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Aluminium toxicity: oxidative stress during germination and early development in purple maize[1]

Toxidez por alumínio: estresse oxidativo durante a germinação e desenvolvimento inicial de milho roxo

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ABSTRACT - Purple maize is a source of natural pigments that can be used for various industrial purposes; however, soil contamination from aluminium affects production. The aim of this study was to evaluate the effect of aluminium toxicity associated with different temperatures on seed germination and initial development in purple maize seedlings, through germination, initial seedling growth and indicators of oxidative damage. The experimental design was completely randomised in a 5 × 2 factorial scheme (concentrations – 0, 25, 50, 75 and 100 mg L^{-1} × temperatures – 25 and 30 ºC). The parameters under evaluation were germination percentage, length and total dry weight of the root system and shoots, and indicators of oxidative damage (chlorophyll *a*, chlorophyll *b* and total chlorophyll, total carotenoids and lipid peroxidation) at the seedling stage. Aluminium chloride concentrations have no effect on seed germination in purple maize. With increasing aluminium concentrations, the length, dry weight, chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids of normal purple maize seedlings are reduced, while lipid peroxidation increases. Aluminium toxicity stress in purple maize seedlings can be detected using biochemical indicators (chlorophyll *a*, chlorophyll *b* and total chlorophyll, total carotenoids and lipid peroxidation).

Key words: *Zea mays*. Physiological potential. Natural pigments.

RESUMO - O milho roxo é fonte de pigmentos naturais que podem ser utilizados para diversas finalidades industriais, no entanto a contaminação dos solos por alumínio afeta sua produção. Diante do exposto, o objetivo foi avaliar o efeito da toxidez por alumínio associado a diferentes temperaturas sobre a germinação das sementes e desenvolvimento inicial de plântulas de milho roxo, por meio da germinação, crescimento inicial das plântulas e indicadores de danos oxidativos. O delineamento utilizado foi inteiramente casualizado, com esquema fatorial 5 × 2 (concentrações – 0, 25, 50, 75 e 100 mg L^{-1} × temperaturas – 25 and 30 ºC). Os parâmetros avaliados foram porcentagem de germinação, comprimento e massa seca total, da parte aérea, do sistema radicular e indicadores de danos oxidativos (clorofilas totais, *a*, *b*, carotenoides totais e peroxidação de lipídeos) na fase de plântula. As concentrações de cloreto de alumínio não interferem na germinação de sementes de milho roxo. Com o aumento das concentrações de alumínio, observa-se redução do comprimento, massa seca, clorofilas totais, a, b e carotenoides totais e acréscimos na peroxidação lipídica em plântulas normais de milho roxo. O estresse por toxidez do alumínio em plântulas de milho roxo é detectado pelos indicadores bioquímicos (clorofilas totais, *a*, *b*, carotenoides totais e peroxidação de lipídeos).

Palavras-chave: *Zea mays*. Potencial fisiológico. Pigmentos naturais.

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INTRODUCTION

Purple maize (*Zea mays* L.), family Poaceae, is a variety native to the Andes region, specifically Peru, whose seeds have of one of the deepest shades of purple found in the plant kingdom; it is widely cultivated and consumed throughout the Andes region of South America, especially Peru, Ecuador, Bolivia and Argentina (LAO *et al*., 2017). Coloured maize cultivars are economically viable sources of natural pigments, which can be used to replace synthetic food colourings (CHEN, 2017). The main pigments found in purple maize are anthocyanins, which are glycosides based on cyanidin, peonidin and pelargonidin (CHATHAM *et al*., 2018).

Purple maize production is essential for the availability of raw materials for the food, pharmaceutical and cosmetics industries, however production can be affected by various factors, including aluminium (Al) toxicity. In acidic soils with a high Al content, toxicity is the most limiting factor to agricultural production (DEL GUERCIO; CAMARGO, 2011), and it is estimated that over 50% of arable agricultural land in the world is acidic (KOCHIAN *et al*., 2015). The absorption of Al by plant cells can lead to the accumulation of ROS (reactive oxygen species), inhibition of photosynthesis, disruption of membrane structures, ionic homeostasis, inhibition of enzymatic reactions and the activation of programmed cell death (TAIZ *et al*., 2017).

