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# RESPOSTAS BIOQUÍMICAS E FISIOLÓGICAS DE *Cucumis sativus* e *Avena sativa* AO ESTRESSE CAUSADO POR ALUMÍNIO

**TESE DE DOUTORADO**

**Luciane Belmonte Pereira**

**Santa Maria, RS, Brasil**
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**Por**

**Luciane Belmonte Pereira**

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de Doutor em Bioquímica Toxicológica

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A Comissão Examinadora, abaixo assinada,
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elaborada por
**Luciane Belmonte Pereira**

como requisito parcial para a obtenção do grau de
**Doutor em Bioquímica Toxicológica**

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**“Pense no poder que há no Universo, que move a**

**Terra e faz as árvores crescerem. Esse**

**mesmo poder existe em você,**

**basta que  tenha coragem**

**e o desejo de usá-lo!”**

**Charles Chaplin**

# LISTA DE ABREVIATURAS

Al – Alumínio

APX – Ascorbato peroxidase

ASA- Ácido ascórbico

CAT – Catalase

ELP – Porcentagem de vazamento de eletrólitos

EROs - Espécies reativas de oxigênio

MDA – Malondialdeído

NPSH – Grupos tióis não proteicos

PBG – Porfobilinogênio

SOD – Superóxido dismutase

δ ALA-D – Delta aminolevulinato desidratase

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# RESUMO


Tese de Doutorado
Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

**RESPOSTAS BIOQUÍMICAS E FISIOLÓGICAS DE *Cucumis sativus* E *Avena sativa* AO ESTRESSE CAUSADO POR ALUMÍNIO**

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Co-orientadora: Vera Maria Morsch
Data e Local de Defesa: Santa Maria, 21 de maio de 2010.

O alumínio (Al) é o metal mais abundante na crosta terrestre, afetando o crescimento e desenvolvimento das plantas. Neste estudo, foram investigados através da análise de parâmetros bioquímicos e fisiológicos, os efeitos do alumínio (Al) em plântulas de pepino (*Cucumis sativus* L.) e em plântulas de aveia (*Avena sativa* L). As plântulas de aveia estudadas pertencem a três diferentes genótipos: UFRGS 930598-sensível ao alumínio, UFRGS 17-tolerante ao alumínio e UFRGS 280-intermediário ao alumínio (resultante do cruzamento entre UFRGS 930598 e UFRGS 17), expostos a 0, 5, 10, 20 e 30 mg/L de Al. Os parâmetros bioquímicos analisados para *C. sativus* foram: a atividade das enzimas antioxidantes (catalse (CAT), ascorbato peroxidase (APX) e superóxido dismutase (SOD)), os níveis de peroxidação lipídica, vazamento de eletrólitos, oxidação de proteínas e conteúdo de clorofila. O aumento na porcentagem de vazamento de eletrólitos e na produção de peróxido de hidrogênio observado está relacionado com a diminuição da eficiência do sistema antioxidante nas concentrações mais altas de alumínio. O sistema antioxidante foi incapaz de impedir a toxicidade, resultando em efeitos negativos, tais como peroxidação lipídica, oxidação de proteínas e diminuição do crescimento das plantas. Plântulas dos três genótipos de aveia foram expostas ao Al em diferentes meios de crescimento. Primeiro a exposição foi em meio de crescimento semi-sólido com agar por 5 dias, com plântulas com 5 dias de desenvolvimento, depois em solução hidropônica por 7 dias com plântulas com 10 dias de desenvolvimento. Foram avaliados o conteúdo de peróxido de hidrogênio, peroxidação de lipídeos, conteúdo de ácido ascórbico e tióis não protéicos (NPSH), a atividade das enzimas CAT, APX e SOD e o conteúdo de Al acumulado nas plântulas. As enzimas do sistema antioxidante SOD e CAT tiveram suas atividades aumentadas nos genótipos UFRGS 17 e UFRGS 280. Mesmo acumulando altas concentrações de alumínio estes dois genótipos não apresentaram altos níveis de peroxidação lipídica e conteúdo de peróxido de hidrogênio quando comparados com o genótipo 930598. Para esse genótipo houve ativação da enzima APX, entretanto os altos níveis de alumínio acumulados na planta causaram um aumento na peroxidação de lipídeos e no conteúdo de peróxido de hidrogênio. O genótipo UFRGS 930598 mostrou ser mais sensível ao alumínio que os genótipos UFRGS 17 e UFRGS 280, o que confirma as análises morfológicas prévias obtidas por FEDERIZZI et al., 2000. Embora tenha ocorrido um aumento da atividade da APX neste genótipo, o sistema antioxidante não foi eficiente na remoção das espécies

reativas de oxigênio (EROS). Com a finalidade de melhor entender esses resultados foi utilizado o meio de crescimento com solução hidropônica onde a mobilidade do metal é maior por um período de exposição de 7 dias com plântulas de aveia com 10 dias de desenvolvimento. Neste experimento além dos parâmetros bioquímicos citados acima, foi feita a análise do crescimento da raiz e parte aérea, matéria seca e fresca, conteúdo de clorofila, atividade da enzima δ –aminolevulinato desidratase (δ-ALA-D) e o monitoramento do pH da solução hidropônica de cada genótipo. O pH da solução hidropônica do genótipo tolerante (UFRGS 17) apresentou um aumento significativo nos valores de pH, enquanto os genótipos sensíveis (UFRGS 930598) e intermediário (UFRGS 280) não apresentaram mudanças significativas nos valores de pH. O genótipo sensível teve inibição do crescimento da raiz e parte aérea nas concentrações mais altas de alumínio enquanto que para o genótipo tolerante e intermediário, não houve modificações significativas do crescimento da raiz e parte aérea. A atividade das enzimas antioxidantes foi aumentada após 7 dias de exposição ao alumínio no genótipo sensível, enquanto que no genótipo tolerante não houve aumento na atividade das enzimas antioxidantes. Nas plântulas do genótipo intermediário houve o aumento na atividade das enzimas CAT, APX e SOD apenas nas maiores concentrações de alumínio (20 e 30 mg/L). Os resultados mostram que os genótipos intermediário e tolerante apresentaram semelhanças quanto ao efeito do alumínio na atividade das enzimas do sistema antioxidante e também nos parâmetros fisiológicos como o crescimento das raízes e parte aérea. Mesmo acumulando altas concentrações de alumínio, o genótipo intermediário não apresentou diminuição do crescimento, o que mostra que ele apresenta mecanismos de resistência, relacionado com a imobilização interna deste metal nos vacúolos. O genótipo tolerante também apresenta mecanismos de resistência ao alumínio, porém estes mecanismos podem ser de exclusão do alumínio pela raiz através da ligação deste metal a ácidos orgânicos. Com a finalidade de investigar quando o sistema antioxidante é ativado nos diferentes genótipos de aveia, as plântulas foram colocadas no meio de crescimento com solução hidropônica e retiradas do meio após 12, 24 e 36 horas de exposição a 20 mg/L de Al. Os genótipos tolerante e intermediário apresentaram aumento na atividade das enzimas do sistema antioxidante após 12 h de exposição ao alumínio, enquanto o genótipo sensível somente após 24 ou 36 horas de exposição. Esta diferença na velocidade de ativação do sistema antioxidante pode ser crucial na manutenção da homeostase celular redox deste genótipo. Nessa fase do desenvolvimento, o genótipo tolerante e o intermediário apresentaram um estímulo do crescimento da raiz enquanto o sensível um atraso do crescimento. Com a finalidade de estabelecer comparações entre as espécies vegetais, o pepino (C*ucumis sativus*) foi colocado em meio de crescimento hidropônico, juntamente com as plântulas de aveia (genótipo sensível e tolerante). O pepino (*Cucumis sativus*) quando colocado em solução hidropônica exposto a 20 mg/L de alumínio juntamente com o genótipo tolerante (UFRGS 17) e o genótipo sensível (UFRGS 930598), apresentou semelhanças com o genótipo sensível tais como o aumento da atividade da enzima catalase após 36 h de exposição ao alumínio. Os níveis de peroxidação lipídica foram elevados após 12, 24 e 36h de exposição ao alumínio e como conseqüência a raiz teve uma diminuição do crescimento. Através da avaliação conjunta, com os genótipos de aveia tolerante, UFRGS 17, e sensível, UFRGS 930598, o pepino (*Cucumis sativus*) pode ser considerado uma espécie sensível ao alumínio.

# ABSTRACT

Doctoral Thesis
Graduate Program in Toxicology Biochemistry
Federal University of Santa Maria

**BIOCHEMICAL AND PHYSIOLOGICAL RESPONSES OF *Cucumis sativus* AND *Avena sativa* TO EXCESS ALUMINUM STRESS.**

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Place and date of the defense: Santa Maria, May 21, 2010.

Aluminum (Al) is the most abundant metal in the Earth's crust, affecting growth and development of plants. The aim of this study was to investigate the effects of Al on seedlings of cucumber (Cucumis sativus L.) and oat (Avena sativa L) through the analysis of biochemical and physiological parameters. Three different genotypes of oat seedlings were studied, namely UFRGS 930598 Al-sensitive, UFRGS 17 Al-tolerant, and 280 Al-intermediate (resulting from the crossing UFRGS 930598 and UFRGS 17), exposed to 0, 5, 10, 20, and 30 mg/L Al. The biochemical parameters analyzed for cucumber were: the activity of antioxidant enzymes (catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD)), the levels of lipid peroxidation, leakage electrolytes, protein oxidation, and chlorophyll content. The increase of electrolyte leakage and the production of hydrogen peroxide observed are related to the decreased efficiency of the antioxidant system at the highest concentrations of Al. The antioxidant system was unable to prevent the toxicity resulting in negative effects such as lipid peroxidation, protein oxidation, and decreased plant growth. Oat seedlings were exposed to Al in different growth media. The first exposure was in the midst of semi-solid growth agar for 5 days, seedlings with five days of development and then to a hydroponic solution for 7 days with 10 days of seedling development. The content of hydrogen peroxide, lipid peroxidation, ascorbic acid and non-protein thiols (NPSH), as well as the activity of the enzymes CAT, APX and SOD, and the content of Al accumulated in the seedlings were evaluated. Enzymes activities of the antioxidant SOD and CAT were increased in UFRGS 17 and UFRGS 280 genotypes. Even when these two genotypes accumulated high concentrations of Al, they did not show high levels of lipid peroxidation and contents of hydrogen peroxide when compared with the 930598 genotype. For this genotype, an activation of the enzyme APX was observed, however, high levels of Al accumulated in the plant caused an increase in the lipid peroxidation and hydrogen peroxide content. The UFRGS 930598 genotype was more sensitive to Al than the UFRGS 17 and UFRGS 280 genotypes, which confirms the previous morphological analysis obtained by FEDERIZZI et al., 2000. Although there was increased activity of APX in this genotype, the antioxidant system was not efficient in the removal of reactive oxygen species (ROS). In order to better understand these results, a growth medium with a hydroponic solution was used in which the mobility of the metal was greater for an exposure period of 7 days with oat seedlings with 10 days of development. Besides the biochemical parameters

mentioned above, the analysis of the growth of roots and shoots, fresh and dry matter, chlorophyll content, activity of the enzyme δ-aminolevulinic acid dehydratase (δ-ALA -D), and the monitoring of the pH of the hydroponic solution of each genotype were performed. The pH of the hydroponic solution of salt-tolerant genotype (UFRGS 17) showed a significant increase in pH, while the sensitive (UFRGS 930598) and intermediate (UFRGS 280) genotypes showed no significant changes. The sensitive genotype presented inhibition of root and shoot growth at the highest concentrations of Al while the tolerant and intermediate genotype showed no significant changes. The activity of antioxidant enzymes was increased after 7 days of exposure to Al in the sensitive genotype, whereas in the tolerant genotype there was no increase in this activity. In seedlings of the intermediate genotype there was an increase in the activity of CAT, APX and SOD enzymes only at higher Al concentrations (20 and 30 mg/L). Results showed that the intermediate and tolerant genotypes showed similar effects not only on the activity of enzymes of the antioxidant system, but also on the physiological parameters such as growth of roots and shoots. Even when accumulating high concentrations of Al, the intermediate genotype did not show a decrease in growth, which shows that it has resistance mechanisms related to the internal immobilization of this metal in the vacuoles. The tolerant genotype also introduces mechanisms of resistance to Al, but these mechanisms may be the exclusion of Al by binding this metal to organic acids. In order to investigate if the antioxidant system is activated in different genotypes of oat, seedlings were placed in growth medium with a hydroponic solution and withdrawn from the medium after 12, 24, and 36 h of exposure to 20 mg/L. Tolerant and intermediate genotypes showed an increase of the enzyme activity of the antioxidant system after 12 h of exposure to Al, while the sensitive genotype presented this increase only after 24 or 36 h. This difference in the rate of activation of the antioxidant system may be crucial in maintaining cellular redox homeostasis of this genotype. At this stage of development, the tolerant and the intermediate genotypes showed a stimulation of root growth while the sensitive type showed significant growth retardation. In order to compare plant species, cucumber was placed in hydroponic growth medium with the oat seedlings (sensitive and tolerant genotypes.) When cucumber was placed in hydroponic solution exposed to 20 mg/L Al along with the tolerant (UFRGS 17) and the sensitive (UFRGS 930 598) genotypes, it showed similarities with the sensitive genotype (increased catalase activity after 36 h of exposure to Al). The levels of lipid peroxidation were high after 12, 24, and 36h of exposure to Al and as a consequence there was a reduction in root growth. Considering the evaluation with tolerant (UFRGS 17) and sensitive (UFRGS 930 598) oat genotypes, the cucumber may be considered an Al-sensitive species.

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# 1. INTRODUÇÃO

A toxidez do alumínio é um fator limitante para a obtenção de altos rendimentos em muitos solos do mundo. Na América do Sul, aproximadamente 500 milhões de hectares apresentam uma extrema acidez e baixa fertilidade. No Brasil, mais de 50 % dos solos são ácidos e apresentam o alumínio tóxico que pode provocar perdas significativas no rendimento das culturas (DELHAIZE et al., 2001; MA et al., 2001).

O alumínio é o terceiro elemento mais abundante na litosfera, após o oxigênio e o silício, participando em 8% na composição da crosta terrestre. O excesso de alumínio, além de inibir a formação normal da raiz, interfere nas reações enzimáticas, na absorção, transporte e uso dos nutrientes pelas plantas. Além disso, o alumínio causa estresse oxidativo, oxidando biomoléculas como lipídeos, proteínas, pigmentos e ácidos nucléicos (YOKEL, 2001, SILVA et al., 2006).

Uma alternativa para contornar este problema é a aplicação de calcário no solo, tornando o alumínio menos disponível pela modificação do pH. Entretanto, nas áreas distantes das fontes de calcário, os gastos de correção da toxidez tornam-se elevados (ZHANG et al., 2007).

A opção mais promissora, neste caso, é a exploração do potencial genético das culturas porque as espécies diferem amplamente na tolerância ao excesso de alumínio tóxico no solo. As plantas respondem diferentemente ao alumínio, e estas respostas são caracterizadas por alterações fisiológicas oriundas de diferentes comportamentos bioquímicos (VITORELLO et al., 2005). O estudo das respostas à toxicidade do alumínio em espécies sensíveis e tolerantes vem sendo realizado em algumas espécies como o trigo desde 1925, sendo que para a cultura de aveia poucos trabalhos foram desenvolvidos (FEDERIZZI et al., 2000).

Estudos desenvolvidos no Departamento de Plantas e Lavoura da Universidade Federal do Rio Grande do Sul (UFRGS), e em outras instituições, mostraram que os genótipos de aveia dos programas de melhoramento apresentam grande variabilidade quanto à tolerância ao alumínio tóxico. No estudo da herança genética estes trabalhos revelaram que a tolerância em aveia é um caráter oligogênico (BENIN et al., 2004). Com o aumento da utilização da aveia para o consumo humano, o estudo do efeito do alumínio no crescimento e desenvolvimento dos diferentes

genótipos dessa cultura é de grande interesse para os programas de melhoramento e também para os impactos ambientais causados por este metal para as mais diversas espécies vegetais (DAVID et al., 2008).

Neste contexto, o presente estudo teve como objetivo analisar o comportamento dos diferentes genótipos de aveia (*Avena sativa*) em relação ao alumínio; analisar o comportamento dos diferentes espécies vegetais como o pepino (*Cucumis sativus*) que é um bioindicador de ambientes contaminados com metais (PEREIRA et al., 2006; CARGNELUTTI et al., 2006; GONÇALVES et al., 2009); os efeitos deste metal no crescimento das plantas, na ativação das enzimas do sistema antioxidante, e também de enzimas envolvidas no metabolismo de nutrientes como a δ–Aminolevulinato desidratase (δ–ALA-D) propondo assim possíveis relações bioquímicas e fisiológicas entre as plantas e a toxicidade causada pelo alumínio.

## 1.1 OBJETIVOS

### 1.1.1 Objetivo geral

Avaliar as respostas bioquímicas e fisiológicas de pepino e de aveia na toxicidade causada pelo alumínio.

### 1.1.2 Objetivos específicos

1) Avaliar os efeitos do alumínio no crescimento de plântulas de pepino e de plântulas dos três genótipos de aveia UFRGS 17, UFRGS 280 e UFRGS 930598 e avaliar se o estresse oxidativo causado pelo alumínio é um sinal inicial que pode desencadear inibição do crescimento da raiz nos diferentes genótipos de aveia.

2) Quantificar o conteúdo de clorofila e analisar a atividade da enzima δ–Aminolevulinato desidratase (ALA-D) de plântulas de pepino e dos três genótipos de aveia expostos ao alumínio.

