inside the cell, aluminum may interfere with core related processes, such as DNA synthesis (VALADEZ-GONZÁLEZ *et al.*, 2007) and cell division (YI *et al.*, 2009). Some studies show that inhibition of root growth due to Al^{3+} ions may occur within hours of exposure to micromolar concentrations (KOCHIAN et al., 2004 and references therein). In maize, aluminum causes oxidative stress (BOSCOLO et al., 2003), reduces photosynthetic capacity and electron transport rates, and negatively affects the accumulation of nutrients in shoots (LIDON; BARREIRO, 1999).

To enable agricultural production to be carried out in acidic soils, farmers lime the soil (YAMADA, 2005). However, without organic matter, the effect of lime is observed only in the first 10 cm of soil and extends to approximately 20 cm when it is bound to organic waste (CASSIOLATO et al., 2000). Considering agricultural inputs, after petroleum, the second most limiting input is phosphorous (ELSER, 2012), however it is pertinent to remember that

lime is also a non-renewable natural resource that can eventually be exhausted. In Brazil, soil acidity occurs through a natural formation process, especially in the region of "cerrado" (Figure 14), which contain high levels of Al^{3+} (MENDONÇA et al., 2006; SILVA et al., 2009).

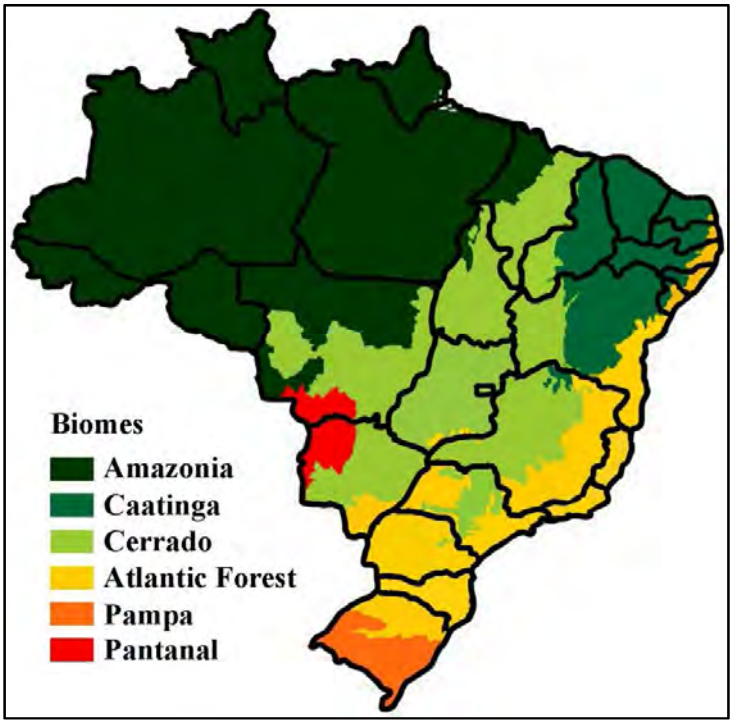

Figure 14 - Biomes of Brazil. In light green the region of "cerrado" which presents the highest Al levels in Brazilian soils. Source: http://siscom.ibama.gov.br/monitorabiomas/

In the United States, beyond natural processes (CIOLKOSZ et al., 1989; LUNDSTRÖM et al., 2000), there is also an effect of pollution from agricultural and industrial activities, particularly in seven states of the Ohio River Valley region (responsible for 41% of the emissions of toxic gases in the United States), which is related to the increased deposition of acidic rain in soils of the northeastern United States (CRETAZ; BARTEN, 2007). The distribution of acidic soils and the pH of rainfall in the United States are illustrated in Figures 15 and 16.

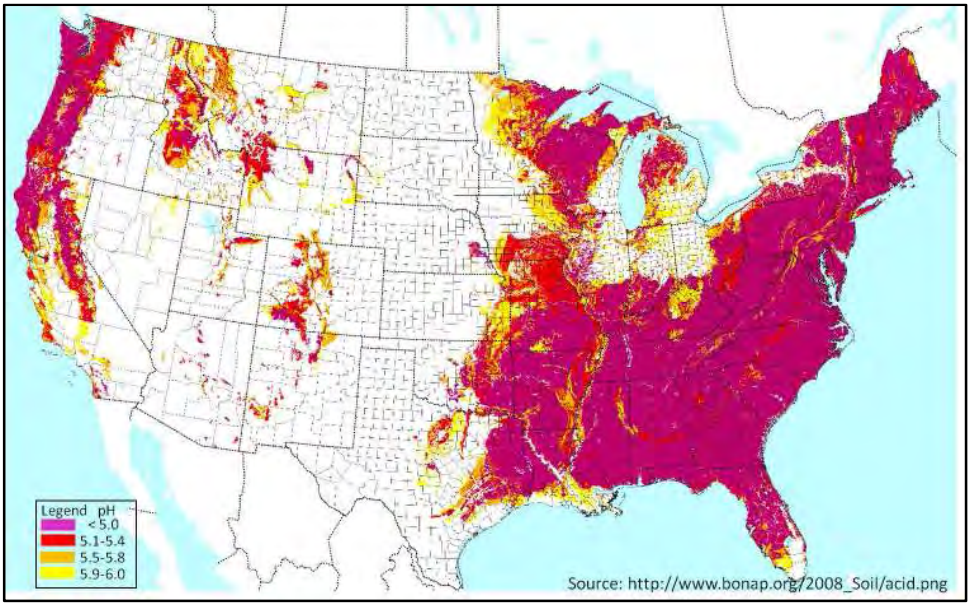

Figure 15 - Distribution of acidic soils in the United States

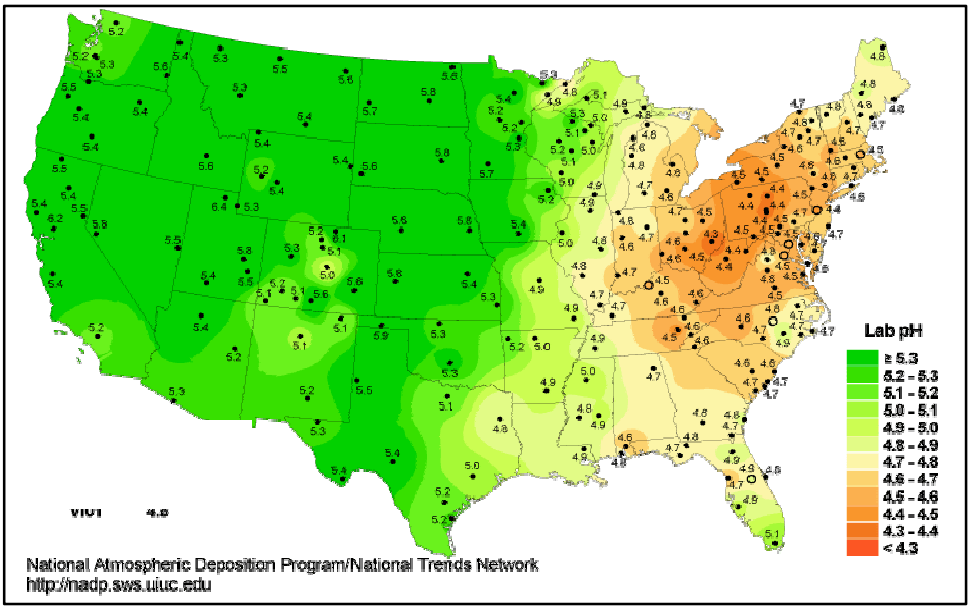

Figure 16 - Distribution of the pH of precipitation in the United States

There are studies showing that some plant species are tolerant to phytotoxic amounts of aluminum (NGUYEN et al., 2003; TAHARA et al., 2008), and differences exist even among cultivars of the same species (BASU et al., 1994). In corn, for example, there are some varieties that are genetically tolerant to such stress (Table 6). However, the line B73, which is the reference genome of maize, is sensitive to aluminum stress (PIÑEROS et al., 2005).

Table 6 - Maize varieties and their characteristics related to Al susceptibility

Varieties	**Source**	**Characteristic**
SA-3	GRANADOS et al. (1995)	Tolerant
SA-8		Tolerant
S1587-17	BOSCOLO et al. (2003)	Sensitive
C100-6		Tolerant
Cateto-Colombia	PIÑEROS et al. (2005)	Tolerant
B73		Sensitive
L53		Sensitive
11 X 723		Sensitive
Tuxpeño	PELLET et al. (1995)	Sensitive
Mo17	PIÑEROS et al. (2002)	Sensitive

In addition to genetic resistance, some studies show that the induction of tolerance to abiotic stresses is possible using a technique that consists of conditioning seeds prior to germinating them under stressed conditions (Table 7). The pre-sowing seed treatment techniques (a.k.a. seed priming) that have been developed to date include hydropriming, halopriming, osmopriming, thermopriming, solid matrix priming and biopriming (ASHRAF; FOOLAD, 2005). The increasing synchronization of germination and improvement of plant vigor caused by seed priming could occur because techniques such as osmopriming activate enzymes including amylases, proteases and lipases that are essential for the early growth and development of embryos (ASHRAF; FOOLAD, 2005) and turn on genes related to the minimization of stress, such as zinc finger proteins, osmotin, catalase, ascorbate peroxidase, glutathione-S-transferase, glutathione reductase, superoxide dismuatse and annexin which is related to cold stress (PATADE *et al.*, 2012). “Plant priming” was previously studied by Gratão et al. (2008), who evaluated a set of plants subjected to a stepwise increase in $CdCl_2$ levels and observed the induction of tolerance at the highest tested concentration of cadmium (1 mM). Currently, there are no available studies on seed priming or “plant priming” for the induction of tolerance to Al stress.

Table 7 - Treatments applied to maize seeds and the respective induced tolerances

Treatments	Tolerance to	Reference
KCl	Cold	FAROOQ et al. (2008a)
Chloric salts	Salinity	ASHRAF; HUMERA (2001)
Ethylene	Salinity	CARVALHO et al. (2011)

3.1.2 Al tolerance mechanisms: the relationship with lignin synthesis

According to Kochian et al. (2004), two mechanisms of Al tolerance can be found in plants: internal tolerance and exclusion. When the ions are tolerated in the cytosol, the mechanism is defined as an internal mechanism, which could involve complexation of Al^{3+} with polypeptides (TAYLOR et al., 1997), compartmentalization of Al in the vacuole and the synthesis of certain proteins (MA et al., 2007). Plants exhibiting internal tolerance mechanisms are usually referred to as hyperaccumulators and mainly comprise tree species from tropical and subtropical regions (VITORELLO et al., 2005). Exclusion mechanisms involve immobilization of Al ions in cell walls through forming a complex with lignin (EZAKI et al., 2005), increased rhizosphere pH levels, causing Al to remain insoluble (DEGENHARDT et al., 1998), and the release of organic acid anions, such as malate and citrate, which complex with Al in the rhizosphere and which is considered the most effective strategy (MA et al., 2001). However, Kochian et al. (2004) and Piñeros et al. (2005) showed that the most Al-sensitive maize variety (Mo17) produced the highest levels of citrate, indicating that other mechanisms of Al tolerance could act simultaneously in plants.

As mentioned previously, the production of lignin may also be related to aluminum tolerance. Ezaki et al. (2005) observed that increased production of lignin in transgenic *Arabidopsis thaliana* roots increased aluminum tolerance and decreased lipid peroxidation. However, excess lignin has negative economic impacts due to properties that present obstacles to chemical pulping, forage digestion and the conversion of polysaccharides into biofuels (UMEZAWA, 2010). Therefore, it is favorable to obtain plant material with lower levels of lignin or that contain lignin which is easily removed, while also being tolerant to aluminum. Thus, understanding how lignin biosynthesis may be affected by Al stress is a subject of great interest.

Lignins are complex aromatic heteropolymers synthesized through the oxidative coupling of p-hydroxycinnamyl alcohols (p-hydroxyphenyl (H), guaiacyl (G) and syringyl

(S)) (BOERJAN et al., 2003). These three units are compounds formed in the phenylpropanoid pathway (Figure 17), which provides the precursors of lignin, flavonoids and anthocyanins (FORNALÉ et al., 2010).

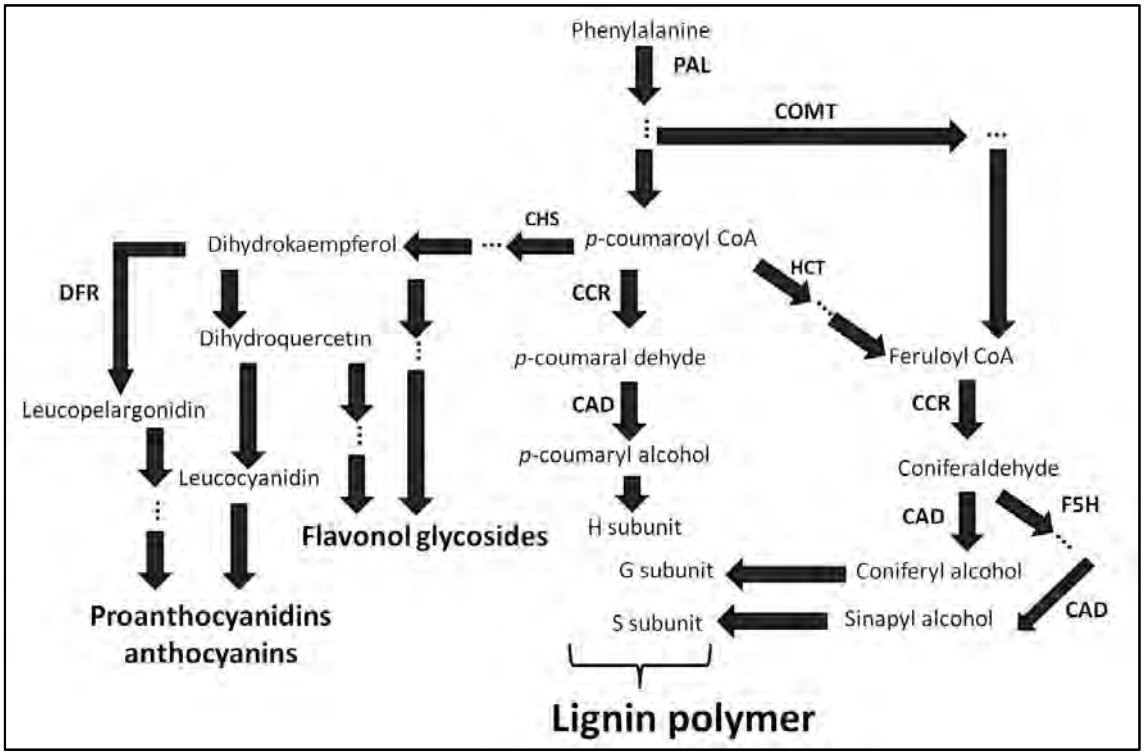

Figure 17 - Phenylpropanoid pathway, adapted from Fornalé et al. (2010). The H, L and S subunits represent p-hydroxyphenyl, guaiacyl and syringyl, respectively, which constitute the lignin polymer

The amount and composition of lignin varies among species, tissues and cell wall layers. For example, dicotyledonous angiosperm lignins possess mainly G and S units and only trace amounts of H units, whereas gymnosperm lignins are generally composed of G units, showing low levels of H units. Lignins from grasses (monocots) incorporate G and S units at similar levels and present more H units than dicotyledonous species (BOERJAN et al., 2003).

In maize, there are two transcription factors belonging to the R2R3-type MYB gene family that could be related in repress genes related to lignin biosynthesis: *ZmMYB*31 and *ZmMYB*42 (FORNALÉ et al., 2010; SONBOL et al., 2009). Over-expression of *ZmMYB*31 in *Arabidopsis thaliana* plants causes repression of genes related to lignin synthesis, including *COMT* and *F5H* (FORNALÉ et al., 2010). Generally, members of the MYB transcription factor family are responsible for regulating secondary metabolism, cell growth and shape and

the responses to hormones and some stresses, such as drought and viral infection (BRAUN; GROTEWOLD, 1999; DUBOS et al., 2010; FELLER et al., 2011). A number of studies have isolated R2R3-MYB genes and characterized their role in the tolerance to some stresses, such as salt, cold, light, dehydration and pathogen attachement (SOITAMO et al., 2008; DUBOS et al., 2010; YANG et al., 2012).

Therefore, characterization of the expression of *COMT* and *F5H* and their transcriptional regulators under conditions of oxidative stress represents a relevant topic of research that aims to provide a better understanding of how lignin biosynthesis is affected.

3.1.3 Objectives

To induce Al tolerance in maize using seed priming and to examine how lignin might potentially be modulated, the objectives of this part of the study were as follows:

a) To verify the effects of seed priming techniques using an Al solution or acidic water;
b) To compare the stress parameters of unprimed and primed seeds;
c) To evaluate the expression of lignin genes;
d) To associate tolerance with stress parameters and the expression of lignin-related genes.

3.1.4 Hypothesis

Based on prior information about the induction of tolerance to abiotic stresses in maize using seed priming techniques and previous studies that have correlated lignin biosynthesis with Al tolerance, the following hypothesis can be formulated:

1) Seed priming using an Al solution could enhance growth under Al stress;
2) Low levels of lipid peroxidation might be observed because of tolerance induction;
3) We can expect the highest expression of lignin-related genes in roots from primed seeds.

3.2 Materials and Methods

3.2.1 Experimental Design

To test the hypothesis that seed priming with an $AlCl_3$ solution can induce Al tolerance in maize, an experiment was conducted using 72 seeds and the following treatments (illustrated in Figure 18):

1) Maize seeds from the inbred line B73 left unprimed (control), sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of distilled water (pH 7.0), in the dark at 25 °C;

2) Maize seeds from the inbred line B73 left unprimed (control), sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of a 50 µM $AlCl_3$ solution, in the dark at 25 ºC;

3) Maize seeds from the inbred line B73 primed with acidic water (pH 4.0) for 6 hours, sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of distilled water (pH 7.0), in the dark at 25 °C;

4) Maize seeds from the inbred line B73 primed with acidic water (pH 4.0) for 6 hours, sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of a 50 µM $AlCl_3$ solution, in the dark at 25 °C;

5) Maize seeds from the inbred line B73 primed with a 50 µM $AlCl_3$ solution (pH 4.0) for 6 hours, sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of distilled water (pH 7.0), in the dark at 25 °C;

6) Maize seeds from the inbred line B73 primed with a 50 µM $AlCl_3$ solution (pH 4.0) for 6 hours, sterilized in 1% sodium hypochlorite for 15 minutes and soaked in water overnight; germination conducted on paper towels (sterile) moistened with 5 ml of 50 µM $AlCl_3$ solution, in the dark at 25 ºC.

The experimental design consisted of three biological replicates in which four seeds were used in each Petri dish.

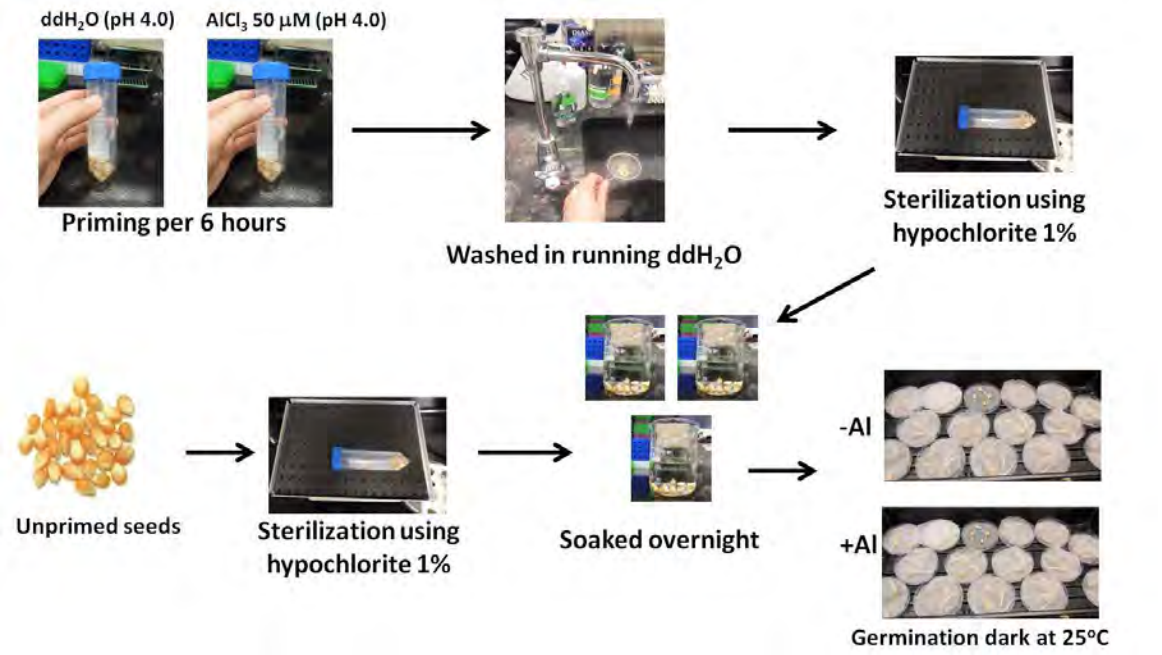

Figure 18 - Scheme of the experiment testing seed priming with acidic water or an Al solution

3.2.2 Evaluation of stress parameters

Initially, we evaluated the percentage of germination and the germination index (GI = $\sum(Gt/Tt)$ where *Gt* is the number of seeds germinated at time *Tt* in days (DEZFULI et al., 2008). After three days of germination, we also evaluated the length of the roots and the absorption of Al in the roots via inductively coupled plasma optical emission (ICP-OES, Optima 3000 DV, Perkin Elmer, at Trace Element Research Laboratory, OH, USA).

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. These readings were performed using a spectrophotometer at 535 and 600 nm, and the concentration of MDA was determined with the following formula:

$$C = [ABS(535 - 600) \div 155] \times 10^6 \quad (1)$$

The results were expressed as nmol MDA·g^{-1} fresh matter.

Statistical analyses of these data were performed using StatSoft software (version 7.0, Tulsa, OK, USA). Significant differences between the averages of the treatments were determined by performing the Duncan test, with a confidence level of 95%.

3.2.3 RNA extraction and cDNA synthesis

Approximately 100 mg of fresh material was macerated in liquid nitrogen, and extraction of total RNA was performed using a kit (#74904, RNeasy Plant Mini Kit, Qiagen). After extraction, the total RNA was treated with DNase (On-Column DNase I Digestion Set, DNASE70-1SET, Sigma) for 20 min at room temperature. Quantification of total RNA was performed using a nanodrop machine. Approximately 100 ng of total RNA was used to obtain cDNA with an Invitrogen kit (SuperScript™ II RT, 18064-014, Life Technologies). Oligo-dT primers were used to bind the polyA tails of the mRNAs to initiate the synthesis of first-strand cDNA with reverse transcriptase. The obtained cDNA samples were treated with RNase. To confirm the effectiveness of reverse transcription, we utilized primers targeting the *ACTIN*, *UBIQUITIN* and *PAL* genes for amplification via conventional PCR. Visualization of the amplicons was performed in 2% agarose gels supplied with ethidium bromide. Amplification through conventional PCR was conducted in a final volume of 25 µl, containing 2.5 µl of 10x buffer supplied with Mg^{2+} (E00007, GeneScript), 0.2 mM dNTPs, 1.0 µM forward primer, 1.0 µM reverse primer, 0.05 U/µl of Taq polymerase and 100 ng of cDNA. The reactions were performed in a Peltier Thermal Cycler, model PTC-225 (MJ Research, Inc.), with the following amplification conditions: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, a melting temperature of 50°C, 57°C or 58°C for 45 s (for the actin, ubiquitin and pal primers, respectively) and 70°C for 1 min, with a final extension at 70°C for 10 min.

3.2.4 qRT-PCR expression analysis

First, the efficiency of the primers targeting sequences of interest was checked by performing quantitative real-time PCR (qRT-PCR) using iQ SYBR Green Supermix (170-8882, Bio-Rad) and two different concentrations of cDNA (10 ng and 100 ng) with iCycler equipment (BioRad). The primers were designed to bind the exons of genes related to lignin biosynthesis (http://www.maizesequence.org) with the help of Dr. Katja Machemer-Noonam. The primers tested for qRT-PCR targeted *UBQ* and *ACT* (reference genes) *PAL*1, *COMT*1,

CCR, *CAD*, *LAR*, *FLS*, *DFR*, *F5H*, *C3H* and *ZmMYB*31 (Table 8). For expression analysis, we selected primers that had only one peak in the melting curve and *E* value between 70% and 130% in qRT-PCR, parameters that indicates the specificity of the reaction and efficiency of the primers, respectively.

Table 8 - Primer sequences used for tests of specificity and efficiency

Gene	**Primer sequence (5' - 3')**	**cDNA product length**
ACT	F: CTTCGAATGCCCAGCAAT R: CGGAGAATAGCATGAGGAAG	153
UBQ	F: GTGAGTCGTGACTGAGCTGGTT R: ATATGCGGTCGCACGATAGTT	73
FLS	F: TGCCCATCCGCCCAGAGACT R: ACGACTTGGAGGCCCTGGCA	303
DFR	F: CGGCGAGAGGACTGGGACGA R: TGCACGAGGCCGACCGTTTC	270
LAR	F: ATTCCGCTGAGCGAGCGAGC R: CCCGTTTCCTCCGGTCACGC	119
CCR	F: GAGCGGGCGCGAGCTAGAAG R: ACCGCCGGCTCCACCATTTG	428
CAD	F: GCGTCGACAAGGGGCTCACC R: CGAACGAACGTTGCACGCGG	223
F5H	F: GCAGGAGTTCTCGAAGCTGT R: TGCTCGTCGATGATCTTGTC	147
C3H	F: CCCTGTTTTGAAACCTGCTG R: TGCAGTACACAAAATGCTCGT	103
*PAL*1	F: CGCGGAGCAGCACAACCAGG R: AGCTCGCCCGAGGGGTTCAT	222
*COMT*1	F: TCTGCGTCGAATTGTCTCTGC R: GAGAGCAATTAAACCGCCATGT	85
*ZmMYB*31	F: GTAGGTGAAAAATACGCGATGG R: AAAATGGAGCAGCAAGAGAGAG	374

After confirming primer specificity and efficiency for phenylpropanoid-related genes, we performed expression analysis of the experimental materials. For each treatment, the relative gene expression (*RGEx*) was calculated by the formula $RGEx = 2^{(Ct_{rg} - Ctpg)}$, adapted from Morohashi; Grotewold (2009), where Ct_{rg} is the Ct value for reference gene and Ct_{pg} is the Ct value for phenylpropanoid related gene.

Statistical analyses of these data were performed using the StatSoft software (version 7.0, Tulsa, OK, USA). Significant differences between the averages of the treatments were determined by performing Duncan's test with a confidence level of 95%.

3.3 Results

3.3.1 Evaluation of stress parameters

Seed priming treatments have shown to improve germination percentages and germination index under abiotic stresses such as cold, drought and salt (FAROOQ et al., 2008b; FAROOQ et al., 2009; MOGHANIBASHI et al., 2012). It is well known that such stresses negatively affect germination; however parameters for the effect of aluminum on the germination of maize seeds is lacking in literature.

No differences were found among the treatments regarding the percentage of germination since we observed 100% of germination for all treatments. However, the germination index (GI) of unprimed seeds under Al stress (GI = 9.5) was lower than primed seeds (GI = 10) (Table 9).

Table 9 - Percentage of germination and germination index (GI) of maize seeds inbred line B73 unprimed and primed with acidic water and Al solution sown with or without Al stress

Treatment of seeds	**Germination**	**% germination**		**GI**
		2^{nd} day	3^{rd} day	
Unprimed	Distilled water	100	100	10
Water pH 4.0		100	100	10
Aluminum pH 4.0		100	100	10
Unprimed	Al solution	91.7	100	9.5
Water pH 4.0		100	100	10
Aluminum pH 4.0		100	100	10

Regarding the length of primary roots, when germination occurred on paper moistened with distilled water, it was observed that seeds primed with acidic water and Al solution promoted better growth (7.88 ± 0.11 cm and 7.48 ± 0.14 cm, respectively) than unprimed seeds (5.20 ± 0.48 cm) (Figure 19). Unprimed seeds germinated with Al solution had the shortest roots (4.33 ± 0.29 cm) and there were significant differences in root length between the treatments (6.23 ± 0.23 cm and 7.13 ± 0.12 cm for seeds primed with acidic water and Al solution respectively) (Figure 20). Notably, the treatment with acidic water was enough to improve growth under these circumstances.

The shortest roots had the largest amount of aluminum absorbed (8.05 ± 0.65 μg $Al \cdot g^{-1}$ dry matter) (Figure 21). There was no difference in the absorption of Al in the treatments with acidic water (5.00 ± 0.40 μg $Al \cdot g^{-1}$ dry matter) and aluminum solution (4.50 ± 0.30 μg $Al \cdot g^{-1}$ dry matter) when the seeds are germinated under stress. The Al-primed seeds germinated without stress possessed roots with small amounts of Al (1.5 ± 0.10 μg $Al \cdot g^{-1}$ dry matter), suggesting that the seeds absorb Al already during the 6 hours immersion treatment.

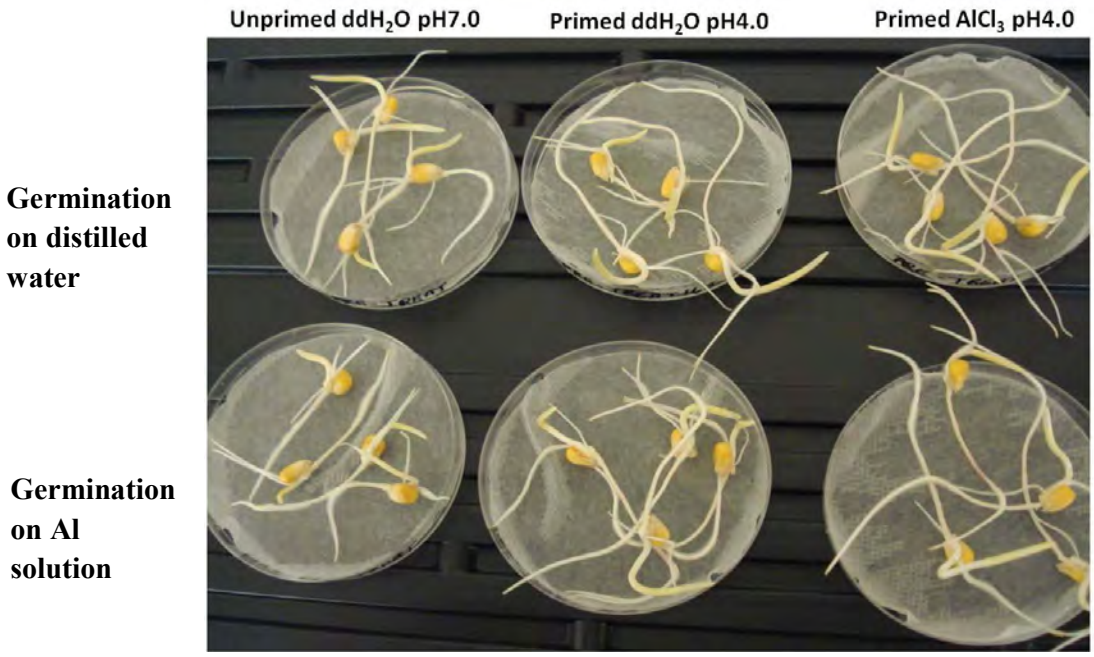

Figure 19 - Germination of maize seeds inbred line B73. Paper towels were humidified with distilled water or with 50 µM $AlCl_3$ solution. Seeds were primed with acidic water and Al solution. Unprimed seeds were used as control. Seedlings at three days after germination

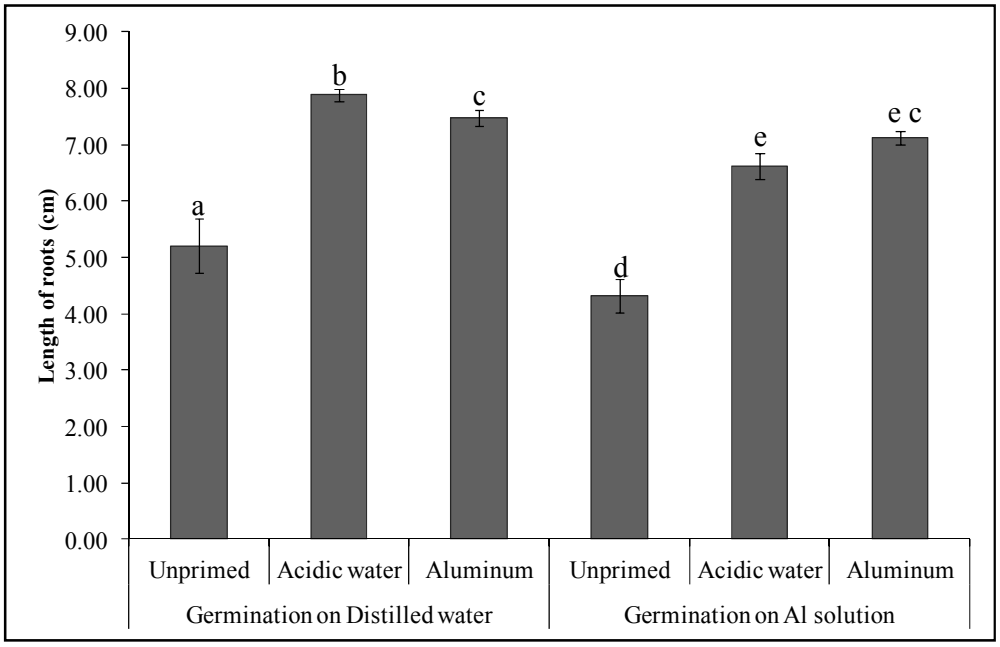

Figure 20 - Root length of maize inbred line B73. Seed treatments: unprimed, primed with acidic water and primed with Al solution. Seeds were germinated on paper towel humidified with distilled water or with 50 µM $AlCl_3$ solution. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n = 5)

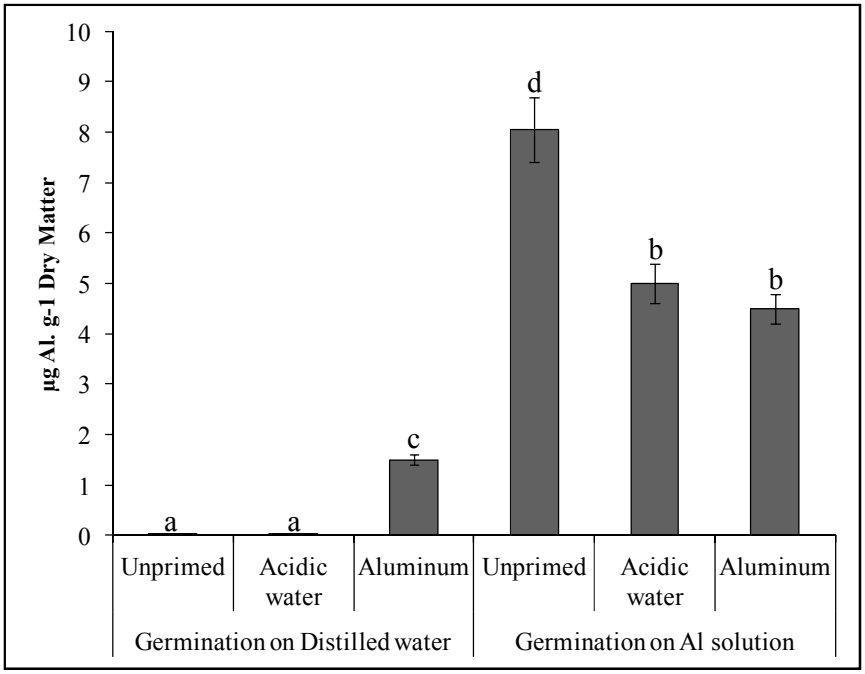

Figure 21 - Aluminum quantification in roots of maize inbred line B73. Seed treatments: unprimed, primed with acidic water and primed with Al solution. Seeds were germinated on paper towel humidified moistened with distilled water or with 50 µM $AlCl_3$ solution. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n = 2)

To confirm the assumption that the seeds were absorbing Al already during treatment, an experiment was conducted in order to observe the amount of aluminum absorbed in the seeds and to check in which tissue aluminum is absorbed. Accordingly, we performed the same treatments for maize seeds; however instead to place them for germination, pericarps were removed, and we separated the endosperm and embryo for Al quantification (Figure 22).

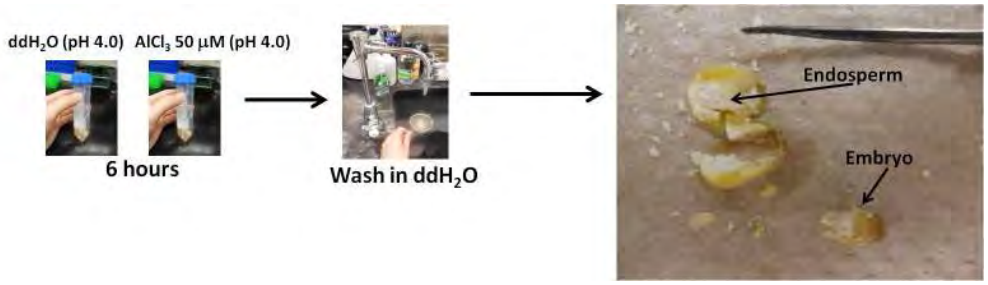

Figure 22 - Scheme of experiment for aluminum quantification in embryo and endosperm of maize seeds inbred line B73

The results show that seeds do absorb aluminum and that embryos absorb more Al (0.73 ± 0.01 µg Al·g^{-1} dry matter) than endosperm (0.41 ± 0.06 µg Al·g^{-1} dry matter) (Figure 23).

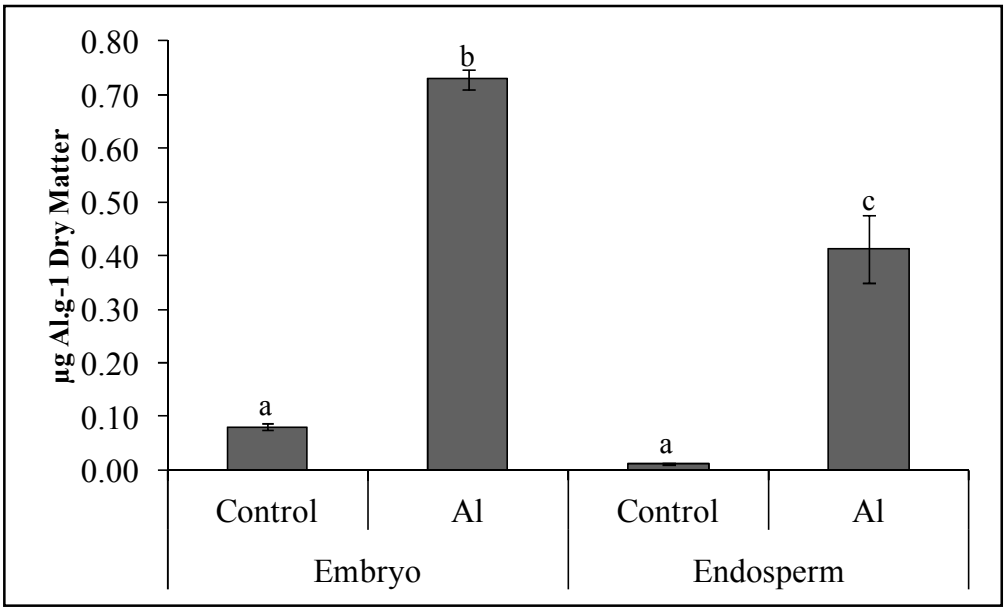

Figure 23 - Aluminum quantification in embryo and endosperm of maize seeds inbred line B73. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n= 3)

Continuing the analysis of maize roots, oxidative damage was assessed based on the quantification of MDA. The roots from unprimed seeds and germinated under Al stress had higher lipid peroxidation (0.034 ± 0.009 nmol MDA.mg^{-1} fresh matter) compared to the roots from seeds primed with acidic water (0.019 ± 0.003 nmol MDA·mg^{-1} fresh matter) or Al (0.016 ± 0.002 nmol MDA·mg^{-1} fresh matter) (Figure 24).