However, plants exposed to high aluminium toxicity demonstrate mechanisms to limit the effects. The most important of these mechanisms is the exclusion of Al via exudation of the acid anion into the rhizosphere, preventing the Al from entering the roots. Internal detoxification of Al by cytosolic chelation with acidic anions or phenolic compounds, and the subsequent compartmentalisation of the Al in vacuoles, is another exclusion mechanism, as is elimination by the plasma membrane activity of ROS and H^+ -ATPase (ZHANG *et al*., 2017).

Given the above, it is essential to understand how aluminium interferes with the germination and initial development of purple maize seedlings. The aim of this study, therefore, was to evaluate the effect of aluminium toxicity associated with different temperatures on seed germination and initial development in purple maize seedlings, through germination, seedling growth and indicators of oxidative damage (chlorophyll *a*, chlorophyll *b*, total chlorophyll, total carotenoids and lipid peroxidation).

MATERIAL AND METHODS

The experiments were conducted in the Biotechnology Laboratories and in the Post-Graduate Program in Plant Production of the Serra Talhada Academic Unit/UAST at the Federal Rural University of Pernambuco/UFRPE. The purple maize seeds, from the 2018/2019 crop, were obtained from a rural producer at the Sabino Farm, in the Tupanaci district of the city of Mirandiba (8°6′43″ S, 38°43′39″ W, altitude 440 m), in the state of Pernambuco, Brazil.

First, the water content of the seeds was assessed using the oven method at 105 ± 3 ºC, for 24 hours (BRASIL, 2009), which recorded a value of 11.76%.

Treatments: During germination and initial growth, the purple maize seeds were submitted to aluminium chloride ($AlCl_3.6H_2O$) at concentrations of 0 (control), 25, 50, 75 and 100 mg L^{-1}, at temperatures of 25 and 30 ºC.

Physiological and morphological evaluations

Germination: A roll of paper towelling was used as substrate, with the sheets previously moistened with different concentrations of the $AlCl_3.6H_2O$ solution at an amount equivalent to 2.5 times the weight of the dry paper; distilled water was used as the control. Prior to sowing, the seeds were disinfected with 2.5% sodium hypochlorite for four minutes, and then rinsed in distilled water (BRASIL, 2009). The prepared rolls were then placed in a B. O. D. (Biochemical Oxygen Demand) germination chamber (Marconi MA 1402/546) for seven days under constant temperatures of 25 and 30 ºC and a photoperiod of 12 hours. The formation of normal seedlings on the seventh day after sowing was adopted as the criterion for germination (BRASIL, 2009). These normal seedlings were later used in the biochemical procedures, i.e. chlorophyll *a*, chlorophyll *b*, total chlorophyll, total carotenoids and lipid peroxidation.

Seedling length: The set-up was similar to that used in the germination test, however, two rows of 10 seeds each were sown on the upper third of the paper sheet per replication (NAKAGAWA *et al*., 1999). The normal seedlings were measured on the seventh day after sowing. The length of the shoots (SL) was evaluated using a millimetre rule, from the collar to the end of the largest leaf. For the length of the root system (RL), the measurement was taken from the collar to the end of the root, with the mean results expressed in cm $seedling^{-1}$.

Seedling dry weight: After evaluating the length, all normal seedlings were cut near the collar to separate the shoots and root system, in addition to removing any traces of caryopses. For each replication, both the shoots and root system were packed in paper bags and placed in a forced air circulation oven for 24 hours at 80 °C (NAKAGAWA *et al*., 1999). The dry weight of the shoots (SDW), root system (RDW) and total dry weight (TDW) of the normal seedlings were then determined.

Indicators of oxidative damage

Levels of photosynthetic pigments: To determine the chlorophyll content (*a*, *b* and total) and total carotenoids, 0.1 gram of fresh leaves was placed into identified test tubes, and the chlorophyll extracted by adding 5 mL acetone (80% v/v). The tubes were hermetically sealed, covered with aluminium foil, and kept under refrigeration for 48 hours. Spectrophotometer readings were then taken at wavelengths of 645, 652 and 663 nm to determine the chlorophyll levels (ARNON, 1949), and at 470 nm (LICHTENTHALER; BUSCHMANN, 2001) to determine the total carotenoids.