3) Avaliar os níveis de peroxidação lipídica e o conteúdo de peróxido de hidrogênio induzidos por alumínio em plântulas de pepino e dos três genótipos de aveia.

4) Analisar a atividade das enzimas superóxido dismutase, ascorbato peroxidase e catalase de plântulas de pepino e aveia expostos ao alumínio e identificar e caracterizar os possíveis mecanismos de tolerância presentes nos diferentes genótipos de aveia.

5) Comparar as respostas encontradas nos genótipos de aveia com os resultados encontrados para o pepino (*Cucumis sativus),* com a finalidade de identificar se o pepino é uma espécie sensível ou tolerante ao alumínio.

# 2. REVISÃO DE LITERATURA

## 2.1 Os solos ácidos

Os solos ácidos ocorrem em aproximadamente 50% da terra em todo o planeta. A acidificação do solo devido ao uso de fertilizantes ou chuvas ácidas, causadas pela poluição industrial, são uma ameaça crescente para a agricultura e para os ecossistemas naturais (YOKEL, 2001).

Os solos ácidos apresentam pH menor ou igual a 5,5 e promovem a inibição do crescimento das plantas, especialmente devido à deficiência de nutrientes e ao estresse causado pelo alumínio (Al). O Al é um metal que compõe cerca de 8% da crosta terrestre e é o terceiro elemento mais abundante depois do oxigênio e do silício. Grande parte deste Al ocorre como óxidos inofensivos e aluminossilicatos. Entretanto, quando os solos tornam-se ácidos como um resultado de processos naturais ou atividades humanas o Al é solubilizado na forma do cátion trivalente tóxico, $Al^{3+}$(DELHAIZE et al., 2001; MORITA et al., 2007).

Este metal afeta aproximadamente 40% das terras aráveis do planeta que são potencialmente usadas para a produção de biomassa e alimentos (Ma et al., 2001). O Al trivalente, a espécie fitotóxica, inibe o crescimento da raiz e a captação de água e de nutrientes, resultando em um atraso no desenvolvimento da planta (KOCHIAN,1995; DELHAIZE et al., 2001). Considera-se a toxidez do Al um dos principais fatores limitantes da produtividade agrícola em solos ácidos (FOY et al., 1992; JEMO et al., 2005; ZHANG et al., 2007).

Ainda mais relevante é o fato de que muitas dessas áreas estão localizadas em países em desenvolvimento da América do Sul, África Central e Sudoeste da Ásia. A acidez dos solos é uma ocorrência natural em solos tropicais e subtropicais e pode resultar do desequilíbrio nos ciclos de nitrogênio, enxofre e carbono (BOLAN & HEDLEY 2003); maior captação de cátions em comparação com ânions e menor fixação de nitrogênio por leguminosas (BOLAN et al., 1991).

Os níveis de acidez do solo aumentam em decorrência das atividades humanas tais como, a liberação atmosférica de poluentes industriais associadas com a lixiviação de solos com chuvas ácidas, atividades de mineração e, no setor agrícola, a

nitrificação subseqüente à aplicação de altas doses de fertilizantes amoniacais (ZHANG, 2007; SILVA et al., 2006).

A maioria dos solos do Rio Grande do Sul e do Brasil são solos ácidos. No Brasil aproximadamente 500 milhões de hectares apresentam solos ácidos, compreendendo dois terços de seu território total – a maior área de solos ácidos dentro de um único país (VITORELLO et al., 2005). Um estudo abrangendo 26 solos de regiões brasileiras mostrou que os valores de pH da camada superficial variaram entre 3,78 e 5,52 e que o $Al^{3+}$ foi o cátion trocável predominante em mais de um terço dos solos com pH inferior a 5,6 (ABREU et al., 2003).

Desta forma as raízes das plantas estão quase sempre expostas ao Al (DELHAIZE et al., 2001). A produção de alimentos, principalmente de grãos é afetada negativamente por estar em solos ácidos; 20% da produção de milho e 13% da produção de arroz do planeta estão em solos ácidos (DELHAIZE et al., 2001). Portanto, os solos ácidos limitam a produtividade de culturas em muitos países em desenvolvimento (KOCHIAN et al., 2004).

### 2.1.1 Alumínio (Al)

O Al é o metal mais abundante e o terceiro elemento mais comum na crosta da terra. A mais rica fonte é o triidrato de alumínio ou bauxita, o minério do qual o Al é derivado. O Al também combina-se com o silício para formar o aluminossilicato, o maior constituinte das rochas, argilas e solos. Apesar de sua disponibilidade difundida, parece que este metal não é requerido para os processos biológicos. Ao mesmo tempo, várias formas químicas de Al são tóxicas para as plantas, peixes e humanos. A toxicidade do Al é o fator limitante primário das plantações em solos ácidos, que compõe aproximadamente 50% da crosta terrestre (KOCHIAN et al., 2004; VITORELLO et al., 2005).

As formas de alumínio que ocorrem na natureza são usualmente não tóxicas e estáveis. Sob certas condições, entretanto, o Al pode tornar-se solúvel, como por exemplo quando um ambiente torna-se ácido ou quando os níveis de matéria orgânica no solo são altos. Tais processos produzem uma ampla variedade de formas solúveis

de Al inorgânico (monômeros tais como, $Al^{3+}$, $Al(OH)_2^+$) e formas orgânicas de Al (KOCHIAN, 1995).

Portanto, a medida que os solos se acidificam, íons Al passam a ocupar as posições de troca catiônica, em superfícies eletronegativas dos colóides, em substituição aos cátions removidos pela lixiviação, onde concentrações de espécies de Al podem alcançar níveis tóxicos para os organismos (ZHANG, 2003).

O interesse em estudar o Al tem aumentado a cada ano devido aos efeitos prejudiciais do Al sobre o ambiente e a saúde humana (KOCHIAN, 1995). O Al entra em contato com o corpo humano através da comida, ar, água, e drogas (YOKEL, 2001). Embora somente uma pequena porção de alumínio seja absorvida pelo trato gastrintestinal, a ingestão oral representa a via de entrada no organismo com maiores implicações toxicológicas (TESTOLIN et al., 1996).

Em plantas, o Al é absorvido pela raiz e o principal sintoma de toxidez é a inibição do crescimento, que parece ser causada por vários mecanismos, incluindo lesões apoplásticas, interações na parede celular, membrana plasmática ou no simplasma da raiz (DIPIERRO, 2005). A inibição do crescimento da raiz resulta em um sistema radicular reduzido que pode levar a deficiência mineral e ao estresse hídrico (KOCHIAN, 1995).

A toxidez causada pelo Al aumenta o rompimento dos dictiossomos e sua função secretória, a vacuolização e o *turnover* de grãos de amido na ponta e nas células meristemáticas da raiz (TAMÁS et al., 2006). A extensão da inibição do crescimento da raiz é comumente usada como uma medida de toxicidade do Al (KOCHIAN, 1995). Entretanto, o crescimento da raiz é um processo complexo e dinâmico. Provavelmente, vários processos bioquímicos e fisiológicos podem já ter sido alterados antes da inibição do crescimento da raiz induzida por Al (ZHANG, 2003). A literatura é rica em relatos mostrando que numerosos processos bioquímicos e fisiológicos são afetados em várias espécies dentro de minutos ou horas após a exposição ao Al, entre eles o estresse oxidativo (YAMAMOTO et al., 2002). Uma característica comum a vários tipos de estresse é a produção de muitas moléculas do radical superóxido, ($O_2^{\cdot -}$), radical hidroxil ($^{\cdot}OH$), e peróxido de hidrogênio ($H_2O_2$) (CHAFFAI, 2005). Essas espécies reativas de oxigênio (EROs) altamente citotóxicas podem causar dano oxidativo a biomoléculas tais como os lipídeos, as proteínas, os

pigmentos e os ácidos nucléicos, levando à peroxidação de lipídeos de membrana, perda de íons, hidrólise de proteínas e até mesmo dano ao DNA (ZHANG et al., 2007). Estudos de toxicidade com o Al em raízes sugerem que a produção de EROs podem contribuir para a inibição induzida por Al do alongamento da raiz (YAMAMOTO et al., 2003).

### 2.2 Estresse oxidativo

O estresse oxidativo corresponde a um estado em que há uma elevada produção de EROs, onde os mecanismos celulares pró-oxidantes superam os antioxidantes. A terminologia espécies reativas de oxigênio (EROs ou ROS: "reactive oxygen species") inclui as espécies chamadas de radicais livres e outras que, embora não possuam elétrons desemparelhados, são muito reativas em decorrência de sua instabilidade (MARRONI, 2002).

O oxigênio molecular ($O_2$) é relativamente não reativo e não tóxico, devido a estrutura estável de elétrons na sua camada externa. Ele é necessário para a sobrevivência de todos os organismos aeróbicos. Assim, a obtenção de energia por estes organismos é feita na mitocôndria através da fosforilação oxidativa onde o $O_2$ é reduzido por quatro elétrons à $H_2O$. Entretanto, alterações na distribuição dos elétrons podem provocar a sua ativação e influenciar os sistemas biológicos. As EROs podem ser geradas dentro das plantas como resultado da excitação do elétron externo, formando oxigênio singlet ($^1O_2$) ou de uma sucessiva adição de elétrons ao oxigênio molecular produzindo $O_2^{\cdot-}$, $^{\cdot}OH$, e $H_2O_2$.. Essas moléculas são ditas reativas porque não necessitam da entrada de energia para reagir com outras moléculas (RESENDE et al., 2003). Abaixo serão descritas espécies reativas de oxigênio que podem ser formadas como resultado da toxicidade causada pelo alumínio:

**Radical Superóxido ($O_2^{\cdot-}$):** pode ser produzido na planta por meio de vários mecanismos, inclusive pela ativação de NADPH-oxidases/sintases ligadas à membrana, peroxidases da parede celular, lipoxigenases e como resultado da transferência de elétrons da mitocôndria ou do cloroplasto. Normalmente, oxida várias moléculas orgânicas, como o ascorbato, ou como redutor de metais como $Fe^{3+}$, nas reações de Haber-Weiss ou Fenton (BREUSEGEM et al., 2001).

**Peróxido de hidrogênio ($H_2O_2$):** a maioria do $H_2O_2$ celular surge da dismutação do $O_2^{\circ-}$ catalisada pela enzima superoxido dismutase. Peróxido de hidrogênio é um oxidante relativamente estável e ausente de carga, o que pode facilitar a passagem através da camada lipídica da membrana celular. Essa capacidade de difundir-se rapidamente através da membrana celular favorece a rápida elicitação da resposta vegetal (MITTLER, 2002; CHAFFAI et al., 2005).

**Radical hidroxila ($^{\circ}OH$):** embora os radicais hidroxila tenham meia-vida curta, são radicais potencialmente fortes e com alta afinidade à biomoléculas no seu sítio de produção (HALLIWELL & GUTTERIDGE, 1989; POSCHENRIEDER et al., 2008), o que dificulta o estudo desses radicais.

**Oxigênio singlet ($^1O_2$):** semelhante aos radicais hidroxila, os $^1O_2$ tem meia-vida curta e são altamente destrutivos, reagindo com a maioria das moléculas biológicas (MITTLER, 2002; GIANNAKOULA et al., 2007), porém a maioria dos danos são próximos aos sítios onde são produzidos. São predominantemente gerados nos cloroplastos, através da transferência de energia de uma clorofila foto-excitada para o elétron do oxigênio molecular. O $^1O_2$ reage facilmente com ligações duplas e têm alta afinidade com "dienos" na membrana e aminoácidos específicos como histidina, metionina, triptofano e cisteína. Sob condições fisiológicas normais, a produção de EROs nas células é baixa e se mantém constante nos cloroplastos. Quando produzidas em excesso elas tornam-se danosas à célula vegetal (MITTLER, 2002).

As EROs também tem a função de sinalizadoras nas células vegetais, e requerem no mínimo dois mecanismos diferentes para regular as concentrações intracelulares destas espécies reativas. Um mecanismo permite a fina modulação de baixos níveis de EROs com o propósito de sinalização e o outro mecanismo permite a remoção do excesso de EROs, especialmente durante o estresse. Os processos oxidativos normais da célula são responsáveis pela geração destas espécies reativas. Durante o transporte de elétrons nos cloroplastos e mitocôndrias, muitos desses elétrons são perdidos, e então captados pelo $O_2$ formando as EROs (DELHAIZE et al., 2009). Fatores abióticos tais como extremos de temperatura, poluentes, estresse osmótico e mecânico, podem aumentar a produção de EROs. Contudo, os elicitores

mais efetivos são macromoléculas derivadas de patógenos e a toxicidade causada por metais (POSCHENRIEDER et al., 2008).

Estudos de toxicidade de Al em raízes sugerem que a produção de EROs pode contribuir para a inibição induzida por Al do alongamento da raiz (YAMAMOTO et al., 2003; MORITA et al., 2007; DELHAIZE et al., 2009).

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### 2.2.1 Enzimas antioxidantes

Como o acúmulo das EROs pode resultar em prejuízos consideráveis, a célula dispõe de vários mecanismos para detoxificar eficientemente essas EROs. Esses mecanismos de proteção foram desenvolvidos pelas plantas durante o processo de evolução, para controlar os níveis dessas moléculas e anular essa toxicidade. Moléculas antioxidantes, enzimas simples e um sistema mais complexo de destoxificação podem estar envolvidos na proteção celular contra as EROs. Conhecida como “scavengers”, várias enzimas reguladoras impedem a ação tóxica das EROs à célula vegetal (BOWLER, 1992; MITLER, 2002). Abaixo serão descritas algumas enzimas antioxidantes presentes nas células:

**Catalase:** são enzimas que convertem o $H_2O_2$ em $H_2O$ e $O_2$ e estão presentes nos peroxissomos e glioxissomas. As catalases são as principais enzimas de detoxificação do $H_2O_2$ em plantas e podem ser divididas em três classes: catalases da classe I que removem o $H_2O_2$ produzido durante a fotorrespiração em tecidos fotossintéticos; catalases da classe II que são produzidas em tecidos vasculares e podem exercer uma função de lignificação, mas sua exata função biológica permanece desconhecida e na classe III estão as catalases presentes abundantemente em sementes e plantas jovens, cuja atividade está relacionada à remoção do $H_2O_2$ produzido durante a degradação dos ácidos graxos no glioxissoma (BREUSEGEM et al. 2001, RESENDE et al., 2003).

**Ciclo do ascorbato/glutationa:** principal sistema de remoção de EROs nos cloroplastos. Nesse ciclo são empregadas quatro enzimas: ascorbato peroxidase (APX), deidroascorbato redutase (DHA), monodeidroascorbato redutase (MDA) e glutationa redutase. O $H_2O_2$ pode ser reduzido e removido pela APX através do ascorbato como

redutor, formando o radical monodeidroascorbato (MDA°) (YOSHIMURA et al., 2000). Esse por sua vez irá dismutar para deidroascorbato (DHA) e ascorbato (MILTTLER 2002; RESENDE et al., 2003).

**Superóxido dismutase:** A SOD participa ativamente na remoção do $O_2^{\circ-}$ gerado através de diferentes processos do metabolismo celular, tais como o transporte de elétrons na mitocôndria e cloroplasto (MITTLER, 2002). O Zn se encontra associado com o cobre (Cu) na isoenzima superóxido dismutase dependente de Cu e Zn (Cu/Zn SOD). A Cu/Zn SOD está localizada nos cloroplastos, citosol e possivelmente no espaço extracelular. Muito provavelmente o átomo de Cu é o componente catalítico e o Zn é o componente estrutural da Cu/Zn SOD. Contudo, na deficiência do Zn, a atividade da Cu/Zn SOD é drasticamente reduzida, mas pode ser restabelecida i*n vitro* pelo suprimento de Zn (INZÉ & MONTAGU, 1995). Portanto, o átomo de Zn é um componente estrutural essencial para o funcionamento normal da Cu/Zn SOD. A redução na atividade da SOD ocorre com um aumento simultâneo do acúmulo de $O_2^{\circ-}$. O radical superóxido é um dos principais oxidantes responsáveis pela peroxidação de lipídeos e conseqüente aumento na permeabilidade das membranas (INZÉ & MONTAGU , 1995).

### 2.3 A enzima delta - aminolevulinato desidratase (δ-ALA-D)

A enzima citoplasmática delta-aminolevulinato desidratase (E.C. 4.2.1.24), também conhecida como porfobilinogênio sintetase (PBGS) foi isolada na década de 50 a partir de fígado bovino (GIBSON et al., 1955). Esta enzima catalisa a condensação assimétrica de duas moléculas de ácido delta-aminolevulínico (ácido 5-aminolevulínico-ALA), com perda de duas moléculas de água, formando o composto monopirrólico porfobilinogênio - PBG (JAFFE, 1995).

As seqüências do gene da δ-ALA-D isolada de diversas fontes tais como humanos, bactérias, camundongos e vegetais apresentam grande similaridade (WETMUR et al., 1986; ECHELARD et al., 1988; BISHOP et al., 1989; BOESE et al.,

1991), sugerindo que a enzima possui estrutura e mecanismo de ação semelhante em diferentes organismos.

No mecanismo proposto para a síntese do porfobilinogênio, um resíduo de lisina do sítio ativo da enzima forma uma base de Schiff com a primeira molécula do substrato (ALA), originando a cadeia lateral P (cadeia propiônica), enquanto uma segunda molécula de ALA origina a cadeia lateral A (acética) do porfobilinogênio (CASTELFRANCO & BATLE, 1983). As duas moléculas de substrato interagem no sítio ativo da enzima, aparentemente, através da formação de uma segunda base de Schiff (ligação carbono-nitrogênio) (JAFFE et al., 1995). As reações enzimáticas do ácido aminolevulínico (ALA) até a protoporfirina IX são comuns para a biossíntese de tetrapirróis em todos os organismos e este caminho leva a biossíntese de cofatores biologicamente importantes, tais como, heme, clorofilas e vitamina B12 (SHOOLINGIN-JORDAN, 1998).