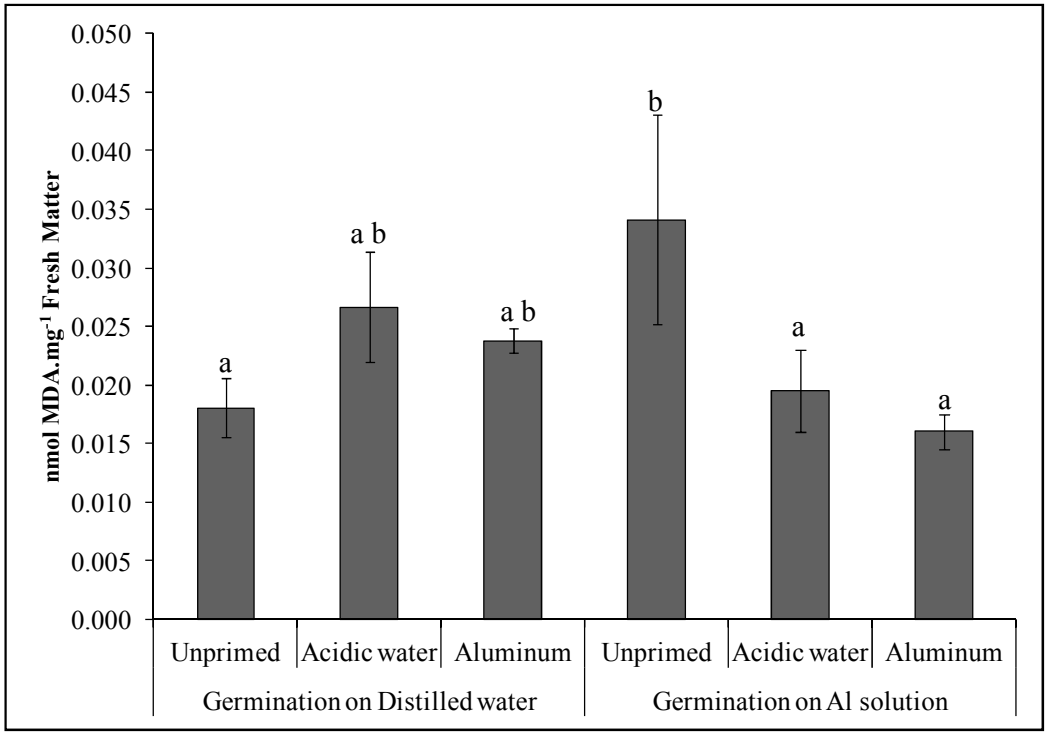

Figure 24 - Quantification of malondialdehyde (MDA) in maize roots inbred line B73. Seed treatments: unprimed, primed with acidic water and primed with Al solution. Seeds were germinated on paper towels humidified with distilled water or with 50 µM $AlCl_3$ solution. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n = 3)

3.3.2 Expression of lignin genes by qRT-PCR

Ezaki et al., (2005) observed in a transgenic line of *Arabidopsis thaliana* that the highest production of lignin is correlated with the lowest lipid peroxidation in the roots when are submitted to Al stress. Because lignin may be related to Al tolerance in the root, we performed an evaluation of gene expression involved in lignin biosynthesis by qRT-PCR. For this purpose, we performed RNA extraction and synthesized cDNA using reverse transcriptase. To confirm the effectiveness of reverse transcription, we performed conventional PCR using *ACTIN*, *UBIQUITIN* and *PAL* genes (Figure 25).

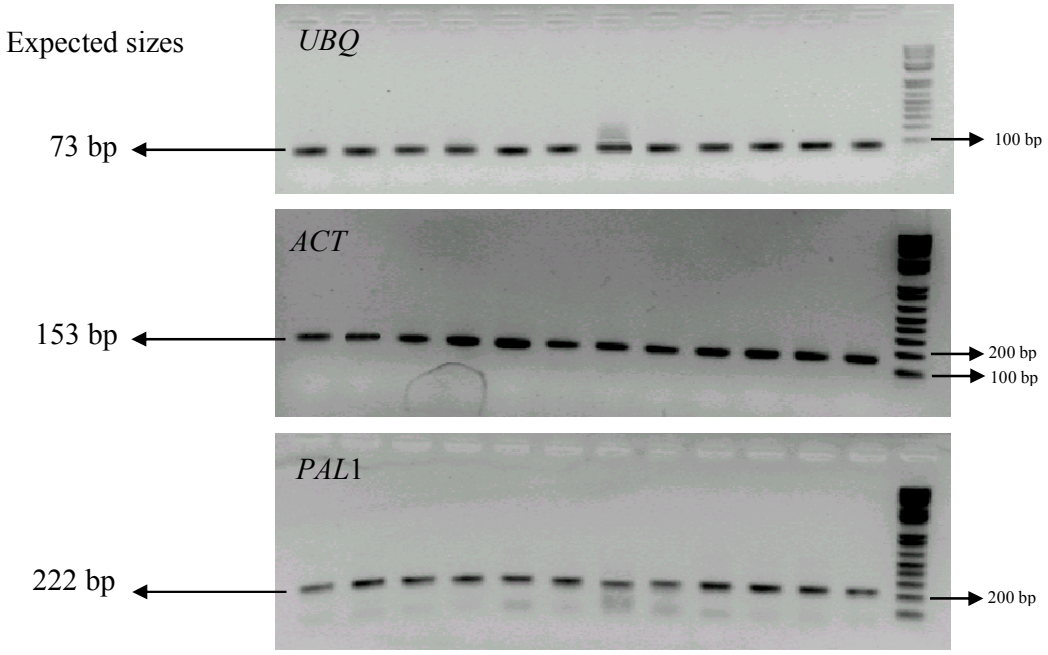

Figure 25 - Agarose gels to visualize amplifications by conventional PCR to confirm effectiveness of reverse transcription. Negative and positive controls are not shown

Furthermore, we tested the efficiencies of the primers targeting *UBQ* and *ACT* (reference genes), *PAL*1, *COMT*1, *CCR*, *CAD*, *LAR*, *FLS*, *DFR*, *F5H*, *C3H* and *ZmMYB31* via qRT-PCR. The test results are provided in Figures 26, 27, 28, 29, 30 and 31. Some primers (i.e., *FLS*) were not used in the expression analysis because they were ineffective and exhibited nonspecific amplification. The actin reference gene (*ACT*) was not used due to the obtained E = 163.7%, which reflects an efficiency that is higher than acceptable. We obtained an acceptable efficiency (E = 117%) for the ubiquitin gene (*UBQ*), which was therefore used as a reference gene in the present study. This decision was also made based on the work of Chen et al. (2011), who noted that *UBQ* is among the best reference genes for comparative expression analyses because of its stability, even following several treatments applied to maize seeds. The primers that exhibited only one peak in the melting curve and efficiencies between 70% and 130% were for the *UBQ* (reference gene), *PAL*1, *COMT*1, *CAD*, *F5H*, *C3H* and *ZmMYB31* genes.

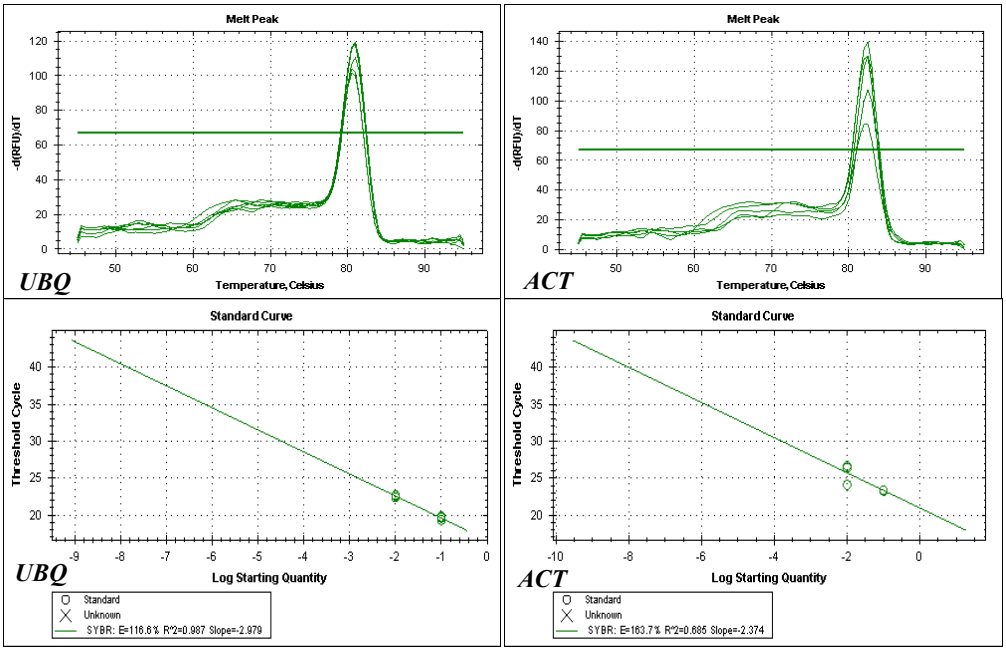

Figure 26 - Primer efficiency for *UBQ* and *ACT*

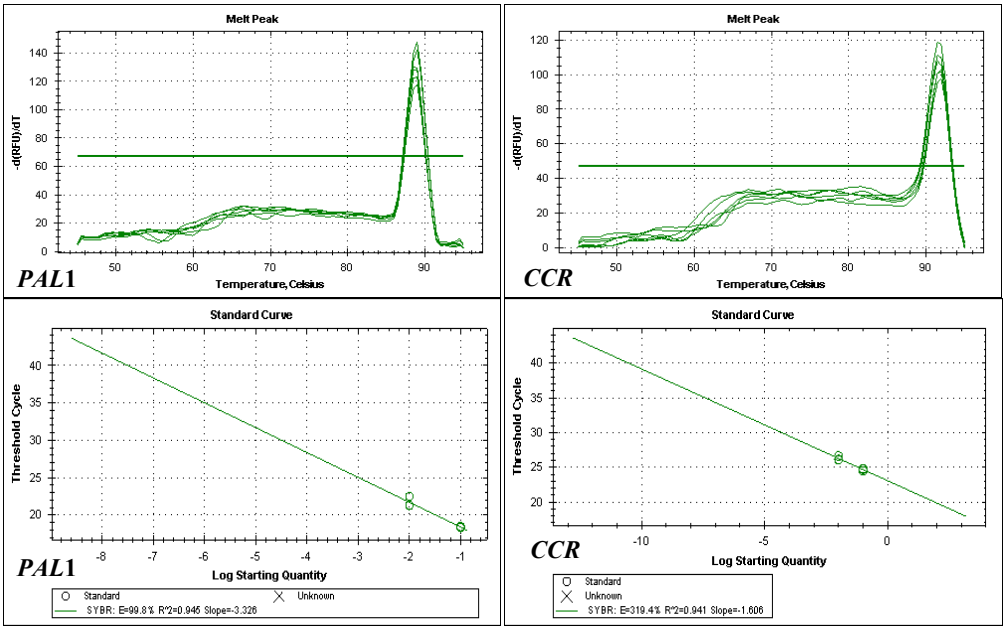

Figure 27 - Primer efficiency for *PAL*1 and *CCR*

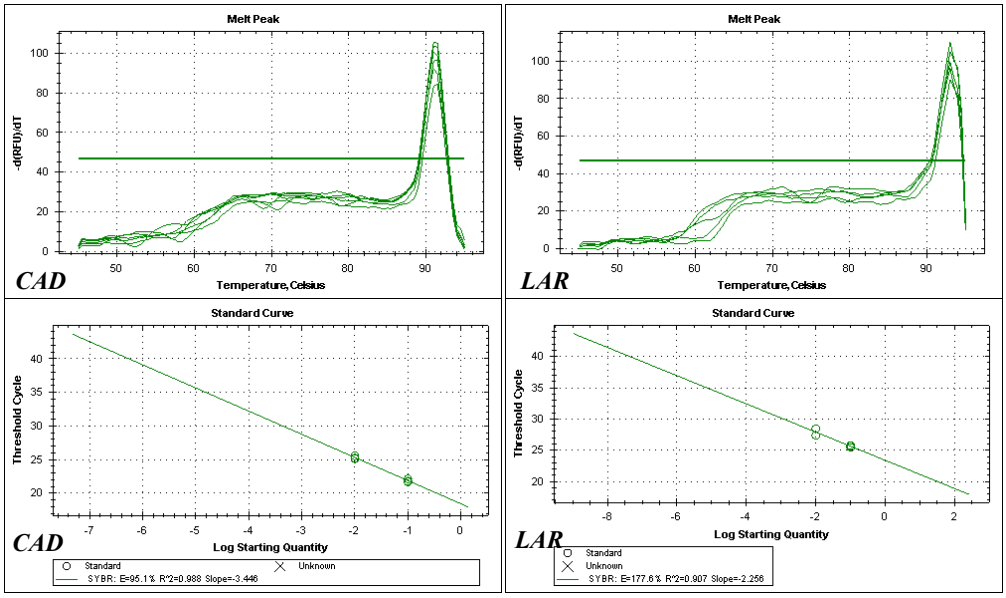

Figure 28 - Primer efficiency for *CAD* and *LAR*

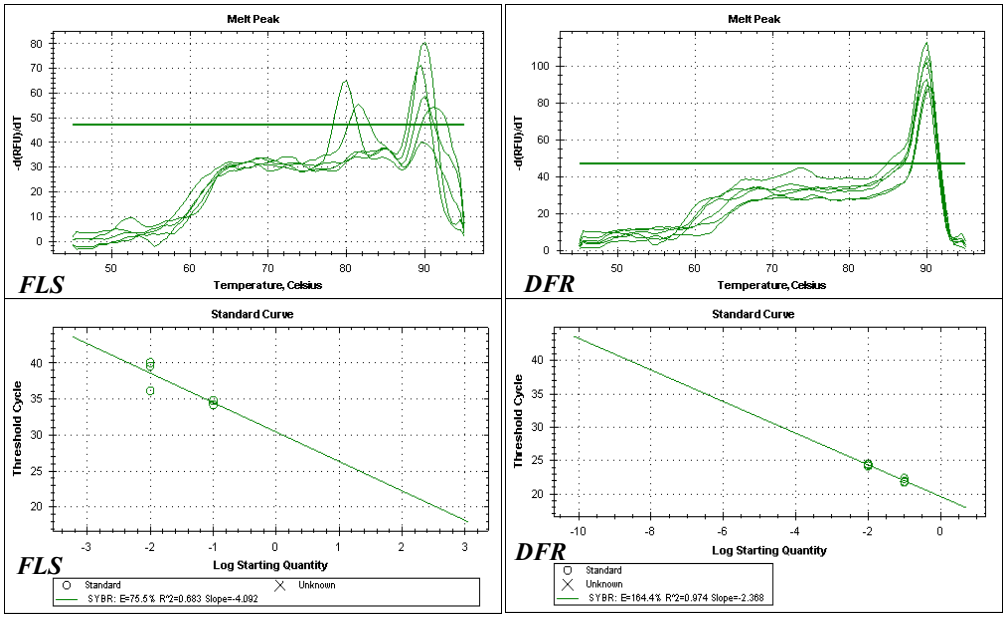

Figure 29 - Primer efficiency for *FLS* and *DFR*

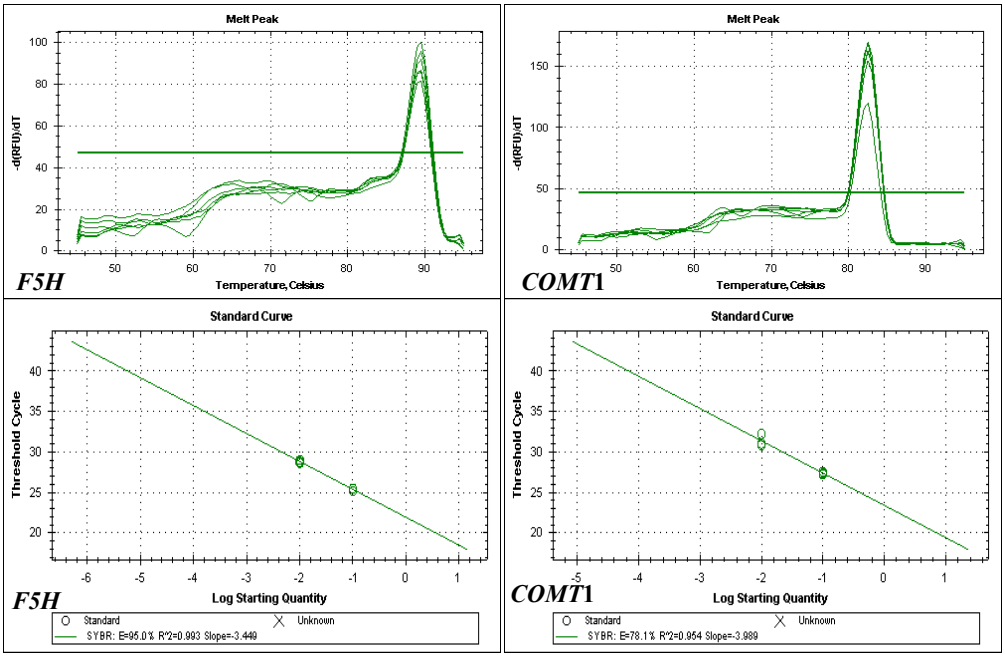

Figure 30 - Primer efficiency for *F5H* and *COMT*1

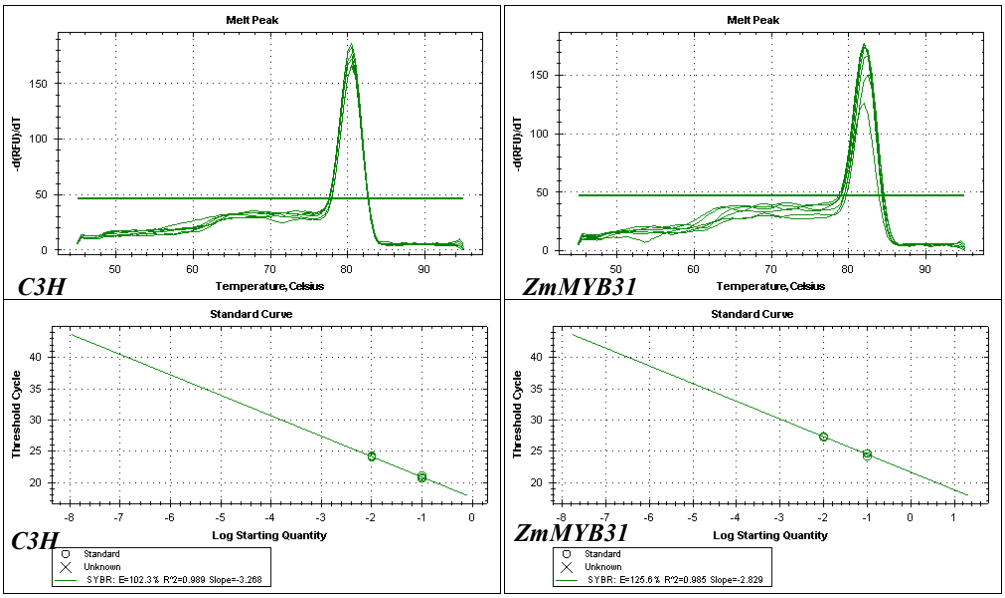

Figure 31 - Primer efficiency for *C3H* and *ZmMYB*31

The expression analysis of experimental materials was performed by qRT-PCR and we used the primers for *UBQ* (as reference gene), *PAL*1, *COMT*1, *CAD*, *F5H*, *C3H* and *ZmMYB31*. Interesting results were obtained for genes *PAL*1, *COMT*1, *F5H* and *CAD* that are shown in Figure 32. For *ZmMYB31*, a transcription factor that could be related to suppress lignin biosynthesis (FORNALÉ *et al.*, 2010), the results are exposed in Figure 33.

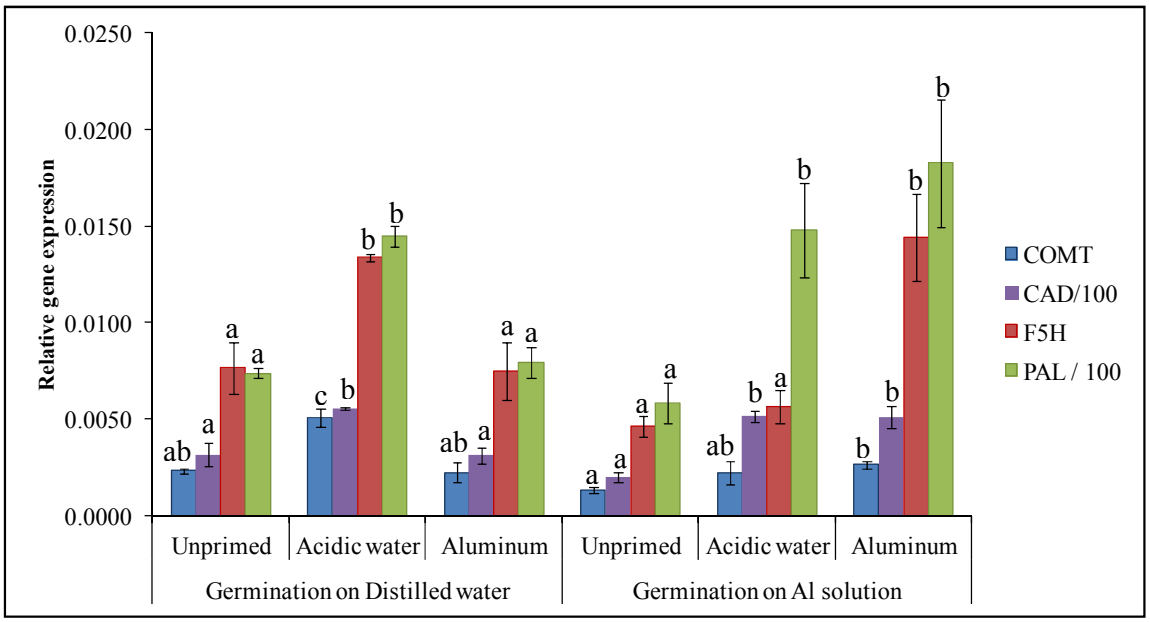

Figure 32 - Expression of genes related to lignin biosynthesis. Data of *PAL*1 and CAD were divided by 100 to simplify the visualization in the graph. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n = 3)

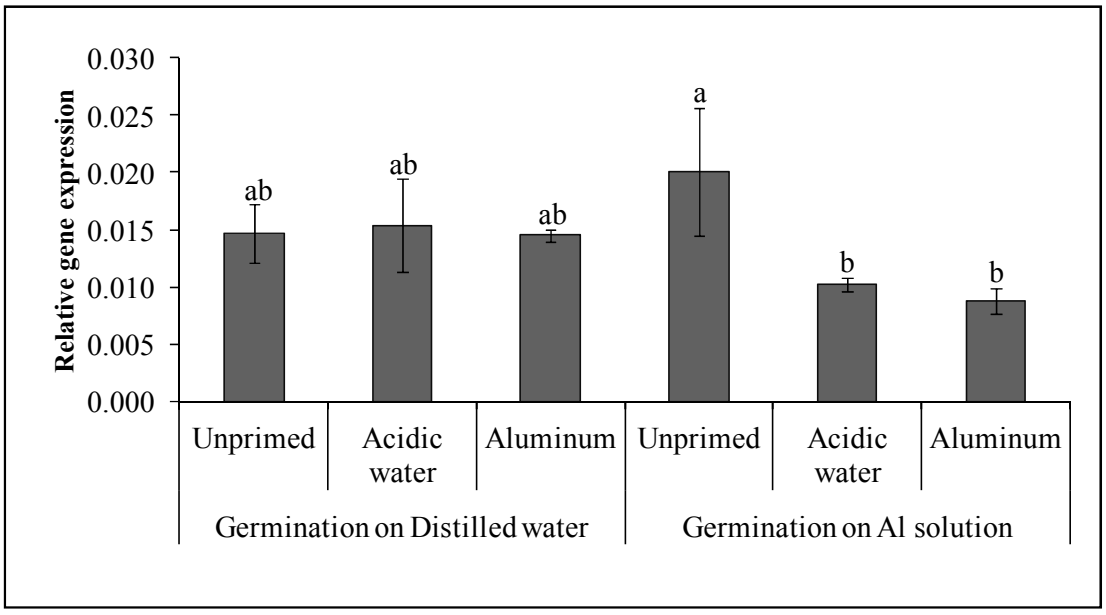

Figure 33 - Expression of transcription factor *ZmMYB*31. Bars with the same letters indicate no significant difference between the means at the 95% confidence level (n = 3)

We note that the expression of *PAL*1, which is responsible for the synthesis of phenylalanine ammonia lyase (PAL), increased when the seeds were primed with acidic water and germinated with Al (from 0.58 ± 0.10 to 1.48 ± 0.24) or without Al stress (from 0.74 ±

0.03 to 1.45 ± 0.05). It also increased when the seeds were primed with Al and germinated under Al stress (from 0.58 ± 0.10 to 1.82 ± 0.33). The specific genes related to lignin synthesis *COMT*1, *F5H* and CAD encoding the enzymes caffeic acid O-methyl transferase, ferulate 5-hydroxylase and cinnamyl alcohol dehydorgenase, respectively, showed an expression pattern similar to *PAL*1. However, the levels of *COMT*1 and *F5H* expression were 100 times lower than that of *PAL*1 and *CAD*.

When germination occurred without stress, there was no difference in the expression of *ZmMYB*31 detected among the treatments. However, when we analyzed gene expression under Al stress, there was consistency in the results observed for genes related to lignin, with the expression of the repressor decreasing (from 0.097 ± 0.0056 to 0.00095 ± 0.00054), and the expression of *COMT*1 and *F5H* increasing in the same treatments.

3.4 Discussion

We observed no differences among the treatments regarding the percentage of germination, most likely because we soaked all of the seeds overnight in water, which is a treatment that is sufficient to enhance seedling emergence, even under saline stress (ASHRAF; FOOLAD, 2005). Another possible explanation is that Al stress does not affect the germination percentage. In contrast, under Al stress, there was a delay in germination, as unprimed seeds exhibited a germination index (GI) that was lower than that of primed seeds. The postponement of germination may be reflected in a decrease of productivity because rapid germination implies early seedling establishment, increasing competitiveness over other species, such as weeds (RICE; DYER, 2001; ASHRAF; FOOLAD, 2005).

Many works have demonstrated that Al can attach to nucleic acids in the cells of the root meristem, affecting cell division (VALADEZ-GONZÁLEZ et al., 2007; YI et al., 2009; QIAO; MA, 2013), and the reduction of root growth observed for unprimed seeds under stress conditions could be occurring because of high levels of absorbed Al. An intriguing observation based on our data is that there was no difference in root length when seeds were primed with Al versus acidic water, suggesting that seed priming using the latter treatment is sufficient to improve growth, with or without Al stress.

The major question arising from these data is: What mechanism is initiated by seed priming with acidic water? A study addressing the role of alterations in the external pH in the regulation of gene expression found these changes to be related to cell wall modification, Ca^{2+}

signaling and hormone responses, i.e., for abscisic acid (ABA), auxin (AIA), brassinosteroids and salicylic acid (LAGER *et al.*, 2010).

We can partially explain our results based on the function of the hormone ABA, which is commonly known as a regulator of growth and stomatal opening (COSTA *et al.*, 2013). However, another important function of ABA is the regulation of seed maturation and dormancy (LIU *et al.*, 2010). During seed formation, the content of ABA can increase considerably within a few days, after which, during ongoing maturation, it might decrease to very low levels (GUTIERREZ *et al.*, 2007). Vivipary in maize was observed due to mutation of a gene that is responsible for reducing ABA levels in the embryo (BURBIDGE et al., 1999 and references there in). The role of ABA in controlling dormancy and quiescence in seeds is well known, although some works have recently demonstrated that the ratio between ABA and GA is also important as well as their antagonistic effects (PISKUREWICZ et al., 2008; LIU et al., 2010).

Hydrogen peroxide functions as a signaling molecule and is involved in a series of processes, including stress responses (SCANDALIOS, 2005), programmed cell death (PANDA *et al.*, 2008) and plant development (LIU *et al.*, 2010). Exogenous H_2O_2 decreases ABA levels by enhancing the expression of key genes encoding enzymes related to ABA catabolism, and H_2O_2 increases GA biosynthesis at the same time (Figure 34). Therefore, until H_2O_2 reaches a certain level, it also exhibits an important function as a promoter of seed germination by oxidizing germination inhibitors (LIU *et al.*, 2010). It is possible that in the present study, seed priming with acidic water could have induced H_2O_2 production, promoting ABA catabolism, which improves the germination index.

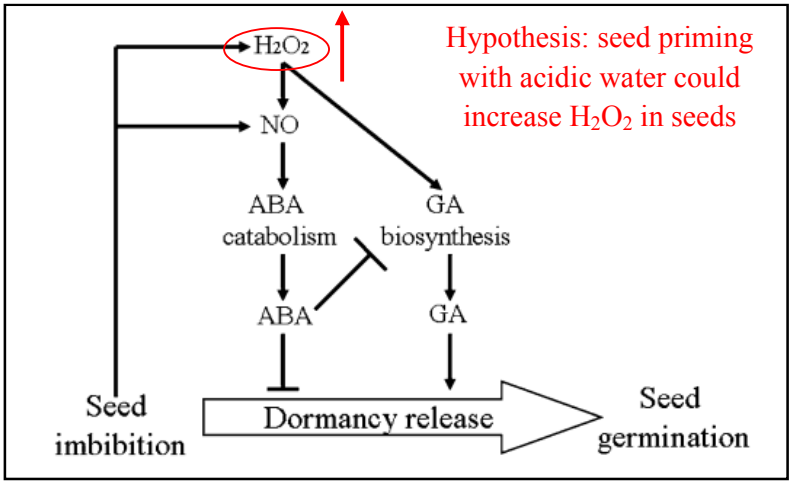

Figure 34 - Model of the regulation of seed germination from Liu et al. (2010). Our hypothesis that seed priming with acidic water increases H_2O_2 is presented in red

To test this hypothesis, an experiment was performed to quantify H_2O_2 levels in the embryo and endosperm. We primed B73 seeds as described in Materials and Methods, section 3.2.1. However, instead allowing the seeds to undergo germination, we opened them and separated the embryo and endosperm for H_2O_2 quantification (see Materials and Methods - Section 2). We noted high contents of hydrogen peroxide inside the embryo when seeds from the inbred line B73 were primed with acidic water (Figure 35). This result confirms our hypothesis that seed priming increases H_2O_2 levels in the embryo, which is an essential signal of seed germination.

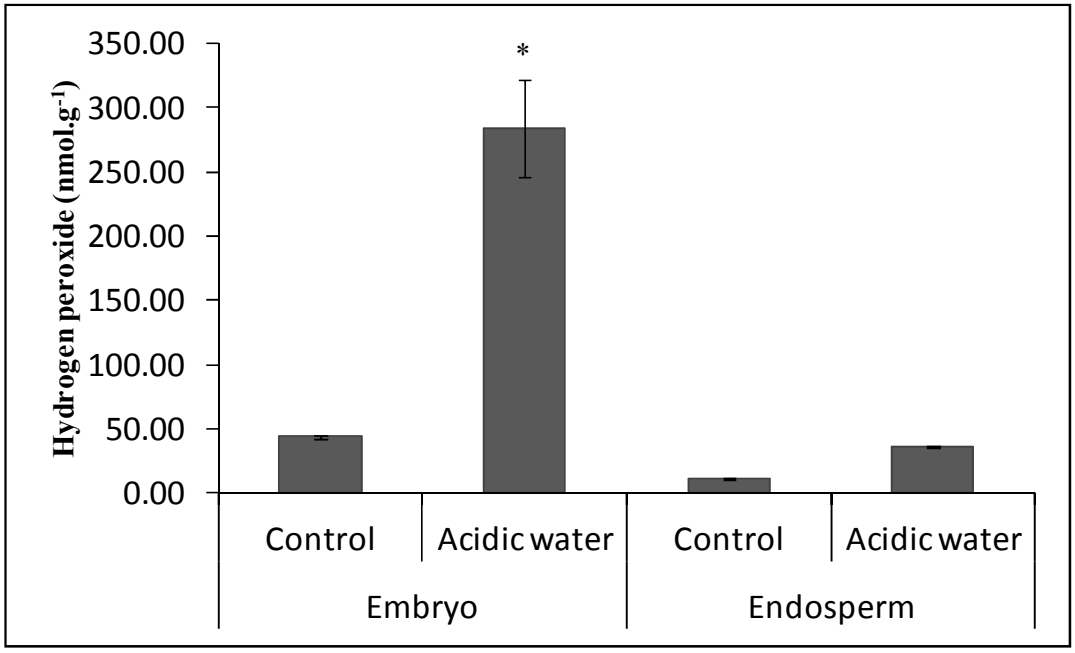

Figure 35 - Quantification of hydrogen peroxide levels in the embryo and endosperm of maize seeds (B73) primed with water at pH 6.0 (control) and seeds primed with water at pH 4.0 (acidic water). The asterisk indicates a significant difference ($p < 0.01$)

Considering Al stress, deposition of lignin in the cell wall of *Arabidopsis thaliana* could be characterized as a tolerance mechanism because it reduces oxidative damage in the roots of transgenic lines over-expressing the *AtBCB* or *NtGDI1* gene (EZAKI et al., 2005). Our data showed that seed priming up-regulated the expression of key genes involved in lignin biosynthesis (*COMT*1, *F5H* and *CAD*) in the roots and improved root growth. Additionally, there was a reduction of MDA content observed in primed seeds compared to unprimed seeds under Al stress, corroborating the findings of Ezaki et al. (2005). The *PAL*1 and *CAD* genes presented the same behavior, though its expression level was 100 times higher than those of *COMT*1 and *F5H*. These difference in expression for *PAL*1 could be explained by the fact that most phenolic compounds are derived from cinnamic acid, which is formed from phenylalanine through the action of phenylalanine ammonia-lyase (PAL), which represents the turning point enzyme between primary and secondary metabolism (HERRMANN, 1995) (Figure 36). Therefore, this enzyme could be required in greater quantities than *COMT*1 and *F5H*. In the case of *CAD*, a specific enzyme for lignin biosynthesis, the difference in expression could be explained by the formation of primary cell wall in young tissues that is composed mainly by H subunits, since the deposition of monoligonols in plant cell walls is spatially and temporally controlled, first being deposited H

subunits, followed by G, and finally S subunits (SANTIAGO et al., 2013). Rapidly growing cells are surrounded by thin primary cell walls while the secondary wall is laid down between the primary wall and the cell membrane when the growth has ceased (DEY et al., 1997). The difference in expression magnitude also could be explained because *CAD* is required for all the final steps of lignin subunits formation (Figure 36).

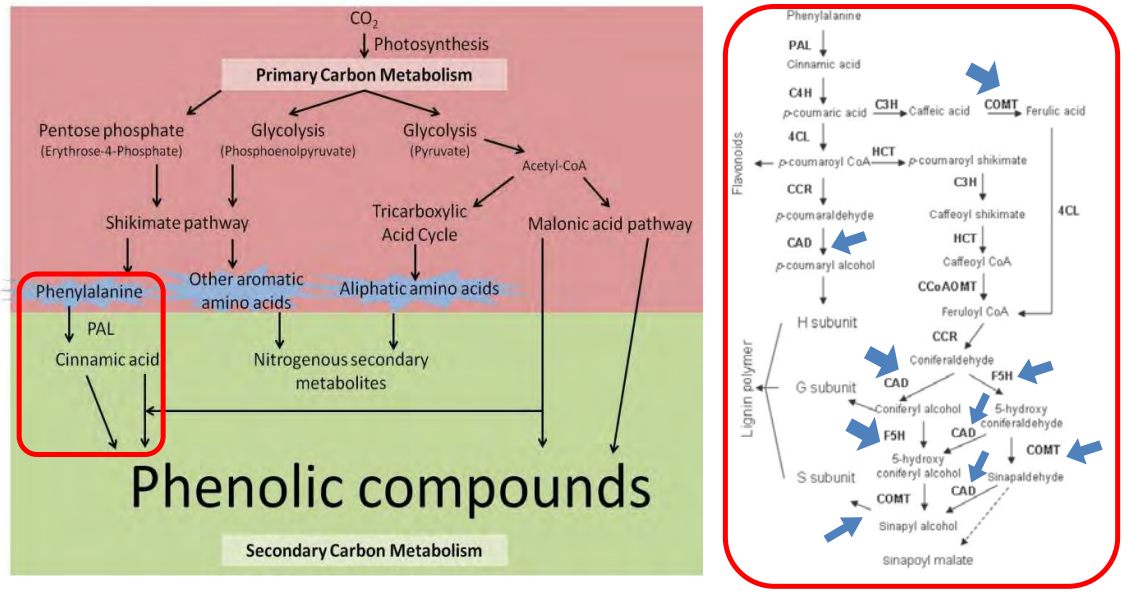

Figure 36 - Biosynthesis of phenolic compounds and their relationship with primary carbon metabolism (TAIZ; ZEIGER, 2009 adapted). The red rectangle highlights the phenylpropanoid pathway, which is shown in detail in the large red rectangle (FORNALÉ *et al.*, 2010). The blue arrows indicate the lignin genes that were focus of this work

It is important to highlight that there are phenolic compounds that are not phenylpropanoids because some of these compounds do not originate from phenylalanine. Changes in the quantity of total phenolic compounds were observed by Mccue et al. (2000) when they primed pea seeds with acidic water (pH 3.0). In this part of the discussion, we will focus on the effect of seed priming with acidic water on lignin as we studied genes related to lignin biosynthesis in the presented work.