Lipid peroxidation: Lipid peroxidation was estimated from the levels of thiobarbituric acid reactive substances (TBARS), as per Heath and Packer (1968). The shoots and the root system of normal seedlings were analysed using 0.1 gram of plant material per replication, which was macerated in a mortar in the presence of liquid nitrogen, followed by the addition of 6% trichloroacetic acid (TCA) and a further 3 min of maceration. The extract was centrifuged at 12,000 xg for 15 min at 4 °C. Then 0.5 mL of the supernatant was added to 2.0 mL 20% TCA solution and 0.5% (w/v) thiobarbituric acid (TBA) solution and heated in a water bath at 95 °C in hermetically sealed tubes for one hour. The reaction was then halted in an ice bath, and readings taken at 532 and 660 nm using a spectrophotometer. The TBARS content was estimated using the molar extinction coefficient of 155 mM^{-1} cm^{-1} after subtracting the absorbance obtained at 660 nm from that obtained at 532 nm.

Statistical analysis

The experimental design was completely randomised in a 5 × 2 factorial scheme (concentrations – 0, 25, 50, 75 and 100 mg L^{-1} × temperatures – 25 and 30 ºC), with four replications of 50 seeds (germination), four replications of 20 seeds (seedling length and dry weight), and three replications of five normal seedlings from the germination test (indicators of oxidative damage). An analysis of variance was then carried out by F-test ($p < 0.05$), with the treatments submitted to regression analysis whenever a significant effect from the interaction was found. In the absence of any significant interaction, the mean values of the variables resulting from the temperatures were compared by Tukey's test ($p < 0.05$), and the mean values of the concentrations were evaluated by regression analysis. All the analyses were carried out using the Sisvar® statistical software (FERREIRA, 2011). The SigmaPlot 10.0 software was used to prepare the graphs.

RESULTS AND DISCUSSION

The analysis of variance (Table 1) shows that there was a significant difference for aluminium concentration in all variables except germination. Regarding the effect of temperature, only the variables of total dry weight, shoot dry weight and root-system dry weight showed any significant difference, whereas only shoot length showed a significant difference for the interaction between aluminium concentration and temperature.

There was a significant difference in relation to chlorophyll *a*, chlorophyll *b*, total chlorophyll, total carotenoids and lipid peroxidation for the aluminium concentrations of the purple maize seedlings. Regarding temperature, and the effect of the interaction between the different concentrations of aluminium and different temperatures, there was a significant difference for lipid peroxidation only (Table 2).

The response found for the germination of purple maize seeds in the present study was similar to that found

Table 1 - Summary of the analysis of variance of the mean values for germination and initial growth in purple maize seedlings subjected to aluminium toxicity stress at different temperatures

Source of variation	DF	Mean square					
		GP	SL	RL	SDW	RDW	TDW
		(%)	(cm seedling^{-1})		(g seedling^{-1})		
Al^{3+} concentrations	4	16.7^{ns}	5.3*	4.8*	0.0005*	0.0008*	0.002*
Temperature	1	16.9^{ns}	0.06^{ns}	0.2^{ns}	0.001*	0.001*	0.01*
Al^{3+} concentration × Temperature	4	11.6^{ns}	2.25*	1.7^{ns}	0.000^{ns}	0.0002^{ns}	0.0002^{ns}
Error	30	48.1	0.6	1.9	0.00009	0.00004	0.0002
Total	39	-	-	-	-	-	-
CV (%)	-	4.64	7.97	3.3	21.68	15.23	15.56

ns, * respectively, not significant and significant at 5% probability by F-test. Legend: DF - Degrees of freedom; GP – Germination percentage (%); SL – Shoot length (cm seedling^{-1}); RL – Root system length (cm seedling^{-1}); SDW – Shoot dry weight (g seedling^{-1}); RDW – Root system dry weight (g seedling^{-1}) and TDW – Total dry weight (g seedling^{-1}) in purple maize seedlings; CV – Coefficient of variation

by Milane *et al*. (2014), who studied the germination of two maize varieties, one conventional and one genetically modified, and found no significant difference in germination percentage at different aluminium concentrations. In general, there was a reduction in shoot length in the purple maize seedlings due to the interaction between the concentrations of the aluminium solutions and the temperatures. The reduction in shoot length was more drastic at 30 ºC compared to 25 ºC (Figure 1A).