NAITO et al., (1980) sugeriram que a biossíntese de clorofila pode ser regulada pela atividade da ALA-D. Diminuída atividade da ALA-D concomitante com reduzido conteúdo de clorofila tem sido relatado em muitas plantas terrestres expostas a várias concentrações de chumbo, cádmio e mercúrio (STOBART et al., 1985). Tendo em vista a importância da enzima delta-aminolevulinato desidratase em plantas e com a finalidade de melhor entender os mecanismos de toxicidade do alumínio denota-se a importância dos estudos do efeito do alumínio na atividade da enzima ALA-D.

### 2.4 Desenvolvimento Vegetal e Estratégias de Resistência ao Alumínio

Nas plantas, o crescimento consiste na conversão de substâncias inorgânicas relativamente simples (água, $CO_2$ e elementos minerais) em quantidades cada vez maiores de proteínas e carboidratos (e também gorduras). A embriogênese inicia o desenvolvimento da planta, mas diferentemente dos animais, é um processo contínuo, estabelecendo o plano básico do corpo vegetal e formando os meristemas que geram os órgãos adicionais no adulto. A maior parte do desenvolvimento vegetal é pós-embrionária e ocorre a partir dos meristemas, os quais podem ser considerados fábricas celulares onde os processos de divisão celular, expansão e diferenciação geram o corpo vegetal. Os meristemas vegetativos são altamente repetitivos produzindo

a mesma ou similar estrutura reiteradas vezes, podendo sua atividade continuar indefinidamente, fenômeno conhecido como crescimento indefinido (TAIZ & ZEIGER, 2004).

O crescimento primário ocorre relativamente próximo às extremidades das raízes e caules. Ele é iniciado pelos meristemas apicais e está primariamente envolvido com a extensão do corpo da planta – o crescimento vertical. Além do crescimento primário, muitas plantas passam por um crescimento adicional que aumenta em espessura o caule e a raiz, tal crescimento é denominado crescimento secundário (RAVEN et al., 2001).

Os estímulos do meio ambiente causam mudanças drásticas no desenvolvimento das plantas. Em contraste, as flutuações ambientais pouco afetam o curso e a maneira do desenvolvimento em animais superiores, a não ser seus ciclos sexuais e seus hábitos de dormência. A razão plausível dessa diferença é que os animais superiores passam apenas uma vez pelo crescimento formativo, não apresentando crescimento progressivo e indefinido; eles não formam uma multiplicidade de centros de crescimento (que são responsáveis pelo crescimento progressivo em vegetais), que a qualquer momento podem reproduzir o comportamento do organismo todo. O crescimento de uma planta pode ser medido de várias maneiras: comprimento total do sistema radicular, comprimento da parte aérea, peso fresco e peso seco total. O fundamento da análise do crescimento é a medida seqüencial do acúmulo de matéria orgânica e a sua determinação é feita considerando o peso seco da planta. A fim de que o crescimento total da planta possa ser estimado, as raízes devem ser consideradas como importantes componentes do vegetal, já que o desenvolvimento da raiz refletirá no desenvolvimento de toda planta (FERRI, 1985; TAIZ & ZEIGER., 2004).

As plantas podem ser classificadas conforme sua sensibilidade ou resistência a toxidez do alumínio. As plantas com baixa resistência ao alumínio são capazes de crescer somente em solos com baixos níveis deste elemento, entretanto plantas com moderada ou alta resistência podem sobreviver em altas concentrações de alumínio (OSAKI et al., 1997). Algumas plantas apresentam mecanismos para tolerar o estresse ao alumínio permitindo que elas cresçam em solos ácidos. Muitas secretam ácidos orgânicos pelas raízes em resposta ao tratamento com alumínio. O citrato, o oxalato e o malato são alguns dos ácidos orgânicos que podem formar fortes complexos com o

$Al^{3+}$. Ao ser absorvido o $Al^{3+}$ é quelado internamente pelo oxalato, formando um complexo (Al-oxalato) que é levado até o xilema, onde o alumínio passa a se ligar ao citrato. Ao chegar na folha, o alumínio é armazenado no vacúolo e o oxalato voltará a se complexar ao $Al^{3+}$ impedindo que este elememto fique livre para se ligar a moléculas biológicas (DELHAIZE et al., 2001). As espécies sensíveis ao alumínio apresentam de forma mais evidente os efeitos da toxicidade do alumínio, tais como a inibição do desenvolvimento e do crescimento do sistema radicular (PUTHOTA et al., 1991).

## 2.5 Mecanismos externos de resistência

### 2.5.1 Ligação ou fixação do alumínio na parede celular

Muitas plantas que crescem em ambientes ácidos não acumulam alumínio em suas folhas e a maioria desse alumínio permanece na parede celular da raiz. Sugere-se que este seja o maior sítio de acúmulo do alumínio e que esta ligação limita o movimento do alumínio no simplasma (YAMAMOTO et al., 2003). O alumínio liga-se principalmente à celulose que é um componente da parede celular, mas as interações precisas do alumínio com os constituintes da parede celular permanecem pouco exploradas (JANSEN et al., 2002). A maioria do alumínio está localizada na epiderme e nas células do córtex periférico da raiz e a penetração do alumínio no cilindro central parece ser prevenida. YAMAMOTO et al. (2003) sugeriram que plantas com uma alta capacidade de troca de cátions na raiz são geralmente mais sensíveis ao alumínio que as plantas que apresentam uma baixa capacidade de troca de cátions. O transporte de alumínio para a parte aérea é feito via xilema, e as paredes do xilema tem uma alta capacidade de troca de cátions o que causa um retardo no movimento dos cátions de alumínio (DELHAIZE et al., 2009).

### 2.5.2 Exudação de ligantes que quelam o alumínio

MA et al. (2001) sugeriram que ácidos orgânicos com alta capacidade de se ligar ao alumínio desempenham uma importante função na detoxificação do alumínio. Estudos tem demonstrado que formas queladas de alumínio são menos tóxicas para o crescimento da planta do que as formas iônicas. DELHAIZE et al. (1993) sugeriram que

o malato liberado protege o ápice da raiz (o sítio de maior toxicidade do alumínio). Vários outros estudos mostram que a resistência ao alumínio está relacionada à excreção de ligantes que quelam o alumínio (ácidos orgânicos) no ápice da raiz como citrato em *Cassia tora* e soja (*Glycine Max)* (YANG et al., 2000), citrato e malato em mutantes de *Arabdopsis thaliana* (LARSEN at al., 1998) e oxalato em *Fagopyrum esculentum* (MARSCHNER, 1995). A análise do xilema em plantas acumuladoras mostra o envolvimento de ácidos orgânicos no transporte do alumínio (DELHAIZE et al., 2001) (Fig. 1).

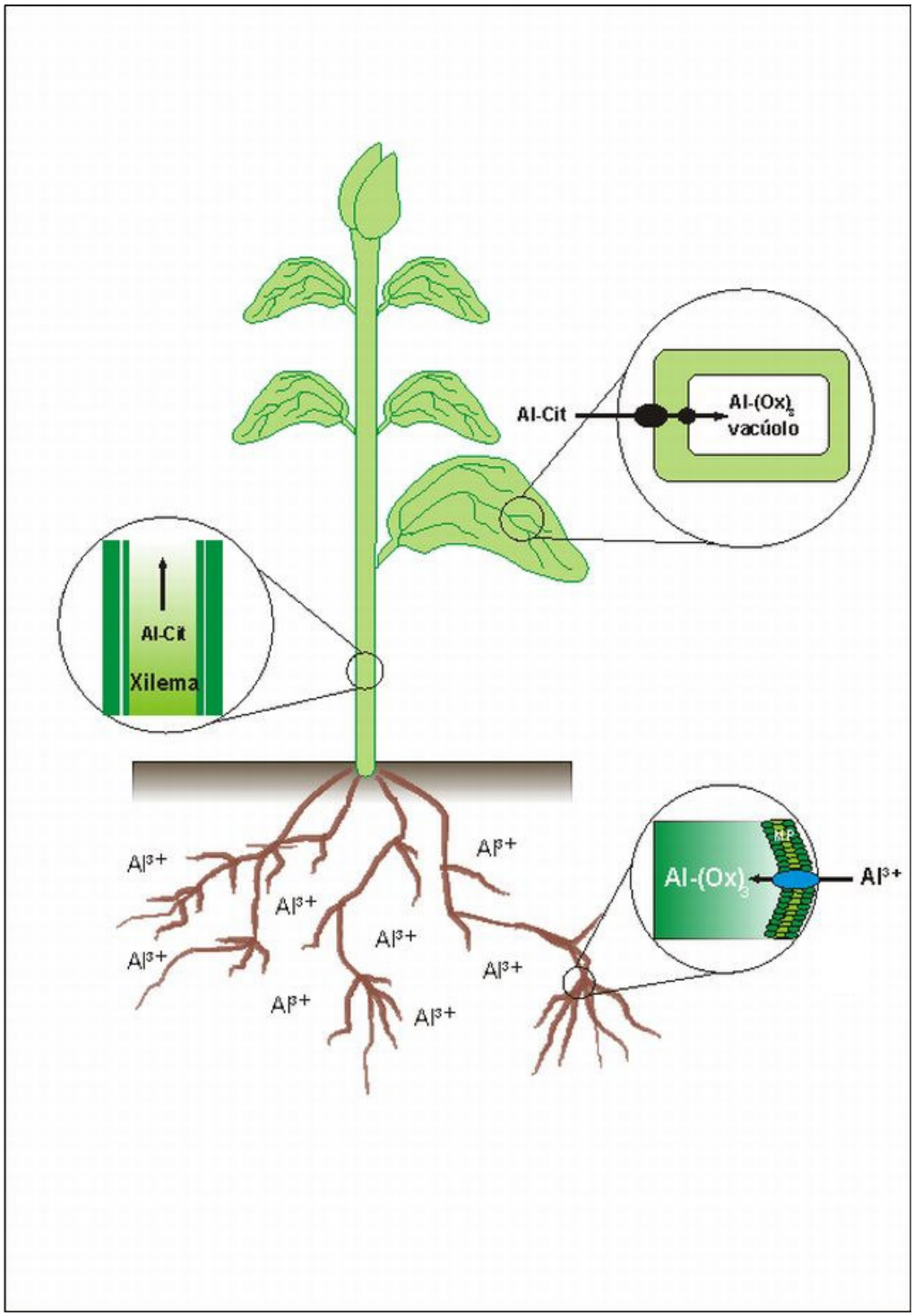


Figura 1. Desenho esquemático dos mecanismos de tolerância ao alumínio. Adaptado de Delhaize et al. (2003).

### 2.5.3 Permeabilidade seletiva da membrana plasmática ao Al

As plantas que acumulam alumínio mostram uma alta concentração deste metal na parte aérea sem efeitos prejudiciais, o que sugere que o alumínio seja transportado através da membrana plasmática pelo simplasma (SILVA et al., 2006).

A principal diferença entre plantas que acumulam alumínio e as que excluem pode ser a permeabilidade das células da endoderme. A endoderme de plantas não acumuladoras limita a entrada do alumínio para o cilindro central, entretanto a endoderme de plantas acumuladoras não limita a entrada do metal. Testes histoquímicos realizados em plantas não acumuladoras mostraram a ausência do alumínio nas células endodérmicas (JANSEN et al., 2002).

## 2.6 Mecanismos Internos de Resistência ao Al

A seguinte hipótese é sugerida para a detoxificação interna de alumínio após sua entrada no citoplasma: formação de agregados de alumínio com ácido orgânico, proteínas ou outros ligantes e compartimentalização do alumínio no vacúolo (KOCHIAN, 1995; MA et al., 2001).

Os ácidos orgânicos são importantes para a detoxificação interna do alumínio porque eles se ligam ao alumínio e impedem que este metal se ligue a outras biomoléculas (MA et al., 2001; DELHAIZE et al., 2001). O alumínio tem uma forte afinidade por compostos doadores de oxigêno como o fosfato inorgânico, ATP, RNA, DNA, proteínas, ácidos carboxílicos e fosfolipídeos. Os mecanismos de detoxificação interna são pré-requisitos para a tolerância ao alumínio em plantas que acumulam este metal. Em *Camellia sinensis* a maioria do alumínio está ligado nas catequinas, compostos fenólicos e ácidos orgânicos. Em folhas de *Hydrangea,* ele está ligado ao ácido cítrico (MORITA et al., 2007). Em *Fagopyrum esculentum* e *Melastoma malabathricum* o alumínio está ligado ao ácido oxálico. Todas estas espécies crescem normalmente e apresentam altas concentrações de alumínio em suas folhas (JANSEN et al., 2002).

### 2.6.1 Mecanismos genéticos de tolerância ao alumínio

A genética da resistência ao alumínio tem sido estudada em várias espécies agrícolas importantes. A tolerância ao alumínio é controlada por um gene em *Hordeum vulgare* e *Triticum* (DELHAIZE et al., 2009) enquanto parece ser controlada por vários genes em *Secale cereale* e outras espécies como *Glycine max* (LARSEN et al., 1998). Outros pesquisadores mostraram que a resistência ao alumínio é um traço que pode ser controlado por um ou mais genes e vários genes menores (POSCHENRIEDER et al., 2008). Em *Arabidopsis thaliana* a resistência ao alumínio é controlada por dois diferentes genes (LARSEN, 1998; EZAKI et al., 2000). Além disso, verificou-se que esses dois genes controlam dois mecanismos distintos: liberação de ácidos orgânicos que se ligam ao alumínio e previnem sua captação pela raiz e o aumento do pH ao redor do ápice da raiz que diminui a concentração de alumínio tóxico. Portanto, é improvável que a resistência ao alumínio seja o resultado de um único mecanismo em todas as espécies vegetais (KOCHIAN, 1995; POSCHENRIEDER et al., 2008; DELHAIZE et al., 2009).

### 2.6.2 Acúmulo do alumínio em angiospermas

Plantas acumuladoras de alumínio ou “aluminum hyperaccumulators” é o nome dado as plantas que acumulam concentrações acima ou iguais a 1000 mg $Kg^{-1}$ (JANSEN et al., 2004). Existem aproximadamente 55 famílias de angiospermas que apresentam espécies acumuladoras de alumínio, sendo que 93% de todos os acumuladores de alumínio que estão descritos pertencem às subclasses Rosidae e Asteridae. Dentro destes grupos os acumuladores de alumínio estão principalmente entre as ordens Myrtales e Gentianales que contam com aproximadamente 42,5% e 35% respectivamente do total de acumuladores conhecidos (Fig. 2). Exemplo de famílias que incluem espécies acumuladoras de alumínio: família Hydrangeaceae, Melatomataceae, Rubiaceae, Theaceae, Symplocaceae e Vochysiaceae. A espécie *Camellia sinensis* da família Theaceae tem grande importância comercial, sendo muito utilizada como chá (chá da Índia). Outra espécie frequentemente citada é a *Hydrangea macrophylla* (conhecida popularmente como hortênsia). A coloração azul ou cor de rosa

das pétalas nas inflorescências da hortência depende da concentração de alumínio na parte aérea das plantas (JANSEN et al., 2002). Nas monocotiledôneas existem espécies que acumulam alumínio principalmente nas famílias Liliaceae e Poaceae (Gramíneas).

A ocorrência do acúmulo do alumínio nestas famílias pode ilustrar a conexão próxima desta característica com a taxonomia. Estudos filogenéticos recentes baseados na sequência molecular procuram desvendar de maneira mais minunciosa a tendência evolucionária de caracteres fitoquímicos (SOLTIS et al., 2000).