Mccue et al. (2000) observed that after 2 days of germination, seed priming using acidic water increased the average total phenolic contents in pea leaves, suggesting that this treatment could temporally enhance lignification. Interestingly, after 10 days of germination, Mccue et al. (2000) found that the same seed treatment decreased the average content of total phenolics (opposite result after 2 days germination) and increased the average biomass of pea leaves. The increase in biomass and decrease of the total phenolic content was much greater

when the seeds were primed with acidic water plus salicylic acid (SA). The authors proposed that leaves originated from primed seeds exhibited a low phenolic content likely because they largely applied their phenolic reserves as antioxidants to oppose free radical generation. They also observed that the activity of a peroxidase (GPOX) was lower in primed plants than unprimed ones, which suggests low lignification.

Our analyses were conducted in the roots of maize plants after 3 days of germination, and we observed high expression of lignin genes in roots caused by seed priming. Therefore, our findings are coherent with those of Mccue et al. (2000) obtained from observations made after 2 days. It is possible that the induction of growth in both studies occurred due to activation of proton pumping across the plasma membrane through proton-ATPase activity, which is essential for nutrient uptake and the generation of turgor (HAGER et al., 1991; SHEN et al., 2006). However, comparison of our results with those obtained by Mccue et al. (2000) at 10 days after germination is intriguing, as these authors observed contradictory behavior, reporting a low content of phenolics in the leaves caused by seed priming. Therefore, the following questions arise from these findings:

1) Do the roots and shoots of maize plants present the same behavior when we apply seed priming?
2) While seed priming modulates expression of lignin genes, is this gene modulation reflected in changes in lignin content?

   We will attempt to answer these questions in Sections 4 and 5.

Considering the findings reported in the literature for maize, under Al stress, among the genes studied in a microarray analysis, it was not possible to observe how Al affects MYB transcription factors (MATTIELLO *et al.*, 2010). Phenylpropanoid genes are regulated by members of the MYB transcription factor family (GRAY et al., 2012 and references therein) that bind AC cis-elements found in the promoters of several genes in the phenylpropanoid pathway (GROTEWOLD et al., 1994; FORNALÉ et al., 2010).

We can make an analogy to *Arabidopsis thaliana*, in which Al stress increases the expression of the *AtMYB52*, *AtMYB81*, *AtMYB23* and *AtMYB53* genes, which belong to the R2R3-MYB transcription factors (Figure 37), and decreases the expression of *AtMYB109* and *AtMYB45* (KUMARI et al., 2008). No changes were observed for *AtMYB3*, *AtMYB4*, *AtMYB7* and *AtMYB32*, which encode transcriptional repressors of sub-group 4 (DUBOS *et al.*, 2010) and are homologous to *ZmMYB31* and *ZmMYB42* (FELLER *et al.*, 2011). Our findings are consistent with those of Kumari et al. (2008), as no changes in the expression of *ZmMYB31* were found to be caused by Al stress. However, a new observation based on our data is that

seed priming decreases the expression of *ZmMYB31* in maize roots when seedlings are grown under stress. Further studies should be conducted to explain the mechanisms whereby seed priming could be influencing *ZmMYB31* expression in roots.

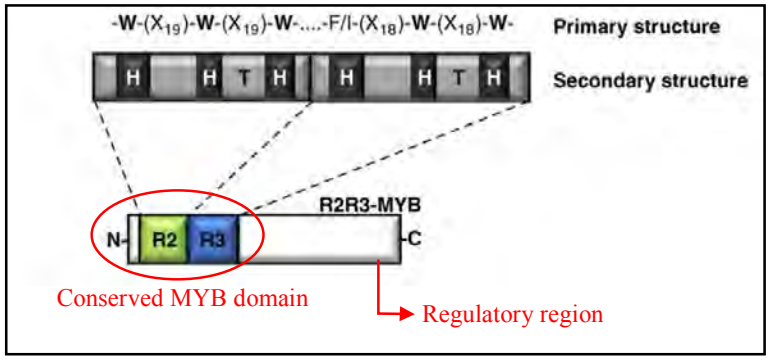

Figure 37 - The plant R2R3-MYB transcription factor (DUBOS *et al.*, 2010). The abbreviations in the primary and secondary structures are as follows: H, helix; T, turn; W, tryptophan; X, amino acid (X). Conserved and regulatory domains are highlighted in red (AMBAWAT *et al.*, 2013)

Under stress, the down-regulation of *ZmMYB31* caused by seed priming was in accord with the up-regulation of *COMT1* and *F5H*, as *ZmMYB31* represses these two genes (FORNALÉ *et al.*, 2010). Other transcriptional factors should be studied to verify their responses to seed priming and correlate their involvement with the up-regulation of lignin genes.

3.5 Conclusions

The results obtained from our objectives and hypotheses generated the following conclusions presented in this section:

Seed priming with an Al solution or acidic water increased the germination index and root growth under Al stress in maize (line B73).

Seed priming with acidic water increased H_2O_2 levels in maize embryos, which could be involved in ABA catabolism and reflected in the improvement of the germination index.

Oxidative damage in roots was reduced when maize seeds were primed and germinated under stress.

In roots, lignin-related genes (*COMT*1 and *F5H*) were up-regulated by seed priming, which is a response that could be involved in the induction of Al tolerance.

Lignin-related genes were up-regulated, while a potential maize repressor *ZmMYB31* was down-regulated in maize roots.

Future studies in the field are necessary to determine which mechanisms of tolerance are being induced in the roots and shoots by seed priming and to verify whether seed priming can affect productivity.

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SECTION 4 - NEW TREATMENTS TO INDUCE AL TOLERANCE IN MAIZE – THE POTENTIAL OF ASCORBIC ACID

In Section 3, we observed that priming of maize seeds with acidic water (pH 4.0) was sufficient for the improvement of the germination index and the growth of roots when seeds were germinated under Al stress. Furthermore, it was observed that unprimed seeds absorb more aluminum and have higher lipid peroxidation when compared to primed seeds germinated under stress. Seed priming promotes an increase in the expression of genes related to lignin biosynthesis in roots, a response that may be related to overall aluminum tolerance. However, it would be interesting to determine how seed priming affects shoots. If lignin expression is also enhanced, it may lead to negative repercussions because of attempts to minimize lignin levels for industrial application of lignocellulosic materials.

Given the above, the following questions were raised:

1) Is it possible to use any acidic powder, which is more easily applied by farmers, for seed priming instead of using acidic water?

2) Regarding the expression of lignin-related genes, will the response in shoots be similar to that observed in roots?

This short section is a continuation of Section 3 and contains preliminary experiments that will support new hypotheses for Section 5 (field experiments).

4 POTENTIAL OF ASCORBIC ACID TO INDUCE AL TOLERANCE AND MINIMIZE LIGNIN GENE EXPRESSION IN SHOOTS OF MAIZE

Abstract

The modulation of lignin biosynthesis is related to the tolerance of biotic and abiotic stresses. However, high lignin content can have a negative effect due to certain properties that present obstacles during chemical pulping and conversion of lignocellulosic biomass into biofuels. Therefore, it is of wide interest to generate plant material with lower lignin content or with easier accessibility for bioenergetically valuable compounds in addition to tolerance of various stresses. In the previous section, we observed that seed priming with acidic water induced lignin gene expression in roots that could be related to Al tolerance induction through Al chelation by lignin. In this current study, we tested two potential seed treatments, using ascorbic acid (AsA) or boric acid powder, in maize seeds in order to verify if either of these treatments present the same response as observed for seed priming with acidic water, and to verify the expression of a key lignin gene (*COMT*1) in roots and shoots. In this experiment, seeds of the maize inbred line B73 were primed with ascorbic acid or boric acid for 6 hours. Five days after germination, we observed that boric acid was not effective for seed priming but that ascorbic acid improved the germination percentage and growth under low pH and Al stress. Quantitative real-time PCR (qRT-PCR) was used to analyze the effect of seed priming on the expression of *COMT*1 and the expression of the transcription factors *ZmMYB*31 and *ZmMYB*42. We observed that the expression of *COMT*1 was increased by seed priming in roots, whereas the expression of the negative transcription regulator *ZmMYB*31 was reduced in roots when germination occurred under low pH and Al stress. In shoots, an antagonistic effect was observed because *COMT*1 was down regulated; however, it was not possible to identify which transcription factor was involved in the response of seed priming. We hypothesize that AsA entails changes in enzymatic activity and suggest more studies to elucidate the association between ascorbic acid effects and lignin biosynthesis.

Keywords: Ascorbic acid; Seed priming; Maize; Germination; *COMT*1

4.1 Introduction

4.1.1 Potential acidic powders to minimize Al stress

In section 3, we reviewed that the acidity of soils leading to Al stress is a relevant issue found in many countries, especially Brazil and the United States. In addition to genetic improvement, there are a number of pre-sowing seed treatment techniques that could be used to improve germination and growth under abiotic stresses, they are known as seed priming (ASHRAF; FOOLAD, 2005). We demonstrated in the previous section that the root length of maize seedlings grown under Al stress was improved by seed priming with acidic water during 6 hours.

In the present section, we will test two potential acids to be used for seed treatment: ascorbic acid and boric acid.

L-ascorbic acid (AsA), also known as vitamin C, is a cofactor for some enzymes, and it is essential for immune cell development and cardiovascular functions and for protection against oxidative stresses in animals. Humans are one of the few mammalian species unable to biosynthetize this vitamin and must acquire it through the ingestion of fruits and vegetables (GALLIE, 2013a).

In plants, ascorbic acid is the most abundant organic antioxidant and is generated by four pathways: 1st) L-galactose (WHEELER; JONES; SMIRNOFF, 1998); 2nd) D-galacturonic acid generated from the breakdown of pectin (VALPUESTA; BOTELLA, 2004); 3rd) D-glucuronic acid generated from *myo*-Inositol (LORENCE *et al.*, 2004); and 4th) regeneration from monodehydroascorbate (MDHA), an oxidized form of ascorbic acid (YIN *et al.*, 2010). The last pathway (ascorbic acid regeneration) plays an important role in minimizing oxidative stresses in plants, and it was previously observed that the maintenance of vitamin C levels is also important for the promotion of plant growth (GALLIE, 2013b). It will be interesting to perform seed treatment using ascorbic acid rather than acidic water due to the applicability of the technique.

Our hypothesis is that ascorbic acid will have the same effect that we observed when seeds were primed with acidic water. This is because in aqueous solution, ascorbic acid ionizes to form ascorbate anions (Figure 38) and hydrogen protons, acidifying the solution weakly (pKa = 4.13) (DAVEY et al., 2002).

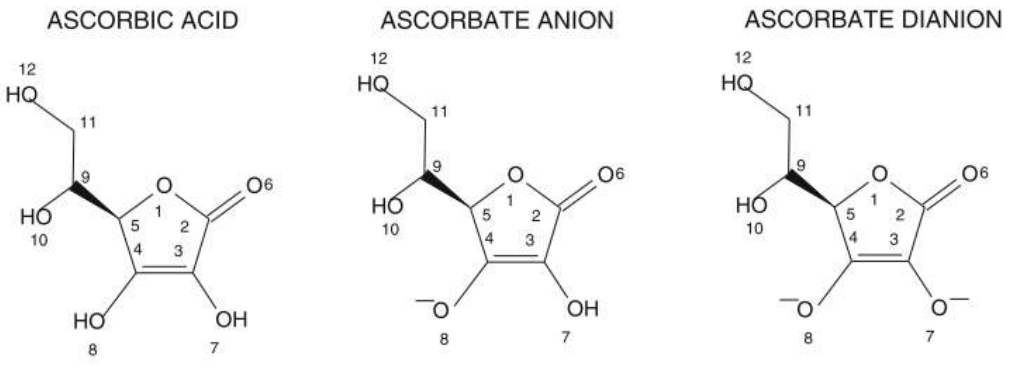

Figure 38 - Chemical structures of ascorbic acid, ascorbate anion and dianion (MUJIKA; MATXAIN, 2013)

A second acid that will be tested for seed treatment is boric acid. Boron (B) is involved in several metabolic pathways important to plant development such as root

elongation, carbohydrate transport and metabolism, nucleic acid synthesis and pollen tube growth (BLEVINS; LUKASZEWSKI, 1998; CAMACHO-CRISTÓBAL et al., 2008). Furthermore, it was previously observed that Al stress inhibits boron uptake (CORRALES et al., 2008 and references therein) and that increased B concentrations could alleviate Al toxicity as it was observed in beans (STASS et al., 2007), pea (YU *et al.*, 2008), citrus (JIANG et al., 2009), cucumber and maize (CORRALES et al., 2008).

4.1.2 The importance of lignin biosynthesis regulation

As stated in Section 3, the modulation of lignin biosynthesis is related to the tolerance of several abiotic stresses, such as aluminum stress. However, high lignin content can have a negative effect due to some properties that present obstacles to chemical pulping, forage digestion, and conversion of lignocellulosic biomass into biofuels (UMEZAWA, 2010). For that reason, it is of wide interest to generate plant material with lower lignin content or facilitating removal of lignin in addition to improving tolerance to various stresses.

Currently, the main source of biofuels in Brazil and the U.S. are sugar and starch, especially from sugarcane (100% in Brazil), corn and sorghum (98% and 2%, respectively in the U.S.) as raw material (BALAT; BALAT, 2009). However, there are efforts from the scientific community to develop new economically viable processes for the utilization of lignocellulosic biomass derived from agricultural residues such as sugarcane bagasse and corn fibers for second-generation biofuel (BALAT; BALAT, 2009; ZHENG et al., 2009; SHRESTA et al., 2012; ACKER et al., 2013).

The expenses for raw materials can greatly affect the costs of biofuel production because feedstock represents 60–75% of the total production cost (BALAT; BALAT, 2009). The technology to obtain ethanol from sugar or starch is fairly well established; thus, the competitiveness of ethanol in fuel markets is more dependent on national policies made to encourage production than on the technology itself. In Brazil, the cost of producing ethanol from sugar is approximately US$ 0.23–0.29 per liter, while in the U.S., the expense of producing starch-derived ethanol is estimated at approximately US$ 0.53 per liter (BALAT; BALAT, 2009).

There is no estimate in the current literature regarding the cost of ethanol production from lignocellulosic material; however, it is known that the expenditure is considerably higher than the cost of production for existing biofuels on the market. Because of this, the

establishment of an efficient processes for fermentation and saccharification is a desirable approach to minimizing costs (HASUNUMA *et al.*, 2013).

Lopez et al. (2009) verified the impact of lignin on the cost of cellulosic pulp production of 10 *Eucalyptus* clones. They observed that the low content of lignin in the wood is highly correlated with the increase in cellulosic pulp yield, demonstrating that the economic impact of lignin occurs on two different facets: (1^{st}) with the presence of more lignin, more wood must be purchased, and (2^{nd}) if more wood is purchased, more wood must be transported from farther distances, causing the cost to rise as a result of the increased transportation.

In *Arabidopsis thaliana*, Acker et al. (2013) showed that lignin gene mutants have an improved saccharification yield by up to 88%, whereas in wild-type, cellulose conversion only reached 18%. However, the absence of lignin also may have a negative impact because this possibly could lead to an increase in susceptibility to pests and diseases (MAHER et al., 1994; WAINHOUSE et al., 1998).

Given the importance of minimizing lignin to optimum levels or making it easier to remove while making plants more tolerant to biotic and abiotic stresses, much work has been performed to identify transcription factors that are involved in its biosynthesis (GOICOECHEA et al., 2005; FORNALÉ et al., 2006, 2010; SONBOL et al., 2009; ZHOU et al., 2009). This area has been a focus because RNA synthesis is the first step of gene expression, and regulation at this level may be the most energy-efficient step in the cell also, transcription factors most of the times regulate several genes or whole branches instead of just one single gene, therefore a much broad effect can occur and not just a shut-off of a specific gene which could lead to accumulation of intermediates (WATSON et al., 2004). In eukaryotic cells, 73% of protein abundance is correlated with mRNA quantity, suggesting that protein levels are determined mostly by transcription regulation (LU *et al.*, 2007). Modulation of the transcription of lignin-related genes may play a central role in cellular adaptation to environmental changes because transcription regulation allows cells to rapidly switch between growth- and stress-related gene expression (LÓPEZ-MAURY et al., 2008). The primary transcription factors that regulate lignin genes belong to the R2R3-MYB family, whose members contain C-terminal transcriptional activation domains; however, repression domains are also be observed in the subgroup 4 of R2R3-MYB family (FELLER et al., 2011 and references there in). In maize, it is possible that *ZmMYB*31 negatively regulates genes involved in lignin biosynthesis, altering carbon flux in the direction of the anthocyanin pathway, although this behavior was just confirmed in transgenic *Arabidopsis* (FORNALÉ *et*

al., 2010). Another example is *ZmMYB*42, which represses lignin synthesis genes, affects cell wall structure and decreases significantly lignin content in transgenic *Arabidopsis thaliana* (SONBOL *et al.*, 2009). Therefore, the use of lignin-regulatory genes could be a powerful tool in biotechnology to improve cellulose productivity, minimize costs and promote sustainable development. Understanding the functions of transcription factors under different conditions is important to give directions in how genes expression could be affected.

4.1.3 Objectives

The general objective of this section is to induce Al tolerance in maize by seed treatment with acidic powders and to examine how lignin is modulated in roots and shoots. The specific objectives of this part of the study are as follows:

a) To verify the effects of seed treatment techniques using ascorbic acid and boric acid;
b) To evaluate the expression of lignin genes in roots and shoots;
c) To study the behavior of the expression of transcription factors related to lignin gene regulation under Al stress and subjection to seed treatment.

4.1.4 Hypothesis

Based on previous data on ascorbic acid and boric acid as potential matrices for seed treatment to induce Al tolerance in maize, and given the importance of studying the modulation of lignin genes, our hypotheses for this study are as follows:

1) Seed treatment using ascorbic acid or boric acid enhances growth under Al stress;
2) Increased expression of lignin-related genes in roots from primed seeds are expected (as observed in section 3);
3) In shoots, the genes related to lignin biosynthesis mimic the behavior in roots;
4) Altered expression of suppressors that regulate lignin biosynthesis is expected.

4.2 Materials and methods

4.2.1 Experimental design

In this experiment we aimed to test two types of acidic powders: L-ascorbic acid and boric acid. The maize seeds that were used in this experiment were not sterilized or soaked overnight to maintain a technique that is more applicable and reproducible for farmers. For this experiment, we used 5 repetitions (each repetition was considered one vase) and two seeds per vase, totalizing 60 seeds in this experiment. For the germination conditions, we used a photoperiod of 16 hours of light at a temperature of 25°C. The seed treatments and soil characteristics are described as follows:

1) Unprimed seeds from inbred line B73 (control in optimum soil) - germinated in soil at pH 6.0;

2) Unprimed seeds from inbred line B73 (control in acidic soil) - germinated in soil at pH 4.0;

3) Seeds from inbred line B73 were primed for 6 hours at a ratio of 3.8 grams ascorbic acid per 25 seeds (enough amount to cover all the seeds) - germinated in soil at pH 6.0;

4) Seeds from inbred line B73 were primed for 6 hours at a ratio of 3.8 grams ascorbic acid per 25 seeds - germinated in soil at pH 4.0;

5) Seeds from inbred line B73 were primed for 6 hours at a ratio of 3.8 grams boric acid per 25 seeds - germinated in soil at pH 6.0;

6) Seeds from inbred line B73 were primed for 6 hours at a ratio of 3.8 grams boric acid per 25 seeds - germinated in soil at pH 4.0;

The experimental setup can be viewed in Figure 39.

Soil acidification was carried out by the addition of acidic distilled water (pH 4.0) to the soil, allowing Al to be more available to the plants in acidic soils (Figure 40).

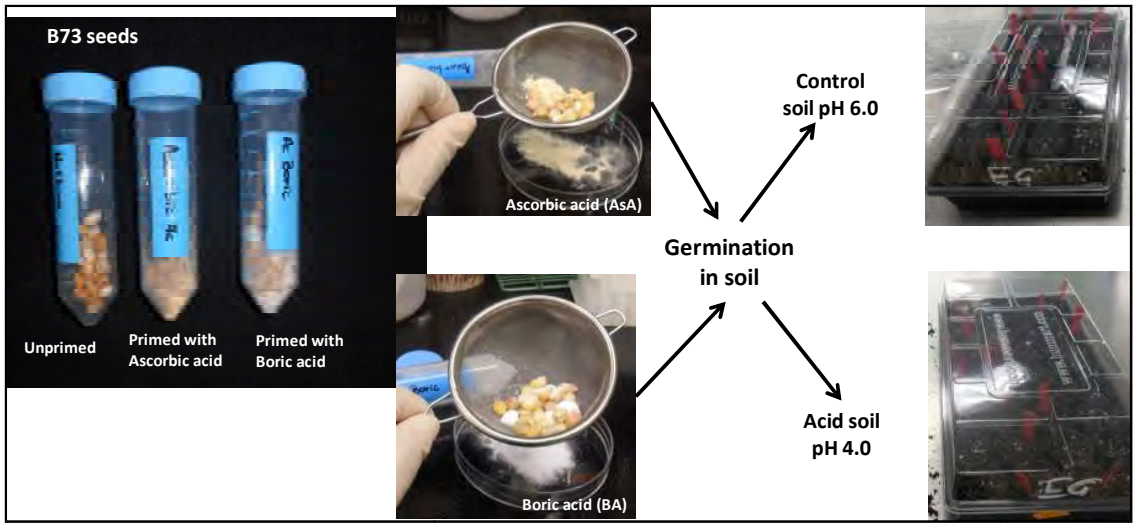

Figure 39 - Scheme of the experiment using acidic powders

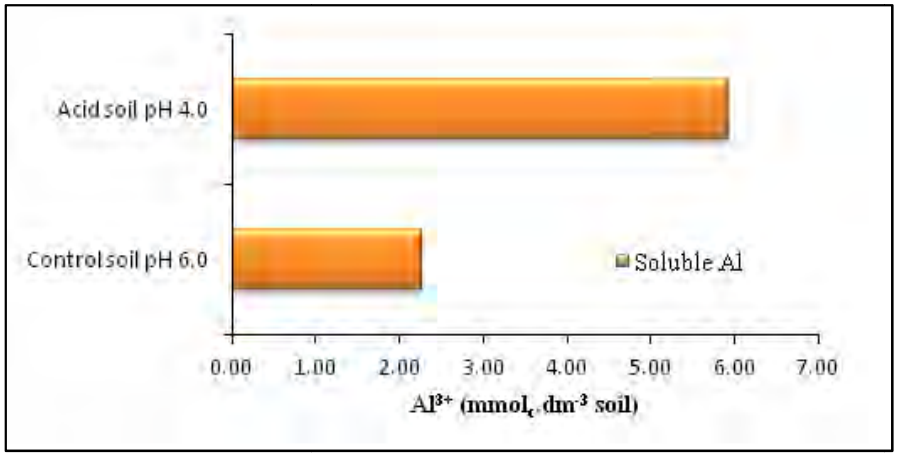

Figure 40 - Soluble aluminum available in acidic soil (pH 4.0) and control soil (pH 6.0)

4.2.2 Evaluation of stress parameters

Five days after germination, we evaluated the percentage of total germination, the lengths of roots and shoots, and the absorption of Al in both tissues via inductively coupled plasma optical emission (ICP-OES, Optima 3000 DV, Perkin Elmer, Trace Element Research Laboratory, OH, USA).

Statistical analyses of these data were performed using StatSoft 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%.

4.2.3 RNA extraction and cDNA synthesis

Approximately 100 mg of roots or shoots were macerated in liquid nitrogen, and extraction of total RNA was performed using a kit (#74904, RNeasy Plant Mini Kit, Qiagen). After RNA extraction, the total RNA was primed with DNase (On-Column DNase I Digestion Set, DNASE70-1SET, Sigma) for 20 min at room temperature. Quantification of total RNA was performed using a nanodrop machine (NanoDrop® ND-1000 UV/VIS, von NanoDrop Technologies). Approximately 100 ng of total RNA was used to obtain cDNA with an Invitrogen kit (SuperScript™ II). Oligo-dT primers were used to bind the polyA tails of the mRNAs to initiate the synthesis of first-strand cDNA with reverse transcriptase. The obtained cDNA samples were primed with RNase. To confirm the effectiveness of the reverse transcription, we utilized primers targeting *UBIQUITIN* gene for amplification via conventional PCR. Visualization of the amplicons was performed in 2% agarose gels supplemented with ethidium bromide. Amplification through conventional PCR was conducted in a final volume of 25 µl, containing 2.5 µl 10x buffer supplied with Mg^{2+} (E00007, GeneScript), 0.2 mM dNTPs, 1.0 µM forward primer, 1.0 µM reverse primer, 0.05 U/µl Taq polymerase and 100 ng cDNA. The reactions were performed in a Peltier Thermal Cycler, model PTC-225 (MJ Research, Inc.), with the following amplification conditions: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, a melting temperature of 57°C for 45 s and 70°C for 1 min, with a final extension at 70°C for 10 min.

4.2.4 qRT-PCR expression analysis

Quantitative real-time PCR (qRT-PCR) was performed at Plant-Microbe Genomics Facility (OSU, Ohio, United States) using routine protocol for BioRad CFX96 (step 1: 95.0°C for 10:00 min; step 2: 95.0°C for 0:15 min; step 3: 60.0°C for 1:00 min; step 4: steps 2-3, 49 more times; step 5: Melt curve of 45.0°C to 95.0°C; Increment of 0.5°C 0:05 min). We utilized the following primers for the expression analysis by qRT-PCR: *ubq* (F: GTGAGTCGTGACTGAGCTGGTT; R: ATATGCGGTCGCACGATAGTT), *COMT*1 (F: TCTGCGTCGAATTGTCTCTGC; R: GAGAGCAATTAAACCGCCATGT), *ZmMYB*31 (F: GTAGGTGAAAAATACGCGATGG; R: AAAATGGAGCAGCAAGAGAGAG and *ZmMYB*42 (F: CTGGGGCTCAGGACCAGCGT; R: GGGAGCAGCTACTGTGGGGAGG). The analyses were performed for roots and shoots of five-day-old maize seedlings. For each treatment, the relative gene expression (*RGEx*) was calculated by the formula $RGEx =$

$2^{(Ct_{ubq}-Ct_{pg})}$, adapted from Morohashi; Grotewold (2009), where Ct_{ubq} is the Ct value for reference gene (*UBQ*) and Ct_{pg} is the Ct value for phenylpropanoid related gene. We used three biological repetitions and three technical replications for gene expression analysis.

Statistical analyses of these data were performed using the StatSoft software (version 7.0, Tulsa, OK, USA). Significant differences between the averages of the treatments were determined by performing Duncan's test with a confidence level of 95%.

4.3 Results

Considering the germination percentage (Table 11) and growth of roots and shoots, the treatment with ascorbic acid led to the best results (Figure 41). We observed that unprimed seeds placed in acidic soil (pH 4.0) presented a reduction in the total percentage of germination by almost 30% (70.8%). Seeds primed with ascorbic acid and germinated in acidic soil had the same germination percentage compared to unprimed seeds germinated in optimum soil (83.3%). Curiously, there was reduction in germination percent from 83.3% to 75% when seeds are primed with ascorbic acid but germinated without stress. Treatment with boric acid was not efficient because it reduced the germination percentage in both optimum soil and acidic soil (approximately 20% and 25%, respectively).

In addition to germination percentage, the treatment with ascorbic acid also significantly improved the growth of root and shoot when the germination occurred in acidic soil. Interestingly, the boric acid treatment was not effective; in contrary, we observed inhibition of growth (Figure 42). Seeds primed with ascorbic acid and germinated in soil at pH 6.0 had no significant difference in the length of roots and shoots (12.0±0.52 cm and 6.0±0.26 cm, respectively) compared to unprimed seeds (12.5±0.60 cm for roots and 6.8±0.38 cm for shoots). However, in soil with pH 4.0, ascorbic acid-primed seeds had longer roots (10.9±0.84 cm) and shoots (6.2±0.05 cm) when compared to unprimed seeds (8.7±0.37 cm and 4.9±0.41 cm for roots and shoots, respectively).

Table 10 - Germination percentage per treatment

Soil pH	Seed treatment	Germination (%)
6.0	Unprimed	83.3
6.0	Ascorbic acid	75.0
6.0	Boric acid	20.8
4.0	Unprimed	70.8
4.0	Ascorbic acid	83.3
4.0	Boric acid	25.0

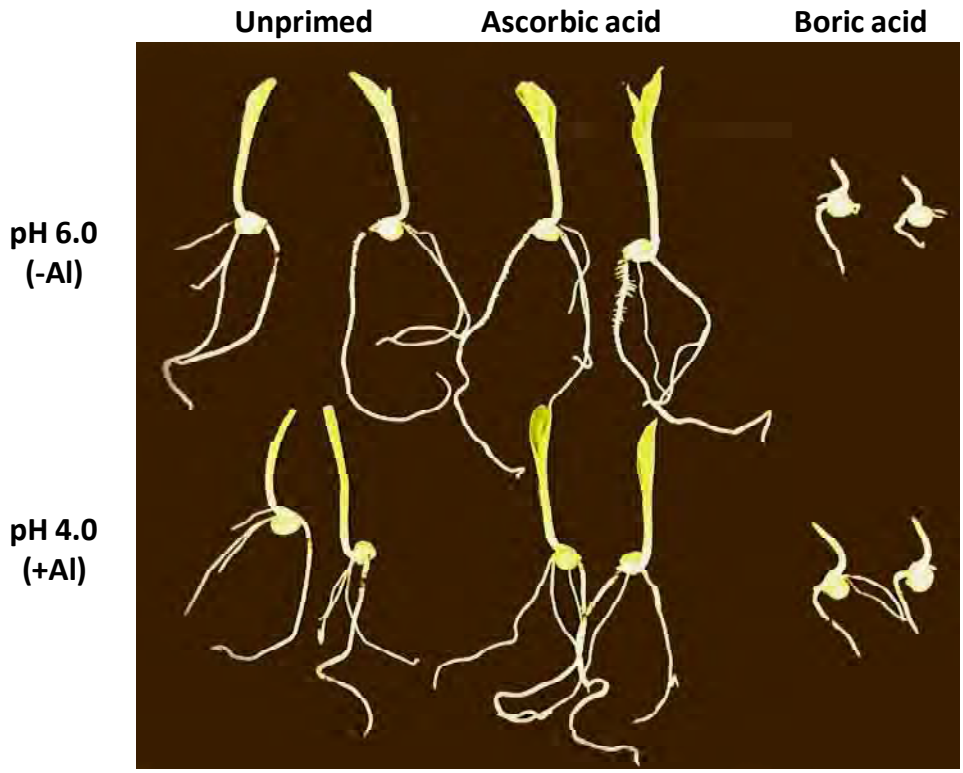

Figure 41- Growth comparison of maize seedlings (inbred line B73) at 5 days old

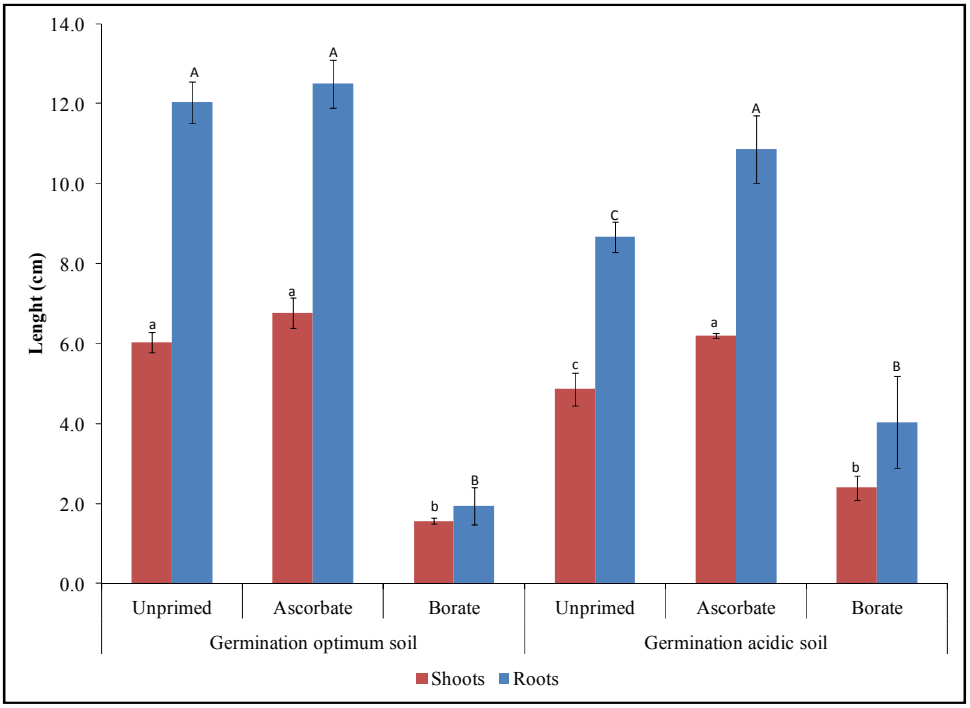

Figure 42 - Length of roots (blue) and shoots (red) of maize seedlings at 5 days old. Bars of the same color that do not have same letters differ significantly from one another ($p < 0.05$)

In regards to the analysis of aluminum absorption, we note that unprimed seeds sown under stress had higher aluminum absorption in roots than seeds primed with ascorbic acid (Figure 43). The aluminum absorption analysis was not performed for the seedlings from seeds primed with boric acid because this treatment resulted in no further growth. Interestingly, seed priming with ascorbic acid reduces the absorption of aluminum in the root (from 169.53±7.26 to 94.09±11.54 µg $Al \cdot g^{-1}$ dry matter). Quantification of aluminum was also performed for shoot tissue; however, there was no significant difference between treatments.

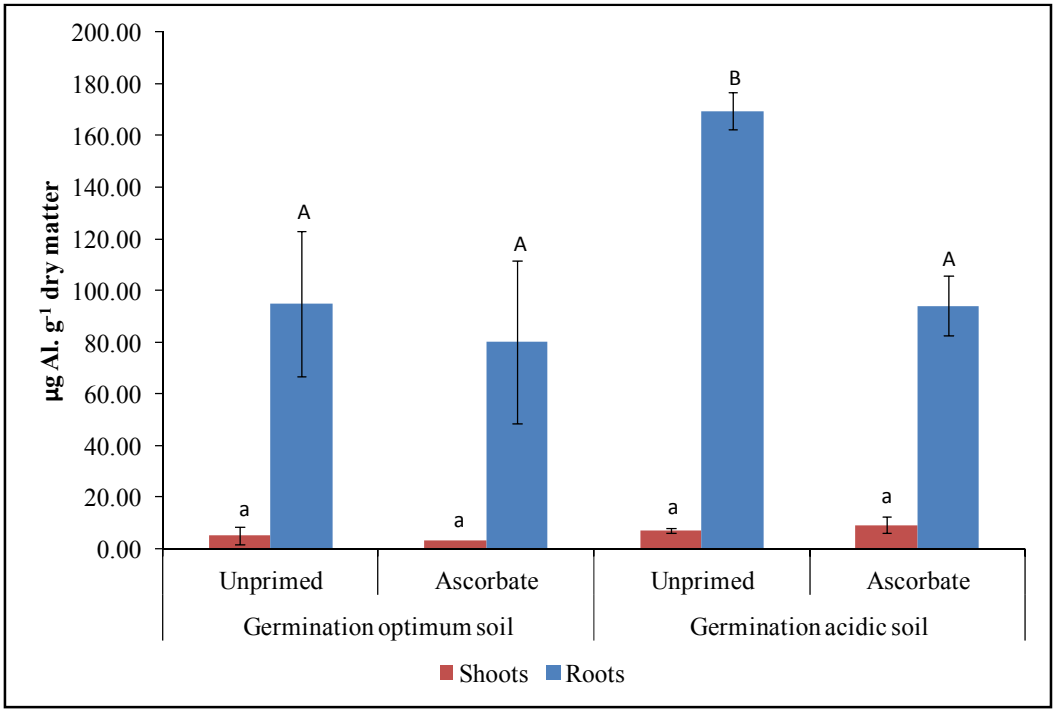

Figure 43 - Quantification of aluminum in root (blue) and shoots (red) of maize seedlings at 5 days old. Bars of the same color that do not have same letters differ significantly from one another ($p < 0.05$)

It was previously shown that lignin biosynthesis may be related to aluminum tolerance in root (Section 3). In the present section, we observed that in roots of unprimed seeds, the key lignin gene, *COMT*1, was over-expressed when the seeds were germinated in acidic soil (relative gene expression increasing from 0.148±0.033 to 0.235±0.032). As expected, with the previous results obtained using seed priming with acidic water (Section 3), the expression of this gene in the roots was much higher when seeds were primed with ascorbic acid and germinated in acidic soil (0.321±0.007). Interestingly, the effect of expression of *COMT*1 in roots was the opposite of that in the shoots because the treatment with ascorbic acid decreased the expression of this gene in the shoots (Figure 44). The treatment with ascorbic acid minimized the expression of *COMT*1 when germination occurred in optimum soil (from 0.299±0.000 to 0.165±0.006) and when it occurred in acidic soil (from 0.347±0.000 to 0.270±0.021).

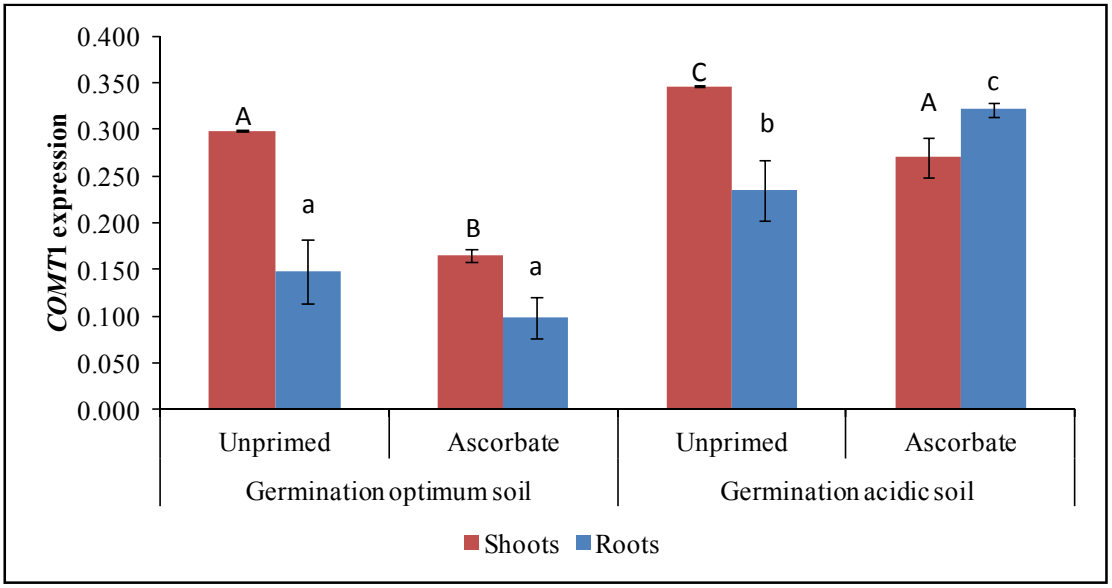

Figure 44 - Gene expression of *COMT*1 in root (blue) and shoots (red) of maize seedlings with 5 days old. Bars of the same color that do not have same letters differ significantly from one another ($p < 0.05$)

Accordingly, the expression of transcription factor *ZmMYB*31, which negatively regulates expression of *COMT*1, was reduced in the root when the seeds were primed with ascorbic acid and germinated under stress with Al. The relative gene expression under stress in roots for unprimed seeds was 0.029±0.003 and for primed seeds was 0.016±0.002 (Figure 45). However, in shoots, the treatment with ascorbic acid did not alter the expression of *ZmMYB*31 under Al stress. Curiously, the expression of another potential repressor in maize, *ZmMYB*42, was not modified in the root but was altered in shoots (Figure 46). Apparently, the expression of *ZmMYB*42 did not account for the modulation of *COMT*1 in shoots, suggesting that other transcription factors are involved in the change of gene expression in the shoots.