The reduction in temperature promotes a slower rate for the biochemical and physiological activities involved in the metabolism of the cells that act in growth and development, as well as the ability to multiply and form tissue, thereby delaying the development of organs such as the leaves (CARVALHO *et al*., 2017). On the other hand, in the present study that employs a situation of stress caused by an increase in aluminium chloride concentrations, the lower temperature minimised the harmful effects on shoot length from the concentration of 50 mg L^{-1} (Figure 1A).

The length of the root system in the purple maize seedlings was reduced with the increase in aluminium chloride concentrations (Figure 1B). The main symptom of Al toxicity in cultivated plants is inhibited root growth (SINGH *et al*., 2017); by inhibiting elongation of the root system, Al reduces the absorption of nutrients and water, limiting production (KOCHIAN *et al*., 2017). The roots have mechanisms for aluminium tolerance: Piñeros and Kochian (2001) report that maize roots release the organic acid, citrate, which is the most common among species in the presence of Al, and is also the most effective among organic acids, as it is a tricarboxylate anion, forming far more stable chelates with $Al3^{+}$ compared to chelates formed by malate (dicarboxylate anion) (HARTWIG *et al*., 2007). The importance of citrate as an organic acid involved in tolerance to toxic Al is therefore obvious.

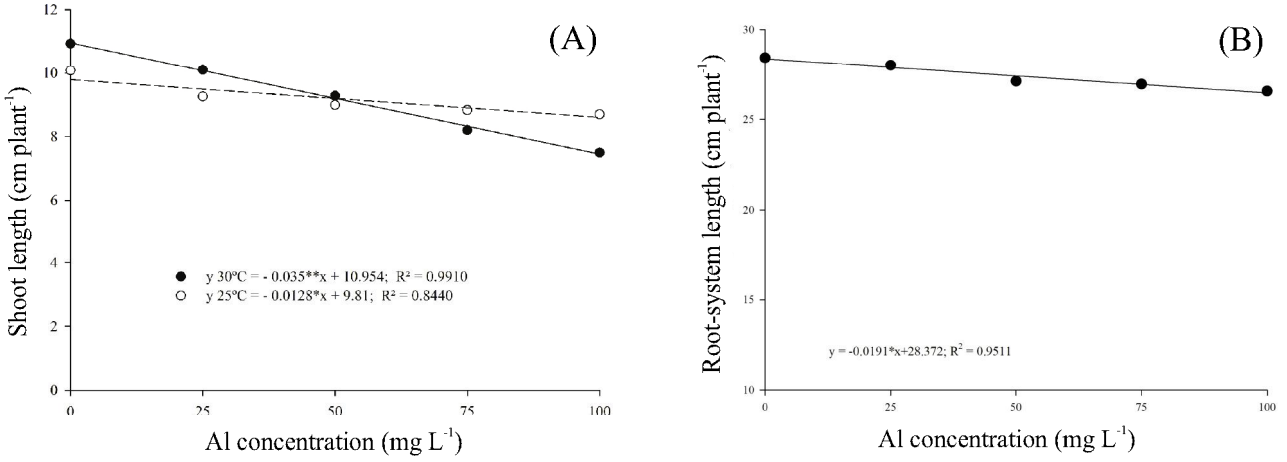
According to Nasr (2013), an increase in the Al content of the soil led to a decrease in the production of shoot and root dry weight in maize, with similar results

Table 2 - Summary of the analysis of variance of the mean values for the indicators of oxidative damage in purple maize seedlings subjected to aluminium toxicity stress at different temperatures