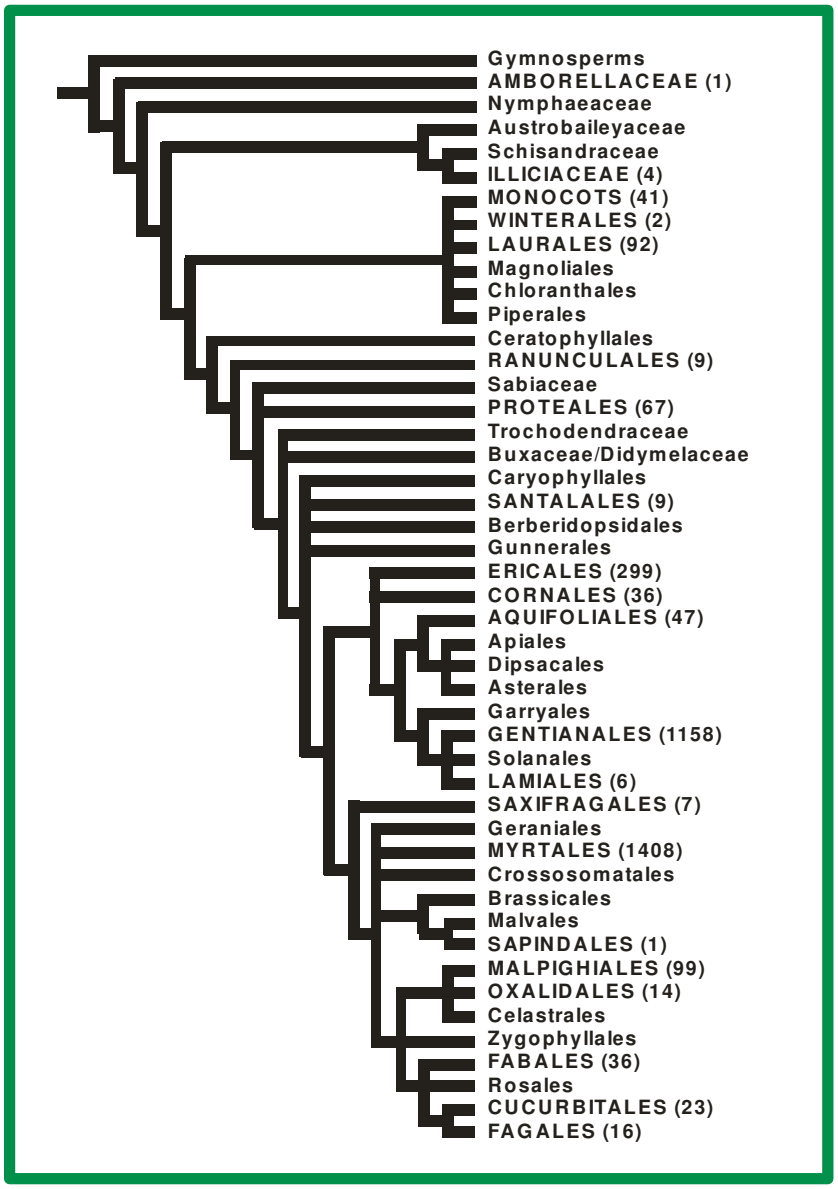


Figure 2. Acúmulo do alumínio em Angiospermas. Dentro do parêntese está o número de espécies conhecidas que acumulam alumínio. (Fonte: **Jansen et al., 2004).**

Entre as dicotiledôneas que apresentam sensibilidade à ambientes contaminados com metais, podemos citar o pepino (*Cucumis sativus*) pertencente a ordem Cucurbitales, que apresenta 23 espécies que acumulam alumínio. A aveia (*Avena sativa*) está entre as monocotiledôneas, que apresentam 41 espécies acumuladoras de alumínio (JANSEN et al., 2004).

### 2.7 Pepino (*Cucumis sativus*)

O pepino é originário das regiões montanhosas da Índia e apropriado para o plantio em regiões tropicais e temperadas. Tem sido cultivado desde a Antiguidade na Ásia, África e Europa. Foi trazido para a América por Cristovão Colombo. A espécie apresenta grande variação, entre os inúmeros cultivares, quanto ao tamanho, forma, cor dos frutos, sabor e características vegetativas. Os pepineiros são plantas trepadeiras, anuais, com folhas lobadas e flor amarela. Os frutos são longos, com casca verde clara com estrias e manchas escuras, a polpa é de cor clara, com sementes achatadas semelhantes as do melão, que é outro membro da família Cucurbitaceae. Embora não tenha grande importância econômica, é utilizado normalmente para alimentação em forma de saladas. A utilização desta espécie em estudos científicos tem aumentado nas últimas décadas devido a rápida germinação das sementes em laboratório (KANG et al., 2002; ZHU et al., 2004) e também por ser um indicador de ambientes contaminados por metais como cádmio, mercúrio e alumínio (CARGNELUTTI et al., 2006; PEREIRA et al., 2006; GONÇALVEZ et al., 2009).

## 2.8 Aveia (*Avena sativa*)

A aveia é um cereal do gênero *Avena*, da família Poaceae (Gramíneas). Foi primeiramente cultivada no norte da Europa. A produção mundial de aveia se mantém em torno de 50 toneladas por ano, o que representa 5 a 7% da produção mundial de grãos. A maior parte da produção, aproximadamente 75% é representada por *Avena sativa* (aveia branca). No Brasil a aveia é cultivada em oito estados: Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Mato Grosso do Sul, Minas Gerais, Rio de Janeiro e Espírito Santo, locais onde a temperatura (20 a 25º C) favorece o seu desenvolvimento vegetativo (WAGNER et al., 2001).

Existem múltiplas possibilidades de utilização da aveia, tais como produção de grãos (alimentação humana e animal), forragem, cobertura de solo, adubação verde e para inibir as infestações de plantas invasoras. Na alimentação humana, a aveia representa um alimento multifuncional, superior em muitos aspectos em comparação com outros tipos de grãos de cereais. Atualmente, mais aveia está sendo moída para consumo humano, tornado-se necessário desenvolver cultivares que contenham nutrientes nas proporções que são consistentes com baixo teor de gordura e alto teor de fibra (FLOSS et al., 1997).

A variabilidade da tolerância ao Al é controlada geneticamente e os mecanismos da herança são diferentes entre espécies e cultivares. Embora a base genética ainda não esteja determinada em aveia, a variabilidade fenotípica observada entre espécies e entre genótipos indica a possibilidade de progresso no melhoramento de aveia para tolerância ao Al tóxico (FLOSS et al., 1997). Trabalhos desenvolvidos no Departamento de Plantas e Lavoura da Universidade Federal do Rio Grande do Sul (UFRGS), e em outras instituições mostraram que os genótipos de aveia apresentaram grande variabilidade quanto à tolerância ao alumínio tóxico. FEDERIZZI et al. (2000) concluíram que o caráter tolerância à toxidez de alumínio é uma característica herdável, controlada por um gene e a ação gênica observada foi a dominância. Avaliando um número maior de cruzamentos WAGNER e colaboradores (2001) obtiveram resultados indicando a presença de um a dois genes dominantes envolvidos na tolerância ao alumínio. A presença dos alelos $A_1$ e $A_2$, levou um maior grau de tolerância nos genótipos estudados. UFRGS 17 foi identificado como um genótipo tolerante, UFRGS

930598 como sensível e UFRGS 280 originário do cruzamento de UFRGS 17 e UFRGS 930598, identificado como intermediário ao alumínio (FEDERIZZI et al., 2000).

## 3. RESULTADOS E DISCUSSÃO

### 3.1 MANUSCRITOS

Os resultados que fazem parte desta Tese estão apresentados sob a forma de um artigo e de quatro manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos próprios manuscritos.

**Artigo I**: Aluminum-induced oxidative stress in cucumber – aceito para publicação na revista Plant Physiology and Biochemistry.

**Manuscrito I**: Differential responses of oat genotypes: oxidative stress induced by aluminum.

**Manuscrito II**: Physiological and oxidative stress responses of three oat genotypes to aluminum grown in hydropony.

**Manuscrito III**: Differential speed of activation in antioxidant system in three genotypes of oat.

**Manuscrito IV:** Physiological and oxidative stress responses of two *Avena sativa* genotypes and *Cucumis sativus* seedlings to aluminum in nutrient solution.

**Artigo I -** ALUMINUM-INDUCED OXIDATIVE STRESS IN CUCUMBER

Plant Physiology and Biochemistry

Running Title: **CUCUMBER SEEDLINGS AND Al EXPOSURE**

Full title: **ALUMINUM-INDUCED OXIDATIVE STRESS IN CUCUMBER**

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**ABSTRACT**

Aluminum (Al) is one of the most abundant elements of the planet and exposure to this metal can cause oxidative stress and lead to various signs of toxicity in plants. Plants are essential organisms for the environment as well as food for humans and animals. The toxic effect of aluminum is the major cause of decreased crop productivity in soils. Thus, the objective of the present study was to analyze the effects of aluminum on the activity of antioxidant enzymes such as catalase (CAT - E.C. 1.11.1.6), superoxide dismutase (SOD - E.C.1.15.1.1) and ascorbate peroxidase (APX - E.C. 1.11.1.11), and on lipid peroxidation, electrolyte leakage percentage (ELP) and chlorophyll and protein oxidation levels in *Cucumis sativus* L (cv. Aodai). Seedlings were grown at different concentrations of aluminum ranging from 1 to 2000 μM for 10 days. The increase in ELP and $H_2O_2$ production observed in the seedlings may be related to the decreased efficiency of the antioxidant system at higher aluminum concentrations. The antioxidant system was unable to overcome toxicity resulting in negative effects such as lipid peroxidation, protein oxidation and a decrease in the growth of *Cucumis* seedlings. Aluminum toxicity triggered alterations in the antioxidant and physiological status of growing cucumber seedlings.

**Keywords:** Aluminum, Ascorbate peroxidase, Catalase, *Cucumis sativus,* Hydrogen peroxide, Superoxide dismutase

**Abbreviations**

APX, ascorbate peroxidase; CAT, catalase; DMSO, dimethylsulphoxide; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetracetic acid; ELP, electrolyte leakage percentage; MDA, malondialdehyde; NPSH, non-protein thiol groups; PBG, porphobilinogen; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; δ-ALA, aminolevulinic acid; δ-ALA-D, δ-aminolevulinate dehydratase; TCA, trichloroacetic acid; TBA, thiobarbituric acid; WC, water content.

## 1. Introduction

Various kinds of environmental stress induce the formation of reactive oxygen species (ROS) in plant cells [1]. Sources of environmental stress include changes in temperature, mechanical shock, UV light, exposure to the ozone, water deficiency, and an excess of metallic ions. Under normal physiological conditions, cells produce ROS by reducing molecular oxygen [2]. However, under environmental stress conditions this production is increased.

Although Al itself is not a transition metal and cannot catalyze redox reactions, the involvement of oxidative stress in Al toxicity has been suggested [2]. Al is a major constituent of soil and, consequently, plants grow in soil environments in which the roots are potentially exposed to high concentrations of aluminum [3]. Al toxicity is one of the major factors limiting crop production on acid soils and, since a large part of the world's total land area consists of acid soil, much attention has been given to Al toxicity [4, 5 and 6].

The first evident symptom of Al toxicity is the inhibition of root growth, which occurs after very brief exposure of roots to Al [1, 3]. Exposure to Al was found to enhance oxidative stress and was a decisive event in the inhibition of cell growth [1]. Cakmak and Horst [7] first reported the relation between ROS and the enhancement of lipid peroxidation and small increases in activities of enzymes such as superoxide dismutase (SOD) and peroxidases caused by Al in root tips of soybean (*Glycine max*), suggesting a generation of ROS. Although many studies have focused on this aspect of toxicity and various mechanisms of action have been proposed, the causes of Al toxicity are still poorly understood.

All cells possess a defense system, consisting of various enzymes such as catalase (CAT - E.C. 1.11.1.6), ascorbate peroxidase (APX - E.C. 1.11.1.11), and superoxide dismutase (SOD - E.C.1.15.1.1). These enzymes reduce ROS under normal conditions, but if complete reduction

does not occur, the result may be a state of oxidative stress leading to the oxidation of biomolecules (lipids, proteins and DNA) [2, 9] or even cell death [10]. *Cucumis sativus* is an environmental bioindicator of ecosystems polluted with metals such as mercury [10], Al [11]. Studies realized in our laboratory have shown that the root system growth is reduced in the presence of Al [11].

The present study describes mainly physiological aspects of Al toxicity focusing on oxidative stress in *Cucumis sativus* seedlings and examines whether Al toxicology affects their growth and development.

## 2. Material and methods

### 2.1 Plant material and growth conditions

*Cucumis sativus* L. seeds (cv. Aodai), commonly known as cucumber, provided by Feltrin Ltd. (Santa Maria, RS) were germinated in glass recipients (100 mL) containing 15 mL of medium with $Al_2(SO_4)_3$ diluted in a 0.5 % agar solution. No nutritive solution was added to the agar. The seedlings made use of the seed nutrition in the initial stage of development, and in a previous experiment, it was verified that up to the tenth day the plants did not suffer any nutrient deficiency (data not shown). Seven different $Al_2(SO_4)_3$ treatments (0, 1, 10, 100, 500, 1000 and 2000 μM) were applied randomly. The medium pH was adjusted to 4.0 by tritation with HCL solutions of 0.1 M and monitored daily. This pH maintained constant throughout the experiment. Each experimental unit consisted of 6 seeds, totalizing 15 replicates per treatment. After germination, the seedlings were maintained in a growth chamber with controlled temperature ($25\pm1^0$C) and photoperiod (16 h light; light intensity of 35 μmol $m^{-2}$ $s^{-1}$ at plant level).

2.2 Metal determination

Al content was determined in the roots and shoot of 10-day-old cucumber seedlings. Approximately 50 mg of roots and shoots were digested with 4 mL $HNO_3$ utilizing the following stages of heating: a) 50 $^0$C for 1 h; b) 80 $^0$C for 1 h; and 120$^0$C for 1 h in a digester block (Velp, Italy). The samples were then diluted to 50 mL with high-purity water. Al concentrations were determined using a Model AAS 5 EA atomic absorption spectrometer (Analytic Jena, Germany) equipped with a transversely heated graphite furnace and an autosampler (MPE 5). The content absorbed was expressed in μg/g dry weight.

2.3 Ascorbate peroxidase (APX - E.C. 1.11.1.11)

For determination of APX activity, cucumber seedlings were homogenized in a 50 mM $KH_2PO_4/K_2HPO_4$ (pH 7.0) containing 1 mM EDTA and 2% PVP, pH 7.8, at a ratio of 1:3 (w/v). The homogenate was centrifuged at 13 000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity, which was assayed according to the modified method of Zhu [12]. The reaction mixture, at a total volume of 2 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM $H_2O_2$ and 100 μL extract. $H_2O_2$–dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm ($E$ = 2.8 $mM^{-1}$ $cm^{-1}$). Ascorbate peroxidase activity was expressed as μmol oxidized ascorbate/min/mg protein.

2.4 Catalase (CAT - E.C. 1.11.1.6)

For the CAT assay, the cucumber seedlings were prepared by the homogenization of fresh tissue material in a solution containing 50 mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 10 g $L^{-1}$ PVP, 0.2 mM EDTA and 10 mL $L^{-1}$ Triton X-100, at a ratio of 1:5 (w/v). After the homogenate was centrifuged at 12 000 x g at 4ºC for 20 min, the supernatant was used for determination of CAT activity, which was assayed according to the modified method of Aebi [13] by monitoring the disappearance of $H_2O_2$ by measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mM $H_2O_2$ in 50 mM $KPO_4$ buffer (pH 7.0) and 30 µL extract. Catalase activity was expressed as ΔE/min/mg protein.

2.5 Superoxide dismutase (SOD - E.C.1.15.1.1)

The activity of superoxide dismutase was assayed according to McCord and Fridovich [14]. About 200 mg fresh tissues were homogenized in 5 mL of 100 mM $KH_2PO_4/K_2HPO_4$ (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered and centrifuged at 22 000 x g for 10 min at 4ºC, and the supernatant was utilized for the assay. The assay mixture consisted of a total volume of 1 ml, containing glycine buffer (pH 10.5), 60 mM epinephrine and enzyme material. Epinephrine was the last component to be added. The adrenochrome formation over the next 4 min was recorded at 480 nm in a UV-Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. SOD activity was expressed as U SOD/mg protein. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the

production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. SOD has been found to inhibit this radical-mediated process.

2.6 Determination of hydrogen peroxide

The $H_2O_2$ contents of both control and treated seedlings were determined according to Loreto and Velikova [15]. Approximately 100 mg of seedlings were homogenized at 4ºC in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000g for 15 min and 0.5 mL of 10 mM $KH_2PO_4/K_2HPO_4$ (pH 7.0) and 1 mL of 1M KI. The $H_2O_2$ content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The $H_2O_2$ content was expressed as µmol/g fresh weight.

2.7 Protein oxidation

The reaction of carbonyls with dinitrophenyl hydrazine (DNPH) was used to determine the amount of protein oxidation, as described by Levine [16]. Cucumber seedlings were homogenized in a 25 mM $KH_2PO_4/K_2HPO_4$ containing 10 ml $L^{-1}$ Triton X-100, pH 7.0, at a ration of 1:5 (w/v). The homogenate was centrifuged at 13 000 x g for 30 min at 4°C. After the DNPH-reaction, the carbonyl content was calculated by absorbance at 370 nm, using the molar extinction coefficient (21 X $10^3$ l/mol cm) and results were expressed as nmol carbonyl/mg protein.

2.8 Estimation of lipid peroxidation and electrolyte leakage percentage (ELP)

The levels of lipid peroxides in the seedlings were determined by measuring malondialdehyde (MDA) content from the thiobarbituric acid (TBA) reaction as described by El-Moshaty [17]. The plants were homogenized in 0.2 M citrate-phosphate buffer, pH 6.5, at a ratio of 1:20 (w/v). The homogenate was filtered through two layers of paper filter and then centrifuged at 20 000 x g at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. MDA values were expressed in nmol MDA/mg protein.

The ELP was measured using an electrical conductivity meter and its determination was based on the method of Lutts [18], with some modifications. Seedling samples were divided into 5 g segments and placed in individual stopped vials containing 50 mL of distilled water after washes with distilled water to remove surface contamination. These samples were incubated at room temperature (25ºC) on a shaker (100 rpm) for 24 h. Electrical conductivity of bathing solution (EC1) was read after incubation. Samples were then placed in a thermostatic water bath at 95ºC for 15 min and the second reading (EC2) was determined after cooling the bathing solutions to room temperature. ELP was calculated as EC1/EC2 and expressed as %.

## 2.9 Chlorophyll determination

0.1 g cotyledons were weighed and used for chlorophyll determination. Chlorophyll was extracted following the method of Hiscox and Israelstam [19] and estimated with the help of Arnon's formulae [20]. Fresh chopped cotyledon samples were incubated at 65ºC in dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 663 and 645 nm in a Spectrophotometer (Celm E-205D). Chlorophyll content was expressed as $\mu g\ g^{-1}$ fresh weight.