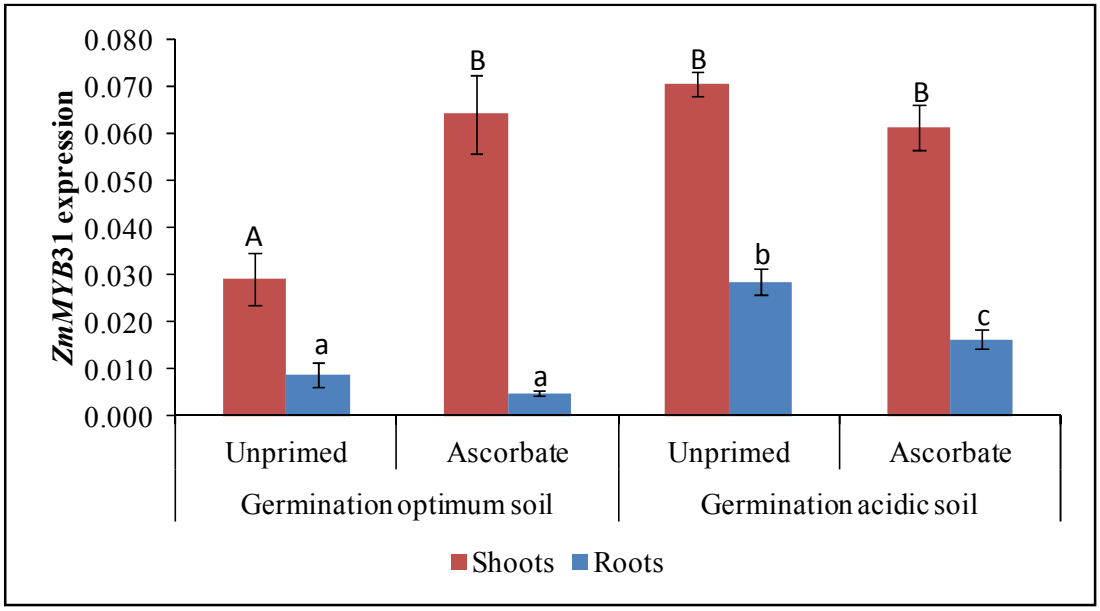

Figure 45 - Expression of the transcription factor *ZmMYB*31 in root (blue) and shoots (red) of maize seedlings at 5 days old. Bars of the same color that do not have same letters differ significantly from one another ($p < 0.05$)

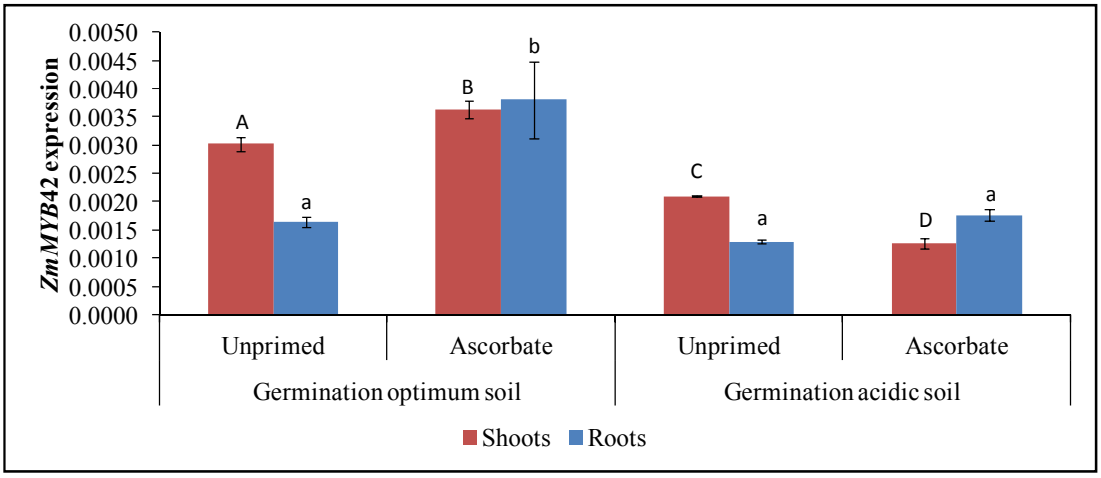

Figure 46 - Expression of the transcription factor *ZmMYB*42 in root (blue) and shoots (red) of maize seedlings at 5 days old. Bars of the same color that do not have same letters differ significantly from one another ($p < 0.05$)

4.4 Discussion

We observed that boric acid was not efficient as a seed priming treatment and inhibits germination percentage and growth probably because the damage was caused by excess of boron that may have toxicity in high amounts (CAMACHO-CRISTÓBAL et al., 2008).

Alternatively, ascorbic acid allowed the recovery of germination at optimum levels under low pH and Al stress and improved root and shoot growth. As we used powdered AsA, it is possible that AsA is adsorbed on the surface of seeds, and consequently is diluted in the soil water, generating ascorbate anions and hydrogen protons around the seed (Figure 47). We can explain the improving in germination and growth under Al stress based on two hypotheses, in the first one seeds primed with ascorbic acid could present similar behaviors as observed for priming with acidic water (Section 3) because of hydrogen proton formation in the soil water could induce the production of hydrogen peroxide (H_2O_2) leading to germination signaling (LIU *et al.*, 2010).

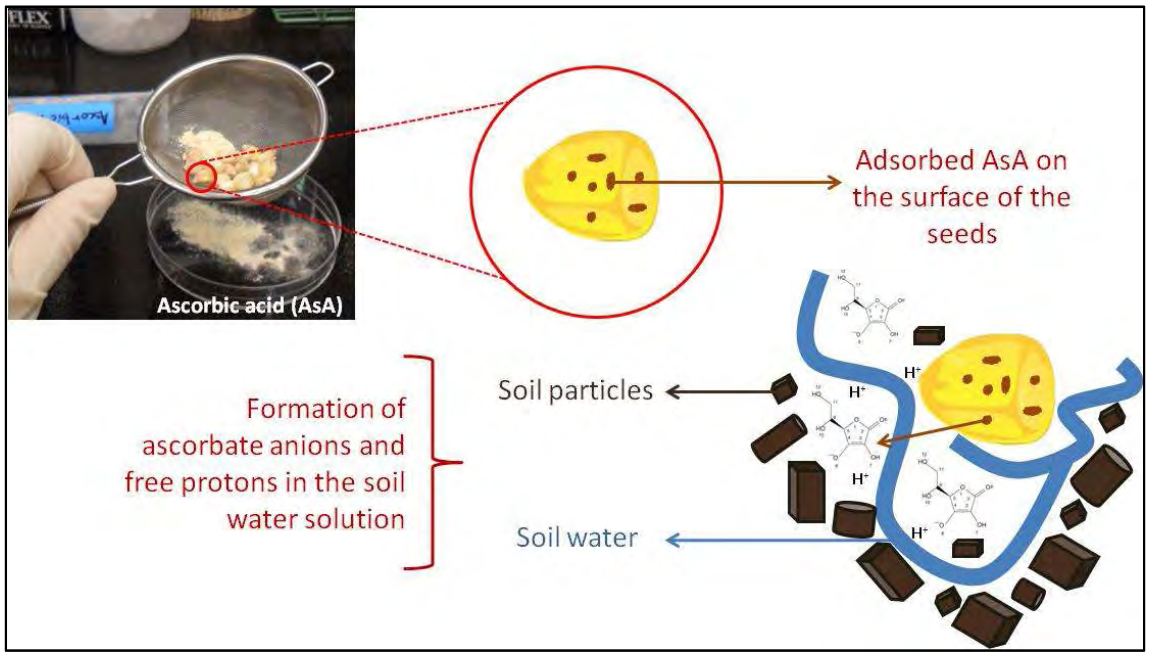

Figure 47- Hypothetical explanation of how the AsA treatment interferes in germination and growth

In Section 3, we observed that seed priming with acidic water improved the germination index because, before sowing, it induced elevated production of hydrogen peroxide in the embryo, a signal that is important for germination. The effect of seed priming using low pH was previously investigated by Mccue et al. (2000), who observed that the increase in the average biomass of pea leaves likely occurs because of proton pumping-ATPase activation. However, we have to highlight an interesting observation: differently to acidic water, the treatment with AsA resulted in a reduced rate of germination when the seeds are placed in optimum soil.

Therefore our second hypothesis is that ascorbic acid without the stress condition could reduce the production of H_2O_2 which is a signal of germination. There are studies that show H_2O_2 promoting seed germination of cereal plants such as wheat and rice, although in certain concentrations, H_2O_2 results in seedling malformation (OGAWA; IWABUCHI, 2001; DIAZ-VIVANCOS et al., 2013 and refrences therein). In our data, the reduction of germination in unprimed seeds sown in soil with Al could occur because Al stress produces hydrogen peroxide at levels that causes oxidative damage in sensitive varieties of maize (GIANNAKOULA et al., 2010). Therefore, it could be interesting to investigate the limit between signalization and oxidation caused by H_2O_2, which could be dose-dependent and species-dependent. For example, Zehra et al. (2013) studied the effect of ascorbic acid on seed germination of grasses and observed that the rate of germination increased for *Eragrostis ciliaris* under saline conditions but was inhibitory for *Dichanthium annulatum*. Furthermore, these authors observed improvement of germination of grasses seeds that are primed with low concentrations of AsA (5 mM), but the treatment was inhibitory when seeds were primed with 10 and 20 mM AsA. In our study, seed priming with ascorbic acid powder improved germination of maize seeds under Al stress possibly because it is reducing H_2O_2 presented at toxic levels, but AsA reduces germination when seeds are placed without stress as it could act in the depletion of H_2O_2 signaling.

Regarding growth improvement, we can focus our discussion based on our observation that the treatment reduced the absorption of Al in the roots. The increase in the expression of the key lignin gene *COMT1* could be involved in the reduction of Al uptake by root cells because the up-regulation of lignin gene expression in the roots was already linked to Al tolerance in previous work (EZAKI et al., 2005; XUE et al., 2008). Interestingly, the reduction in expression of the repressor *ZmMYB*31 caused by ascorbic acid was coherent to the increase of *COMT*1 expression in roots. Therefore, this transcription factor is by some means affected by seed priming, and is partially responsible for the increase in lignin gene expression in roots.

Curiously, we observed a different behavior of lignin expression in shoots. When we primed the seeds with ascorbic acid, the expression of *COMT*1 is reduced with and without stress. Interestingly, when we analyze unprimed seeds, the germination under stress caused the up-regulation of *COMT*1 expression. If we examine the expression of repressor *ZmMYB*42, it seems as though this transcription factor responds to Al stress in the shoots as there is a total reduction in expression that is coherent with the induction of *COMT*1 under Al stress. However, following ascorbic acid treatment, the expression of *ZmMYB*42 is not

correlated, suggesting that other transcription factors are involved in the response in the shoots.

If we examine the graph of *COMT*1 expression for primed seeds germinated in optimum soil, the shoots had the lowest expression levels of lignin genes among all the treatments and, at the same time, the highest expression levels of transcription factors *ZmMYB*31 and *ZmMYB*42 when we analyze the two repressors together which is coherent to Fornalé et al. (2006).

Our data also corroborate the findings of Mccue et al. (2000), who observed a decrease in total phenolics in pea leaves after 10 days germination when seeds were primed with acidic water. The reduction of phenolics was much more drastic when they primed the seeds with salicylic acid (SA). In our work, ascorbic acid caused the down regulation of *COMT*1 expression in maize shoots at 5 days old. Our data contribute to the observation of an antagonistic response occurring between root and shoot that could be explained by the trade-off caused by carbon allocation to the roots while reducing the expression of lignin genes in the shoots.

It will be interesting to quantify the lignin in shoots to verify if the response of gene expression results in the diminution of the final product in which industry has economic interest. This experiment will be performed in Section 5.

In addition to the avoidance of Al absorption caused by induction of lignin genes in the roots, it is possible that the growth improvement caused by AsA treatment is also occurring through the activation of important enzymes that are related to the minimization of oxidative stress, leading to the promotion of plant development (GALLIE, 2013b). Farooq et al., (2013) observed that seed priming using ascorbic acid in solution improved growth in wheat plants under drought stress and suggest that it could be occurring because of the decrease in oxidative damage.

In plants, AsA is essential for detoxifying reactive oxygen species originating from photosynthesis and from endogenous or exogenous sources, and it plays an essential role in regulating cell division and flowering as a cofactor in multiple enzymatic reactions (BARTH et al., 2006).

Under drought stress, AsA regeneration is more important than AsA synthesis (BARTOLI *et al.*, 2005). This behavior was also observed by Yin et al. (2010), who showed AsA regeneration during Al stress using transgenic tobacco plants over expressing dehydroascorbate reductase (DHAR) or monodehydroascorbate reductase (MDAR) from *Arabidopsis thaliana*. The oxidized form of ascorbic acid, known as MDHA, is produced in

plants by two molecules of AsA and one enzyme, ascorbate peroxidase (APX), that reduces H_2O_2 to produce water (Figure 48, apoplast side). For regeneration of AsA, MDHA is reduced by the enzyme MDAR using NADH/NADPH as an electron donor. However, MDHA is an unstable radical, and if it is not rapidly reduced to AsA by MDAR, it could generate dehydroascorbate (DHA), which can also be reduced to AsA by DHAR, which uses glutathione (GSH) as a reducing substance (Figure 48, symplast side). Yin et al. (2010) observed Al tolerance induction in DHAR-expressing transgenic tobacco, but not in MDAR-expressing plants, suggesting that the over expression of DHAR is more important for conferring the maintenance of AsA levels for Al tolerance.

Based on Yin et al. (2010) model, we suggest the hypothesis that during seed germination, the increased amount of AsA available in the root zone could increase the quantity of AsA in root apoplasts, altering the activity of APX and leading to a more efficient antioxidant system. However, to verify this hypothesis, the enzyme activities of the antioxidant system will be examined in Section 5. In the next Section, we will also try to answer the question of how AsA could change the relationship between the activity of antioxidant enzymes and lignin polymer biosynthesis.

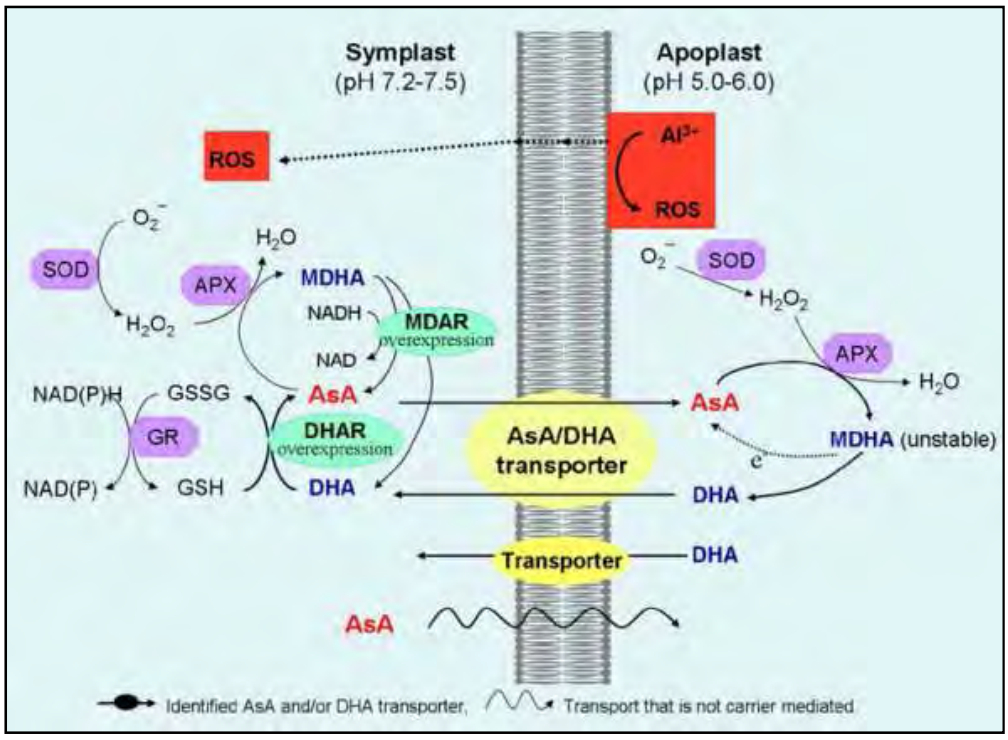

Figure 48 - Model of interactions between Al-induced ROS and the depletion/regeneration of AsA (YIN *et al.*, 2010). In our hypothesis, the increase in AsA in the apoplast changes the activity of APX

4.5 Conclusion

Some of our results corroborate with the initial hypotheses, although some were refuted. Our conclusions based on our objectives were as follows:

Seed priming with ascorbic acid (AsA) increased the germination percentage, root growth and shoot growth under Al stress in maize (line B73).

Seed priming with boric acid was not efficient and inhibited growth.

In general, there was up-regulation of *COMT*1 when germination occurred under stress.

Seed priming with AsA increased the expression of a lignin-related gene (*COMT*1) in roots, a response that could be involved in the induction of Al tolerance.

An antagonistic response was observed in shoots which presented down regulation of *COMT*1. This response may reflect low lignin content in shoots.

The up-regulation of *COMT*1 in roots caused by seed priming could be related to the down-regulation of the potential maize repressor *ZmMYB31* in maize roots.

The up-regulation of *COMT*1 caused by low pH and Al stress could be related to total down-regulation of the potential maize repressor *ZmMYB42* under stress.

Other transcription factors could be involved in the response of down-regulation of *COMT*1 in shoots because the expression of *ZmMYB*31 and *ZmMYB*42 do not account for the reduction of lignin gene expression in shoots.

Since AsA could be directly related to changes in enzyme activity, more experiments are necessary to clarify the relationship between antioxidant enzymes and lignin biosynthesis, and determine a pathway that could explain the connection between seed priming with ascorbic acid and its effects on phenylpropanoids pathway.

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SECTION 5 - SEED PRIMING WITH ASCORBIC ACID – A FIELD EXPERIMENT PERFORMED IN BRAZIL

In Section 4, we observed that treatment of maize seeds with ascorbic acid was efficient in improving plant growth (shoots and roots) in the Al-sensitive line B73 when grown in acidic soil with Al stress; furthermore, interesting results were obtained with respect to the expression of the key gene related to lignin biosynthesis (COMT1) and transcription factors (ZmMYB31 and ZmMYB42) from the Myb family responsible for suppressing the expression of this gene. In this section, we describe results from a similar experiment; however, field conditions with a high Al content (cerrado region) were used. We used another Al-sensitive line (Mo17) and one Al-tolerant commercial variety from Dow AgroSciences (DA) to verify whether ascorbic acid affects productivity in both genotypes and also to verify whether there are changes in the lignin content caused by the seed priming. During the experiments, we observed an attack of porcupines primarily in the blocks where we planted primed seeds. Following this observation, more questions were raised regarding the kernel quality. The results and a discussion of this field experiment are provided in this final section. Although some questions remain to be answered, this section provides a direction for future research.

5 THE POTENTIAL OF ASCORBIC ACID TO INCREASE PRODUCTIVITY AND IMPROVE GRAIN QUALITY IN AL-TOLERANT MAIZE

Abstract

There is a great necessity to improve maize productivity, particularly when exposed to Al stress. In this section, we describe attempts to verify whether seed priming with ascorbic acid in an Al-sensitive line and an Al-tolerant variety improves growth (as observed in previous sections) and, consequently, whether it enhances productivity. We performed this experiment in field conditions of "*cerrado*" soils (Brazil) that present as main characteristic, the high content of exchangeable Al. We used one Al-sensitive line (Mo17) and one Al-tolerant variety (DA) for this experiment. The ascorbic acid treatment was not sufficient to induce Al tolerance in the Mo17 line; however, it increased the productivity of the DA variety. Because we observed that porcupines attacked the blocks containing maize from primed seeds, we also decided to analyze whether this treatment affected the quality of the kernels. Interestingly, we observed that ascorbic acid treatment increased the contents of boron, vitamin C, carbohydrates and some amino acids, although the methionine levels were reduced. Our work provides insight into how ascorbic acid interferes with growth in an Al-sensitive line and an Al-tolerant maize variety because the enzyme activities and metabolite content were very different between the genotypes. The seed priming reduced Al absorption and stress metabolites (MDA) in Mo17, but these parameters did not change in the DA variety. In the DA variety, the ascorbate content in leaves increased, suggesting that in this variety, this metabolite is directly related to growth.

Keywords: Vitamin C; Maize; Productivity; Carbohydrate; Amino acid

5.1 Introduction

5.1.1 The importance of increasing maize productivity

In Section 3, we reviewed data showing that approximately 97% of potentially arable land in Brazil is composed of acidic soils with Al^{3+} toxicity. In the U.S., this problem is present in approximately 50% of the land area, within which 20% of all corn is planted (KOCHIAN *et al.*, 2009). We also reviewed data showing that although the U.S. and Brazil are the primary global sources of corn, they do not possess the highest yields when compared with other countries. This problem can cause yield losses up to 80% (BARINAGA, 1997). Therefore, acidic soils together with Al stress are some of the factors that limit corn productivity in the U.S. and in particular, in Brazil (Section 3 – see item 3.1.1).

In Section 4, we demonstrated that the Al-sensitive variety B73 showed improved growth when seeds were primed with ascorbic acid. This response may be due to the reduced absorption of Al by roots that show the potential of this technique for improving growth under

this type of stress. Because we observed this behavior in maize at 5 days of age, in this section, we will examine the same treatment (seed priming with ascorbic acid) to verify whether it affects productivity significantly.

There have been great efforts placed in improving corn yield because demand is growing exponentially. In the U. S. total corn production has been stimulated over the past 10 years by the industrial use of alcohol for fuel (Figure 49).

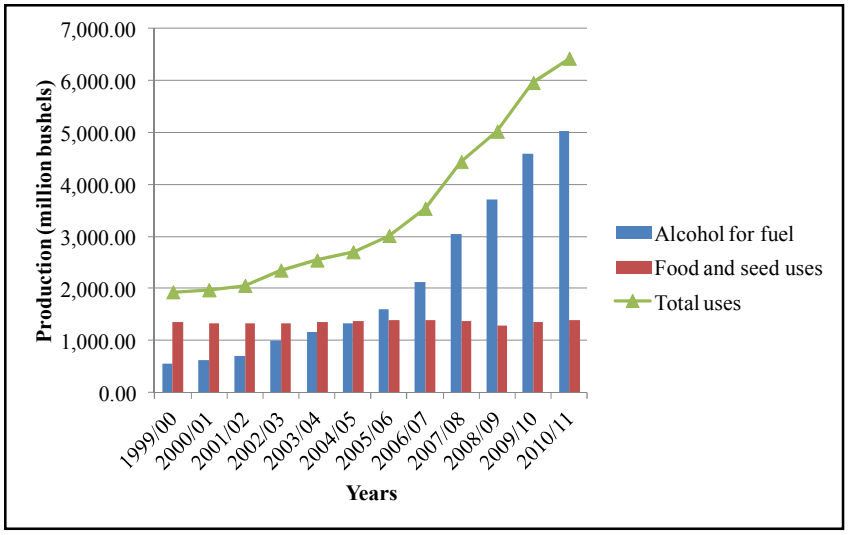

Figure 49 - Corn use in the U.S. in recent years. Source: http://www.ers.usda.gov/

In Brazil, the majority of maize production is oriented toward the domestic market, in particular, animal feed. The segments that consume the most corn are swine and aviculture (Figure 50). However, the surplus in production in Brazil is exported, primarily to the U.S., due to the increase in demand for biofuels from maize (ALVES; AMARAL, 2011). Furthermore, due to policies that have improved income in the Brazilian population, it has been predicted that meat growth consumption will increase by 24% over the next 10 years (Federação das Indústrias do Estado de São Paulo-FIESP, 2013). Thus, it is important to increase corn productivity similarly to supply the increased demand and avoid deforestation.

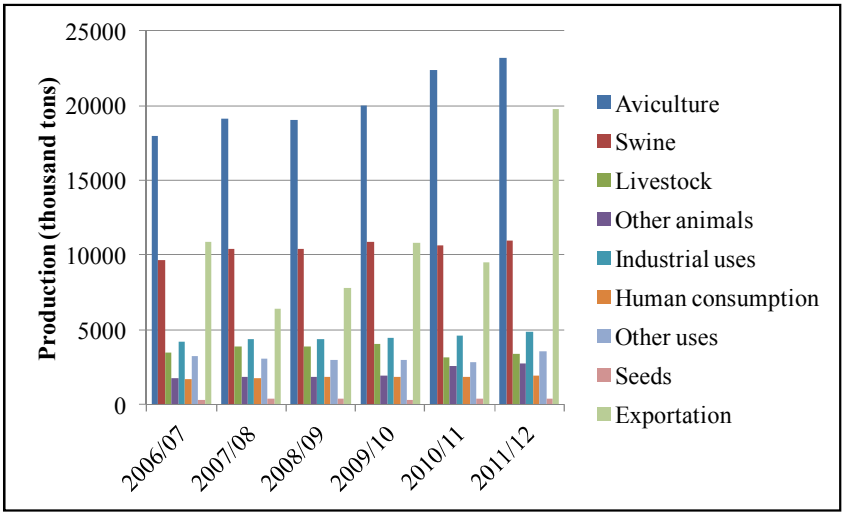

Figure 50 - Recent uses of corn in Brazil. Source: http://www.abimilho.com.br/estatistica

5.1.2 The role of antioxidant defenses in maize growth

There is an interesting and paradoxical property of oxygen (O_2) in aerobic organisms: this molecule provides life and at the same time causes ageing and death (DAVIES, 2000; SCANDALIOS, 2005). Together with the valuable properties of O_2 is its ability to form reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), that may interact with proteins, lipids, and nucleic acids, causing molecular damage (Figure 51) (SCANDALIOS, 2005; GILL; TUTEJA, 2010).

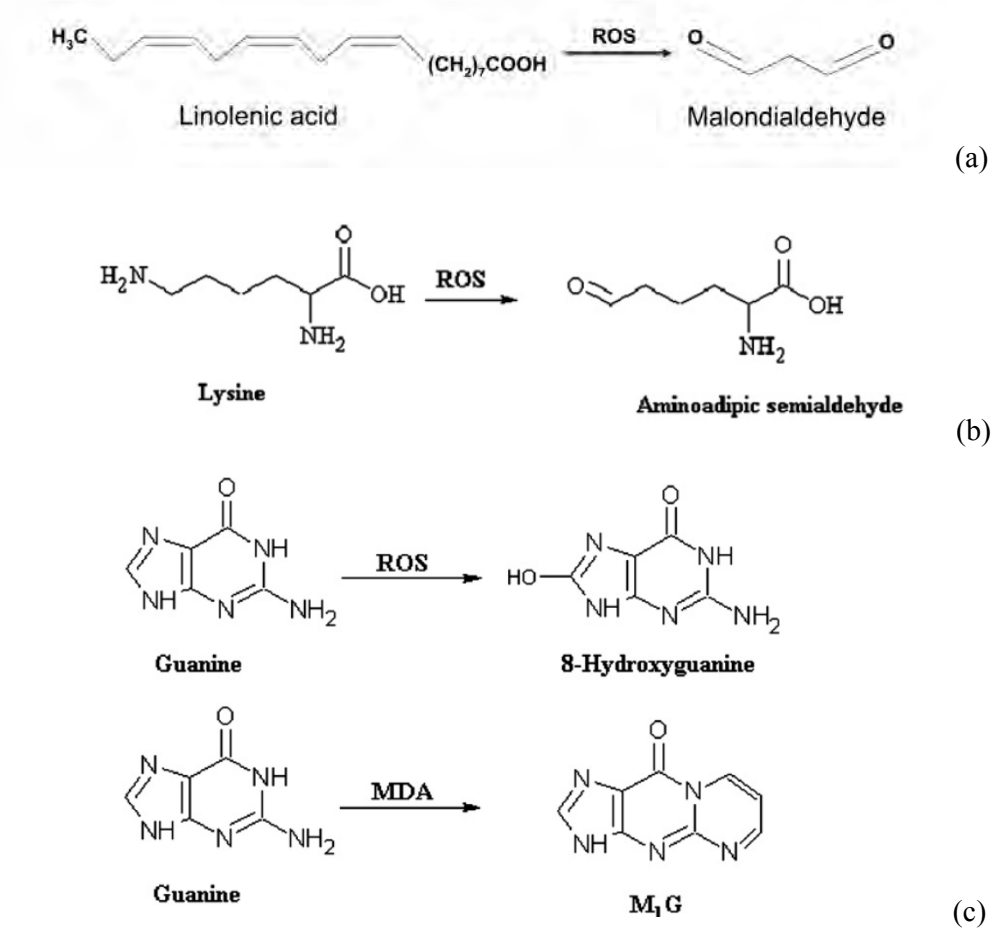

Figure 51 - Examples of molecular damage caused by reactive oxygen species (ROS). (a) Lipid peroxidation generating malondialdehyde (MDA); (b) protein oxidation and (c) DNA oxidation caused by ROS or MDA (GILL; TUTEJA, 2010)

Aerobic organisms evolved antioxidant defenses to minimize the harmful effects of ROS generated from oxygen that include non-enzymatic compounds, such as anthocyanins, glutathione and ascorbic acid, and also enzymatic systems, such as superoxide dismutases (SOD), catalases (CAT), glutathione S-transferases (GST), and glutathione reductase (GR) as well as ascorbate peroxidase (APX) and guaiacol-type peroxidases (GPOX) in the case of plants (ASSADA, 1992; CAO et al., 1997; HIRAGA et al., 2001; SCANDALIOS, 2005; COSIO; DUNAND, 2009; PANDA et al., 2009).

In an optimal environment, the oxidant/antioxidant system is balanced. However, under conditions where an excess of ROS is present due to stimuli that may include, for example, exposure to ozone (AZEVEDO et al., 1998), heavy metals (GRATÃO et al., 2008), mineral deficiency (LIDON; BARREIRO, 1999), diseases (LAMB; DIXON, 1997) and lack of water (CIA et al., 2012), oxidative stress occurs. Therefore, an imbalance in the redox state

of the cell is deleterious to cell integrity and metabolism and should be ameliorated to enable organism tolerance and growth.

With respect to the enzymatic system, the first line of defense is superoxide dismutase (SOD; EC 1.15.1.1.), which is found in all aerobic organisms and acts to detoxify of superoxide radical ($O_2^{\bullet-}$) in a dismutation reaction, thereby generating hydrogen peroxide (H_2O_2) and O_2 (Reaction 1) (MCCORD; FRIDOVICH, 1969; GILL; TUTEJA, 2010).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

In the online maize sequence (www.maizesequence.org), we found 13 isozymes of SOD; however, depending on the maize genotype, Kernodle; Scandalios (2001) noted that only four or five SOD isozymes are detected using electrophoresis. These isozymes are classified into three groups based on the cofactor involved: 1) copper/zinc (Cu/Zn-SOD), found in the cytoplasm and chloroplasts; 2) manganese (Mn-SOD), found in mitochondria; and 3) iron (Fe-SOD), found only in chloroplasts (WHITE; SCANDALIOS, 1988; KERNODLE; SCANDALIOS, 2001; SCANDALIOS, 2005).

In contrast, the univalent reduction of $O_2^{\bullet-}$ produces H_2O_2, which is moderately reactive (GILL; TUTEJA, 2010) and therefore other types of enzymes are necessary to convert hydrogen peroxide into a non-harmful compound, the most crucial of which are the catalases (CAT; EC.1.11.1.6.) (DOMÍNGUEZ et al., 2014 and references therein).

Catalases primarily convert hydrogen peroxide generated via SOD-mediated dismutation reactions or oxidative stresses (Reaction 2) to water and oxygen (GILL; TUTEJA, 2010). This reaction is very efficient and occurs in two steps. In the first step, the heme iron of catalase interacts with H_2O_2 to form oxygen-rich iron peroxide, referred to as compound I (CAT-Fe-OOH + water), and in the second step, under high concentrations of H_2O_2, compound I reacts with a second H_2O_2 molecule (Figure 42) to eventually produce two molecules of water and one of oxygen (SCANDALIOS et al., 1997).

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \quad (2)$$

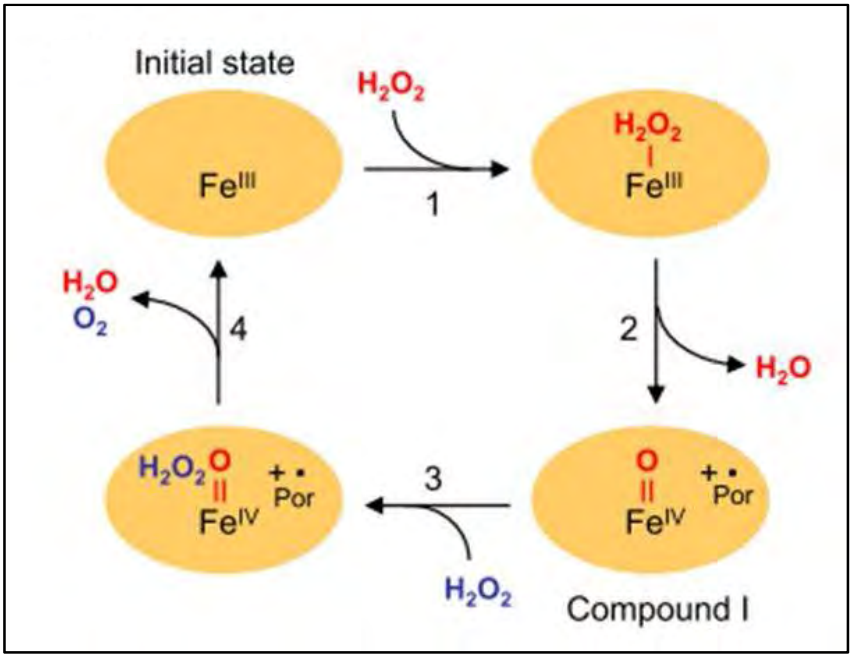

Figure 52 - Simplified schematic of catalase catalytic mechanisms (MHAMDI *et al.*, 2010)

In maize, there are three catalase isozymes (CAT-l, CAT-2 and CAT-3) encoded by three distinct and independent genetic loci (*Cat*1, *Cat*2 and *Cat*3) (ABLER; SCANDALIOS, 1993). Their activity is tissue-specific, for example, CAT-1 is not found in maize mature leaves, although this isozyme is present in the pericarp and endosperm. CAT-3 is most active in maize stem, whereas in leaves, only two isozymes (CAT-2 and CAT-3) exist, although only one, CAT-3, is present in some genotypes (SCANDALIOS et al., 1980; ACEVEDO; SCANDALIOS, 1991; SCANDALIOS, 2005). Each of the maize catalase isozymes is composed of four approximately 50-70 kDa subunits, and they are structurally similar to catalases found in other organisms (Figure 53) (SCANDALIOS et al., 1997).

According to Scandalios et al. (1980), CAT-3 is associated with mitochondria although the gene is located in the nucleus (chromosome 4; GRMZM2G079348). The gene products of maize *Cat*1 (chromosome 5; GRMZM2G088212) and *Cat*2 (chromosome 1; GRMZM2G090568) are localized to the cytosol and peroxisomes, and their sequences contain a conserved Ser-Arg-Leu repeat, whereas in *Cat*3, the Ser is replaced by Thr (MCCLUNG, 1997; GILL; TUTEJA, 2010 and references therein).

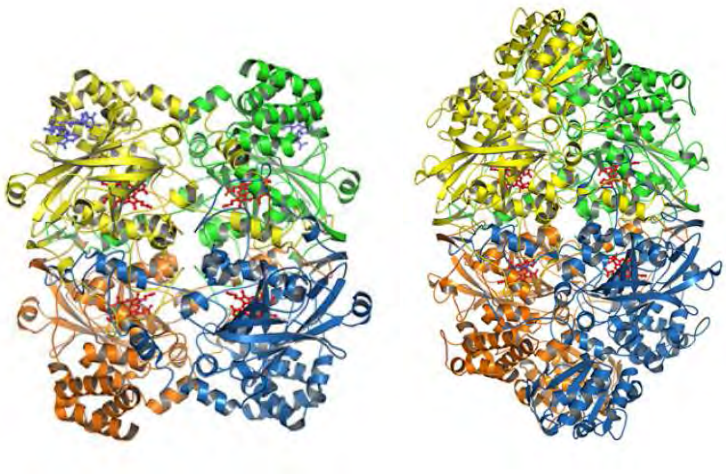

Figure 53 - Human (right) and *Escherichia coli* (left) catalase structures (DÍAZ *et al.*, 2012)

Although the structure of the domains of this enzyme is conserved, recent studies have shown that the activities of catalases may vary markedly. For example, under Al stress, CAT activity increased in *Hordeum vulgare* (ACHARY et al., 2012); however, it decreased in *Alium cepa* (ACHARY et al., 2008). Lidon and Barreiro (1999) noted that for maize (cv.XL-72.3), catalase activity decreased under Al-stress. Boscolo et al. (2003) observed that degradation of H_2O_2 generated from Al stress occurred due to the activation of other peroxidases rather than catalase. Data also varies with respect to the acclimation of maize plants to other stresses: an increase in CAT activity was observed in maize during acclimation to salt stress using H_2O_2 foliar spraying (GONDIM *et al.*, 2012), but no changes in CAT activity was observed when maize seeds were primed with hormones for acclimation to salt stress (CARVALHO *et al.*, 2011). These differences in response may have been caused by the fact that CAT activity can vary depending on: 1) species or variety, 2) period of evaluation while under stress, 3) circadian clock, 4) magnitude of stress and 5) different types of acclimation.