Source of variation	Mean square						
	DF	Total chlorophyll	Chlorophyll *a*	Chlorophyll *b*	Total carotenoids	TBARS content	
						SH	RS
Al^{3+} concentrations	4	0.06*	0.05*	0.001*	0.00009*	47252.1**	43277.5**
Temperature	1	0.0^{ns}	0.0^{ns}	0.0003^{ns}	0.00002^{ns}	12352.5*	12670.3**
Al^{3+} concentration × Temperature	4	0.004^{ns}	0.003^{ns}	0.0009^{ns}	0.00002^{ns}	4068.1^{ns}	5133.20**
Error	20	0.005	0.005	0.0002	0.00002	2077.3	1011.7
Total	29	-	-	-	-	-	-
CV (%)	-	22.45	25.78	27.11	28.72	29.24	22.48

ns, **, * respectively, not significant and significant at 1% and 5% probability by F-test. Legend: DF - Degrees of freedom; SH – shoots; RS – Root system; CV – Coefficient of variation

Figure 1 - Shoot length – SL (A) in purple maize seedlings submitted to different concentrations of aluminium chloride and different temperatures; and root system length – RL (B) in purple maize seedlings submitted to different concentrations of aluminium



being found in the present study (Figure 2A). In acidic soils, Al is solubilised in the soil solution, taking the form of a trivalent cation (Al^{+3}), which becomes available to the plant and results in toxicity (KOPITTKE *et al*., 2015). Al toxicity causes disruption of homeostasis in reactive oxygen/Ca^{2+} species and Ca^{2+}-mediated signalling, considered key events in inducing DNA damage or cell death in plants (ACHARY; PARINANDI; PANDA, 2013).

The total dry weight, shoot dry weight and root-system dry weight of the purple maize seedlings showed greater accumulation when exposed to a temperature of 30 ºC (Figure 2B), since lower temperatures reduce the capacity of the seed to absorb water, slowing down the germination process. Although 25 ºC is not considered a low temperature for germination in maize seeds, and is suggested when carrying out the standard germination test (BRASIL, 2009), using a temperature of 30 ºC may have resulted in greater dry-weight accumulation in the purple maize seedlings due to favouring greater speed in the germination process (Figure 2B). When studying different maize batches, Sbrussi and Zucareli (2015) found that a temperature of 34 ºC gave the greatest dry weight accumulation for both the shoots and root system of maize seedlings.

The levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids were negatively affected by the increased concentrations of aluminium chloride (Figures 3A and 3B). Similar results were seen by Zhao *et al*. (2017), who found an aluminium-induced reduction in leaf photosynthesis in maize plants. Higher concentrations of aluminium therefore result in lower levels of chlorophyll, limiting the metabolic potential of the plant. The reduction in photosynthetic performance suggests that Al^{3+} can destroy protein complexes, chloroplasts and the photosynthetic apparatus (JIANG *et al*., 2008). The authors showed that a lower electron transport capacity in the photosynthetic apparatus, together with a lack of reducing equivalents, were the main factors that contributed to the lower assimilation of CO_2 in plants exposed to Al^{3+}.

Figure 2 - Total dry weight – TDW, shoot dry weight – SDW and root-system dry weight – RDW in purple maize seedlings submitted to different concentrations of aluminium chloride (A) and different temperatures (B). Mean values followed by the same letters do not differ by Tukey's test ($p < 0.05$)