## 2.10 Magnesium concentration

The cucumber seedlings were separated into shoot and roots and then were oven-dried at 65°C until reaching a constant weight. The plant material was ground with a stainless steel grinder and then digested in a mixture of $HNO_3$ - $HClO_4$ at a ratio of 4:1 (v/v). Magnesium ($Mg^{2+}$) concentrations were measured with a GBC 932AAS atomic absorption spectrophotometer (GBC Scientific Equipment Pty Ltd, Victoria, Australia). The macronutrient ($Mg^{2+}$) is expressed as g/kg dry weight.

## 2.11 Protein determination

In all the enzyme preparations, protein was measured by the Coomassie blue method according to Bradford [21] using bovine serum albumin as standard.

2.12 Statistical analysis

The experiments were carried out through a randomized design. The analyses of variance were computed for statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± S.D. of at least three independent replicates. The mean differences were compared utilizing the Tukey test. Three pools of 5 replicates each (n=3) were taken for all analyses from each set of experiments.

## 3. Results

3.1 Metal determination

The Al content was determined in the roots and shoots of 10-day-old cucumber seedlings (*Table I*). The roots and shoots showed an increase of Al at 10, 100, 500, 1000 and 2000 μM when compared with the control. More aluminum was accumulated in the roots than in the shoot. The maximum accumulation of Al was 34.16 μg/g dry weight in roots treated with 2000 μM (*Table I*).

**Table I**

**Concentration of aluminum in root and shoots of cucumber.**

Values are the mean ± SD of three independent experiments. Letters indicate statistical differences at $p<0.05$.

| *Al concentration* | *Root (μg / g dry weight)* | *Shoot (μg / g dry weight)* |
|---|---|---|
| 0 μM | $7.48 \pm 0.19^{d}$ | $5.17 \pm 0.04^{e}$ |
| 1 μM | $9.67 \pm 0.33^{d}$ | $6.36 \pm 0.05^{e}$ |
| 10 μM | $11.75 \pm 1.39^{c}$ | $8.85 \pm 0.13^{d}$ |
| 100 μM | $18.64 \pm 0.32^{c}$ | $10.54 \pm 0.14^{c}$ |
| 500 μM | $28.22 \pm 1.65^{b}$ | $12.69 \pm 1.45^{b}$ |
| 1000 μM | $32.46 \pm 0.98^{a}$ | $17.69 \pm 0.59^{a}$ |
| 2000 μM | $34.16 \pm 1.89^{a}$ | $18.33 \pm 0.59^{a}$ |

3.2 Activities of antioxidant enzymes

The presence of Al in the substrate caused an increase in CAT activity of about 18%, 18% and 20% at concentrations of 10, 100 and 500 μM, respectively ($p<0.05$). On the other hand, CAT activity was reduced to basal levels at 2000 μM when compared with the control (*figure 1A*).

APX activity was increased by about 10%, 68% and 12% at 10, 100 and 500 μM $Al_2(SO_4)_3$, respectively ($p<0.05$). Nonetheless, APX activity was inhibited at 1000 and 2000 μM (*figure 1B*). The maximum APX activity was 0.70 μmol ascorbate oxidate/min/mg of protein at 100 μM $Al_2(SO_4)_3$.

Figure 1C shows the SOD activity of cucumber seedlings. At the lower concentrations (1 – 500 μM $Al_2(SO_4)_3$), a significant increase in SOD activity was observed. However, at the highest aluminum concentration (2000 μM) there was a decrease in SOD levels.

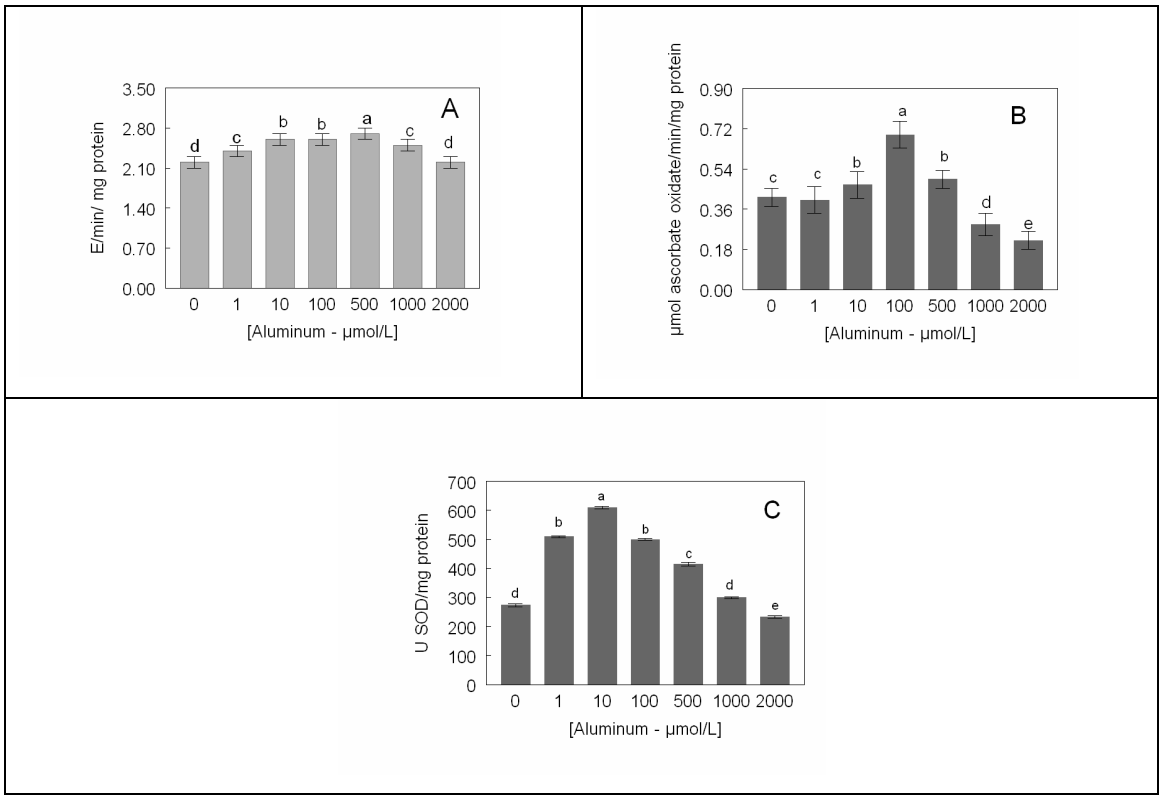


**Fig. 1** Effect of $Al_2(SO_4)_3$ on CAT activity (**A**), APX activity (**B**) and SOD activity (**C**) in 10 day old cucumber seedlings. Data represent the mean ± SD of three different experiments. Letters indicate statistical differences at $p<0.05$.

3.3 Lipid peroxidation, protein oxidation, electrolyte leakage and hydrogen peroxide levels

MDA content in the whole plant increased by 90% at levels of up to 500 µM in comparison with the control, while it significantly decreased, at 1000 and 2000 µM, to levels near to the control (*figure 2A*). Electrolyte leakage percentage (ELP) was significantly enhanced (*figure 2B*) at all concentrations tested, except at 1 µM $Al_2(SO_4)_3$. A significant increase of more than 50% was observed for protein oxidation at all concentrations, whereas at 2000 µM there was an increase of 84% (*figure 2D*).

The effect of $Al_2(SO_4)_3$ on $H_2O_2$ content is shown in figure 2C. The levels of endogenous $H_2O_2$ increased by about 70% in comparison to control plants at 100 µM $Al_2(SO_4)_3$. At the higher concentrations (1000 and 2000 µM), there was an increase in $H_2O_2$ content of about of 34% and 55%, respectively.

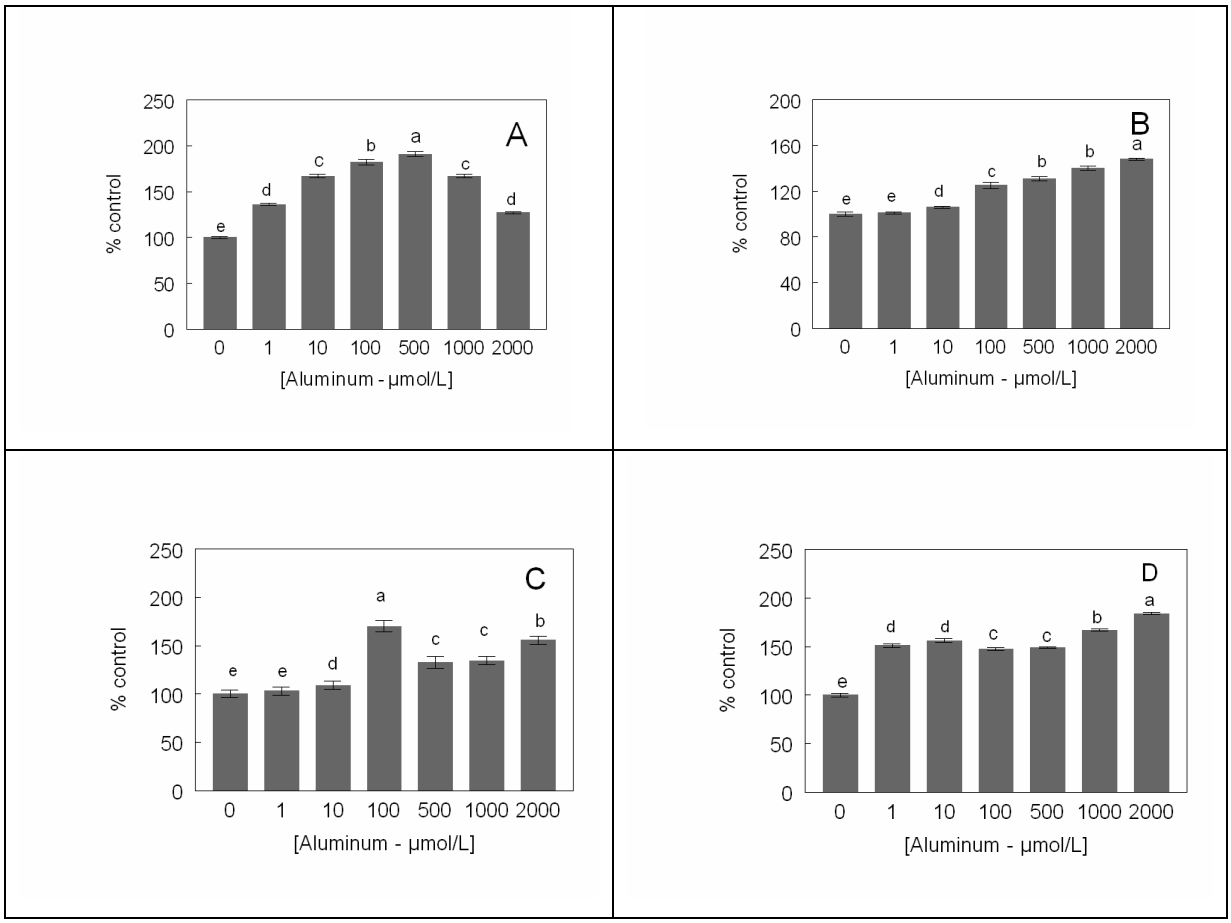


**Fig. 2** Effect of $Al_2(SO_4)_3$ on lipid peroxides (**A**), electrolyte leakage (**B**) $H_2O_2$ content (**C**) and Protein oxidation (**D**) in 10 day old cucumber seedlings. Data represent the mean ± SD of three different experiments. The control specific activity (without aluminum) that represents 100% was 0.23 ± 0.79 nmol of MDA/mg of protein, 0.128 ± 0.08 µs/cm, 2 ± 3.96 µmol/L g fresh weight and 11.39 ± 1.74 nmol of carbonyl/mg of protein. Letters indicate statistical differences at $p<0.05$.

3.4 Determination of Chlorophyll and Magnesium contents

Chlorophyll and magnesium contents were determined in cucumber cotyledons (*figure 3A and B*). The chlorophyll content decreased with increasing concentrations of aluminum (*figure 3A*). An inhibition of the chlorophyll content of 60% was observed at 2000 µM $Al_2(SO_4)_3$. At the lower concentrations of aluminum (1 and 10 µM $Al_2(SO_4)_3$), the magnesium content was increased. Conversely, a decrease in the magnesium level was detected at 100, 500, 1000 and 2000 µM $Al_2(SO_4)_3$ (*figure 3B*).

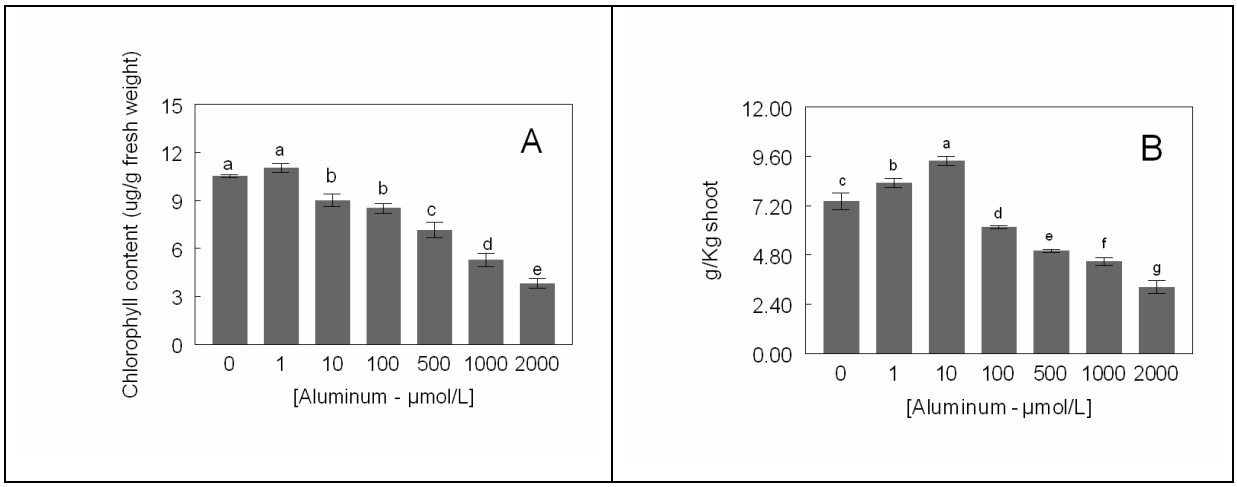


**Fig. 3** Effect of $Al_2(SO_4)_3$ on chlorophyll content (**A**) and magnesium concentration in shoot of cucumber seedlings (**B**). Data represent the mean ± SD of three different experiments. Letters indicate statistical differences at $p<0.05$.

## 4. Discussion

A common feature of several stresses including Al toxicity is perturbation of the cell redox homeostasis and, as a consequence, the enhanced production of reactive oxygen species (ROS) [8, 22 and 23]. Studies of Al toxicity in roots suggest that production of ROS may significantly contribute to Al-induced inhibition of root elongation [23].

Previous studies from our laboratory have shown that the growth, dry weight and fresh weight of roots and shoots of *Cucumis sativus* were decreased at 100, 500, 1000 and 2000 μM $Al_2(SO_4)_3$ [11]. Probably, the growth of root cells was affected by aluminum, causing a decrease in cell wall synthesis because aluminum inhibits the secretory function of the Golgi apparatus [2, 4, 11 and 24].

It is generally accepted that Al accumulates in root apices including the root cap and in the meristematic and elongation zones. Many researchers have reported that the major portion of absorbed Al, ranging from 30% to 90% of total Al, is localized in the apoplast [25, 26]. As Al is a polyvalent cation, under acidic conditions it binds strongly to negative charges in the Donnan free space of root cell apoplasm. The negative charges are mostly free carboxyl groups of pectic material in cell walls [41]. The pectin matrix is the main target of Al accumulation and thus Al toxicity. The pectin matrix with its different degree of pectin esterification (DE) seem to play a fundamental role in the expression of Al toxicity and resistance. High degrees of esterification of pectins would thus be an indicator of Al-resistance as the high DE decreases the binding strength of Al to the pectin matrix and favours it release/desorption by organic acids [41, 42, 43]. The aluminum sorption affects the conformation of the Ca- pectates complex, both aluminum and calcium seem to interact with the carboxylate groups as well as to the anomeric oxygens of pectins [42].

Soluble Al can exist in many different ionic forms in aqueous solution depending on the pH. In acidic solutions (pH<5.0), $Al^{3+}$ exists as the octahedral hexahydrate, $Al(H_2O)_6^{3+}$, which by convention is usually called $Al^{3+}$[4, 34] Al species that are relevant to phytotoxicity can be categorized into several different classes. With regard to the solution bathing the root, these classes include free or mononuclear forms as $Al^{3+}$, polynuclear Al, and Al as low molecular weight complexes. At low pH values the main species is $Al(H_2O)_6^{3+}$ , however, as the pH increase, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ are gradually formed and the neutral pH amorphous $Al(OH)_3$ precipitates: at basic pH this precipitate dissolves to form $Al(OH)_4$. In the cellular cytoplasm, Al in either reversible or irreversible macromolecular complexes should also considered. Polynuclear Al is defined as any species, complex, or aggregation (including solid-phase $Al(OH)_3$) that contains more than one Al atom. $Al_{13,}$ or other polynuclear species, can arise if the [Al] increases or if the pH rises in acid solution or falls in basic solutions [40]. It has been suggested that Al toxicity was better correlated with either the sum of all of the monomeric hydroxyl-Al species or a combination of $Al^{3+}$ and certain other monomeric hydroxyl-Al species, instead of $Al^{3+}$ alone [4]. It also has been suggested that for dicotyledonous, either $Al(OH)^{2+}$ or $Al(OH)_2^{+}$ were phytotoxic species, and $Al^{3+}$ was hypothesized to be much less toxic [34]. This difference in aluminum response between monocots (which are most sensitive to $Al^{3+}$) and dicotyledonous is puzzling but more studies were necessary and this can depends on the plant species [40]. In our experiments the pH was monitored daily and maintained to 4.0 in all experiment time and there is consensus that trivalent cationic $Al^{3+}$ in acid environments is the most relevant toxic form to plants [40]. Some studies have demonstrated that Al in solutions remain mononuclear for many days when $\{Al^{3+}\}/\{H^{+}\}^3 \leq 10^{8.8}$, where braces denote ion activities [4, 34 and 40].