In addition to catalase, H_2O_2 may also be converted to water via "peroxidases", a term denoting the enzyme-catalyzed reduction of hydrogen peroxide and the oxidation of a variety of substrates (Reaction 3) (HIRAGA *et al.*, 2001).

$$H_2O_2 + AH_2 \xrightarrow{\text{Peroxidase}} H_2O + A \qquad (3)$$

The plant, fungal and bacterial peroxidase superfamily may be divided into three classes. The first includes the intracellular enzymes originating from prokaryotes (they show

similar structures – see Figure 54), cytochrome *c* peroxidase (EC 1.11.1.5), catalase-peroxidase (EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11) (HIRAGA *et al.*, 2001). The latter, the focus of our study, oxidizes ascorbate to form dehydroascorbate (DHA) and water (Reaction 4) (ASADA, 1992; ZÁMOCKÝ et al., 2000).

$$2H_2O_2 + AsA \xrightarrow{APX} 2H_2O + DHA \qquad (4)$$

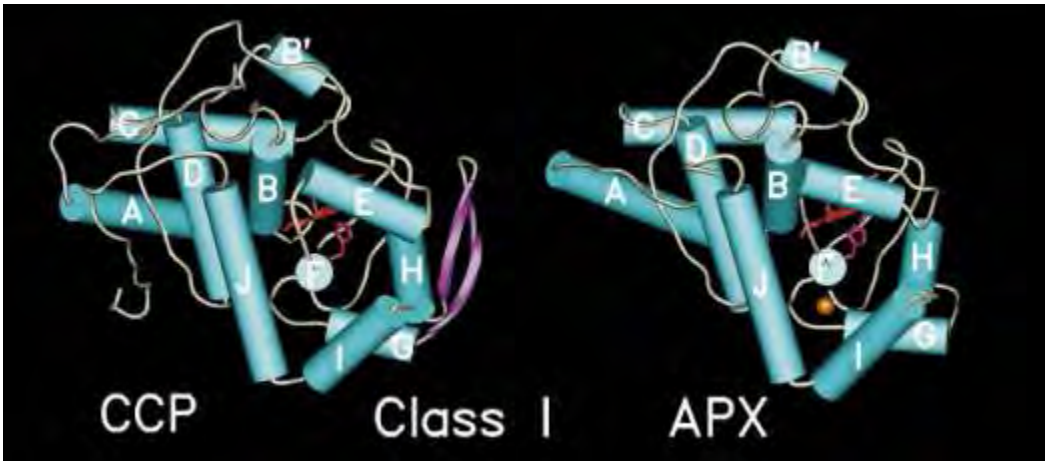

Figure 54 - Examples of the conserved overall fold within Class I peroxidases. (Left) Yeast cytochrome *c* peroxidase (CCP); (right) pea cytosolic ascorbate peroxidase (APX) (SCHULLER *et al.*, 1996)

Class I peroxidases are present in all three domains of life (Archaea, Bacteria, and Eukarya); however, due to divergent physiological requirements, specialized subfamilies have largely evolved following the division of domains (ZÁMOCKÝ; JANECEK; KOLLER, 2000). For example, APX is found in the chloroplasts, mitochondria, cytosol and cell wall of plants, in eukaryotic algae and in many cyanobacteria (WELINDER, 1992; MITTLER et al., 2004), whereas cytochrome *c* peroxidase is found in yeast, fungi and in plant mitochondria (PRASAD et al., 1995), and bifunctional catalase-peroxidase is found only in bacteria and fungi (SANTONI *et al.*, 2004). Although both catalase and catalase-peroxidase degrade H_2O_2, monofunctional catalase employs H_2O_2 as both an electron donor and acceptor (Reaction 2), whereas bifunctional catalase-peroxidase uses organic substrates as the electron donor to reduce H_2O_2 (in addition to reaction 2, it can also perform reaction 3) (MHAMDI *et al.*, 2010).

The Class II enzymes are extracellular peroxidases found in fungi (WELINDER, 1992), and the Class III enzymes are classical secretory plant peroxidases that include the most famous guaiacol-type peroxidase (GPOX; EC 1.11.1.7) (WELINDER, 1992; ZÁMOCKÝ et al., 2000). This enzyme utilizes several substrates to convert H_2O_2 to water, for example, lignin precursors and auxins (Reaction 5) (HIRAGA et al., 2001; BLOKHINA, 2003 and references there in).

$$2H_2O_2 + \text{Monoligonols} \xrightarrow{\text{GPOX}} 2H_2O + \textit{Lignin polymer} \quad (5)$$

Generally, for this type of multiple-substrate enzyme, the activity most widely used assay uses guaiacol as a substrate (the name GPOX comes from this type of assay) (ASADA, 1992). In contrast to APX, which is a Class I peroxidase, GPOX belongs to Class III peroxidases because they have three different helices instead of one specific helix (Figure 55), and it oxidizes various substances, from small molecules to macromolecules (HIRAGA *et al.*, 2001).

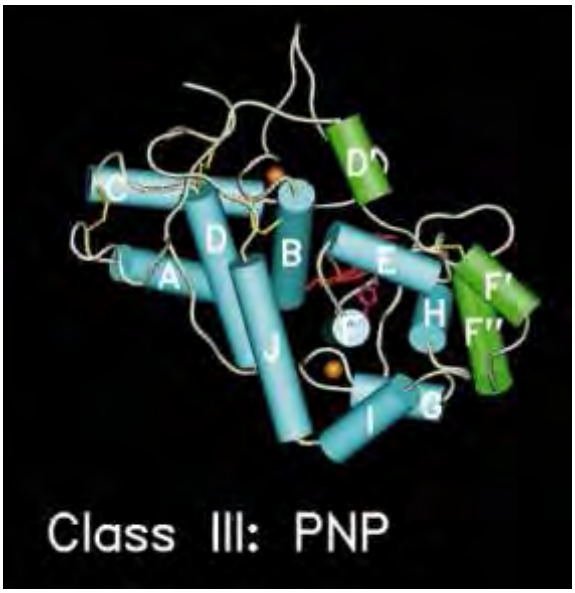

Figure 55 - Structure of a Class III peroxidase found in the peanut. In green, the three distinct helices (D', F' and F") found in this Class compared with Class I peroxidases are shown (SCHULLER *et al.*, 1996)

GPOX plays a critical role in plant land colonization because in addition to adapting the organisms to the more oxygenated environment, it also provides more rigid structures (COSIO; DUNAND, 2009). Class III peroxidases also perform other important functions;

however, in this section, we will focus on the scavenging of ROS generated from abiotic stress and lignification.

Hydrogen peroxide is utilized as a substrate during the process of lignin polymerization (OGAWA et al., 1997). The lignin polymers grow following the coupling of the monolignols in the “nucleation sites”, which are dependent on peroxidase reactions occurring in the middle lamella and in the corners of the cell (BOERJAN et al., 2003).

In maize, GPOX may be present in the apoplast of root maize cells as demonstrated by Mika; Luthje (2003); moreover, SOD4 may participate in the processes of lignification because an increase in the expression and activity of this type of SOD was observed during stem development (ACEVEDO; SCANDALIOS, 1991). SOD4 belongs to the Cu/Zn-SOD family that also may localize to the apoplast, which is attached to plasma cell membrane (Figure 56) (OGAWA et al., 1997).

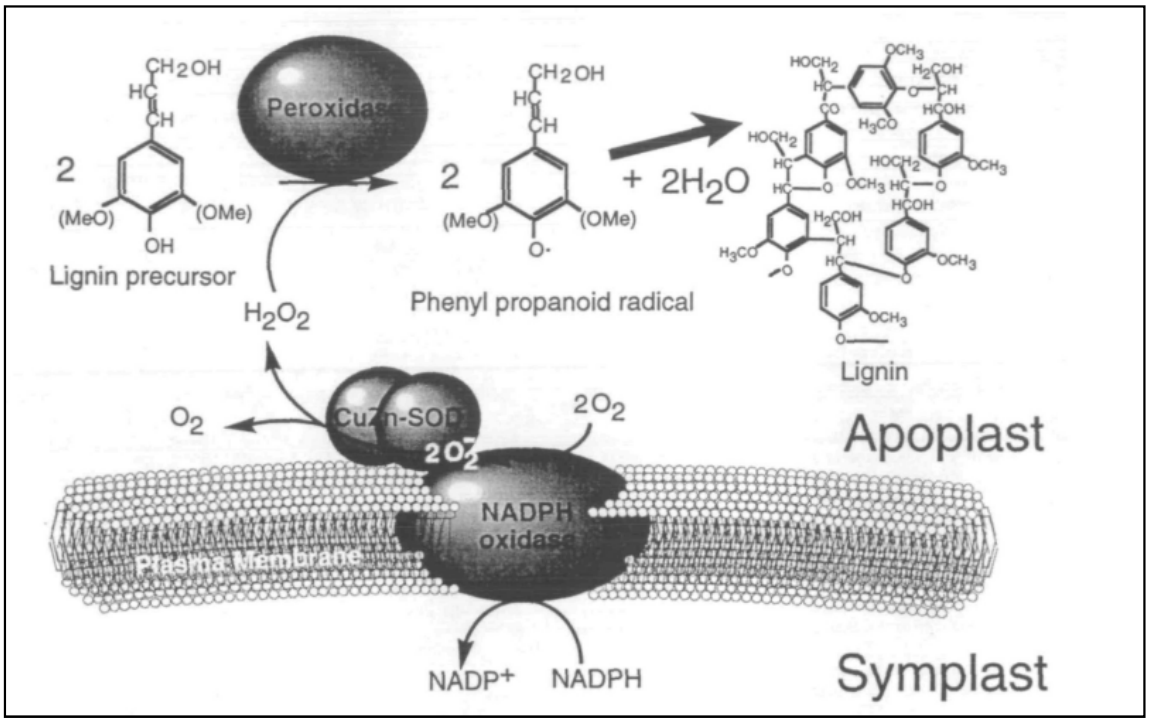

Figure 56 - The relationship between Class III peroxidases and Cu/Zn-SOD during lignification (OGAWA et al., 1997)

As previously reviewed, the activity of APX consumes AsA and generates DHA that can be regenerated into AsA via an enzymatic reaction mediated by dehydroascorbate reductase (DHAR) using glutathione (GSH) as a reducing substance (YIN *et al.*, 2010). Therefore, another enzyme to be studied is glutathione reductase (GR; EC 1.8.1.7), which

plays an important role in the antioxidant defense machinery by reducing the oxidized form of glutathione (GSSG), thereby recuperating GSH (ADAMIS et al., 2007) (Figure 57).

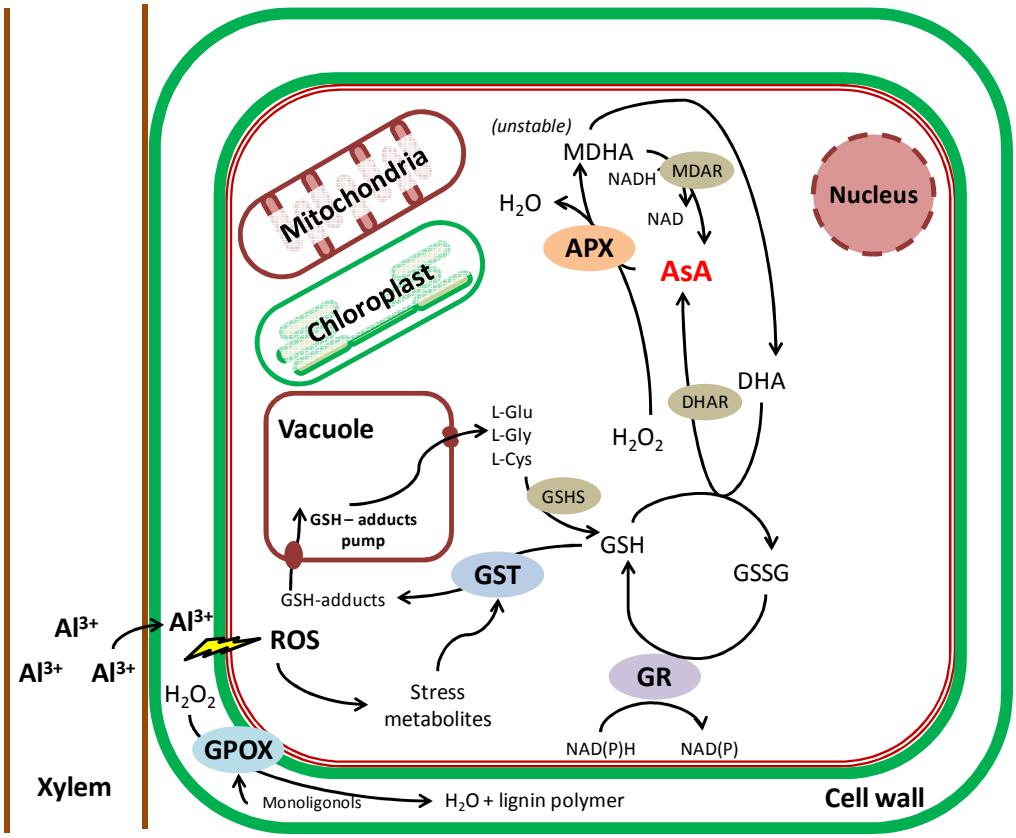

Figure 57 - Schematic of the role of glutathione reductase in the antioxidant machinery and ascorbate regeneration mediated by glutathione (GSH). The enzymes involved are: ascorbate peroxidase (APX); guaiacol-type peroxidase (GPOX); glutathione reductase (GR); and glutathione-S-transferase (GST). The compound in red is ascorbic acid (AsA)

In addition to the regeneration of AsA, glutathione also plays an important role in the conjugation of stress metabolites. In addition to the superoxide dismutase, catalases and peroxidases involved in ROS scavenging, ROS may eventually generate stress metabolites. Here, GSH-conjugate formation is mediated by glutathione S-transferase (GST; EC 2.5.1.18), an enzyme that contains active sites that bind GSH to cytotoxic compounds or hydrophobic ligands (MARRS, 1996). GST is important for the catalysis of GSH conjugation to: 1) phytochemicals, such as anthocyanins, which tags them for vacuolar sequestration (MARRS et al., 1995); 2) electrophilic xenobiotics, such as herbicides (EDWARDS et al., 2000); and 3) metabolites of oxidative damage, such as 4-hydroxynonenal (ESTERBAUER et al., 1991) and malondialdehyde (MDA) (BLAIR, 2010). In maize, 42 GSTs have been discovered to date

that can be divided in type I, II or III based on sequence similarities (MCGONIGLE *et al.*, 2000). All GSTs possess a conserved GSH-binding site (G-site) located in the N-terminal domain, although they may exclusively recognize a specific substrate in the C-terminal domain (EDWARDS et al., 2000). GSTs also have catalytic roles as carriers for endogenous compounds, including indole-3-acetic acid (IAA) and flavones (MARRS, 1996 and DIXON et al., 2002), which are involved in plant growth and defense against herbivores and pathogens.

Therefore, GSTs are important in inactivating toxic chemicals and in enabling plant development, and some of these chemicals enable protective strategies that have been adopted by plants but that can also be harmful to the cells that produce them (MARRS, 1996). The study of how ascorbic acid interferes in the activity of enzymes involved in reducing ROS and the activity of GSTs responsible for detoxifying damaging compounds is essential for understanding how cells and organisms tolerate and grow while under stress.

5.1.3 Objectives

The main objective of the experiments described in this section was to test seed priming with ascorbic acid under field conditions of high natural Al content and to verify whether this treatment affects maize productivity. The specific objectives of these experiments are as follows:

a) To verify whether seed priming using ascorbic acid in the field affects productivity;
b) To determine the effect of ascorbic acid on nutrient absorption;
c) To quantify lignin content in maize shoots and
d) To study enzyme activity related to antioxidant defenses when ascorbic acid is applied as seed priming.

5.1.4 Hypothesis

Based on previous results using ascorbic acid as a potential seed priming to induce Al tolerance in maize (Section 4) and given the importance of enhancing corn productivity, our hypotheses for this section are as follows:

1) Seed priming using ascorbic acid increases maize growth and consequently enhances corn productivity;

2) Because we expected growth improvements caused by seed priming, we expect that the priming would increase nutrient absorption;
3) Reduction in lignin content in shoots of primed seeds was expected (as observed in Section 4);
4) Reduction of oxidative stress was expected in plants originating from primed seeds and
5) Changes in antioxidant enzyme activity were expected.

5.2 Material and Methods

5.2.1 Plant material and field experiment

For the field experiment, we utilized seeds of the maize (*Zea mays* L.) inbred line Mo17 (Al-sensitive) and the commercial transgenic (Bt gene) simple hybrid 2B587PW (Al-tolerant from Dow AgroSciences – in this study, abbreviated to DA). The experiment was performed in the Genetics Experimental Station of "Escola Superior de Agricultura Luiz de Queiroz – Universidade de São Paulo" (Figure 58) located in a *cerrado* region, the Anhumas district (Lat. 22°45' - 22°50' S; Long. 48°00' - 48°05' W; 460 m altitude), Brazil, and conducted using a randomized block design with five repetitions over a total area of 690 m^2. Each block was composed of 3 rows (8 plants per row), and to guarantee the germination of all plants per row with the aim of analyzing productivity, we sowed 2 seeds per fissure. The thinning steps occurred after 1 month and 20 days of germination. Seeds were sown on February 06, 2013, and harvested on July 17, 2013. Unprimed and ascorbic acid-primed maize seeds (3.8 g of ascorbic acid/ 25 seeds) were cultivated in soil type Yellow Red Latosol (Oxisol) with the following characteristics: pH 4.6 in H_2O; 27 $mmol_c \cdot Kg^{-1}$ of Al content; 18 $mg \cdot Kg^{-1}$ of phosphorus; 2.6 $mmol_c \cdot Kg^{-1}$ of potassium; 5 $mmol_c \cdot Kg^{-1}$ of calcium; 3 $mmol_c \cdot Kg^{-1}$ of magnesium; bases saturation of 19% and changeable aluminum of 72%. We supplemented the soil with nitrogen, phosphorus and potassium using NPK 08:28:16 250 $Kg \cdot ha^{-1}$ prior to sowing. For this experiment, the soil was not limed to maintain the acidic characteristic to test the efficiency of the ascorbic acid treatment on the maize seeds. To control weeds, we utilized Primatop SC (Atrazine, 250 $g \cdot L^{-1}$ and Simazine, 250 $g \cdot L^{-1}$) at a dose of 7.0 $L \cdot ha^{-1}$. To control insects, we used three applications of Tracer insecticide at a dose of 100 $mL \cdot ha^{-1}$.

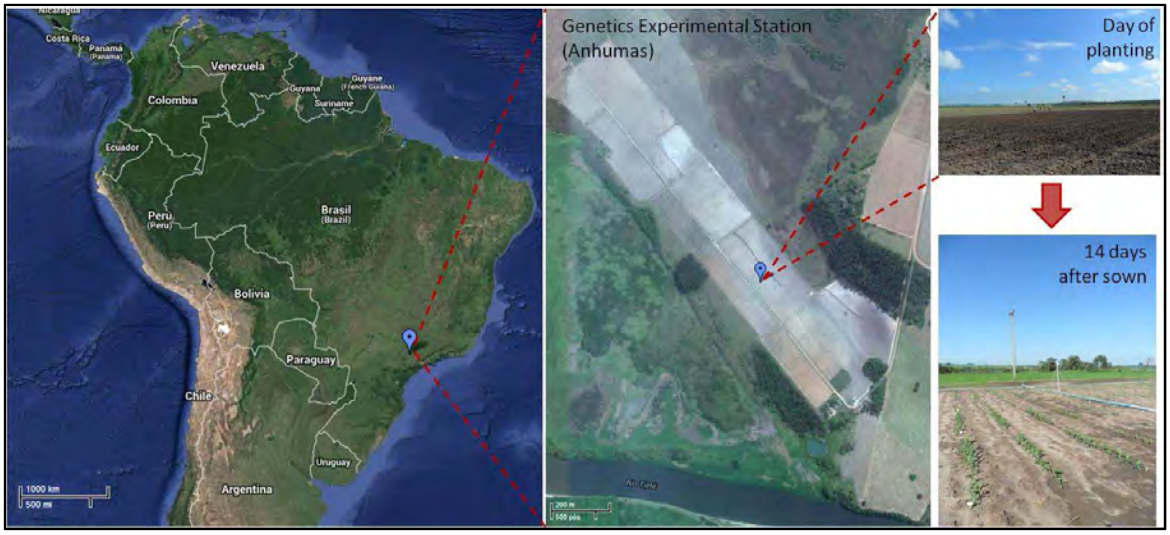

Figure 58 - (Left) Localization of the experimental area in Brazil, and (middle) a zoomed in view of the Genetics Experimental Station in the Anhumas district (Google maps). (Right and up) A zoomed in view of the planting area and (right and down) the maize seedlings growing in the area (14 days after being sown)

5.2.2 Maize growth and grain yield

For the productivity parameters, we considered the following measurements: total height of the maize plants (cm), height to flag leaf (cm) and yield of grain ($t.ha^{-1}$). We utilized a measuring tape to measure total heights and height to flag leaf. Three plants were measured to obtain the average per block (Figure 59 shows the point of measurements). To obtain the grain yields, we harvested all maize ears for each block, and using random sampling, we obtained 35 cobs from each treatment, measured the weight of 1000 kernels and estimated the mean grain number per block. To calculate the yield of grain per hectare, we utilized the equation (1):

$$Yield\ (t\ ha^{-1}) = \frac{\frac{Weight\ of\ 1000\ kernels\ (g)}{1000} \times Number\ of\ grains\ per\ block}{10^6 \times block\ area\ (ha)} \quad (1)$$

The coefficient of variation was provided by the formula (2):

$$CV\ (\%) = 100\ \times \frac{\sqrt{MS_{res}}}{\bar{x}} \quad (2)$$

In which MS_{res} is the residual mean square and $\bar{x}$ is the total average of the variable.

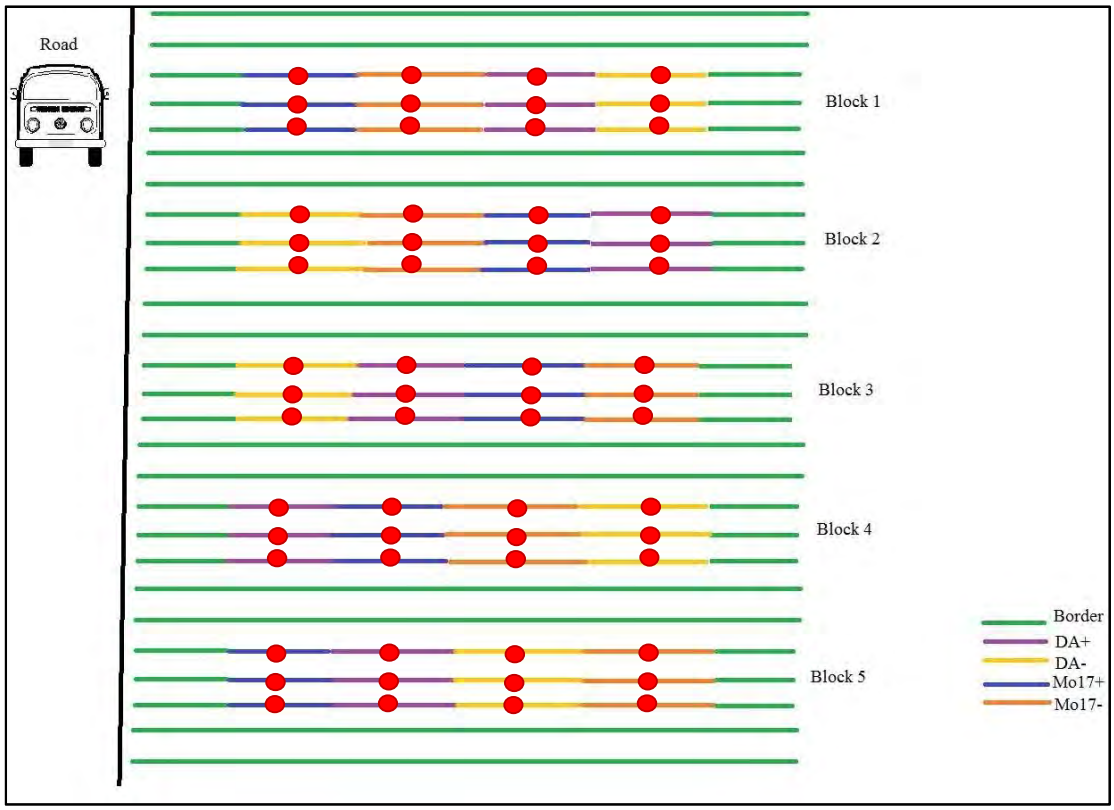

Figure 59 - Schematic of the experimental area. Red dots denote plants used for height measurements

5.2.3 Nutrient absorption

Samples of maize shoots were obtained during the thinning step (1 month 20 days old), and kernels were sent to the Laboratory of Vegetal Tissues Analyses located at the Soil Department of "Escola Superior de Agricultura Luiz de Queiroz – Universidade de São Paulo". Nutrient content measurements were performed according to Malavolta et al. (1997), a report that describes the techniques routinely used in that lab.

5.2.4 Lipid peroxidation

Oxidative damage in maize leaves obtained during thinning (1 month 20 days old) was quantified biochemically based on methods described in a previous publication (CAKMAK; 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. Readings at

535 and 600 nm were performed using a spectrophotometer, and the concentration of MDA was determined using the formula (3):

$$C = [ABS(535 - 600) \div 155] \times 10^6 \quad (3)$$

The results were expressed as nmol MDA·g^{-1} fresh matter.

5.2.5 Hydrogen peroxide

We determined levels of hydrogen peroxide using maize leaves obtained during thinning (1 month 20 days old) according to methods described by Alexieva et al. (2001). Maize leaves were homogenized in 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 *g* for 15 min at 4 °C. Following centrifugation, 200 µL of the supernatant was added to 200 µL of 100 mM potassium phosphate buffer (pH 7.0) and 800 µL of 1 M KI. Readings were taken at a wavelength of 390 nm.

5.2.6 Ascorbate quantification

The content of ascorbate was determined as described by Arakawa et al. (1981). To perform the extraction of ascorbate from leaf tissue, 0.2 g of vegetal material was macerated in liquid nitrogen using 1 mL of extraction buffer containing 5% (w/v) trichloroacetic acid (TCA).

The homogenate was centrifuged at 15,000 *g* for 15 min at 4 °C, and an aliquot of 50 µL of supernatant was collected and diluted in 70 µL of TCA (5%). To the diluted sample, we added 125 µL of pure ethanol and 125 µL of Na_2HPO_4 (0.2 M pH 8.0). This mixture was incubated at 25 °C for 10 min. Subsequently, the following reagents (all dissolved in pure ethanol) were added to the same tube: 125 µL 0.24% (w/v) N-ethylmaleimide, 125 µL pure ethanol, 125 µL 4% (v/v) H_3PO_4, 250 µL 0.5% (w/v) bathophenanthroline and 150 µL 0.03% (w/v) $FeCl_3$, resulting in the final volume of 1.145 mL. The tubes were vortexed, and the mixture was incubated at 30 °C for 90 min. The reactions were then stopped by placing the tubes in an ice bath, and the absorbance of the mixtures was measured at 534 nm. The ascorbate content in the samples was calculated based on a standard curve obtained using known concentrations of ascorbic acid (Figures 60 and 61).

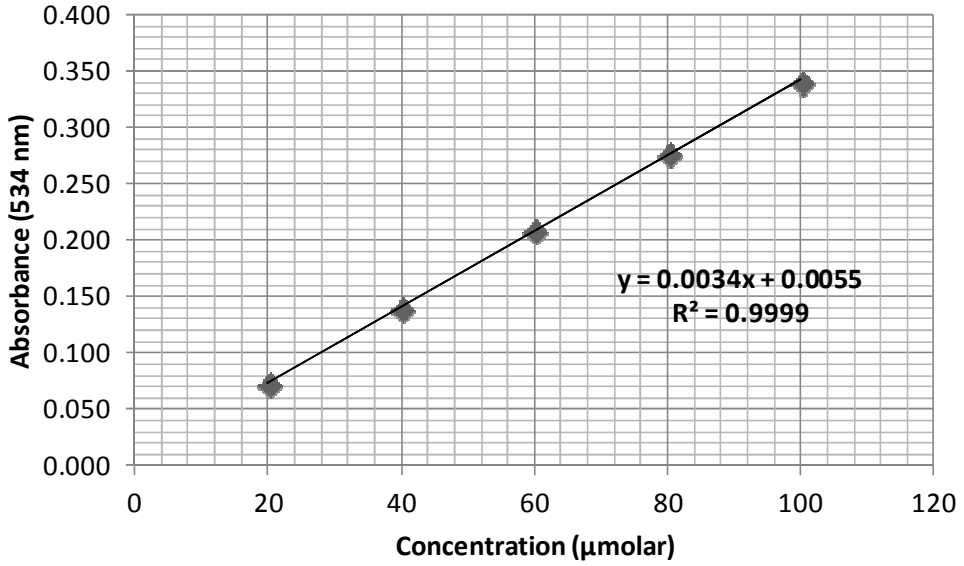

Figure 60 - The standard curve used for ascorbate quantification

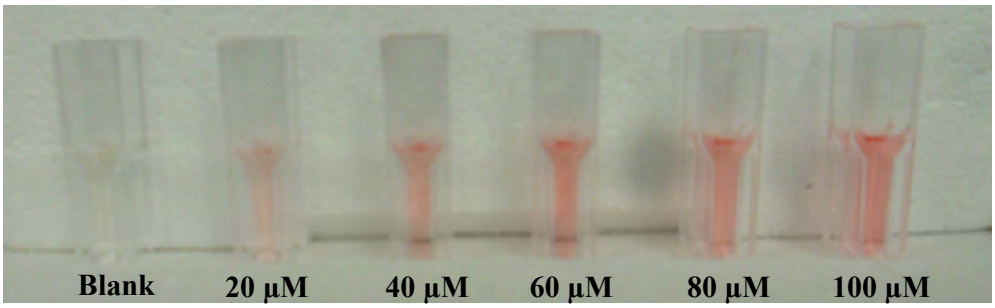

Figure 61 - Demonstration of the colorimetric reaction used for ascorbate determination

5.2.7 Protein extraction and quantification

We collected maize leaves from just below the flag leaf from plants aged 1 month 20 days. The leaves were macerated in liquid nitrogen and homogenized using a mortar and pestle in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTT and 4% (w/v) insoluble PVPP (AZEVEDO et al., 1998) at a ratio of 5 : 1 (buffer volume : fresh weight). The homogenate was centrifuged at 10,000 *g* for 30 min at 4 °C (GRATÃO et al., 2008), and the supernatant was stored in 200-µl aliquots at -80 ℃ for further analyses of SOD, CAT, GR, GST, GPOX and APX. Protein concentrations for all samples were determined using the Bradford method (1976) at a wavelength of 595 nm.

5.2.8 Non-denaturing polyacrylamide gels

To prepare 10% non-denaturing polyacrylamide gels, we utilized 2.5 ml of a 40% acrylamide/bis-acrylamide solution (Sigma) and 2.5 ml of 2.9 M TRIS buffer (pH 8.9) in 5 ml of deionized water, 19 µL of TEMED and 25 µL of ammonium persulfate (10%). After polymerization, approximately 4.5 ml of stacking gel was added to above the main gel. To prepare the stacking gel, we added 0.5 ml of 40% acrylamide/bis-acrylamide and 1.25 ml of 500 mM TRIS buffer (pH 6.8) to 2.75 ml of deionized water, 10 µL of TEMED and 50 µL of ammonium persulfate (10%). For the enzyme assays, electrophoresis was performed at 4 °C at a constant current of 15 mA per plate (running time depending on the enzyme). The amount of protein sample loaded was 20 µg for all assays (except for SOD isozymes determination we used 70 µg). For enzyme activity analyses, we also performed densitometry analyses using ImageJ software, version 1.47 (National Institutes of Health, U.S.A; http://imagej.nih.gov/ij).

5.2.9 Enzyme assays

Superoxide dismutase - SOD activity analyses were performed using spectrophotometry and electrophoresed proteins in 10% polyacrylamide gels. The former assay was performed according to Giannopolitis; Ries (1977), and the gel-based method was performed according to Beauchamp & Fridovich (1971). We separated plant proteins by electrophoresis over a period of 4 hours. The gels were then incubated in the dark at 25 °C in a solution containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM NBT and 0.3% TEMED. Following 30-min incubation, the gels were washed using deionized water and then placed in the light for several minutes until bands appeared with a negative dark background. The photo-oxidation was terminated by soaking the gels in a solution of acetic acid (7%). SOD isozymes were determined as follows:

1. At the end of electrophoresis, we divided the gels into three equal vertical parts;
2. The first part was typically used to determine SOD activity;
3. The second and third sections were incubated for 20 minutes in 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM KCN or 100 mM potassium phosphate buffer (pH 7.8) containing 5 mM H_2O_2, respectively.
4. Both sections were then washed and stained for SOD activity; and

5. Pretreatment of H_2O_2 and KCN gels prior to the development of SOD enabled the classification of SOD isozymes as follows: Mn-SOD is resistant to both inhibitors; Fe-SOD is resistant to KCN and inhibited by H_2O_2; and Cu/Zn-SOD is inhibited by both inhibitors (Figure 62).

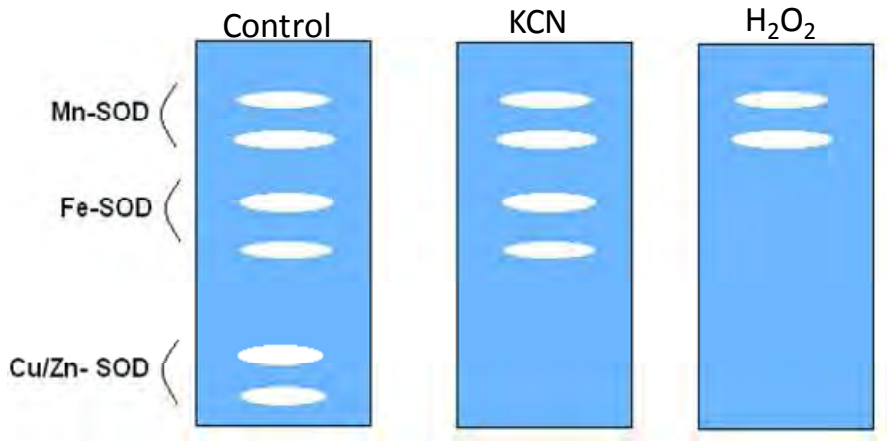

Figure 62 - Schematic of SOD isozyme determination

Catalase - CAT activity analyses were performed using a 10% non-denaturing polyacrylamide gel and spectrophotometry. For the spectrophotometric method, CAT activity was measured by incubating reaction mixtures at 25 ℃, and they contained 10 ml of potassium phosphate buffer (100 mM, pH 7.5) and 25 µl of H_2O_2, based on Azevedo et al. (1998). The reaction was initiated by adding 25 µl of plant protein extract, and CAT activity was determined by monitoring the degradation of H_2O_2 at 240 nm (absorption peak of H_2O_2) in quartz cuvettes over 1-second intervals up to 60 seconds. The results were expressed as $\mu mol.min^{-1}.mg^{-1}$ of protein. To determine CAT isoforms, we separated plant proteins by electrophoresis over a period of 24 hours. CAT activity was revealed after washing the gel for 45 minutes in deionized water (3 x 15 minutes) and incubating for 10 minutes in a solution containing H_2O_2 (0.003%) at room temperature (gentle and constant agitation). The gels were then washed quickly with deionized water and placed for 15 min in a solution containing $FeCl_3$ (1% w/v) and $K_3Fe(CN)_6$ (1% w/v) with gentle agitation. The solution was then removed and the gels washed with deionized water. Gels were fixed using acetic acid (7%).

Glutathione reductase - GR activities were measured using 10% non-denaturing polyacrylamide gels and spectrophotometry. After separating the proteins by electrophoresis over a period of 3 hours and 30 min, GR staining was performed as described by Medici et al. (2004). For the spectrophotometric method, mixtures were incubated at 30 ℃ and contained 3

mL of 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5,5'-dithiozbis(2-nitrobenzoic acid), 1 mM GSSG and 0.1 mM NADPH. The reaction was initiated by adding 50 µL of enzyme extract. The rate of reduction of GSSG was followed by monitoring changes in absorbance at 412 nm over a period of 2 min (AZEVEDO et al., 1998).

Glutathione-S-transferase - GST activity was assayed spectrophotometrically according to the method of Marrs et al. (1995), with modifications. Briefly, reaction mixtures were incubated at 30 ℃ and contained 900 µL of 100 mM potassium phosphate buffer (pH 6.5), 50 µL of 0.1 mM GSH, 25 µL of 0.04 M 1-chloro-2, 4-dinitrobenzene (CDNB) and 25 µL of enzyme extract. GST activity was measured by measuring the rate of change in absorbance at 340 nm over a period of 1 minute.

Guaiacol peroxidase - GPOX activity was evaluated by spectrophotometry as described by Gratão et al. (2008) with some modifications. The reaction mixture contained 390 µL of phosphate-citrate buffer pH 5.0 (dibasic sodium phosphate 0.2 M: citric acid 0.1 M), 10 µL of enzyme extract and 25 µL of 0.5% guaiacol, which was vortexed and incubated in water bath at 30 ℃ for 15 min. The reaction was terminated by quickly cooling in an ice-water bath, followed by the addition of 25 µL of 2% sodium metabisulfide. The reaction mixture was incubated for 10 min, and the GPOX activity was then evaluated by quantifying the absorbance at 450 nm.

Ascorbate peroxidase - APX activities were measured in 10% non-denaturing polyacrylamide gels and determined spectrophotometrically. For the spectrophotometric method, APX activity was measured according to Durner & Klessig (1995), with some modifications. A reaction mixture of 1 ml was used that contained 650 µL of potassium phosphate buffer (80 mM, pH 7.0), 100 µL of AsA (5 mM), 100 µL EDTA (1 mM) and 100 µL of H_2O_2 (1 mM). The amount of protein extract used for each assay was 50 µL. Oxidation of AsA was followed by measuring the decrease in absorbance at 290 nm (extinction coefficient: 2.8 $mM^{-1}cm^{-1}$) over 1 minute. The activity of APX in gels was performed according to a previous publication (MITTLER; ZILINSKAS, 1993).

5.2.10 SDS-polyacrylamide gel

We performed one-dimensional electrophoresis using 10% SDS-polyacrylamide gels to analyze the total protein profiles of maize leaves (1 month 20 days old). To prepare these gels, we added 2.5 ml of 40% acrylamide/bis-acrylamide (Sigma) to 2.5 ml of 2.9 M TRIS

buffer (pH 8.9), 5 ml o deionized water, 100 µL of SDS (10%), 19 µL of TEMED and 25 µL of ammonium persulfate (10%). After polymerization, approximately 4.5 ml of stacking gel was added to above the main gel. To prepare the stacking gel, 0.5 ml of 40% acrylamide/bis-acrylamide was added to 1.25 ml of 500 mM TRIS buffer (pH 6.8), 2.75 ml of deionized water, 50 µL of SDS (10%), 10 µL of TEMED and 50 µL of ammonium persulfate (10%). Electrophoresis was performed at room temperature at a constant current of 20 mA per plate over a period of 4 hours. The amount of protein loaded was 20 µg for all samples. Gel staining was performed using 0.5 g of Coomasie blue solution (R-250) diluted in bleach solution (40% methanol and 7% glacial acetic acid) overnight. After staining the gels, the bleach solution was added for manual visualization.