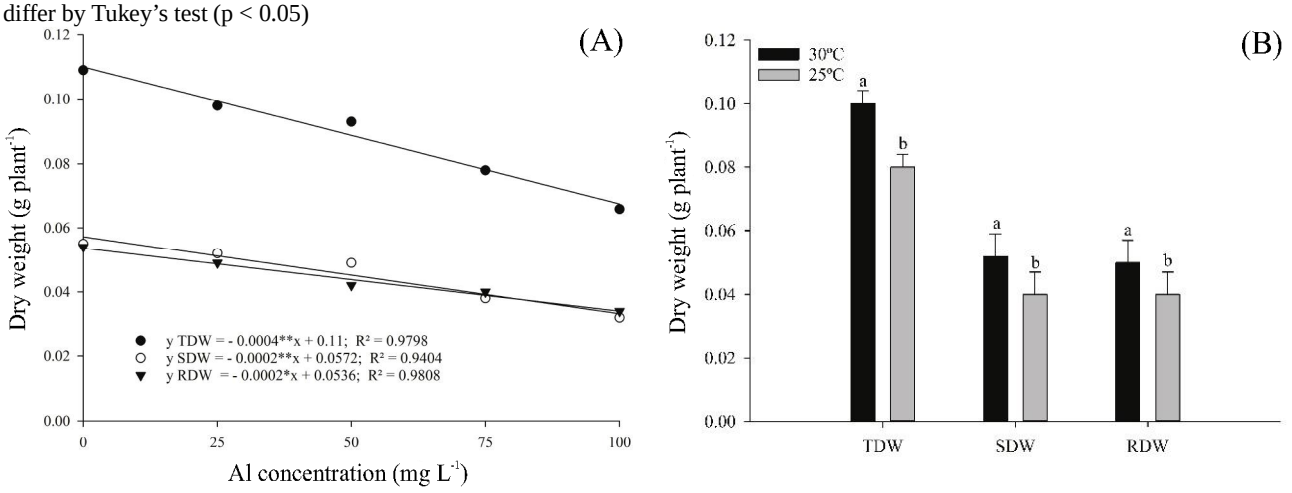
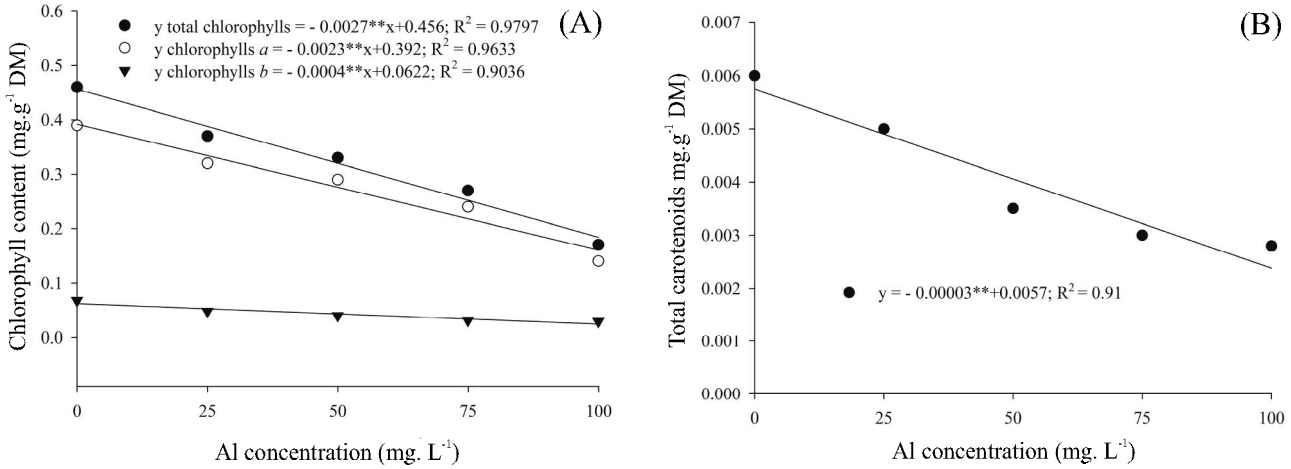


Figure 3 - Total chlorophyll, chlorophyll a and chlorophyll b (A) and total carotenoids (B) in the leaves of purple maize seedlings submitted to different concentrations of aluminium chloride



The shoots of the purple maize seedlings subjected to different concentrations of aluminium showed an increase in lipid peroxidation (Figure 4A). Similar results were seen by Giannakoula *et al*. (2010), who studied two varieties of maize, one sensitive and the other tolerant to aluminium, and found that as the aluminium concentration increased, there was an increase in the levels of malondialdehyde (MDA).

Oxidative stress is caused by an imbalance in the production of ROS and the elimination of antioxidants from plant tissue that results from any situation of stress. As a result of ROS production, phytotoxic reactions, such as lipid peroxidation, protein degradation and DNA mutation, occur in plants (TANOU; MOLASSIOTIS; DIAMANTIDIS, 2009). These reactions interfere directly in plant development and growth. The temperature of 30 ºC resulted in greater lipid peroxidation (TBARS) in the shoots of the purple maize seedlings, compared to the temperature of 25 ºC (Figure 4B).