In this study, Al content in the tissues of cucumber seedlings was higher in the roots than in the shoots (*Table I*). This result shows that the cucumber root system serves as a partial barrier to the transport of aluminum to the shoots. The transport to the shoots probably only played a minor role. A major role may be played by the export of Al from the roots into the substrate, as well as the exclusion of Al from uptake into the roots. It has been reported that one specific response to Al stress in tolerant plants is the secretion of malate or citrate. Malate and citrate might be released by plants either to prevent Al sorption by the roots or to desorption Al already present in the root apoplast [26, 28]. However, cucumber has proved not to be an Al tolerant plant [11].

One of the important targets of Al at the cellular level might be the plasma membrane. This is supported by the interference of Al in membrane lipids which is caused by the increased production of highly ROS. Cakmak and Horst [7] found the highest lipid peroxidation in the root tips (<2cm) of soybean at a longer duration of Al exposure. A close relationship exists between lipid peroxidation and inhibition of the rate of root elongation induced by Al. These observations, and others showing an increase in MDA and accumulated Al, indicate that cucumber seedlings experience substantial oxidative damage when exposed to high concentrations of $Al_2(SO_4)_3$ for ten days (*figure 2A*). Al also affects the membrane electrophysiological properties of plant species that differ in their tolerance to Al [27, 29]. Changes of the membrane lipid architecture induced by Al can lead to modification of membrane permeability [30, 31 and 32].

The results of the present study indicate that in cucumber seedlings, electrolyte leakage percentage (ELP) levels were significantly enhanced, and this enhancement was concentration-dependent (*figure 2B*). Another study showed that in cucumber plants exposed to $HgCl_2$, ELP content also increased [10]. Stab and Horst [27] proposed that binding of Al induces a stronger

association of membrane phospholipids and a higher packing density of phospholipids, reducing membrane permeability.

Major ROS-scavenging enzymes in seedlings include SOD, APX and CAT [29, 30 and 33]. The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide [30]. The different affinities of APX (μM) and CAT (mM) for $H_2O_2$ suggest that they belong to two different classes of $H_2O_2$ – scavenging enzymes: APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress.

Hydrogen peroxide also appears to play an important role in signal transduction during plant abiotic stress. $H_2O_2$ produced from oxidative burst functions, such as a local trigger of programmed cell death of challenged cells, causes a rapid cross-linking of cell wall proteins [31]. Results of the present study clearly indicate that Al induced an increase in $H_2O_2$ content in cucumber seedlings (*figure 2C*), which coincided with the increase in CAT activity (*figure 1A*). CAT is only present in peroxisomes, but it is indispensable for ROS detoxification during stress, during which time high levels are produced [29, 31].

APX is thought to be the most important $H_2O_2$ scavenger operating both in the cytosol and chloroplasts. APX uses ascorbic acid as a reducing substrate and forms part of a cycle, known as the ascorbate-glutathione or Halliwell-Asada cycle [32]. Because CAT does not require a supply of reducing equivalents for its functioning, it might not be affected during stress, unlike other mechanisms, such as APX [33]. The present investigation indicated that higher concentrations of Al (1000 and 2000 μM $Al_2(SO_4)$ decreased APX activity (*figure 1B*). Thus, seedlings with suppressed APX production induce increased SOD and CAT to compensate for loss of this enzyme [33].

An increase in SOD and APX activities was observed in response to low levels of Al (1 μM and 10 μM) in substrate (*figure 1B and 1C*). However, a decline in APX activity at 2000 μM suggests a possible delay in the removal of $H_2O_2$ and hence an enhancement of lipid peroxidation. Low SOD levels at 2000 μM may be related to the increase of $H_2O_2$ levels, because $H_2O_2$ may inactivate enzymes by oxidizing their thiol groups [30]. $Al^{3+}$ has great affinity for SH groups of endogenous biomolecules such as SOD and acid delta aminolevulinic (ALA-D) enzyme.

One important finding observed in this study is related to the fact that aluminum has been associated with biphasic or hormetic responses of different physiological parameters, where the low dose is stimulatory and high dose is inhibitory. Hormesis is a dose-response phenomenon reported for various chemical-physical stressors [34]. Aluminum-induced growth stimulation of $H^+$-sensitive varieties may be brought about by $Al^{3+}$ which, as a trivalent cation, would reduce the cell surface negativity and, in consequence, the $H^+$ activity at the cell membrane surface [34, 35]. The hormetic effect and the Al-induced alleviation of $H^+$ toxicity is considered an important starting point for the investigations into the mechanisms of Al- and proton-induced inhibition of root elongation in relation to Al species and their toxic effects on the plasma membrane and can be identified as an adaptative response of cells following an initial disruption in homeostasis [35].

The decrease in chlorophyll content (*figure 3A*) observed in the present study may be due to the inhibition of ALA-D activity shown in previous studies [11]. The enzyme δ-aminolevulinic acid dehydratase (ALA-D) is sensitive to metals due to its sulfhydrylic nature [36] and catalyzes the asymmetric condensation of two molecules of δ-aminolevulinic acid (ALA) to porphobilinogen [37]. The synthesis of porphobilinogen promotes the formation of porphyrins, hemes and clorophylls, which are essential for adequate chlorophyll aerobic metabolism and for photosynthesis [37, 38]. In line with this, it has been reported that ALA-D activity in plants

increases during chloroplast development, which is a period of rapid chlorophyll accumulation [37, 39] and a role of ALA-D in the regulation of chlorophyll has been proposed by Naito et al. [39]. $Mg^{2+}$ is not essential for plant ALA-D activity but causes a significant increase in the *V*max of the enzyme [37]. The binding data for Mg(II) indicate that plant ALA-D can bind up to 3 Mg(II)/subunit. The kinetic data support the existence of a required Mg(II), an allosteric Mg(II) and an inhibitory Mg(II), but data are insufficient to address the individual stoichiometries of these three types of Mg(II) [38]. In addition, aluminum may reduce the amount of almost all organic nutrients of plants [4] and may interfere with the absorption, transport and use of several cations such as calcium and magnesium [7]. The results of the present study indicate a continuous decrease in the content of magnesium in the cucumber shoots (*figure 3B*). This suggests that the reduction in chlorophyll content in the presence of aluminum is caused by a decrease of chlorophyll biosynthesis, ALA-D activity and magnesium content.

Lipids and proteins are common targets of oxidative damage in tissue under environmental stress [14]. Carbonyl content is a sensitive indicator of oxidative damage to proteins [16], and levels of carbonylated proteins in plants demonstrate oxidative stress associated with heavy metals [2], drought [32] and low temperatures [17]. The data from the present study indicate that the differences in protein oxidation at the higher concentrations of Al in cucumber seedlings are related to low levels of antioxidant defenses (*figure 2D*). The accumulation of carbonyls in cucumber seedlings, thus, indicates that the quantity of free radicals exceeds the capacity of the antioxidant defense system. Therefore, aluminum indeed induced the production of ROS, such as the hydroxyl radical, since neither $H_2O_2$ nor $O_2^{\bullet -}$ were reactive enough to provoke oxidation.

In a previous study, Pereira et al. [11] reported that Al rapidly induced drought stress in cucumber seedlings, which probably contributes to the induction of oxidative stress [22]. This Al-

induced ROS production can activate signal transduction pathways and lead to cell death [11] as a general symptom of Al-treated plants [2].

In conclusion, the increase in $H_2O_2$ production in cucumber seedlings may be related to the fact that the antioxidant system was not be able to overcome the toxicity caused by higher levels of Al. This resulted in negative effects such as lipid peroxidation and ELP which affected membrane protein oxidation and brought about a decrease in the growth of cucumber seedlings. These results demonstrate that the toxic effects of Al are harmful for plant development and affect the quality of crop productivity.

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**Manuscrito I -** DIFFERENTIAL RESPONSES OF OAT GENOTYPES: OXIDATIVE STRESS INDUCED BY ALUMINUM

# DIFFERENTIAL RESPONSES OF OAT GENOTYPES: OXIDATIVE STRESS INDUCED BY ALUMINUM

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**ABSTRACT**

The phytotoxic effects of aluminum and the mechanisms of genetically-based Al tolerance have been widely investigated, as reported in many papers and reviews. However, investigations on many Al-sensitive and Al-resistant species demonstrate that Al phytotoxicity and Al-resistance mechanisms are extremely complex phenomena. The objective of the present study was to analyze the effects of aluminum on the activity of antioxidant enzymes such as catalase (CAT - E.C. 1.11.1.6), superoxide dismutase (SOD - E.C.1.15.1.1) and ascorbate peroxidase (APX - E.C. 1.11.1.11). Also was evaluated the lipid peroxidation, $H_2O_2$ content, levels of ascorbic acid (ASA), non-protein thiols (NPSH) and Al content in three genotypes of oat, *Avena sativa* L (UFRGS 930598, UFRGS 17 and UFRGS 280). The genotypes were grown in different concentrations of Al ranging from 5 to 30 mg $L^{-1}$ for 5 days. The antioxidant system was unable to overcome toxicity resulting in negative effects such as lipid peroxidation and $H_2O_2$ content in UFRGS 930598. The results showed that UFRGS 930598 was the most sensitive genotype. UFRGS 17 and UFRGS 280 were more resistant to Al toxicity. These results suggest that UFRGS 17 has mechanisms of external detoxification and UFRGS 280 has mechanisms of internal detoxification. The different behavior of enzymatic and non-enzymatic antioxidants of the genotypes showed that aluminum resistance in UFGRS 17 and UFRGS 280 may be related to oxidative stress.

**Keywords:** *Avena sativa,* Superoxide dismutase, hydrogen peroxide, ascorbate peroxidase, catalase, aluminum content, oxidative stress.

## Introduction

All aerobic organisms including plants require molecular oxygen for survival, which can be easily reduced to reactive oxygen species (ROS) through various biochemical reactions (Morita et al., 1999). ROS include the superoxide anion radical ($O_2$•-), hydrogen peroxide ($H_2O_2$), the hydroxyl radical (•OH) and singlet oxygen ($^1O_2$) which are reactive species and can oxidize and damage cellular components (De Biasi et al., 2003). The cells possess a defensive system, consisting of various enzymes such as catalase (CAT - E.C. 1.11.1.6), ascorbate peroxidase (APX - E.C. 1.11.1.11), and superoxide dismutase (SOD - E.C.1.15.1.1), that reduce ROS under normal conditions, but if complete reduction does not occur, the result may be a state of oxidative stress leading to the oxidation of biomolecules as lipids, proteins and DNA (Mittler, 2002). The antioxidative system falls into two general classes: (1) low molecular weight antioxidants, which consist of lipid-soluble membrane associated antioxidants such as a-tocopherol and b-carotene, and water-soluble reductants such as glutathione and ascorbate and (2) antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Gratão et al. 2005).

The toxicity caused by aluminum in plants in acid soils is a well-known example of environmental stress (for a review, see Ma, 2000; Matsumoto, 2000; Ma et al., 2001; Ryan and Delhaize, 2001). Although Al itself is not a transition metal and cannot catalyze redox reactions, the involvement of oxidative stress in Al toxicity has been suggested. $Al^{3+}$, the most toxic of the soluble forms of Al (Yamamoto et al., 2001) induces oxidative stress, and a number of studies have reported its participation in various processes, including an increase in enzyme activity (SOD) related to ROS and lipid peroxidation in soybeans (*Glycine max*) (Cakmak and Horst, 1991) peas (*Pisum sativum*) (Yamamoto et al., 2001), changes in the expression of various genes induced by Al in *Arabidopsis* (Sugimoto and Sakamoto, 1997; Richards et al., 1998), tobacco

(Ezaki et al., 2000) and wheat (*Triticum aestivum* L.) (Snowden and Gardner, 1995; Cruz-Ortega et al., 1997; Hamel et al., 1998). Comparative studies of Al tolerance in 22 species from seven families have established that plants can resist the toxic effects of Al. There are many proposed mechanisms of Al tolerance in plants involving external avoidance or internal tolerance (Kochian, 1995). The phytotoxic effects of aluminum and the mechanisms of genetically-based Al tolerance have been widely investigated, as reported in many papers and reviews (Kochian, 1995; Delhaize & Ryan, 1995; Ciamporová, 2002; Gonçalves et al., 2009). Evidence corroborating the relation between aluminum stress and oxidative stress in plants has been obtained with transgenic *Arabidopsis* plants (Ezaki et al., 2000). However, investigations on many Al-sensitive and Al-resistant species demonstrate that Al phytotoxicity and the mechanisms of Al-resistance are extremely complex phenomena. When comparing the physiology and biochemistry of genotypes that differ in Al tolerance, it is preferable that the genetic backgrounds be similar so as to eliminate differences that are unrelated to Al tolerance (Ezaki et al., 2000).

In this study, we have used three genotypes of oat (*Avena sativa* L) that differ in Al tolerance .These genotypes, UFRGS 17, UFRGS 930598 and UFRGS 280, were previously classified as tolerant, sensitive and intermediate to aluminum respectively in accordance with morphological characteristics suck as root length (Federizzi et al., 2000). Measurements of the effects of Al were carried out after 5 days of exposure, and allowed us to reveal whether there are any biochemical differences between UFRGS 17, UFRGS 930598 and UFRGS 280 and their classification as tolerant, sensitive and intermediate respectively in accordance with previous morphological analysis was confirmed. It was also shown that aluminum resistance may be related the oxidative stress.

## Material and Methods

### Plant material and growth conditions

*Avena sativa* L. seeds of three genotypes (UFRGS 17 Al-tolerant; UFRGS 930598 Al-sensitive, UFRGS 280 Al-intermediate), commonly known as oat, provided by Programa de Melhoramento Genético de Aveia from the Universidade Federal do Rio Grande do Sul (UFRGS) were germinated in plastic recipients (1000 mL) containing 250 mL of medium with $Al_2(SO_4)_3$ diluted in a 0.5% agar solution. No nutritive solution was added to the agar. The seedlings made use of the seed nutrition in the initial stage of development, and in a previous experiment, it was verified that up to the tenth day the plants did not suffer any nutrient deficiency (data not shown). Five different $Al_2(SO_4)_3$ treatments (0, 5, 10, 20 and 30 mg $L^{-1}$) were applied randomly. The medium pH was adjusted to 4.0. Each experimental unit consisted of 30 seeds, totalizing 15 replicates per treatment. After germination, the seedlings were maintained in a growth chamber with controlled temperature (25 ± $1^0$C) and photoperiod (16 h light; light intensity of 35 μmol $m^{-2}$ $s^{-1}$ at plant level). On the fifth day the seedlings were separated into root and shoot and prepared for homogenization.

### Metal determination

Al content was determined in the roots and shoot of 5-day-old oat seedlings. Approximately 50 mg of roots and shoot were digested with 4 mL $HNO_3$ utilizing the following stages of heating: a) $50^0$C for 1 h; b) $80^0$C for 1 h; and $120^0$C for 1 h in a digester block (Velp, Italy). The samples were then diluted to 50 mL with high-purity water. Al concentrations were determined using a Model AAS 5 EA atomic absorption spectrometer (Analytic Jena, Germany)

equipped with a transversely heated graphite furnace and an autosampler (MPE 5). The content absorbed was expressed in mg/Kg dry weight.

Antioxidant enzyme activities

Catalase (CAT)

For the CAT assay, the oat root and shoot seedlings were prepared by the homogenization of fresh tissue material in a solution containing 50mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 10g $L^{-1}$ PVP, 0.2mM EDTA and 10 mL $L^{-1}$ Triton X-100, at a ratio of 1:5 (w/v). After the homogenate was centrifuged at 12 000 x g at 4ºC for 20 min, the supernatant was used to determine CAT activity, which was assayed according to the modified method of Aebi (1984) by monitoring the disappearance of $H_2O_2$ by measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mM $H_2O_2$ in 50 mM $KPO_4$ buffer (pH 7.0) and 30 µL extract. CAT activity was expressed as ΔE/min/mg protein.

Ascorbate peroxidase (APX)

For determination of APX activity, oat root and shoot were homogenized in a 50 mM potassium phosphate buffer containing 1mM EDTA and 2% PVP, pH 7.8, at a ratio of 1:3 (w/v). The homogenate was centrifuged at 13 000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity, which was assayed according to the modified method of Zhu (2004). The reaction mixture, at a total volume of 2 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM $H_2O_2$ and 100 µL extract. $H_2O_2$–dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm ($E$ = 2.8 $mM^{-1}$ $cm^{-1}$). APX activity was expressed as µmol oxidized ascorbate /min/mg protein.

Superoxide dismutase (SOD)

The activity of SOD was assayed according to McCord and Fridovich (1969). About 200 mg fresh tissues were homogenized in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered and centrifuged at 22 000 x g for 10 min at 4ºC, and the supernatant was utilized for the assay. The assay mixture consisted of a total volume of 1 ml, containing glycine buffer (pH 10.5), 60 mM epinephrine and enzyme material. Epinephrine was the last component to be added. The adrenochrome formation over the next 4 min was recorded at 480 nm in a UV-Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. SOD activity was expressed as U SOD/mg protein. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. SOD has been found to inhibit this radical-mediated process.