5.2.11 Klason lignin determination

For Klason lignin determination, dried maize shoots obtained during thinning (1 month 20 days old) were ground using a mill knife and passed through a 60-mesh sieve. All of the results obtained were expressed relative to 1 g of absolute dried material. First, small pans were incubated at 105 ± 3 °C overnight and then placed into desiccators until cool and then weighed using a balance with an accuracy of 0.001 g (*pw*). Second, the powdered samples (*ms*) were weighed. The pans with samples were placed in an oven at 105 ± 3 °C overnight, and the following day, the samples were placed in a desiccator until cooled and then weighed on the balance. The new mass was annotated (*nms*) to determine the percentage of absolute dried material (Formula 4):

$$\text{Abs dried mat (\%)} = 100 \times \left(\frac{\text{nms} - \text{pw}}{\text{ms} - \text{pw}}\right) \quad (4)$$

The quantity of mass equivalent to 1 gram of absolute dried material was provided by the formula 5:

$$equivalent\ mass\ to\ 1\ g\ absolute\ dried\ material = \frac{1}{Abs\ dried\ mat\ (\%)} \quad (5)$$

We weighed the mass equivalent to 1 g of absolute dried material and placed it into filter paper bags (gramature 70) to obtain extracts.

The extraction was performed using 700 ml of toluol:ethanol solution (2:1) over a period of 8 hours and then 700 ml of pure ethanol also over 8 hours. For the last extraction, we removed the samples from the paper bags and placed them into Erlenmeyer flasks with 100 ml of deionized water and incubated them in a hot water bath (100 °C) for 1 hour. The samples and water were then filtered using porous glass and the filtrate residue was dried in an oven at 105 ± 3 °C overnight. The dried samples without extracts (*dswe*) were then weighed, and the percentage of extracts was provided using equation 6:

$$\text{Extracts content (\%)} = 100 \times (1 - dswe) \quad (6)$$

For acid hydrolyses, we weighed 300 mg of dried sample without extracts and added 3 ml of 72% H_2SO_4. This mixture was incubated in a water bath at 30 °C for 1 hour. Next, we added 84 ml of deionized water and placed the flasks into an autoclave at 120 °C for another hour. After acid hydrolysis, the samples were filtered using glass microfiber (GF-1; D 47 mm), and the acid-insoluble lignin was determined gravimetrically, whereas the acid-soluble lignin was determined spectrophotometrically at 215 nm and 280 nm (KIRK; OBST, 1988). Figure 63 shows the steps used for lignin determination.

The percentage of insoluble lignin was provided using equation 7:

$$\text{Insoluble lignin (\%)} = 100 \times \left[\left(\frac{\text{acid insoluble residue (g)}}{0.3}\right) \div \left(1 - \frac{\text{Extracts (\%)}}{100}\right)\right] \quad (7)$$

And the percentage of soluble lignin was determined using formula 8:

$$\text{Soluble lignin (\%)} = 100 \times \left[\left(\frac{(4.53 \times \text{Abs 215 nm}) - \text{Abs 280 nm}}{300 \times \frac{0.3}{\left(1 - \frac{\text{Extractives (\%)}}{100}\right)}}\right)\right] \quad (8)$$

We determined holocellulose content based on the difference between total mass and extracts plus total lignin (Equation 9):

$$\text{Holocellulose (\%)} = 100 - [\text{extracts (\%)} + \text{total lignin (\%)}] \quad (9)$$

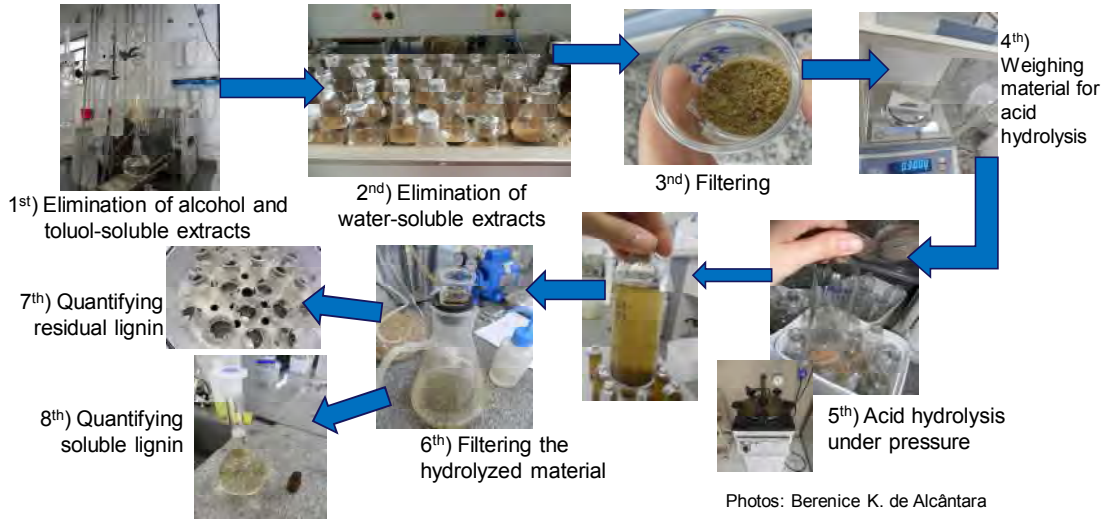

Figure 63 - Schematic of the lignin extraction

5.2.12 Carbohydrate quantification

For carbohydrate quantification, dried maize kernels were ground using a mill knife and filtered through a 60-mesh sieve. All results obtained were relative to 1 g of absolute dried material; thus, we first incubated small pans at 107 °C overnight, which were then placed in a desiccator until cooled and were then weighed using a balance with an accuracy of 0.001 g (*pw*). Second, we weighed the powdered kernels (w*k*). The pans and kernel powder were placed in an oven at 107 °C overnight, and the following day, the samples were placed in a desiccator until cooled and were then weighed using the balance. The new weight was annotated *nwk* to determine the percentage of absolute dried material (Equation 10):

$$\text{Abs dried mat for kernels (\%)} = 100 \times \left(\frac{\text{wk} - \text{pw}}{\text{nwk} - \text{pw}}\right) \quad (10)$$

The weight equivalent to 1 gram of absolute dried kernel powder was provided using formula 11:

$$equivalent\ weight\ to\ 1\ g\ absolute\ dried\ kernels = \frac{1}{Abs\ dried\ mat\ (\%)} \quad (11)$$

Starch is insoluble in toluol, alcohol and cold water (BROWN, 2005); however, it is soluble in hot water. Therefore, after the procedures described above, we weighed the mass equivalent to 1 g of absolute dried powder grains and placed it into filter paper bags (gramature 70) to obtain extracts. The extraction was performed using 700 ml of a toluol:ethanol solution (2 : 1) over a period of 8 hours and in 700 ml of pure ethanol for another 8 hours. For the final extraction, we removed the samples from the paper bags and placed them into Erlenmeyer flasks with 100 ml of Milli-Q water (22 °C) and incubated with constant agitation (160 rpm) over a period of 48 hours. The starch and water was filtered using porous glass, and the residue of the filtrate (pure starch) was dried in an oven at 107 °C overnight. For starch hydrolyses, we weighed 300 mg of dried pure starch without extracts and added 3 ml of 72% H_2SO_4. This mixture was incubated in a water bath at 30 °C for 1 hour. Next, we added 84 ml of Milli-Q water and placed the flasks into an autoclave at 120 °C for 1 hour (TAPPI, 1991a; TAPPI, 1991b). The hydrolyzed starch was then filtered with glass microfiber (GF-1; D 47 mm) and diluted in Milli-Q water at a ratio of 1 mL: 100 mL. The sugars were quantified using liquid chromatography (Dionex, model DX 500). Figure 64 shows the steps used for starch hydrolysis, and Figure 65 shows the carbohydrate chromatogram used for the calibration curve.

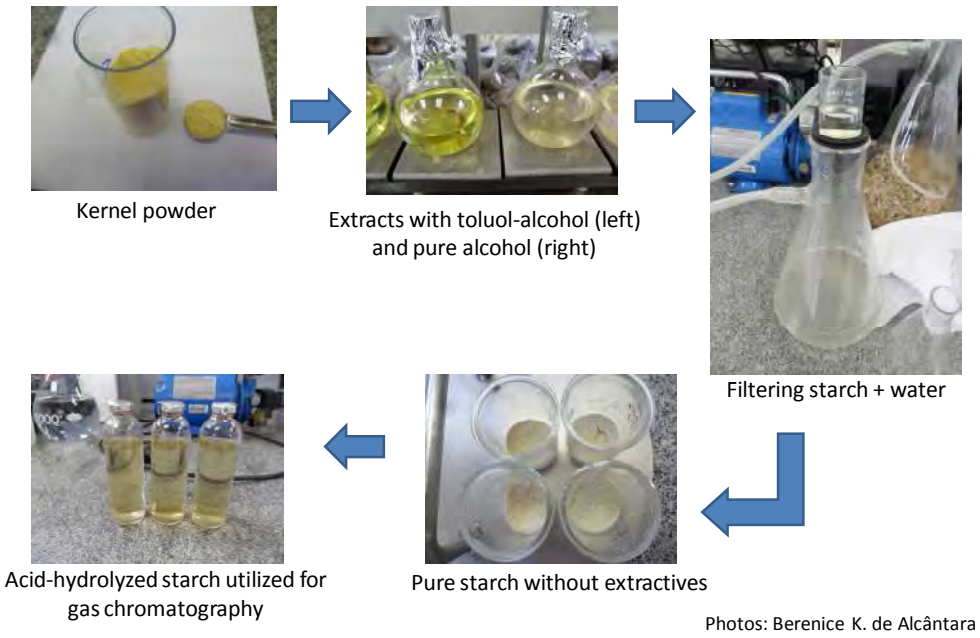

Figure 64 - Schematic of the starch hydrolysis used for carbohydrate quantification

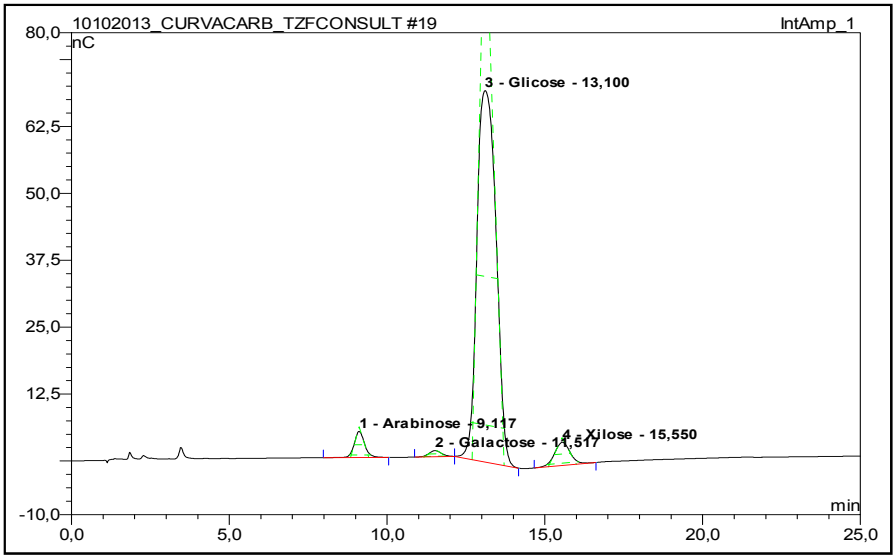

Figure 65 - Example of a carbohydrate chromatogram

5.2.13 Characterization of soluble amino acids and their quantification

Soluble amino acids were extracted following the method of Bieleski and Turner (1966) using three technical replicates and five biological replicates. Kernel powder was filtered through 60-mesh sieves, and 200 mg of this thin flour was homogenized in 2 ml of MCW (chloroform:methanol:water; 12:5:3). The mixture was incubated at 4 °C overnight and centrifuged at 10,000 *g* for 20 minutes. We removed the supernatant and added 0.5 ml of pure chloroform and 0.75 ml of Milli-Q water for each 2 ml of MCW. Another centrifugation step was performed, and the aqueous phase formed was removed carefully. The tubes containing the remaining phase were placed in a water bath and incubated at 38°C for 1 hour; however, the lids were left open to allow the alcohol to evaporate. The remaining phase was lyophilized, and the amino acids were resuspended in 200 µl of Milli-Q water. The analysis of amino acids was then performed using an Ultra Performance Liquid Chromatography (UPLC) Acquity system (Waters) with a BEH C18 column (2.1 x 100 mm, 17 µm) at 43 °C.

For derivatization, we utilized 70 µl of borate buffer, 10 µl of sample and 20 µl of derivatization reagent (AccQ-fluor Reagent Kit Waters). We mixed the reagents using a vortex, and we then placed the mixture in a water bath at 55 °C for 10 minutes. The injection volume was 1 µl, and the wavelength used for amino acid detection was 260 nm.

We utilized a specific gradient of mobile phases (Table 11) for amino acid determination.

Table 11 - Mobile phases used for amino acid characterization

Time (min)	Flow ($ml.min^{-1}$)	A* (%)	B (%)	C (%)	D (%)
0	0.7	10	0	90	0
0.29	0.7	9.9	0	90.1	0
5.49	0.7	9	80	11	0
7.1	0.7	8	15.6	57.9	18.5
7.3	0.7	8	15.6	57.9	18.5
7.69	0.7	7.8	0	70.9	21.3
7.99	0.7	4	0	36.3	59.7
8.59	0.7	4	0	36.3	59.7
8.68	0.7	10	0	90	0
10.2	0.7	10	0	90	0

* A: AccQ-Tag, Eluent A from Waters; B: 10% acetonitrile; C: 100% Milli-Q water; D: 100% acetonitrile

The concentrations of amino acids in the samples were determined using a standard curve constructed from known concentrations of histidine, serine, arginine, glycine, aspartate, glutamate, threonine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine (Figure 66). Some of the calibration curves are shown in Figure 67.

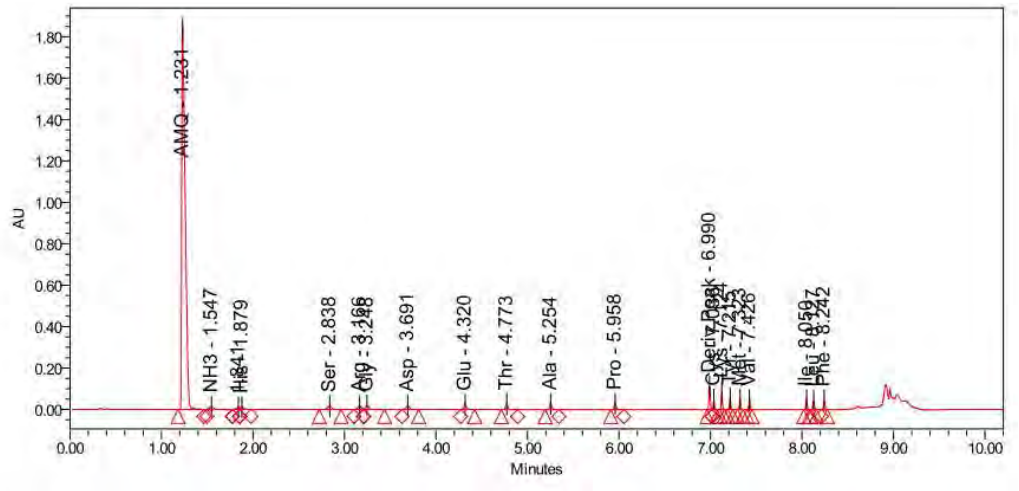

Figure 66 - Amino acids were quantified based on known concentrations of standards (15 ρmol. μL^{-1})

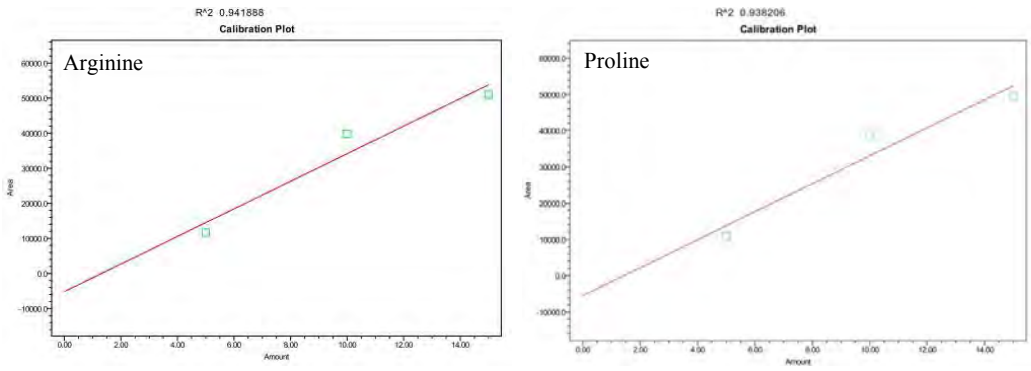

Figure 67 - Example calibration curves for arginine and proline

5.2.14 Statistical analyses

Statistical analyses for all data were performed using StatSoft software (version 7.0, Tulsa, OK, USA). Significant differences between the mean of treatments were determined using the Duncan test, at a confidence level of 95%.

5.3 Results

5.3.1 Maize growth and grains yield

Measurements of the total height of the maize plants and their height at the flag leaf (Figure 68) were taken on April 14th, 2013 (2 months 18 days after sowing). The total height of unprimed seeds of the Al-tolerant commercial hybrid (DA) averaged 120.56 ± 9.57 cm, whereas primed seeds were 150.99 ± 6.19 cm on average. For the sensitive line (Mo17), the mean total height of plants of unprimed seeds was 37.96 ± 7.15 cm and was 52.02 ± 6.98 cm for primed seeds. The average height to the flag leaf of plants from unprimed seeds of the DA variety was 94.44 ± 7.80 cm, whereas for DA primed seeds, the height was 121.28 ± 5.64 cm. For the sensitive inbred line Mo17, the mean height to flag leaf of plants from unprimed seeds was 31.59 ± 5.20 cm and for primed seeds was 41.36 ± 5.25 cm.

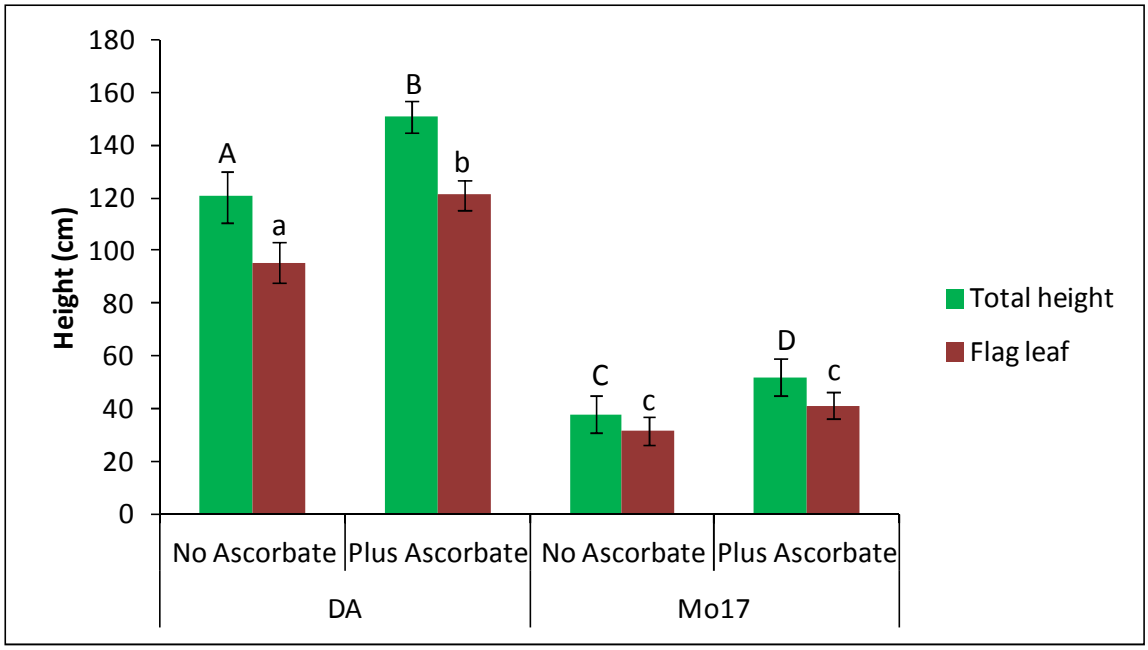

Figure 68 - The total height of maize plants (green) and height at the flag leaf (red) at 2 months 18 days after sowing. Similar letters above the bars of the same color show that the means are not significantly different at a confidence level of 95% (n = 5)

Grain productivity was analyzed for the Al-tolerant variety (DA) only because all Mo17 plants died within 3 months and 15 days after sowing (Figure 69). Ascorbic acid was obviously not sufficient to induce Al-tolerance in the sensitive Mo17 line; however, curiously, the seed priming improved the productivity of the DA variety, as shown in Table 12 and Figure 70.

Figure 69 - (Left) Maize line Mo17 died without any production within 3 months and 15 days after sowing. (Right) DA variety (Al-tolerant), for which the grain yield was measured

Table 12 - Productivity parameters for the maize variety DA

Seed Treatment	Weight of 1000 kernels (g)	Number of grains per block	Yield of grains ($t.ha^{-1}$)
Unprimed	263.98	15701 a	1.63 a
Ascorbic acid	339.02	19953 b	2.65 b
CV (%)	16.88	6.37	22.2

Different letters in the same column denote statistically significant differences at a confidence of 95% (n=5).

Figure 70 - Differences in the maize cob sizes of plants originating from unprimed seeds (left) or primed seeds primed with ascorbic acid (right)

We observed no significant difference in weight of 1000 kernels; 263.98 ± 21.54 g for kernels originating from unprimed seeds and 339.02 ± 16.50 g for kernels originating from primed seeds. However, there was a significant difference in number of grains per block: 15701 ± 458 grains for kernels originating from unprimed seeds and 19953 ± 770 grains for kernels originating from primed seeds. Furthermore, we observed differences in yield of grains per hectare where 1.63 ± 0.16 $t.ha^{-1}$ were obtained from kernels originating from unprimed seeds and 2.65 ± 0.21 $t.ha^{-1}$ from kernels originating from seeds primed with ascorbic acid.

5.3.2 Nutrient absorption and Al absorption

To verify whether ascorbic acid affected the absorption of nutrients, maize shoots obtained during thinning step (1 month 20 days) were sampled and sent to the Laboratory of Vegetal Tissues Analyses. The results are shown in Table 13.

The nutrients absorbed more by the DA (Al-tolerant) variety compared with the Mo17 (Al-sensitive) were magnesium and manganese; however, curiously, the amount of phosphorus and sulfur were higher in the Mo17 strain than in the DA strain. The amount of Al absorbed was consistently and significantly higher in the Mo17 inbred line (194.55 ± 12.52 $mg.Kg^{-1}$) than in the DA variety (103.20 ± 3.17 $mg.Kg^{-1}$).

Surprisingly, we observed that seed treatment did not affect nutrient uptake, and the differences in nutrient levels were more correlated with genotype than seed treatment. With respect to the toxic element aluminum, the tolerant variety DA did not show differences in Al uptake; however, interestingly, seed priming decreased Al absorption significantly (from 215.30 ± 8.80 $mg.Kg^{-1}$ to 173.80 ± 1.20 $mg.Kg^{-1}$) in the inbred line Mo17 (Al-sensitive).

Table 13 - Nutrient and aluminum uptake in the maize shoots of Al-tolerant (DA) and Al-sensitive (Mo17) genotypes at 1 month 20 days of age

Genotype	Treatment	Macronutrients (g.Kg^{-1})					
		N	**P**	**K**	**Ca**	**Mg**	**S**
DA	Unprimed	10.44 ± 0.40	2.04 ± 0.20	19.64 ± 1.02	1.00 ± 0.06	0.67 ± 0.03	0.72 ± 0.01
	Ascorbic acid	10.18 ± 0.30	2.07 ± 0.10	21.42 ± 1.59	0.95 ± 0.23	0.73 ± 0.07	0.76 ± 0.03
	Average	10.31 ± 0.23	2.06 ± 0.10*	20.53 ± 0.94	0.98 ± 0.10	0.70 ± 0.04*	0.74 ± 0.02*
Mo17	Unprimed	10.91 ± 1.35	3.02 ± 0.01	16.45 ± 0.38	0.75 ± 0.00	0.50 ± 0.00	0.97 ± 0.11
	Ascorbic acid	11.10 ± 1.37	2.81 ± 0.29	17.21 ± 2.68	0.78 ± 0.08	0.55 ± 0.05	1.02 ± 0.08
	Average	11.01 ± 0.79	2.92 ± 0.13*	16.83 ± 1.13	0.76 ± 0.03	0.53 ± 0.02*	0.99 ± 0.06*

Genotype	Treatment	Micronutrients (mg.Kg^{-1})					Toxic element (mg.Kg^{-1})
		B	**Cu**	**Fe**	**Mn**	**Zn**	**Al**
DA	Unprimed	7.61 ± 0.06	3.33 ± 0.33	70.67 ± 1.69	69.83 ± 2.33	22.00 ± 2.18	99.33 ± 3.40
	Ascorbic acid	8.45 ± 0.23	3.00 ± 0.29	68.83 ± 5.95	66.67 ± 2.24	22.00 ± 2.02	107.07 ± 4.88
	Average	8.03 ± 0.22	3.17 ± 0.21	69.75 ± 2.80	68.25 ± 1.61*	22.00 ± 1.33	103.20 ± 3.17*
Mo17	Unprimed	10.16 ± 0.29	2.75 ± 0.75	66.25 ± 1.75	47.75 ± 3.75	20.25 ± 0.25	215.30 ± 8.80 a
	Ascorbic acid	11.52 ± 4.55	2.25 ± 0.25	60.75 ± 1.75	50.50 ± 2.50	23.25 ± 4.75	173.80 ± 1.20 b
	Average	10.84 ± 1.90	2.50 ± 0.35	63.50 ± 1.88	49.13 ± 2.00*	21.75 ± 2.13	194.55 ± 12.52*

Observation: means with asterisk denote significant differences between genotypes. The significant differences among Mo17 seed treatments are shown in letters

We also quantified nutrients in kernels from genotype DA, and we observed an interestingly result: boron was present in higher levels in kernels from seeds primed with ascorbic acid (from 1.41 ± 0.42 to 2.80 ± 0.40 mg.Kg^{-1} dry matter; Table 14).

Table 14 - Nutrient and Al content in the grains of maize variety DA (Al-tolerant)

Treatment	**Macronutrients (g.Kg^{-1})**					
	N	**P**	**K**	**Ca**	**Mg**	**S**
Unprimed	15.74 ± 0.53	2.98 ± 0.10	2.96 ± 0.17	0.04 ± 0.00	0.87 ± 0.07	0.64 ± 0.03
Ascorbic acid	15.12 ± 0.55	2.99 ± 0.29	2.91 ± 0.19	0.04 ± 0.01	0.83 ± 0.05	0.62 ± 0.04
Treatment	**Micronutrients (mg.Kg^{-1})**					**Toxic (mg.Kg^{-1})**
	B	**Cu**	**Fe**	**Mn**	**Zn**	**Al**
Unprimed	1.41 ± 0.42*	1.60 ± 0.10	29.0 ± 1.15	5.6 ± 0.37	23.2 ± 0.78	57.80 ± 1.72
Ascorbic acid	2.80 ± 0.40*	1.90 ± 0.19	28.0 ± 3.12	6.1 ± 0.60	23.8 ± 0.97	56.14 ± 5.95

Observation: the means with asterisk denote significant differences at a confidence level of 95%

5.3.3 Lipid peroxidation and hydrogen peroxide

To estimate oxidative damage in the maize shoots, we quantified malondialdehyde (MDA), which is a product of lipid peroxidation (Figure 71). We noted that seed priming reduced the production of MDA in Mo17 shoots (from 9.87 ± 1.48 to 6.57 ± 0.36 nmol MDA.g^{-1} fresh matter); however, the treatment did not change lipid peroxidation in the tolerant DA variety. The mean MDA production did not differ between genotypes; however, interestingly, the production of hydrogen peroxide was significantly higher in Mo17 shoots (Al-sensitive) compared with DA (Al-tolerant) shoots (Figure 72).

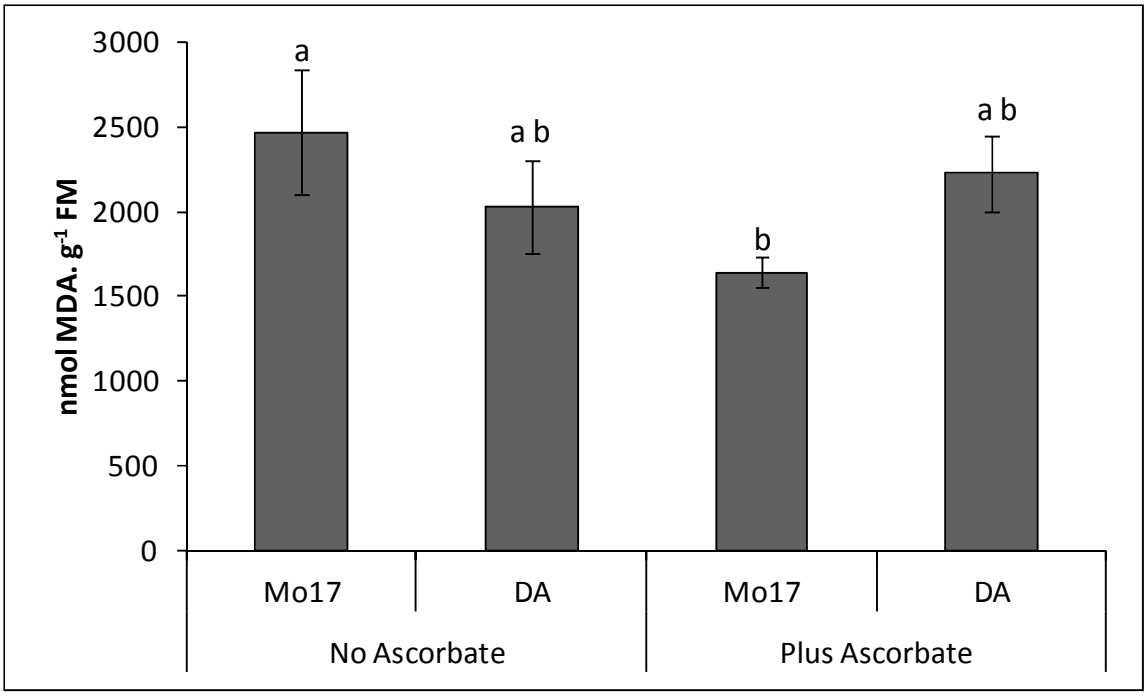

Figure 71 - Malondialdehyde (MDA) quantification. Similar letters above the bars denote means that are not significantly different at a confidence level of 95%

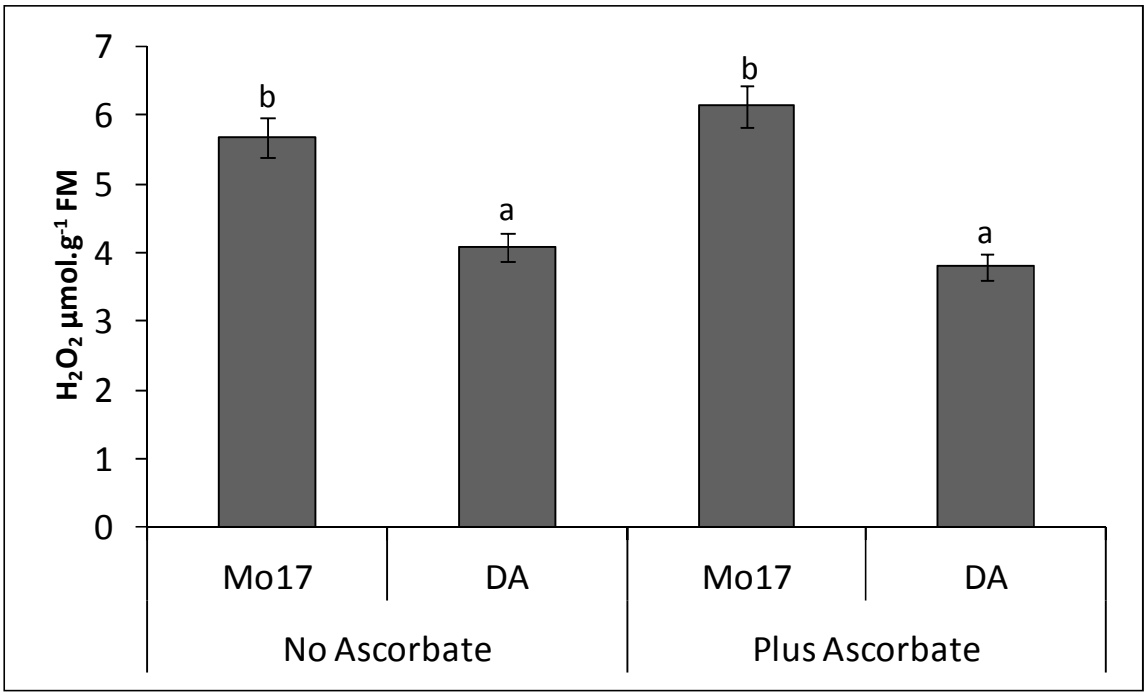

Figure 72 - Hydrogen peroxide (H_2O_2) quantification. Similar letters above bars denote means that are not significantly different at confidence level of 95%

5.3.3 Ascorbic acid determination

To verify whether treating seeds with ascorbic acid interfered ascorbic acid content in the maize leaves, we quantified ascorbate using the method of Arakawa et al. (1981) (Figure 73). We noted that seed priming increased ascorbic acid in the DA variety from 36.9 ± 7.32 to 89.10 ± 11.16 µM of ascorbate.g^{-1} fresh matter; however, the treatment did not change ascorbate content in the leaves of the sensitive inbred Mo17 line (Figure 74). Ascorbate levels in the kernels were also quantified for the DA variety, and we observed greater levels of vitamin C in maize plants primed with ascorbic acid (Figure 75).

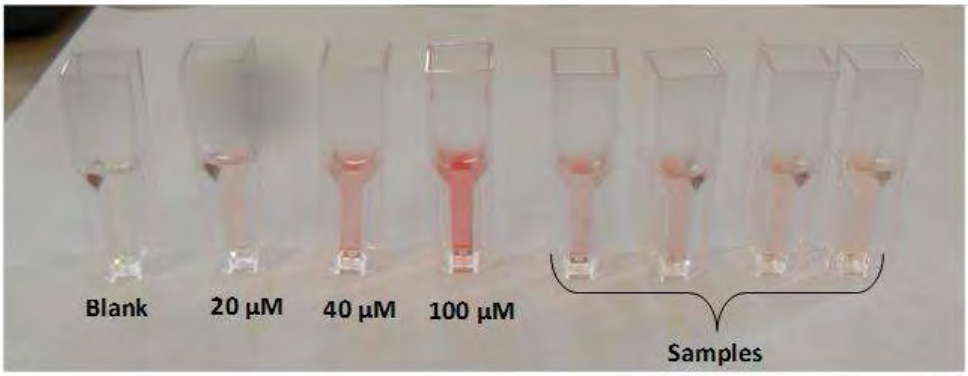

Figure 73 - Demonstration of the color reaction used for ascorbate determinations in the samples

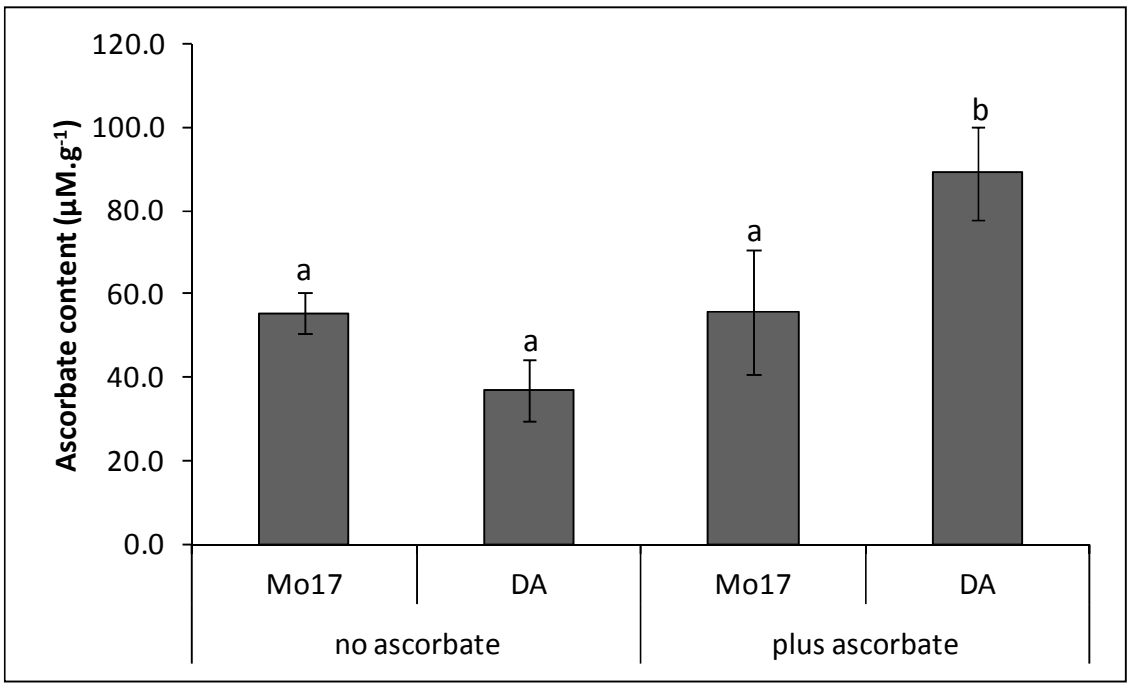

Figure 74 - Spectrophotometric analysis of the ascorbate content of maize leaves. Similar letters above the bars denote means that are not significantly different at a confidence level of 95%

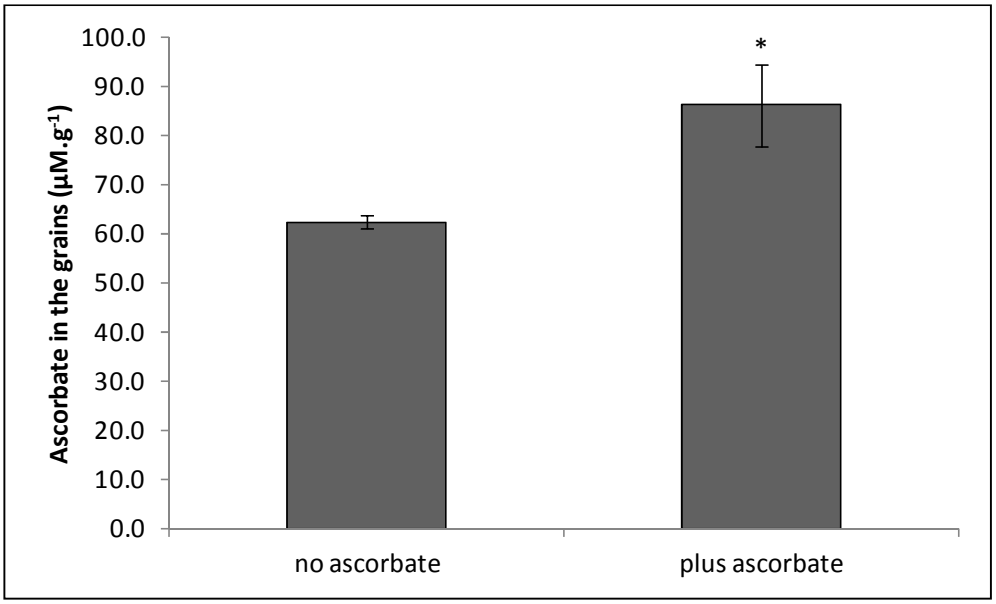

Figure 75 - Spectrophotometric analysis of ascorbate content of maize grains for the Al-tolerant (DA) variety. The mean values are significantly different at a confidence level of 95%

5.3.4 Enzyme assays and protein profiles

To verify whether seed priming with ascorbic acid interfered with the enzymatic antioxidant systems of Al-sensitive (Mo17) and Al-tolerant (DA) genotypes, we determined enzyme activities using spectrophotometric methods and, for some enzymes, gel assays. We utilized leaves just below the flag leaf from plants aged 1 month 20 days. All enzyme assays were performed using the same amount of protein (20 µg), except for SOD isozymes determination we utilized 70 µg.

a) Superoxide dismutase (SOD)

The first enzyme that we analyzed was superoxide dismutase (SOD) because this enzyme is the first be activated in the antioxidant system. However, surprisingly, we did not observe any differences in activity of this enzyme (Figure 76).