Figure 4 - Lipid peroxidation in the shoots of purple maize seedlings submitted to different concentrations of aluminium chloride (A) and different temperatures (B). Mean values followed by the same letters do not differ by Tukey's test ($p < 0.05$)

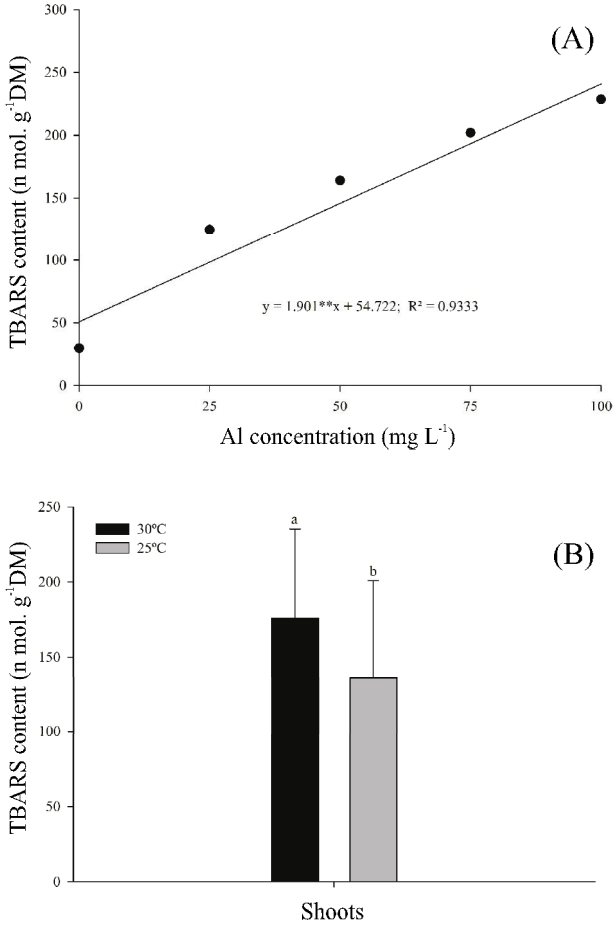
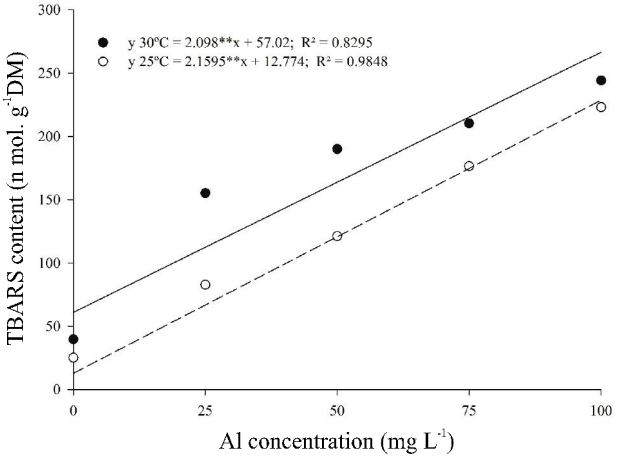


Figure 5 - Lipid peroxidation of the root system of purple maize seedlings submitted to different concentrations of aluminium chloride and different temperatures



The interaction of the different aluminium chloride concentrations and different temperatures caused greater lipid peroxidation in the root system of the purple maize seedlings, however the temperature of 30 ºC, even when not suggested as thermal stress for the species in question, resulted in greater lipid peroxidation compared to the temperature of 25 ºC (Figure 5). ROS-induced peroxidation in lipid membranes is a reflection of stress-induced damage at the cellular level (JAIN *et al*., 2001).

CONCLUSIONS

1. Aluminium chloride concentrations have no effect on seed germination in purple maize;
2. With increasing aluminium concentrations, the length, dry weight, chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids of normal purple maize seedlings are reduced, showing an increase in lipid peroxidation;
3. Aluminium toxicity stress in purple maize seedlings can be detected using biochemical indicators (chlorophyll *a*, chlorophyll *b*, total chlorophyll, total carotenoids and lipid peroxidation).

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