Determination of hydrogen peroxide ($H_2O_2$)

The $H_2O_2$ contents of both control and treated seedlings were determined according to Loreto and Velikova (2001). Approximately 100 mg of root and shoot were homogenized at 4ºC in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000g for 15 min and 0.5 mL of 10 mM potassium phosphate buffer pH 7.0 and 1 mL of 1M KI. The $H_2O_2$ content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The $H_2O_2$ content was expressed as µmol/g fresh weight.

Estimation of lipid peroxidation

The levels of lipid peroxides in the seedling were determined by measuring malondialdehyde (MDA) content from the thiobarbituric acid (TBA) reaction as described by El-Moshaty (1993). The root and shoot were homogenized in 0.2 M citrate-phosphate buffer, pH 6.5, at a ratio of 1:20 (w/v). The homogenate was filtered through two layers of paper filter and then centrifuged at 20 000 x g at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. MDA values were expressed in nmol MDA/mg protein.

Non-protein thiol (NPSH)

NPSH content in root and shoot (mg) was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). The reaction was read at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). Treated seedlings were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g at 4 ºC for 10 min, and supernatants were used for total thiol group determination. NPSH groups were determined in the fraction obtained after mixing 1 volume of supernatant with 1 volume of 4% trichloroacetic acid followed by centrifugation and neutralization (to pH 7.5) with 1 M Tris as described by Jacques-Silva et al. (2001). A standard curve using cysteine was used to calculate the content of thiol groups in samples, and was expressed as µmol SH/g fresh weight.

Ascorbic acid (AsA)

AsA determination was performed as described by Jacques-Silva et al. (2001). Briefly, root and shoot were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g for 10 min and protein was removed by dilution with 1 volume of 4% trichloroacetic acid followed by centrifugation. An aliquot of the sample was incubated at 37ºC in a medium containing 4.5 mg/ml dinitrophenylhydrazine, 0.6 mg/mL thiourea, 0.075 mg/mL $CuSO_4$, and 0.675 mol/L $H_2SO_4$ (final volume 1 mL). After 3 h, 1 mL of 65% $H_2SO_4$ was added and samples were read at 520 nm and were expressed as µg AsA/g fresh weight. A standard curve was constructed using ASA.

Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± S.D. of at least three independent replicates. The mean differences were compared utilizing Tukey's range test.

**Results**

Metal determination

The roots of all genotypes showed the highest concentration of aluminum. The roots of the sensitive (UFRGS 930598) and intermediate (UFRGS 280) seedlings showed an increase of aluminum content at 5, 10, 20 and 30 mg $L^{-1}$ when compared to control (Table 1). In the tolerant seedling root (UFRGS 17), there was also a significant increase at all concentrations, but this increase was lower than that observed in the sensitive seedling root. The maximum accumulation of Al was 8950 mg/kg dry weight in roots treated with 30 mg $L^{-1}$ in the sensitive seedling. More aluminum was accumulated in the roots than in the shoot. The content of aluminum was significant in all genotype shoots, but was greatest in the intermediate seedling with a dry weight

of 7056 mg/kg at 30 mg $L^{-1}$. In the sensitive seedling shoot, the aluminum content was 6678 mg/kg dry weight at 30 mg $L^{-1}$. The tolerant seedling shoot accumulated the least amount of aluminum.

**Table 1**
**Concentration of aluminum in root and shoots of three genotypes of oat (UFRGS 17 Al-tolerant; UFRGS 930598 Al-sensitive and UFRGS 280 Al-intermediate).**

| Al concentration | Root (mg / Kg dry weight) | Shoot (mg / Kg dry weight) |
|---|---|---|
| | **UFRGS 930598** | **UFRGS 930598** |
| 0 mg $L^{-1}$ | 3648 ± 200[d] | 1893 ± 200[d] |
| 5 mg $L^{-1}$ | 4096± 250[c] | 2993 ± 500[c] |
| 10 mg $L^{-1}$ | 5870 ± 300[b] | 4568 ± 450[b] |
| 20 mg $L^{-1}$ | 6520 ± 500[b] | 5194 ± 600[b] |
| 30 mg $L^{-1}$ | 8950 ± 350[a] | 6678 ± 500[a] |
| | **UFRGS 17** | **UFRGS 17** |
| 0 mg $L^{-1}$ | 3700 ± 200[d] | 1766 ± 290[d] |
| 5 mg $L^{-1}$ | 4533 ± 290[c] | 2394 ± 350[c] |
| 10 mg $L^{-1}$ | 5006 ± 200[b] | 3458 ± 380[b] |
| 20 mg $L^{-1}$ | 6142 ± 300[a] | 4083 ± 342[a] |
| 30 mg $L^{-1}$ | 6370 ± 350[a] | 4478 ± 300[a] |
| | **UFRGS 280** | **UFRGS 280** |
| 0 mg $L^{-1}$ | 3756 ± 200[d] | 1926 ± 200[e] |
| 5 mg $L^{-1}$ | 4580 ± 350[d] | 3478 ± 250[d] |
| 10 mg $L^{-1}$ | 5089 ± 300[c] | 3996 ± 200[c] |
| 20 mg $L^{-1}$ | 6890 ± 290[b] | 5720 ± 250[b] |
| 30 mg $L^{-1}$ | 7145 ± 250[a] | 7056 ± 190[a] |

Different letters are statistically different ($p<0.05$).

Activities of antioxidant enzymes

The activities of antioxidant enzymes, namely CAT,APX and SOD were determined in 5-day-old roots and shoots of the sensitive, intermediate and resistant genotypes (Figs. 1, 2 and 3 respectively). As a result of Al stress, root CAT activity increased in all three genotypes (Fig. 1A), while shoot CAT activity of the sensitive seedling was decreased at 20 and 30 mg $L^{-1}$ when compared between the genotypes. By contrast, root CAT activity increased to a greater extent in the tolerant seedling than in the sensitive seedling. There was no significant change in shoot CAT activity in the resistant seedling (Fig. 1B). Shoot CAT activity in the tolerant seedling was increased only at 20 and 30 mg $L^{-1}$. In the sensitive seedling shoot, CAT activity increased at 5 and 20 mg $L^{-1}$ while in the intermediate shoots it was increased only at 20 mg $L^{-1}$.

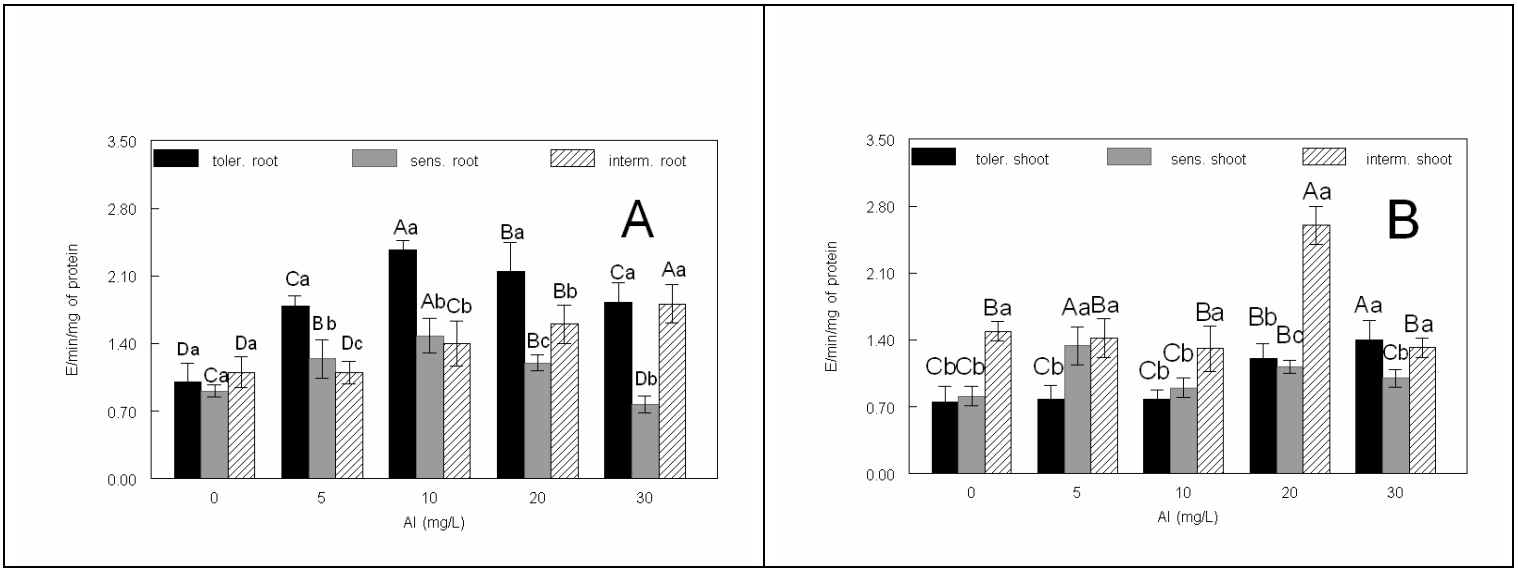


**Fig. 1** Effect of increasing concentration of $Al_2(SO_4)_3$ on CAT activity, in root (**A**) and shoot (**B**) in 5 day old oat seedlings of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

Root APX activity was increased in all seedlings (Fig. 2A). Nonetheless, shoot APX activity was inhibited in the intermediate seedling in all treatments, while in the sensitive seedling shoots APX activity was increased in all treatments (Fig. 2B). The maximum APX activity was 3.46 µmol ascorbate oxidized/min/mg protein at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in sensitive shoots (Fig. 2B) and 6.25 µmol ascorbate oxidized/min/mg protein at 10 mg $L^{-1}$ $Al_2(SO_4)_3$ in sensitive root (Fig. 2A).

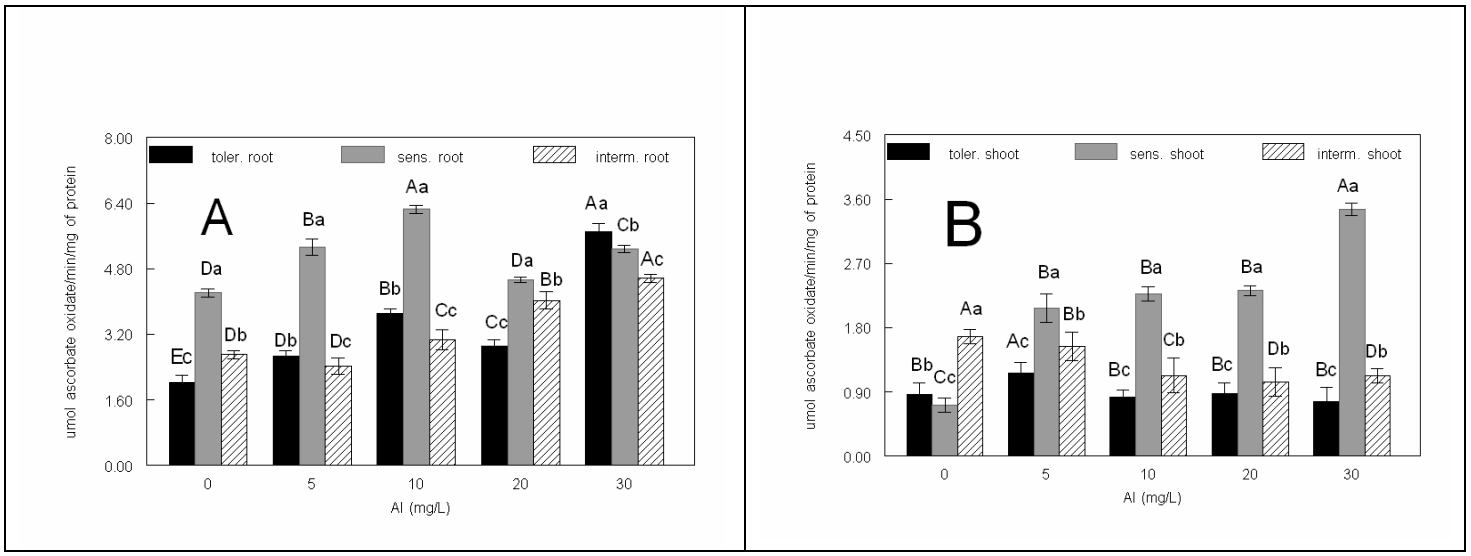


**Fig. 2** Effect of increasing concentration of $Al_2(SO_4)_3$ on APX activity, in root (**A**) and shoot (**B**) in 5 day old oat seedlings of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$) A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

Fig. 3 shows the SOD activity of oats seedlings. Root SOD activity was increased at 10 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in the resistant seedling (Fig. 3A) and decreased at 20 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in the sensitive and intermediate seedlings. Root SOD activity increased to a greater extent in the resistant seedling during Al stress. Nonetheless, shoot SOD activity in the intermediate seedling was increased at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ when compared with the shoot of

resistant and sensitive seedlings (Fig. 3B). The maximum SOD activity was 35249 U SOD/mg protein at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in resistant roots (Fig. 3A) and 6500 at U SOD/mg protein at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in intermediate shoots (Fig. 3B).

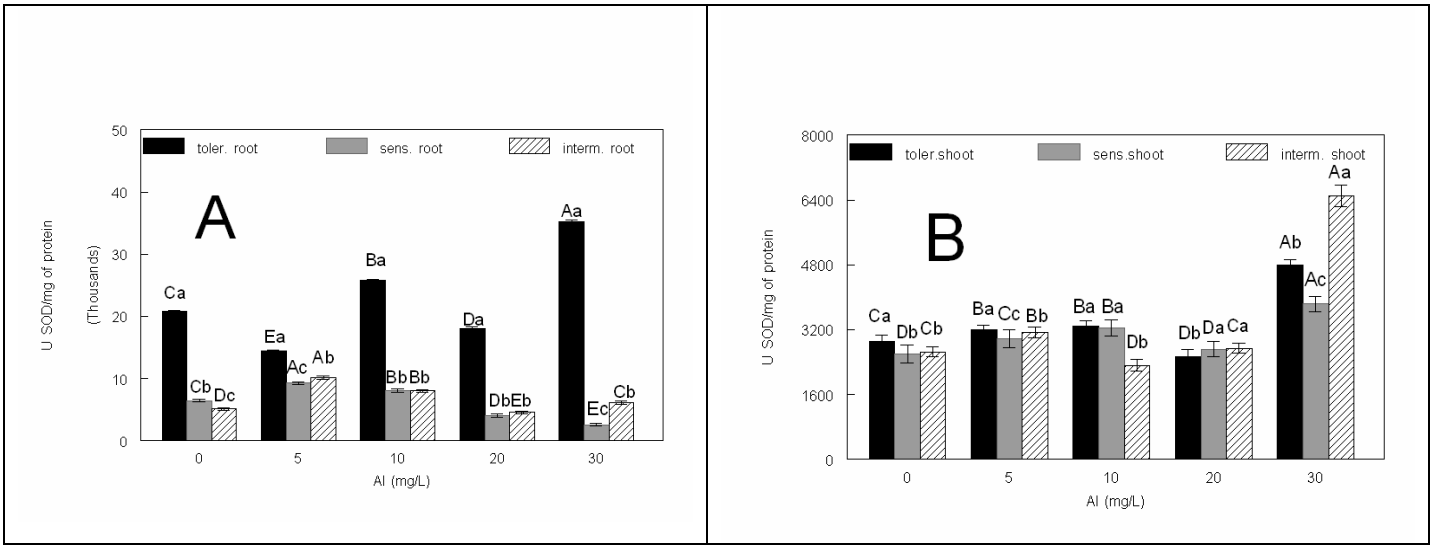


**Fig. 3** Effect of increasing concentration of $Al_2(SO_4)_3$ on SOD activity, in root (**A**) and shoot (**B**) of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

Lipid peroxidation and hydrogen peroxide levels

Al showed an increased root MDA content in all three seedlings when compared to control (Fig. 4A). The root MDA content increased to a greater extent in sensitive and intermediate seedlings if compared to the resistant seedling during Al stress. However, shoot MDA content of the resistant seedling did not change (Fig. 4B), while shoots MDA content showed significant changes in the sensitive and intermediate seedlings. At 30 mg $L^{-1}$ $Al_2(SO_4)_3$, intermediate shoot MDA content was reduced (Fig. 4B).