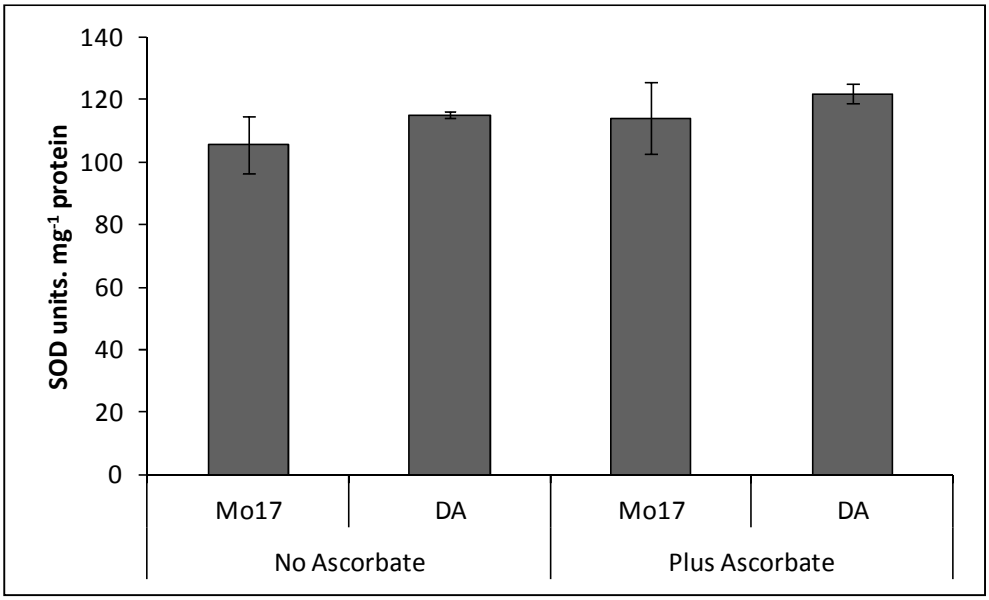

Figure 76 - Spectrophotometric analysis of the activity of superoxide dismutase (SOD) in maize leaves. The means were not significantly different at a confidence level of 95%

Gel analysis (Figure 77) revealed three different isozymes of SOD for both genotypes (20 µg of protein loaded in each lane). To classify the SOD isozymes, we performed an experiment where pieces of the gels were primed with KCN or H_2O_2 prior to gel development; however, here, we used 70 µg of protein and 12% polyacrilamide gels. The MnSOD isoforms are resistant to KCN and H_2O_2, whereas FeSOD is resistant to KCN and sensitive to H_2O_2. The most abundant isozyme is Cu/Zn-SOD, which is sensitive to both treatments (Figure 78).

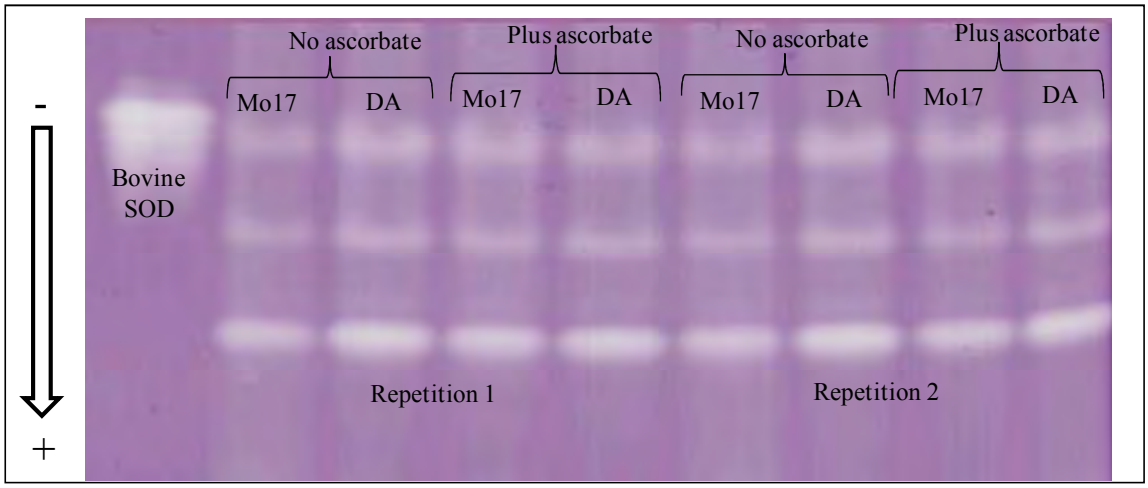

Figure 77 - Visualization of cupper/zinc superoxide dismutase activity (Cu/Zn-SOD) from maize leaves in a 10% polyacrylamide gel (20 µg of protein loaded per lane)

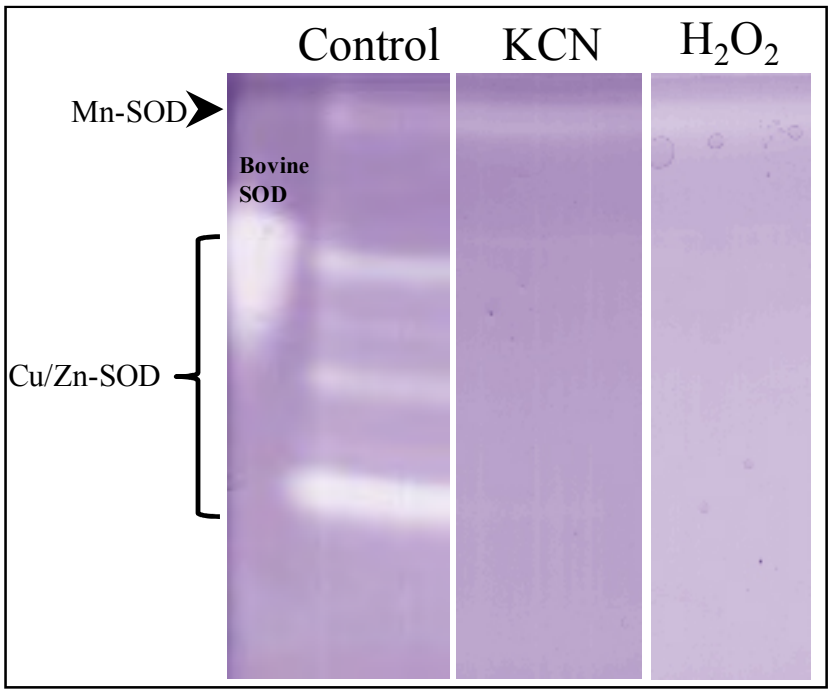

Figure 78 - Isozyme characterization of superoxide dismutase (SOD) of maize leaves in 12% polyacrylamide gels (70 µg of protein loaded per lane)

b) Catalase (CAT)

Although we did not observe differences in SOD activity, interestingly, the activity of the enzyme catalase was changed with respect to seed priming. Because the spectrophotometric analysis did not work, we will discuss results obtained from the gel analyses. We observed that Mo17 apparently expresses two CAT isozymes that differ in activity levels, whereas the DA variety expresses only one isozyme (Figure 79). Based on the molecular weight observed by Scandalios (2005), we identified these isozymes as CAT-3 and CAT-2. To determine whether the isozymes showed differences in activity, we performed densitometric analyses of the gel, and we observed that CAT-3 from the Al-tolerant variety (DA) was more active than CAT-3 from the Al-sensitive line (Mo17).

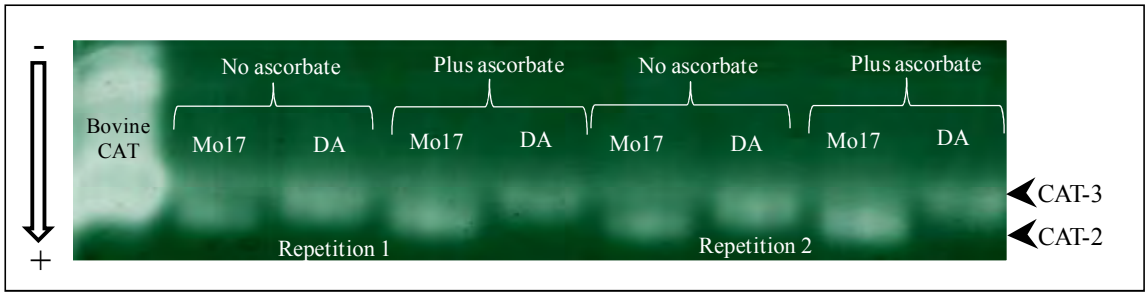

Figure 79 - Visualization of catalase activity (CAT) from maize leaves determined from 10% polyacrylamide gels (20 µg of protein loaded per lane)

Notably, seed priming reduced CAT-3 activity significantly in the Al-tolerant variety (DA; from 71.53 ± 1.38 to 54.43 ± 1.86 µmol $H_2O_2.min^{-1}.mg^{-1}$ protein; Table 15).

Table 15 - Densitometric analysis of catalase (CAT) gels. Activities are presented as µmol H_2O_2 $.min^{-1}.mg^{-1}$ protein. Similar letters denote means that are not significantly different at a confidence level of 95%

	No ascorbic				**Plus ascorbic**			
	Mo17		**DA**		**Mo17**		**DA**	
CAT-3	28.57 ± 0.21	a	71.53 ± 1.38	C	35.42 ± 0.63	a	54.43 ± 1.86	b
CAT-2	33.47 ± 0.67	a	0	-	50.16 ± 1.75	b	0	-

c) Glutathione reductase (GR)

With respect to the activity of glutathione reductase, we observed that seed priming in the Al-sensitive line (Mo17) resulted in a significant increase in GR activity (from 0.174 ± 0.04 to 0.383 ± 0.04 $units.mg^{-1}$ protein). Using a spectrophotometer, no significant changes were observed for the Al-tolerant variety (DA, Figure 80). Moreover, results from the gel analysis were consistent with the quantitative study (Figure 81 and Table 16).

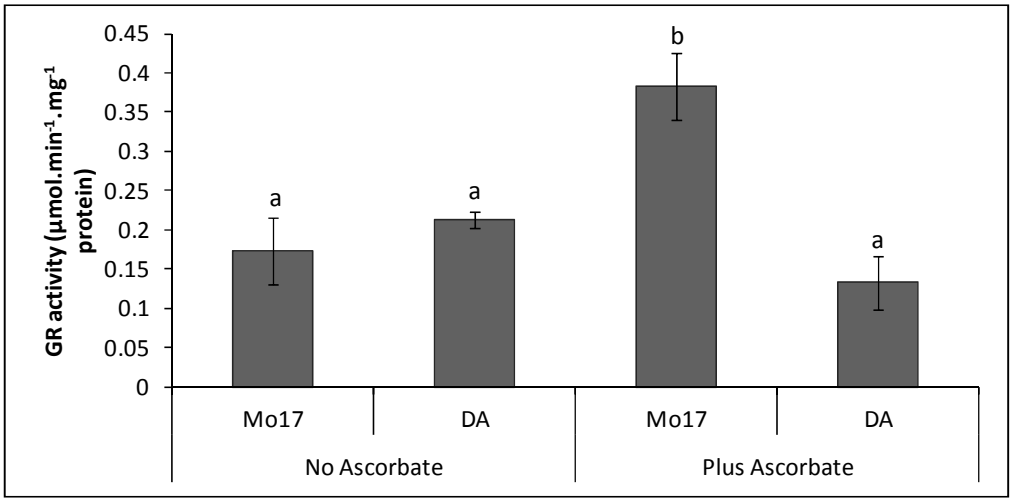

Figure 80 - Spectrophotometric analysis of the glutathione reductase (GR) activity of maize leaves. Similar letters denote means that are not significantly different at a confidence level of 95%

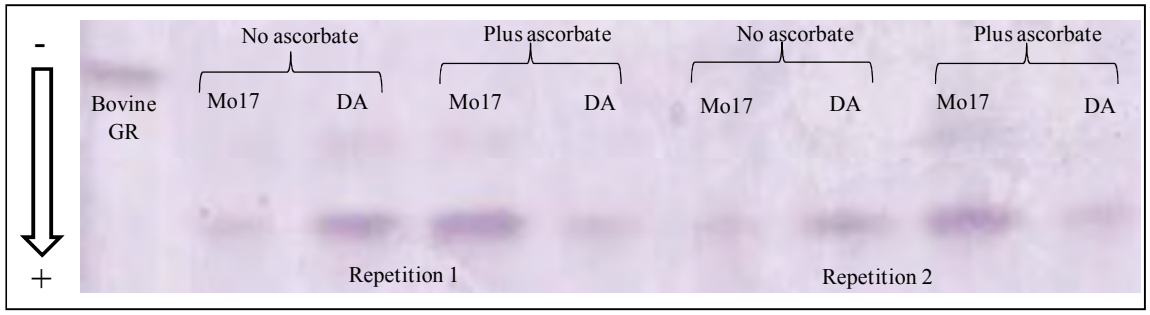

Figure 81 - Visualization of glutathione reductase activity (GR) in maize leaves using 10% polyacrylamide gels (20 µg of protein loaded per lane).

Table 16 - Densitometric analysis of the glutathione reductase (GR) gel-based assay. Activities are presented as a percentage of the standard (1 U). Similar letters denote means that are not significantly different at a confidence level of 95%

	No ascorbic				**Plus ascorbic**			
	Mo17		**DA**		**Mo17**		**DA**	
GR	74.64 ± 8.53	a	80.99 ± 7.02	A	117.52 ± 14.48	b	74.15 ± 0.8	a

d) Glutathione-S-transferase (GST)

With regard to glutathione-S-transferase activity, surprisingly, we observed no effect on GST activity in the Al-sensitive line (Mo17). However, in the Al-tolerant variety (DA), a significant reduction in activity was caused by seed priming (from 0.0747 ± 0.003 to 0.0471 ± 0.004 GST units.mg^{-1} protein; Figure 82).

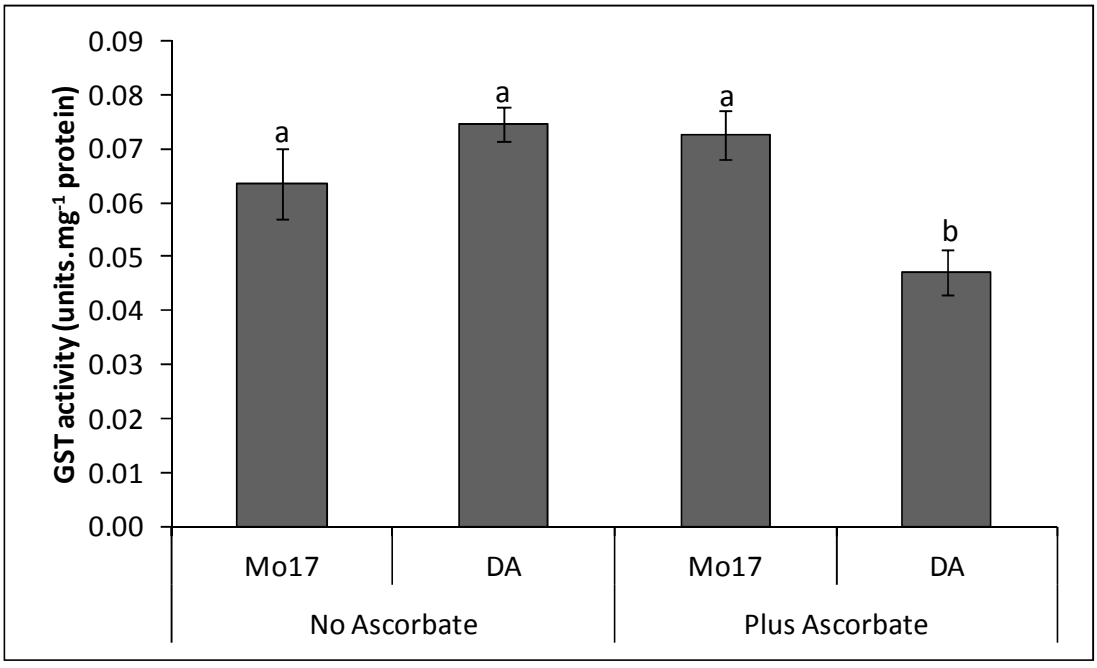

Figure 82 - Spectrophotometric analysis of glutathione-S-transferase (GST) activity in maize leaves. Similar letters denote means that are not significantly different at a confidence level of 95%

e) Guaiacol-type peroxidase (GPOX)

In previous research, guaiacol-type peroxidase was noted to participate in the process of lignification (OGAWA et al., 1997; MCCUE et al., 2000; COSIO; DUNAND, 2009). The activity of GPOX was determined using a spectrophotometric assay. We observed that ascorbic acid significantly reduced GPOX activity (from 13.23 ± 0.16 to 7.60 ± 0.74 μmol H_2O_2.min^{-1}.mg^{-1} protein) in the Al-sensitive genotype (Mo17); however, no significant changes were observed in GPOX activity in the Al-tolerant variety (DA; Figure 83).

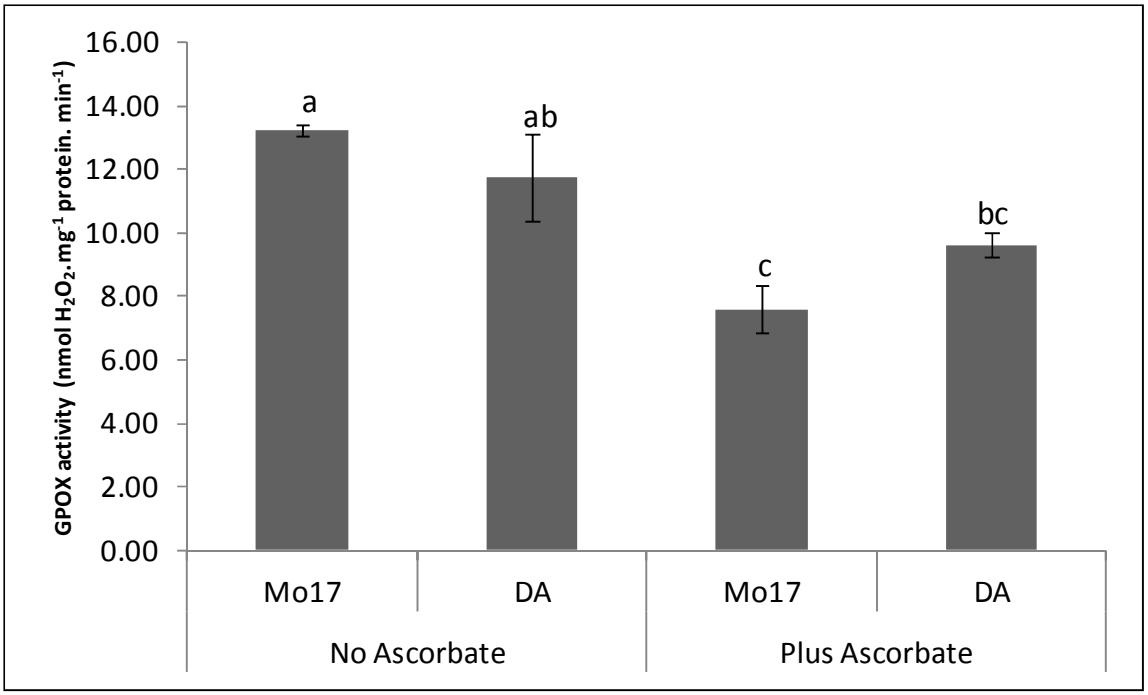

Figure 83 - Spectrophotometric analysis of guaiacol-type peroxidase (GPOX) activity in maize leaves. Similar letter denote means that are not significantly different at a confidence level of 95%

f) Ascorbate peroxidase (APX)

Ascorbate peroxidase is an antioxidant enzyme found in plants that reduces H_2O_2 to water by oxidizing AsA. The data obtained in spectrophotometric analysis was not coherent and therefore we just discussed that obtained in the gel analysis. Gel analyses (Figure 84) showed that seed priming with ascorbic acid did not change APX activity, and only one difference was observed between the unprimed Mo17 and the primed DA genotypes (Table 17). The data observed for APX activity was very coherent to GR activity.

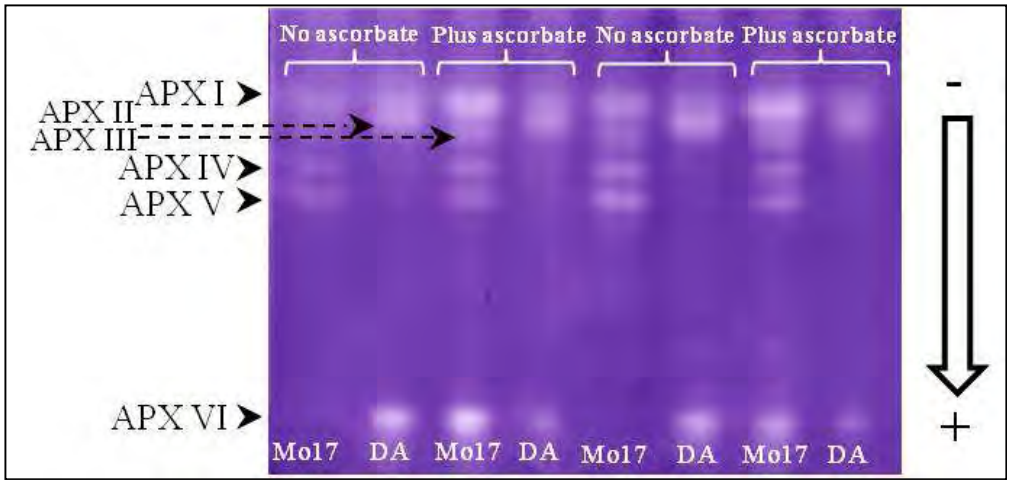

Figure 84 - Gel analysis of ascorbate peroxidase (APX) activity in maize leaves (10% polyacrylamide gel)

Table 17 - Densitometric analysis of ascorbate peroxidase (APX) activity gel assays. The relative activity of the isoenzymes is provided as a percentage. Similar letters in the same line denote means that are not significantly different at a confidence level of 95%

Isoenzymes		No ascorbic				Plus ascorbic			
		Mo17		DA		Mo17		DA	
% relative to total activity	APX I	22.16 ± 2.3	a	18.35 ± 0.43	a	23.27 ± 0.41	a	19.63 ± 1.14	a
	APX II	-	-	23.13 ± 3.55	a	-	-	23.3 ± 0.29	a
	APX III	16.35 ± 2.19	a	-	-	12.82 ± 2.08	a	-	-
	APX IV	12.06 ± 3.85	a	-	-	8.77 ± 1.11	a	-	-
	APX V	15.12 ± 4.5	a	-	-	7.93 ± 1.11	a	-	-
	APX VI	5.84 ± 0.13	a	31.77 ± 0.9	b	24.6 ± 3.71	bc	18.15 ± 4.56	c

Gel analysis revealed six isoenzymes, which we named APX I, II, III, IV, V and VI. The data obtained from the gels provided interesting information about isoenzymes activities that differ between genotypes. Moreover, APX I shows the highest activity among the isoenzymes and APX VI seems to respond to ascorbate treatment more than the others isoenzymes. Interestingly, Al-tolerant genotype (DA) had one isoenzyme that is not present in Al-sensitive genotype (Mo17). Based on the gel analysis, APX II could be playing an important role in tolerance of DA genotype.

g) Protein profiles

To analyze the effect of ascorbic acid on the protein profile of maize leaves from the Al-tolerant variety (DA) and the Al-sensitive line (Mo17), we performed a gel analysis using

SDS-PAGE. We observed no differences in protein profiles with respect to seed priming. However, when genotypes were compared, it appeared that Mo17 expresses one heavy protein (higher than 220 KDa) that is absent in the DA variety (Figure 85).

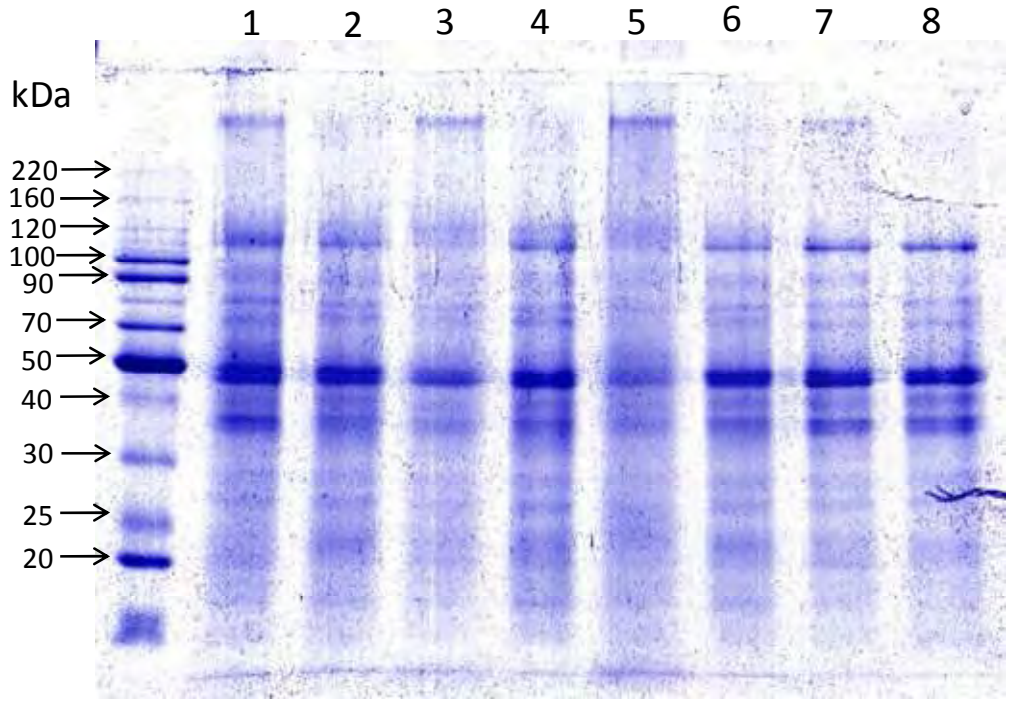

Figure 85 - Protein profile of maize leaves at 1 month 20 days after the sowing of the Al-sensitive line (Mo17) and the Al-tolerant variety (DA). (1and 5) Mo17 leaves from unprimed seeds; (3 and 7) Mo17 leaves from seeds primed with ascorbic acid; (2 and 6) DA leaves from unprimed seeds; (4 and 8) DA leaves from seeds primed with ascorbic acid

5.3.5 Klason lignin determination

In Section 4, we observed that treatment with ascorbic acid decreased the expression of the key lignin gene *COMT*1 in the Al-sensitive line at 5 days of age. To verify whether the treatment affected gene expression in mature plants, here, we quantified total lignin in maize shoots at 1 month 20 days of age. We observed that the treatment decreased lignin in the Al-sensitive line (Mo17); however, in the Al-tolerant line (DA), the treatment did not change the relative lignin content (Table 18). Interestingly, the soluble lignin levels did not change; however, residual (or acid-insoluble) lignin was reduced significantly by ascorbic acid (from 10.01 ± 0.33% to 8.81 ± 0.30%). The reduction in total lignin in maize plants originating from primed seeds was 9.98%.

Table 18 - Extracts, lignin and holocellulose content of maize shoots from Al-tolerant (DA) and Al-sensitive (Mo17) genotypes at 1 month 20 days of age (data in percentage relative to 1 gram absolute dried material)

Variety	Treatment	Extractives	Soluble lignin	Residual lignin	Total lignin	Holocellulose
DA	Unprimed	25.47 ± 0.57	2.93 ± 0.04	8.94 ± 0.09	11.87 ± 0.12	62.66 ± 0.61
	Ascorbic acid	25.33 ± 0.68	2.86 ± 0.12	9.10 ± 0.23	11.96 ± 0.34	62.72 ± 0.43
Mo17	Unprimed	24.87 ± 1.03	2.73 ± 0.08	10.01 ± 0.33*	12.73 ± 0.41*	62.40 ± 1.44
	Ascorbic acid	26.44 ± 0.73	2.65 ± 0.05	8.81 ± 0.30*	11.46 ± 0.35*	62.10 ± 1.08

Observation: the means with asterisk denote significant differences at a confidence level of 95%

5.3.6 Carbohydrate and amino acid quantification in maize kernels

During the field experiment, we observed some attacks to the maize ears (Figure 86). The field workers noted that the evidence suggested that porcupines were the cause of the attack, and we observed that the blocks sowed with primed seeds were more affected (Table 19). Therefore, to verify whether seed priming with ascorbic acid interfered with kernel quality, we quantified carbohydrates (Table 20) and amino acids (Table 21).

Photo: Berenice K. de Alcântara

Figure 86 - Evidence of porcupine attacks on the maize plants

Table 19 - Quantification of maize attacks in the Al-tolerant variety (DA)

Treatment	Block	Maize ears attacked
Unprimed	1	0
Unprimed	2	1
Unprimed	3	1
Unprimed	4	0
Unprimed	5	0
Ascorbic acid	1	3
Ascorbic acid	2	0
Ascorbic acid	3	4
Ascorbic acid	4	5
Ascorbic acid	5	1

Table 20 - Total carbohydrate content determined from the hydrolyzed pure starch of maize kernels of the Al-tolerant variety (DA) and the respective percentages of arabinose, galactose, glucose and xylose

Treatment	Total Carbohydrate ($mg.g^{-1}$ pure starch)	Arabinose	Galactose	Glucose	Xylose
Unprimed	936.25 ± 6.31*	4.15 ± 0.23	1.10 ± 0.06	88.92 ± 0.43	5.83 ± 0.25
Ascorbic acid	974.97 ± 5.48*	5.17 ± 0.36	1.19 ± 0.08	86.78 ± 0.97	6.87 ± 0.53

Observation: The means with asterisk denote significant differences at a confidence level of 95%.

We observed the treatment with ascorbic acid increased total carbohydrate levels in maize kernels (from 936.25 ± 6.31 to 974.97 ± 5.48 $mg.g^{-1}$); however, the relative sugar content did not change.

With regard to quantification of soluble amino acids in grains from the Al-tolerant variety (DA), we observed that the histidine, glycine, threonine, leucine and phenylalanine content did not change (Table 21). In contrast, amino acids that increased in content were: serine (1.7x), arginine (1.4x), aspartic acid (2.3x), glutamate (2.2x), alanine (2.2x), proline (2.7x), lysine (1.7x), tyrosine (1.4x), valine (2.2x) and isoleucine (1.5x). Curiously, seed priming caused a decrease in the content of one amino acid: methionine (from 65.18 ± 2.5 to 35.93 ± 4.68 $\mu g.g^{-1}$ dry matter).

Table 21 - Soluble amino acids in maize kernels from the Al-tolerant variety (DA; µg.g^{-1} DM)

Treatment	**His**	**Ser**	**Arg**	**Gly**
Unprimed	155.98 ± 12.02	107.19 ± 4.85*	123.44 ± 10.83*	23.31 ± 5.73
Ascorbic acid	204.77 ± 17.43	178.51 ± 14.01*	170.47 ± 5.62*	39.83 ± 6.42
	Asp	**Glu**	**Thr**	**Ala**
Unprimed	148.21 ± 15.79*	600.05 ± 28.48*	57.03 ± 20.77	245.59 ± 7.13*
Ascorbic acid	333.52 ± 46.03*	1312.59 ± 186.37*	51.69 ± 32.36	534.43 ± 81.1*
	Pro	**Lys**	**Tyr**	**Met**
Unprimed	1484.92 ± 137.94*	88.47 ± 5.38*	431.82 ± 23.72*	65.18 ± 2.5*
Ascorbic acid	4022.09 ± 460.36*	153.92 ± 16.96*	606.40 ± 51.12*	35.93 ± 4.68*
	Val	**Iso**	**Leu**	**Phe**
Unprimed	100.98 ± 7.65*	61.10 ± 3.78*	72.43 ± 9.04	124.89 ± 9.19
Ascorbic acid	225.63 ± 18.65*	89.79 ± 2.72*	103.07 ± 9.23	170.55 ± 26.72

Observation: the means with asterisk are significantly different at a confidence level of 95%

5.4 Discussion

Interesting results were obtained with regard to ascorbic acid (AsA) treatment on growth, productivity, lignin content and kernel quality. Therefore, in general, the results of the treatment with ascorbic acid in maize were very positive, demonstrating promise for future use. With respect to plant growth, a recent publication demonstrated that seed priming with an ascorbic acid solution (2 mM) increased root and shoot lengths and improved the foliar area of wheat under drought conditions (FAROOQ et al., 2013). In our study, seed priming using ascorbic acid powder also improved growth in Al-sensitive and Al-tolerant genotypes growing in soil with a high Al content; however, the seed priming was not sufficient to induce Al tolerance in the sensitive line with respect to production, showing that even though the treatment improved aspects of growth, genetic effects are irreplaceable. In contrast, another interesting aspect of our results was that seed priming with ascorbic acid increased grain yield in an Al-tolerant variety and also improved kernel quality, advocating the use of this treatment in complementary forms of improvement.

In Section 4, we also observed growth enhancement caused by seed priming in the Al-sensitive line B73, and we focused our discussion on the reduction in Al absorption. With regard to the Al accumulation in maize shoots at 1 month 20 days of age, we observed that the Al content was consistent with levels observed in previous reports that quantified Al in shoots of maize (GIANNAKOULA et al., 2008; GIONGO; BOHNEN, 2011). Furthermore, our data shows that the Al-sensitive line (Mo17) accumulated more Al and H_2O_2 than the tolerant variety (DA). Indeed, CAT3 may play an important role in minimizing levels of H_2O_2 in the DA variety because it was more active than in the Mo17 line, which is consistent with data from a previous publication that compared Al-tolerant and Al-sensitive maize varieties and observed similar results (GIANNAKOULA et al., 2008).

The novel observation raised by our data was demonstrated in section 4.3.2, which reveals a significant reduction in Al absorption caused by the ascorbic acid treatment in the Al-sensitive line (Mo17) at 1 month 20 days of age. A similar result was also observed in Section 4 in the Al-sensitive line (B73), where a reduction in Al absorption in the roots at 5 days of age was noted. In Section 4, we suggested that the increase in lignin gene expression (*COMT*1) in the roots may underlie the reduction in Al absorption because previous reports have already elucidated this relationship (EZAKI et al., 2005) however, in contrast, the reduction in *COMT*1 expression in shoots may be due to the increased allocation of carbon to roots for lignin biosynthesis (trade-off behavior).

Consistent with this previous result, in this section, we observed that seed priming with ascorbic acid reduced the total lignin content in shoots and minimized Al absorption in the sensitive Mo17 line and also that the stress metabolite, malondialdehyde (MDA), was reduced when seeds were primed with AsA, which could be due to the decreased absorption of Al. In contrast, these parameters remained unchanged in the Al-tolerant variety. There was no difference in the MDA content in the DA variety, likely because there was no difference in Al absorption. Interestingly, the data are correlated with GPOX activity, the enzyme involved in lignification (MCCUE *et al.*, 2000), because we observed that seed priming decreased GPOX activity in Mo17 shoots but did not cause changes in the DA variety.

This study clearly indicates that different mechanisms are initiated by seed priming in Al-sensitive and Al-tolerant genotypes. Figures 87 and 88 show a summary of these changes. In the case of the Al-sensitive line (Mo17), seed priming with ascorbic acid reduces Al absorption; however, in the Al-tolerant variety (DA), the content of Al uptake remained unchanged. Thus, ascorbic acid interferers with lignin synthesis in sensitive genotype, but a different mechanism occurs in the tolerant variety. The critical question raised from these data is that if ascorbic acid neither changes lignin synthesis nor Al absorption in the DA variety, how does this treatment improve growth and productivity in this tolerant variety?

We may answer this question by a first hypothesis based in the ascorbate content in leaves of the DA variety. We observed that ascorbate content increased in the DA variety, but no significant differences were observed for the Mo17 line. Ascorbate is a cofactor for a number of important enzymes, such as proline and lysine hydroxylases, that catalyze the hydroxylation of proline and lysine residues in plants, thus modifying a number of proteins including the extensins (glycoproteins involved in cell wall expansion) (DAVEY et al., 2000). Thus, the increase in ascorbate content in DA leaves is likely directly involved in the growth improvement in this variety.

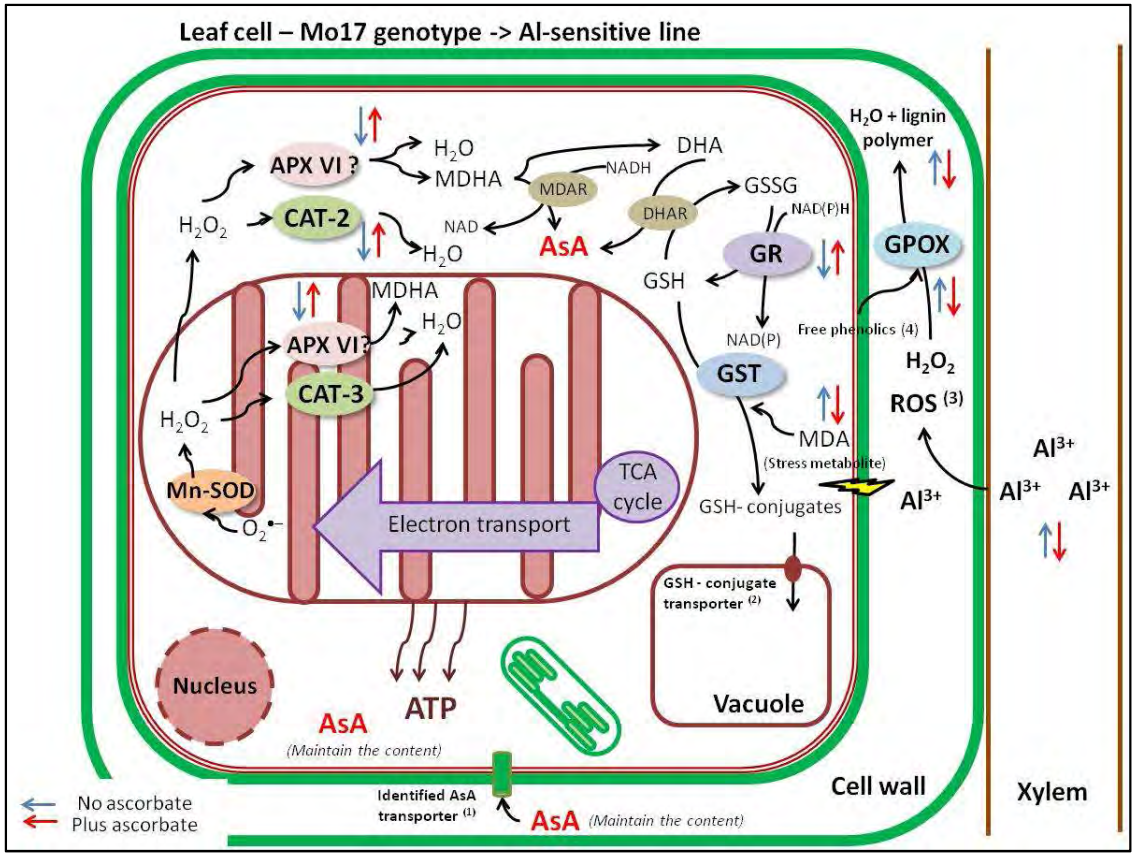

Figure 87 - Summary of the responses in the enzyme activity and metabolite content in the leaves of maize. Responses in a leaf cell of Al-sensitive genotype (Mo17). The arrows in blue and red symbolize the results obtained in our research. Arrows in blue denote the magnitude of the activity/content of enzymes/metabolites in unprimed seeds compared with primed seeds (red arrows)

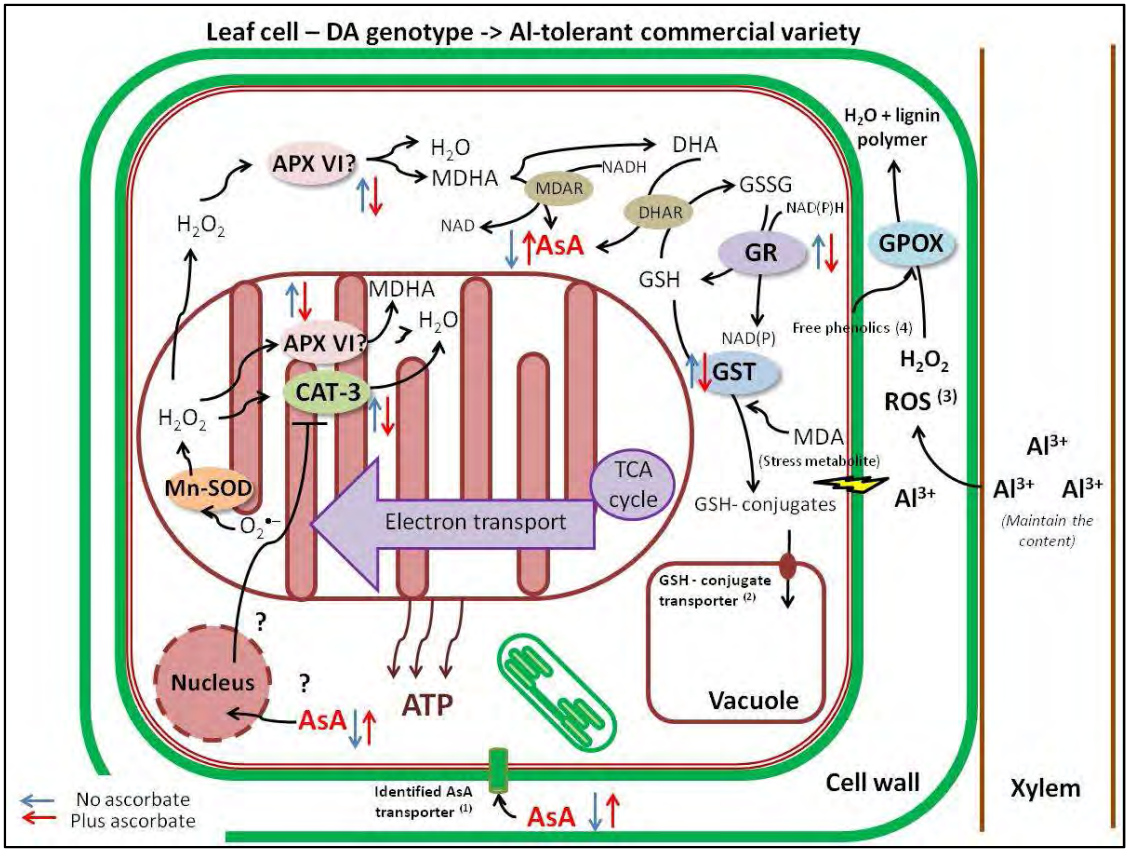

Figure 88 - Summary of the responses in the enzyme activity and metabolite content in the leaves of maize. Responses in a leaf cell of Al-tolerant genotype (DA). The arrows in blue and red symbolize the results obtained in our research. Arrows in blue denote the magnitude of the activity/content of enzymes/metabolites in unprimed seeds compared with primed seeds (red arrows)

A second and most possible explanation could be elucidated in the Figure 89. The ascorbic acid powder that was adsorbed on the surface of maize seeds could form ascorbate anions and hydrogen protons in the soil water changing the interactions between soil microorganisms and maize plants. However the effect of ascorbic acid in soil microorganisms and their interactions with plants remain to be elucidated in future works.

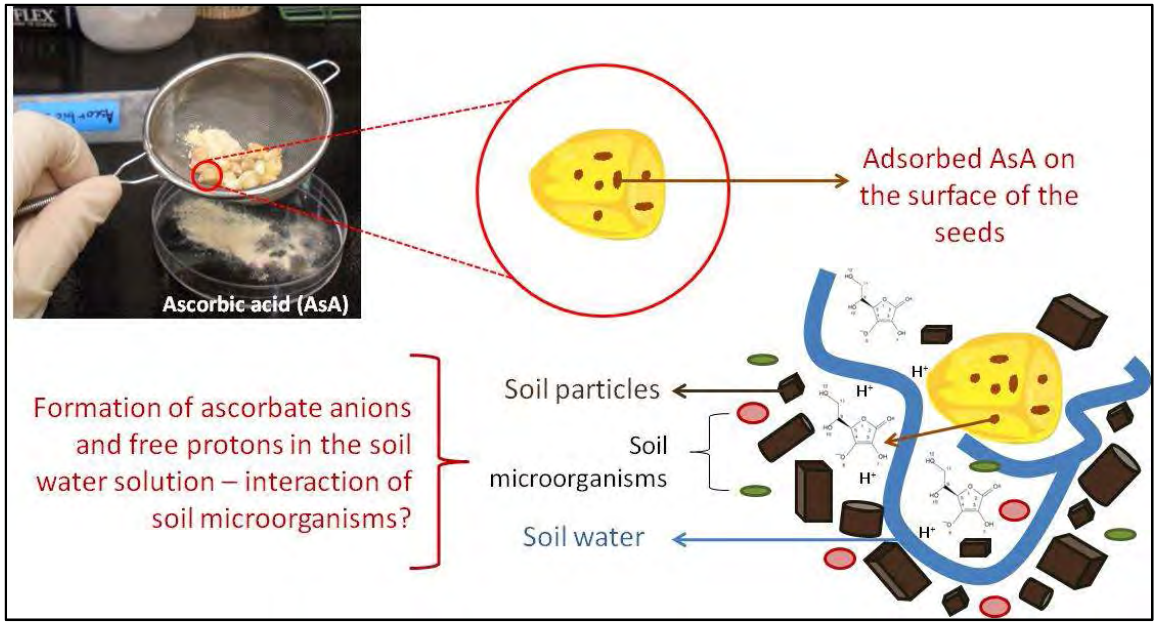

Figure 89 - Second hypothesis for growth and productivity improvement observed in DA genotype.

We also hypothesize that in the Al-tolerant variety, the mechanism of Al-tolerance is already efficient; thus, ascorbic acid "prefers" to modulate mechanisms other than lignin synthesis. However, before we begin to explain the mechanisms that ascorbic acid affects in DA plants, we must confirm that this DA property is likely related to an avoidance of Al absorption because our data show that the magnitude of Al absorption in DA plants was lower than in Mo17 plants.

In attempting to understand the effect of ascorbic acid on the Al-tolerant variety (DA), we should begin to explain why the activity of CAT-3 and APX VI were reduced by ascorbic acid treatment if we did not observe a seed priming-mediated reduction in H_2O_2. The novel observation of our study suggests that in maize, ascorbic acid may signal for the down-regulation of the gene encoding CAT-3 and APX VI to reduce its activity possibly because ascorbic acid is already acting as a non-enzymatic antioxidant and that it is energetically unnecessary to have the CAT-3 and APX VI enzyme functioning in this context. The role of vitamin C in the regulation of transcriptional factors has already been observed in humans in whom ascorbate induces the expression of the collagen gene (ARRIGONI; DE TULLIO, 2002) and also down-regulates a transcription factor that is responsible for tumor growth and apoptosis (TRABER; STEVENS, 2011). In plants, AsA appears to be associated with flowering time, developmental senescence, programmed cell death, and responses to pathogens because it affects the signaling process networks (PAVET et al., 2005; BARTH; DE TULLIO; CONKLIN, 2006). Ascorbic acid is a model antioxidant because it reacts with a

variety of free radicals and it is also suitable for regeneration (ARRIGONI; DE TULLIO, 2002). However, the hypothesis that ascorbic acid modulates transcription factors related to *Cat*3 expression should be studied in future research.

As observed in our results, seed priming also did not change the MDA levels in the tolerant variety (DA), likely because this treatment did not change Al absorption. Curiously, GST showed reduced activity when seeds were primed with ascorbic acid. In future experiments, it may be interesting to analyze the effects of ascorbic acid on anthocyanin transport because besides MDA, GST is also correlated with anthocyanin transport to the vacuole (MARRS et al., 1995).

When comparing the responses of GR and APX VI activities for both genotypes, we observed coherent responses. Using gel analysis, for Al-sensitive genotype (Mo17), the treatment with ascorbic acid seems to induce GR and APX VI activities. For Al-tolerant (DA), an opposite behavior, since we observed reduction in GR and APX VI activities caused by AsA treatment. It would be interestingly to quantify glutathione (GSH) and dehydroascorbate (DHA) to understand this behavior in all the process of ascorbate-glutathione cycle. Also it would be interesting in future works to determine in which cell compartment APX VI is functional.

Regarding the translocation of mineral elements, the tolerant variety (DA) possessed a consistently lower Al content and hydrogen peroxide levels than the Al-sensitive (Mo17) line (see summary in Figure 90 and 91).

However, when comparing nutrient absorption in the tolerant and sensitive genotypes at 1 month 20 days of age, we observed that the DA variety absorbed less phosphorus (P) and sulfur (S) than the Mo17 inbred line. Sulfur and phosphorus are essential elements required for plant growth. Thus, this result was unexpected because the DA variety showed better growth than the Mo17 line. Our results show that DA plants absorb less P and S than Mo17 plants and that the tolerant variety grows better than sensitive lines likely because the use of these nutrients in tolerant plants is more efficient than in sensitive plants. Genotypic differences in S efficiency during the vegetative stage in mustard (AHMAD *et al.*, 2005) and canola (BALINT; RENGEL, 2009) have been reported previously. Moreover, substantial differences in P utilization have been shown in maize (SHENOY; KALAGUDI, 2005; CORRALES et al., 2007). Furthermore, some studies have already linked P efficiency to Al tolerance (KOCHIAN et al., 2004 and references therein). Therefore, in addition to Al tolerance, DA is S and P efficient, a characteristic that is increasingly desirable, in particular

with respect to P, given the limitation of mineral phosphate fertilizers (SHENOY; KALAGUDI, 2005).

Continuing with our comparisons of nutrient absorption, we observed that the differences were more correlated with genotype than ascorbic acid treatment. Interestingly, the DA variety absorbed more magnesium (Mg) and manganese (Mn) than the Mo17 line, which are essential micronutrients for photosynthesis. Mg^{2+} is the central atom of the chlorophyll molecule, and fluctuations in its levels in the chloroplast regulate the activity of key photosynthetic enzymes (SHAUL, 2002 and references therein); Mn^{2+} is involved in photosynthetic water oxidation to produce 1 molecule of oxygen, 4 electrons and 4 protons (YACHANDRA; SAUER; KLEIN, 1996).

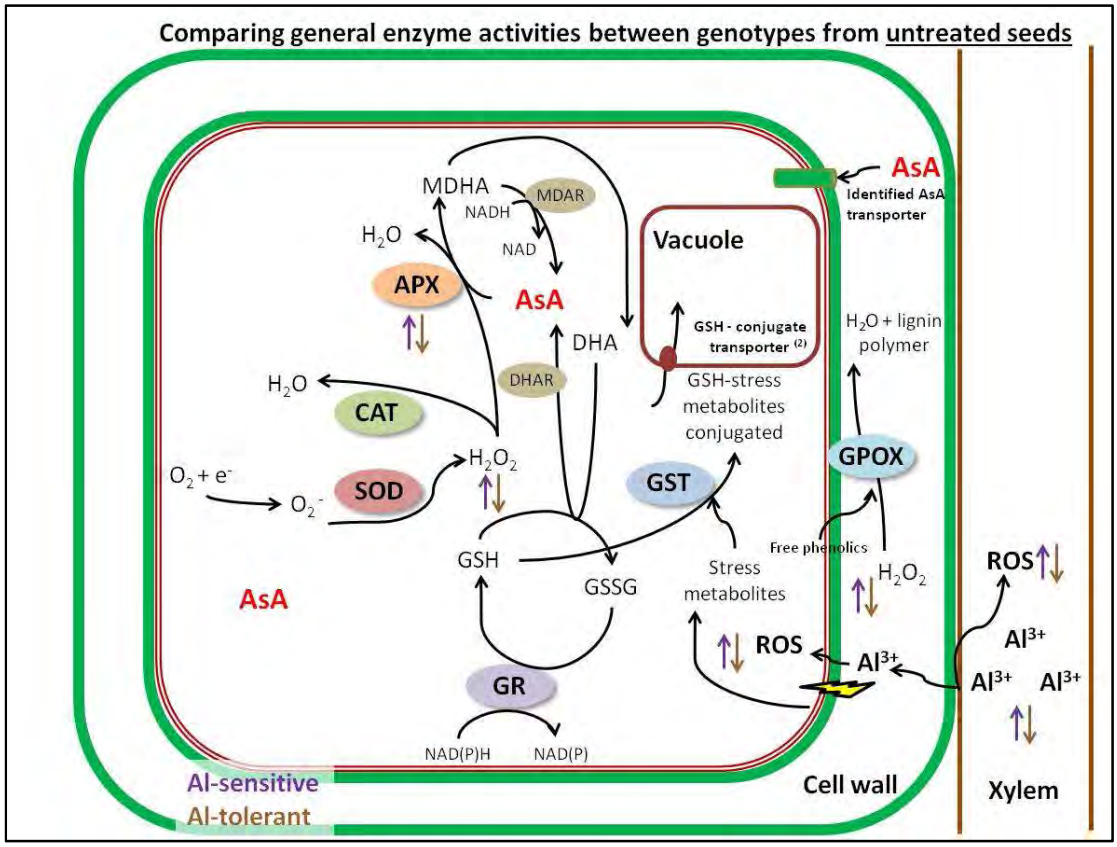

Figure 90 - Summary of the responses in the enzyme activity and metabolite content in the leaves of maize from unprimed seeds. The arrows in purple denote the direction of changes in the Al-sensitive line (Mo17) compared with the Al-tolerant variety (DA; light-brown arrows)

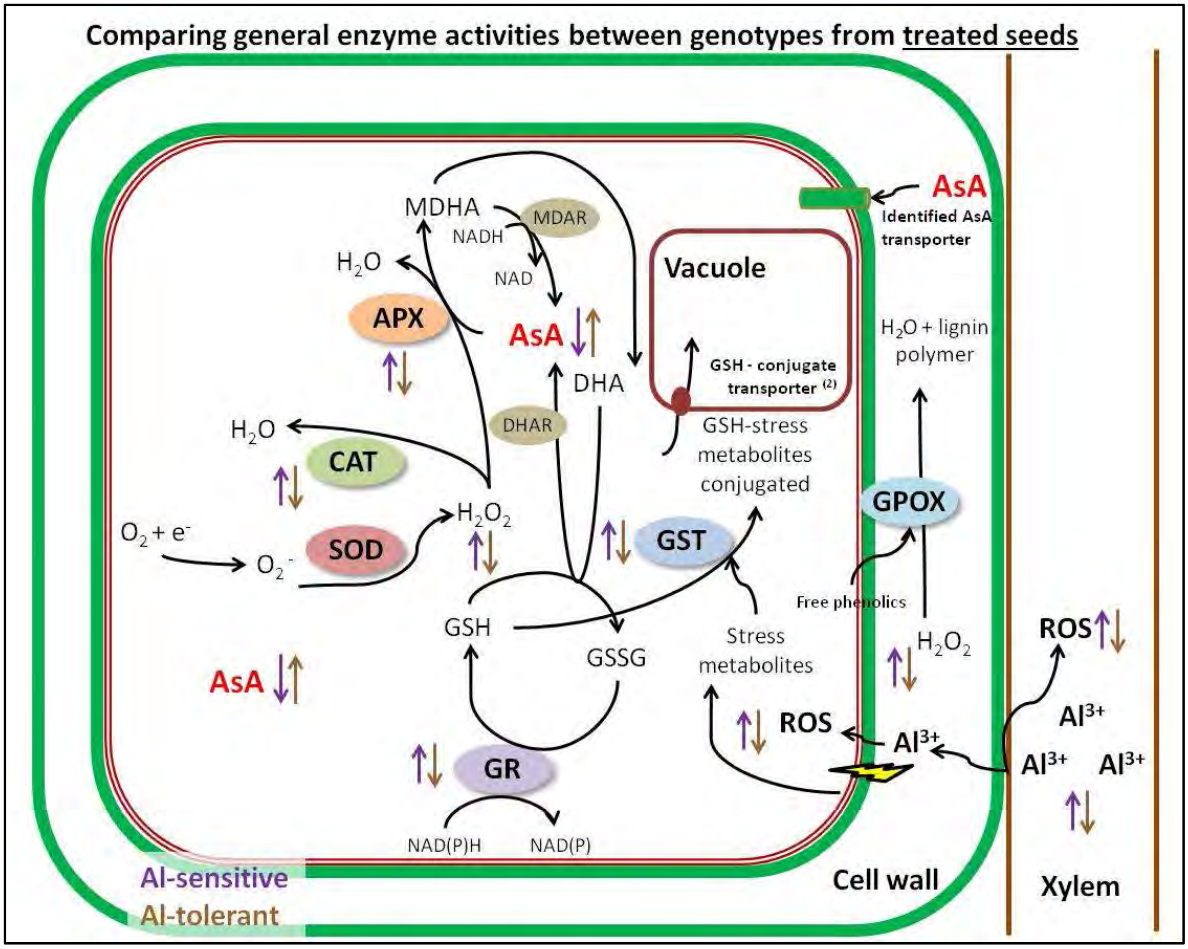

Figure 91 - Summary of the responses in the enzyme activity and metabolite content in the leaves of maize from AsA primed seeds. The arrows in purple denote the direction of changes in the Al-sensitive line (Mo17) compared with the Al-tolerant variety (DA; light-brown arrows)

With regard to the chemical elements in grains, we were greatly surprised by the magnitude of Al compared with other micronutrients (B, Cu, Fe, Mn, Zn). A previous study that quantified Al in corn noted 31-40 mg $Al.Kg^{-1}$ in grains from plants growing in fields irrigated with Al-contaminated lake water and 17-26 mg $Al.Kg^{-1}$ in grains from plants irrigated with non-contaminated water (KHAN et al., 2012). Our experiments revealed approximately 50-60 $mg.Kg^{-1}$ in plants growing in soil containing high Al content although no differences were noted between unprimed and ascorbic acid-primed seeds.

Under typical conditions, the gastrointestinal absorption of aluminum is low in humans and generally in the range of 0.1–0.4% because aluminum is readily excreted in the urine (LITOV *et al.*, 1989). However, large doses of aluminum may be a concern for health in patients with compromised kidney function (AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY - ATSDR, 2008).

The Joint FAO/WHO Expert Committee on Food Additives established a provisional tolerable weekly intake (PTWI) of 2 mg of $Al.kg^{-1}$ body weight (The Aluminum Association, 2008). Based on ATSDR (2008), in the U.S., the estimated daily aluminum intakes are: 0.10 mg of $Al.kg^{-1}$ of body weight per day for 6-11-month-old infants; 0.30-0.35 mg of $Al.kg^{-1}$ of body weight per day for 2-6-year-old children; 0.11 mg of $Al.kg^{-1}$ of body weight per day for 10-year-old children; 0.15-0.18 mg of $Al.kg^{-1}$ of body weight per day for 14-16-year-olds; and 0.10-0.12 mg of $Al.kg^{-1}$ of body weight per day for adults. Users of medications containing aluminum may ingest much larger levels of aluminum than that obtained from their diet, possibly as high as 12-71 mg of $Al.kg^{-1}$ body weight per day.

In the United States, a typical adult consumes approximately 7-9 mg of Al per day from their food (ATSDR, 2008), a range that is markedly below the tolerable limit (20 mg per day). Because Brazilians have a different diet than citizens from the U. S., more detailed studies are required to determine the Al ingestion in Brazil; however, in view of the mean levels of corn consumed in the Brazilian diet (18 kg *per capita* per year) (EMBRAPA, 2005-2011), we estimate that we consume approximately 2.5-3.0 mg of Al from corn per day (in view of our values obtained for the DA variety grown in soil containing high levels of Al). This range is also far below the tolerable limit established by the FAO and WHO; thus, no risks are noted with respect to human consumption.

With respect to the improvement in kernel quality in the tolerant commercial variety (DA), interestingly, we observed that although seed priming did not affect Al absorption, this treatment significantly increased the allocation of boron (B) to the kernels, an element that may alleviate the effects of absorbed Al, as some studies have previously shown (CORRALES et al., 2008; JIANG et al., 2009). An increase in carbohydrates caused by improved boron supply was also observed in a previous study (PANDEY; GUPTA, 2013). Thus, the increased total carbohydrate levels observed in our study may be a side effect caused by the increase in boron in the grains because this element is involved in sugar translocation and carbohydrate metabolism (CAMACHO-CRISTÓBAL et al., 2008).

Gauch (1953) demonstrated that one function of boron involves a reaction with sugar that forms a sugar-borate complex that moves through cellular membranes more readily than non-borated sugar molecules. Boron is transported initially by the xylem, and after reaching the leaves, it is transported by the phloem together with sugar (Figure 92), in particular during reproductive stages, which are periods that show higher concentrations of boron in phloem exudates than in xylem sap (HUANG et al., 2008; TAKANO et al., 2008).

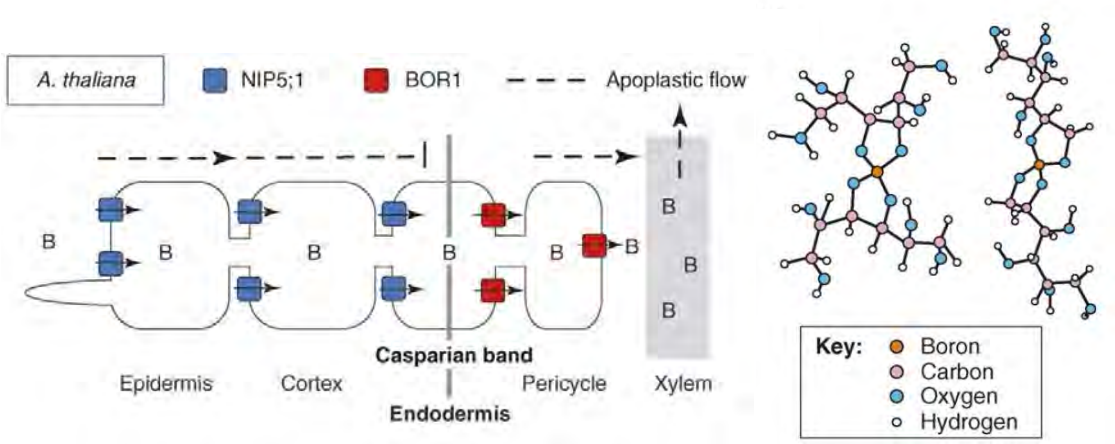

Figure 92 - Boron uptake and transport. (Left) Model of boron radial transport in *Arabidopsis thaliana* roots. (Right) Predicted structures of bis-mannitol borate in phloem (TAKANO; MIWA; FUJIWARA, 2008)

Because boron is important for sugar transport and carbohydrate metabolism in grain, the important question raised by our data is what is the influence of ascorbic acid on boron translocation?

The relationship between boron absorption and AsA has already been studied. We observed greater ascorbate content in DA leaves growing from ascorbic acid-primed seeds compared with those from unprimed seeds. Based on the work of Blevins and Lukaszewski (1998) and the references therein, there is substantial evidence supporting an association between boron and ascorbate metabolism. An interesting report showed that in squash (*Curcubita pepo* L.), boron deficiency caused reduced root growth and ascorbate content in root tips (LUKASZEWSKI; BLEVINS, 1996). These latter authors also verified that supplementation with ascorbic acid in medium lacking boron rescued growth in these plants, and they suggested that the lack of boron affected vitamin C metabolism and thus supplementation with AsA compensated for boron and enabled root elongation. The novel observation raised from our data is that seed priming with ascorbic acid induces boron translocation during kernel production likely because AsA availability is increased in DA variety leaves. Thus, supplementation with AsA in vegetative tissues may induce boron translocation to other parts of the plant, such as the reproductive tissues, because sugar translocation requires complexation with this element.

Plants produce AsA in mitochondria via L-galactose (WHEELER; JONES; SMIRNOFF, 1998), D-galacturonic acid (VALPUESTA; BOTELLA, 2004) and D-glucuronic acid generated from *myo*-Inositol (LORENCE *et al.*, 2004) (Figure 93). Based on

Horemans et al. (2000) and the references therein, AsA is present in several cell compartments, for example, in chloroplasts, the cytosol, vacuoles and the apoplast (Figure 94). Because AsA biosynthesis does not occur in the apoplast, the presence of AsA transporters has been suggested to play an important role in the translocation of AsA from the cytosol to the apoplast (MOZAFAR; OERTLI, 1993). Interestingly, subsequent studies have shown that ascorbate is also transported in the reverse direction (apoplast to the cytoplasm) (LUWE, 1996).

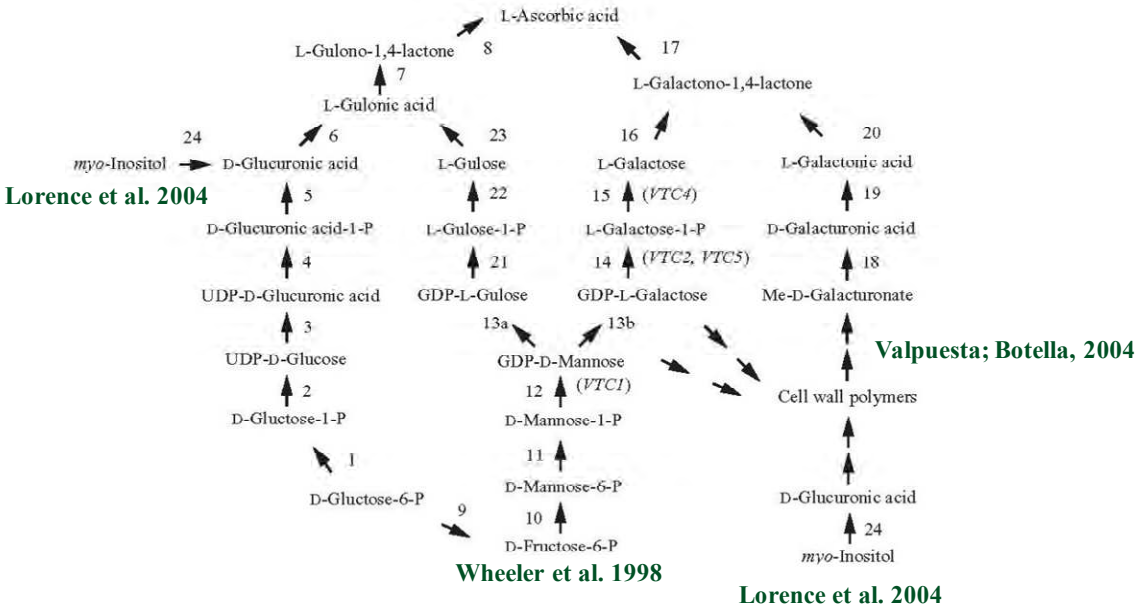

Figure 93 - L-ascorbic acid synthesis pathways. The pathway in animals is shown as reactions 1-8, whereas the pathways in plants are shown as reactions 9-24 (GALLIE, 2013a)

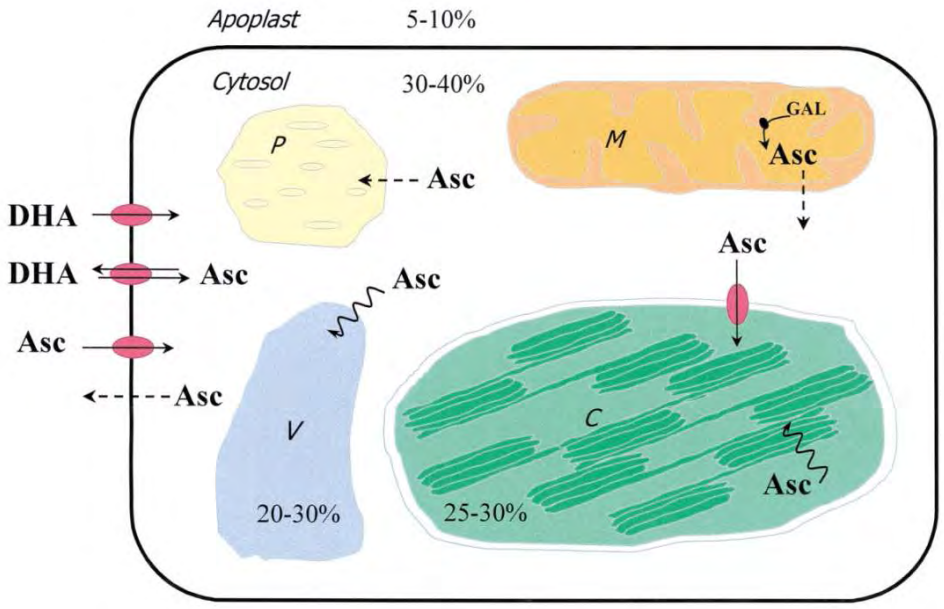

Figure 94 - Schematic of a plant cell with different cell organelles and compartments (V, vacuole; M, mitochondrion; C, chloroplast; P, peroxisome). The relative occurrence of ascorbate (Asc) is provided as a percentage. Peroxisomal and mitochondrial Asc levels are included as a percentage of the cytosol content (HOREMANS *et al.*, 2000)

With respect to amino acid metabolism, we observed that ascorbic acid increased the contents of ten free amino acids in grains: serine, tyrosine, alanine, valine, glutamate, arginine, proline, aspartate, lysine and isoleucine. The free amino acids are the soluble amino acids in MCW (methanol:chloroform:water) that are not incorporated into proteins. It is already known that ascorbate is a cofactor of some enzymes, and with regard to amino acid metabolism, AsA is a cofactor of 4-hydroxyphenylpyruvate dioxygenase, an enzyme involved in tyrosine metabolism (DAVEY *et al.*, 2000). Furthermore, it is possible that treatment with ascorbic acid redirects the flow of D-glucuso-6-P to amino acid metabolism (Figure 95) because the same source of carbon is used for ascorbic acid biosynthesis (WHEELER; JONES; SMIRNOFF, 1998).

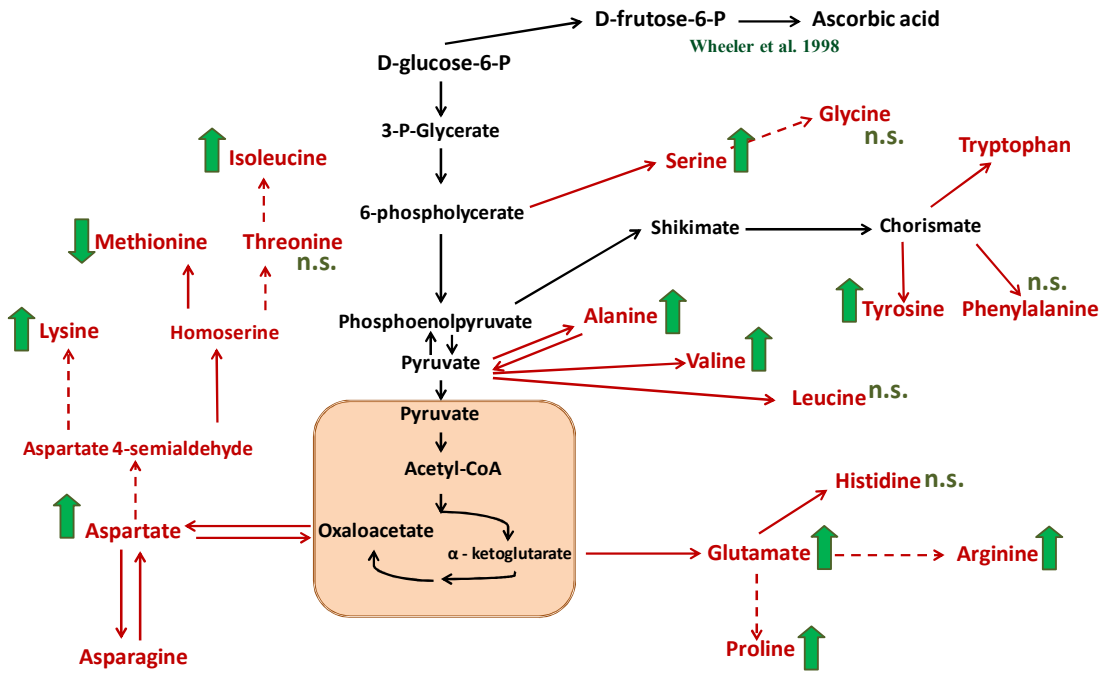

Figure 95 - Relationship between ascorbic acid biosynthesis and amino acid metabolism. Adapted from Wang and Larkins (2001). The orange square contains the reactions occurring in the mitochondrial matrix. Red arrows denote amino acid metabolism. Continuous arrows relate to biosynthesis, whereas dashed arrows denote degradation. Green arrows denote the results obtained in our study. Arrows pointing upwards represent the amino acid content increased by seed priming, arrows pointing downwards represent the amino acid content decreased by seed priming and n.s. denotes no significant difference

Our study is the first to examine the effect of ascorbic acid seed priming on amino acid biosynthesis. We observed that only one amino acid was decreased by ascorbic acid: methionine, an amino acid that shares the same biosynthetic route of lysine, threonine and isoleucine (AZEVEDO; LEA, 2001). The lysine and isoleucine content increased, whereas the methionine content decreased. This response may be explained as an increase in lysine that results in the feedback inhibition of specific isozymes of aspartate kinase (AK) that are responsible for methionine synthesis (GALILI; LARKINS, 1998; AZEVEDO et al., 2006).

The sensitivity of AK to feedback inhibition is a major limiting factor for lysine synthesis enhancement; mutations conferring isozyme resistance to lysine may be interesting to improve the amino acid content in maize kernels. Some limited success in increasing the concentration of methionine in legume seeds has been obtained by expressing a feedback-

insensitive AK (AZEVEDO et al., 2006 and references therein). It may be interesting to study the effect of ascorbic acid treatment in maize plants expressing this type of mutation in the AK enzyme. To select plants with a mutation in the AK enzyme, it is recommended to treat the plants with lysine and threonine; thus enabling the selection of feedback-insensitive AK mutants because there are two AK isozymes that are inhibited by lysine and threonine. Wild-type plants growing in medium culture containing lysine and threonine die following methionine starvation due to inhibition of total AK activity (CORUZZI; LAST, 2000), therefore it would be interesting to test this seed priming in maize plants expressing AK mutants because the increase in lysine caused by seed priming may reduce the methionine content in grain via feedback inhibition.

There is also a second hypothesis that could be related to the increase of isoleucine route, since methionine has the same precursor (homoserine). We hypothesize that ascorbic acid treatment somehow is allocating homoserine to isoleucine formation, reducing the flow for methionine synthesis.

Furthermore, it is important to highlight that we quantified soluble amino acids and we did not analyze amino acids incorporated into proteins; thus, another hypothesis is that because ascorbic acid could be directly involved in plant growth, it is possible that methionine may be used more for protein biosynthesis (therefore, reducing levels of soluble methionine).

5.5 Conclusions

Some of our results confirm our initial hypotheses; however, other hypotheses were not confirmed. Based on our objectives, our conclusions are as follows:

Seed priming with ascorbic acid (AsA) increases growth in the Al-sensitive line (Mo17) and the Al-tolerant variety (DA); however, it was not capable of inducing Al-tolerance in Mo17 plants until the phase of production.

Seed priming reduced Al absorption, MDA content, GPOX activity and lignin content in the Al-sensitive line (Mo17). However, in the Al-tolerant variety (DA), Al absorption remained unchanged, as did the lignin content and the stress metabolite MDA.

The cause of the improvement in growth in DA plants was not explained by nutrient absorption because the nutrient levels remained unchanged. In DA leaves, the AsA content increased, which may be directly related to the improvements in growth in this variety

because AsA is a cofactor for extensin biosynthesis (a glycoprotein important for cell expansion).

With regard to enzyme activity, AsA caused different effects in Al-sensitive (Mo17) and Al-tolerant (DA) genotypes: in the case of the Mo17 line, it reduced the GPOX activity. In the case of the DA variety, the activities of GPOX did not change although the treatment decreased the CAT-3 and APX VI activities, suggesting that the increase in AsA content may down-regulate *Cat-3* and APX VI gene expression.

The behavior of GR and APX VI activities were very similar when seeds were treated with AsA. In the case of Mo17, AsA induced both GR and APX VI, while in DA genotype, AsA reduced the activities of these enzymes.

Because only the DA variety reached the age of production, seed priming improved the kernel quality with respect to boron allocation, total carbohydrate content and also increased the content for some soluble amino acids, such as serine, tyrosine, alanine, valine, glutamate, arginine, proline, aspartate, lysine and isoleucine, although the methionine levels were decreased.

Seed priming with ascorbic acid induced some interesting effects that may have future applications. However, studies on the economic viability of this technique are necessary. Moreover, it would be interesting to test this seed priming in maize plants expressing aspartate kinase mutants (AK) because the increase in lysine caused by seed priming may reduce the methionine content in grain via feedback inhibition. However, we also have another hypothesis that should be tested: the pool of free methionine decreased due to the increase in its utilization for protein synthesis in the DA variety because ascorbic acid improved growth and productivity.

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