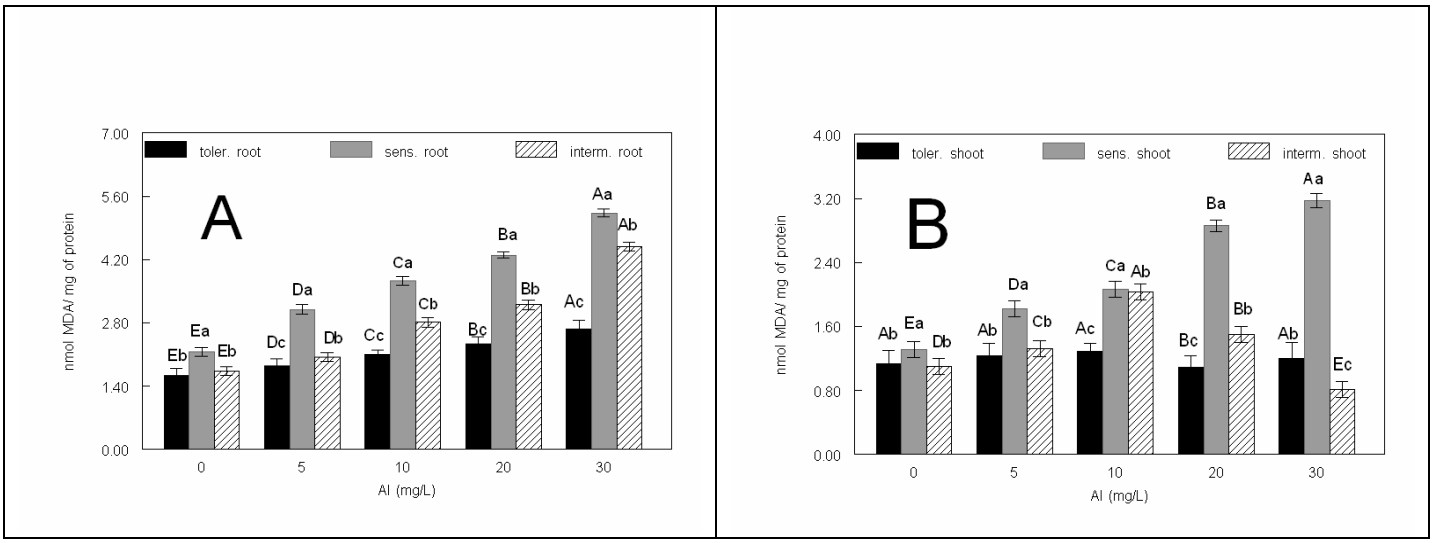


**Fig. 4** Effect of increasing concentration of $Al_2(SO_4)_3$ on level of lipid peroxides, in root (**A**) and shoot (**B**) of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

The effect of $Al_2(SO_4)_3$ on $H_2O_2$ content is shown in Fig. 5. Root endogenous $H_2O_2$ increased by about 70% when compared to control in tolerant and sensitive seedlings at 20 mg $L^{-1}$ $Al_2(SO_4)_3$ (Fig. 5A). Moreover, shoot $H_2O_2$ content increased in sensitive and intermediate seedlings, but no change was observed in the tolerant seedling at any Al concentration (Fig. 5B).

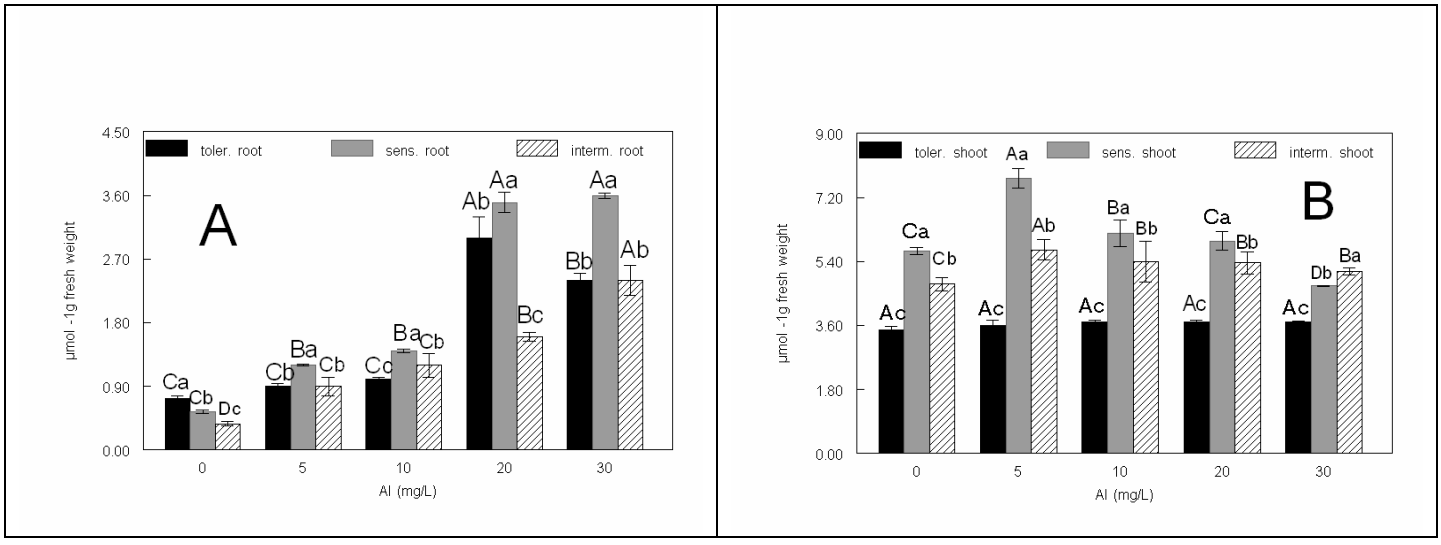

**Fig. 5** Effect of increasing concentration of $Al_2(SO_4)_3$ on $H_2O_2$ content, in root (**A**) and shoot (**B**) of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

Non-protein thiol group (NPSH) concentrations and Ascorbic acid (AsA)

NPSH content increased upon Al exposure. However, at the highest Al concentrations (20 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$), root NPSH content was decreased in the resistant seedling (Fig. 6A). Al effects on shoot NPSH content are shown in Fig. 6B. Shoot NPSH content showed an increase in the resistant seedling at 5 and 10 mg $L^{-1}$ $Al_2(SO_4)_3$ and a decrease at all concentrations in the sensitive seedling shoot.

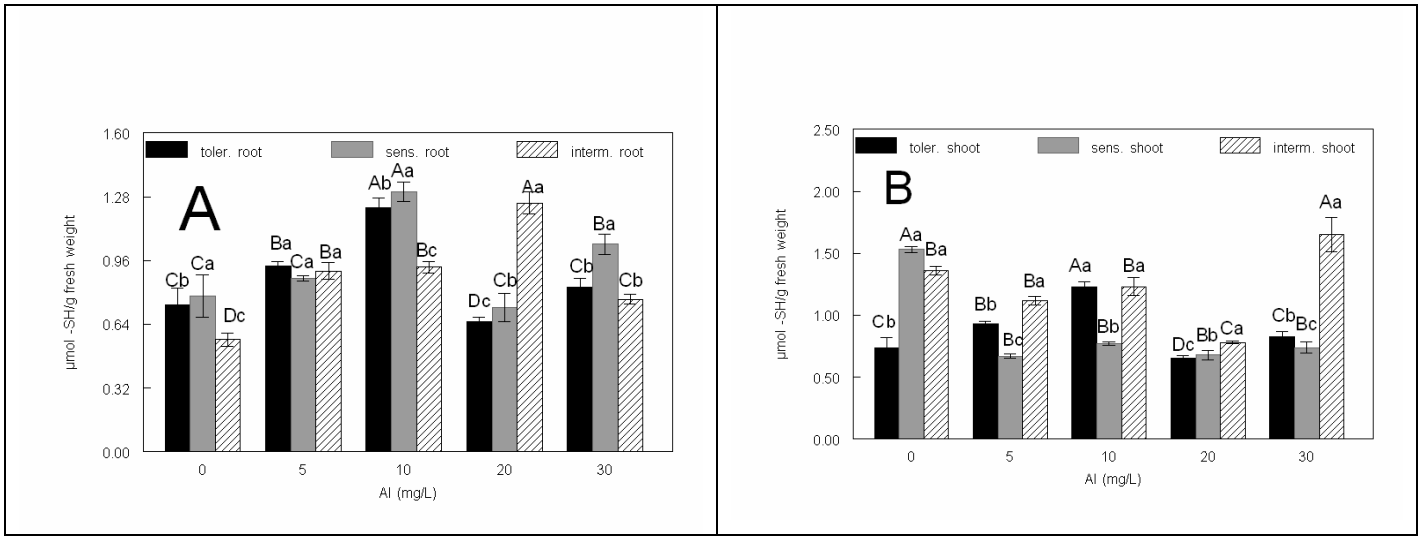


**Fig. 6** Effect of increasing concentration of $Al_2(SO_4)_3$ on NPSH content, in root (**A**) and shoot (**B**) of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference into genotypes in same concentration.

AsA content increased as a function of Al concentration in roots and shoots of all seedlings (Fig. 7A and 7B). AsA content increased to a greater extent in shoots than in root during Al stress. The maximum accumulation of ASA was 385.83 µg $AsA^{-1}$g fresh weight in intermediate seedling roots treated with 20 mg $L^{-1}$ $Al_2(SO_4)_3$ (Fig. 7A) and 745.63 µg $ASA^{-1}$ g fresh weight in sensitive seedling shoots treated with 30 mg $L^{-1}$ $Al_2(SO_4)_3$ (Fig. 7B).

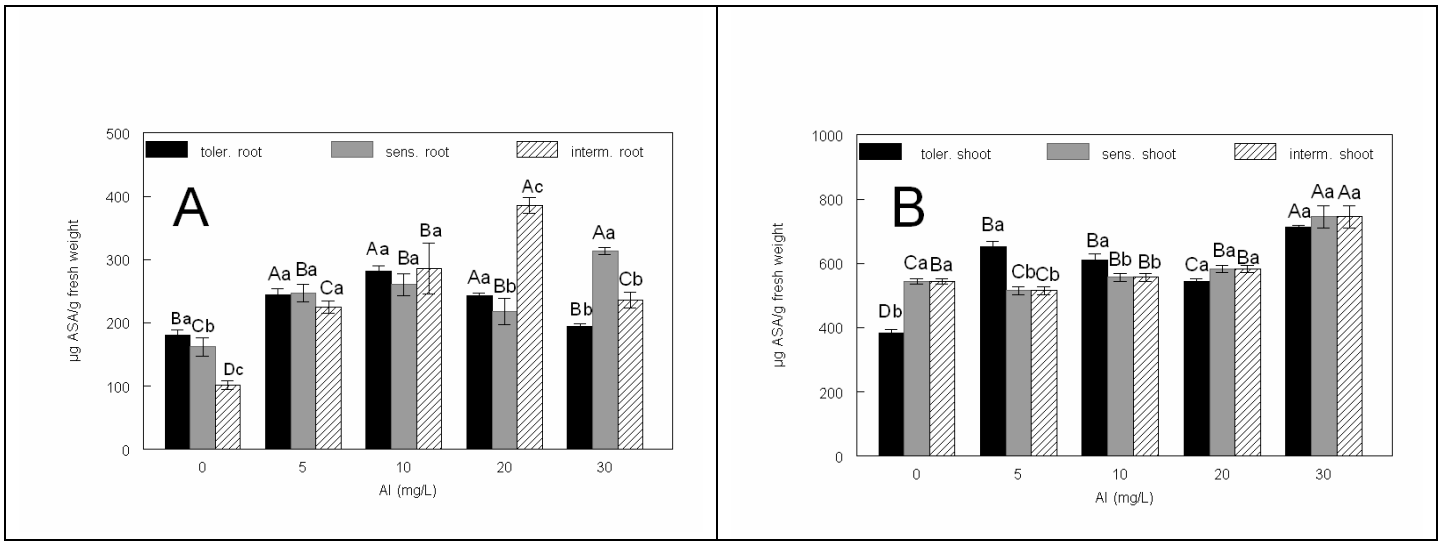


**Fig. 7** Effect of increasing concentration of $Al_2(SO_4)_3$ on AsA content, in root (**A**) and shoot (**B**) of the three genotypes (tolerant, sensitive and intermediate). Data represent the mean ± SD of three different experiments. Different letters are statistically different ($p<0.05$). A=difference into concentrations (0, 5, 10, 20 and 30) and a=difference between genotypes in same concentration.

**Discussion**

A common feature of stresses, such as Al toxicity, is perturbation of cell redox homeostasis and, as a consequence, enhanced production of ROS (Mitler, 2002). During stress the impairment of electron transport chains on injured membranes leads to the formation of ROS and to subsequent activation of the antioxidant defense system (Tamás et al., 2006). This study showed that CAT and SOD activities had a more protective effect in the tolerant (UFRGS 17) and intermediate seedlings (UFRGS 280) than in the sensitive seedling (UFRGS 930598) (Fig. 1A and B; 3A and B). The sensitive seedling showed lower CAT activity than the other seedlings (Fig. 1A and 1B). However, APX had a more protective effect on the sensitive seedling than on the other seedlings (Fig. 2A and 2B).

APX, CAT and SOD are major ROS-scavenging mechanisms in plants. The balance between these enzymes is crucial for determining the steady-state level of ROS excess. Different affinities of APX (μM range) and CAT (mM range) to $H_2O_2$ suggest that they belong to two different classes of $H_2O_2$- scavenging enzymes: APX might be responsible for the fine modulation of ROS in the sensitive seedling, showing that even small quantities of $H_2O_2$ caused oxidative stress in this genotype. This indicates that this genotype is, in fact, more sensitive than the tolerant genotype, which is in accordance with Federizzi et al. (2000) who showed differences between seedling through the decreased root growth in the sensitive genotype (UFGRS 930598) when compared with the others two genotypes.

In the intermediate seedling, CAT and SOD activities were increased to a greater extent in shoots than in root at 20 and 30 mg $L^{-1}$, respectively when compared with shoots of others seedling. Hydrogen peroxide is a stable ROS and its synthesis has been shown to increase under a variety of stressful conditions (Sharma & Dubey, 2004). It can be converted to more toxic molecules such as $^{\circ}OH$, if it is not quickly removed from plant cells (Wang & Zhang, 1999). The

$H_2O_2$ content was increased in roots and shoots of both the sensitive seedling and the resistant seedling (Fig. 5A and 5B), but this increase was lower in the resistant seedling. These results suggest that the antioxidant system was more efficient in the resistant seedling, but that it was also activated in the sensitive seedling, although in a differential way.

Plants possess $H_2O_2$- scavenging enzyme APX that converts $H_2O_2$ to $H_2O$ using ascorbate as an electron donor. A recent investigation revealed that ascorbate content regulates plant defense gene expression and modulates plant growth and development via phytohormone signaling (Pastori et al., 2003). ASA is involved in the regulation of photosynthesis, cell expansion, root elongation, and trans-membrane electron transport (Smirnoff, 2000). ASA is found in millimolar concentration in leaves and plays an important role in plant tolerance to stresses as a component of the antioxidant system (Noctor & Foyer, 1998). In line with this, our results show that the tolerant seedling accumulated more ASA in shoot and root than did the sensitive seedling (Fig. 7A and 7B). Antioxidants such as ASA and glutathione, which are found at higher concentrations in chloroplasts and other cellular compartments (5-20 mM ascorbic acid and 1-5 mM glutathione) are crucial for plant defense against of oxidative stress (Noctor & Foyer, 1998). The glutathione levels in oat were higher in the sensitive seedling than in the tolerant seedling (Fig. 6A and 6B). This suggests that aluminum activated the antioxidant system in the sensitive seedling. However, the antioxidant system may not have been efficient as greater lipid peroxidation was observed in this genotype (Fig. 4A and 4B).

Mutants with suppressed ASA levels and transgenic plants with altered content of glutathione are hypersensitive to stress conditions. It is generally believed that maintaining a high reduced per oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells. In addition, the overall balance between different antioxidants has to be tightly controlled. Enhanced glutathione biosynthesis in chloroplasts can result in oxidative damage to

cells rather than their protection, possibly by altering the overall redox state of chloroplasts (Creissen et al., 1999). In the present study, this hypothesis also may be valid for oats seedling sensitive to Al.

The cell plasma membrane, the ultimate obstacle for free access of Al ions into the symplast, is believed to be a primary target of rhizotoxic Al (Barceló et al., 1996). Aluminum ions bind with high affinity to negative charges on carboxyl and phosphate groups in the plasma membrane (Chaffai et al., 2005). Membranes of root cells exposed to Al present several indications of substantial disturbance (Barceló et al., 1996). The most prominent indicator of plasma membrane damage is Al-stimulated lipid peroxidation. Aluminum ions bound to the membrane modify lipid packaging and structure, facilitating lipid peroxidation by Fe (Oteiza et al., 1994). Lipid peroxidation levels in roots and shoots of sensitive seedlings were higher when compared to the tolerant genotype (Fig. 4A and B). Root lipid peroxidation in the tolerant genotype may have been due to direct contact with the metal. In shoots of tolerant seedlings, lipid peroxidation was not detected. Regulation of lipid membrane composition and adjustment of the unsaturation level of membrane fatty acids are extremely important in dealing with metal toxicity and make a considerable contribution to plant survival. Changes in properties of cellular membranes occur to ensure the proper function of processes that take place within them, and lead to ameliorative growth contributing to plant adaptation (Chaffai et al., 2005).

Through our measurements of Al effects on seedlings, it can be suggested, in agreement with Federizzi et al. (2000), that UFRGS 17 was more tolerant than UFRGS 930598. Al showed more toxic effects in UFRGS 930598, but this genotype also showed an activation of the antioxidant system, though less efficient if compared to that of the tolerant genotype (UFRGS 17).

UFRGS 280 (intermediate) showed some results that were similar to the tolerant genotype and others that were similar to the sensitive genotype. However, the intermediate seedling was the genotype that accumulated the most Al in shoots, although lipid peroxidation and $H_2O_2$ content were not the highest.

In conclusion, this result suggests the existence of mechanisms of internal detoxification for UFRGS 280, and mechanisms of detoxification by exudation of Al-ligating compounds for UFRGS 17. Therefore, there are biochemical differences between the three seedlings. However, the intermediate seedling may be more tolerant than was shown by morphological evaluations, as it showed greater aluminum resistance. We suggest that the intermediate seedling is in fact a tolerant genotype, but in a transition stage. The different behavior of enzymatic and non-enzymatic antioxidants showed that Al resistance in the tolerant seedling (UFGRS 17) and the intermediate seedling (UFRGS 280) may be related to oxidative stress.

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**Manuscrito II -** PHYSIOLOGICAL AND OXIDATIVE STRESS RESPONSES TO ALUMINUM OF THREE OAT GENOTYPES GROWN HYDROPONICALY

# PHYSIOLOGICAL AND OXIDATIVE STRESS RESPONSES TO ALUMINUM OF THREE OAT GENOTYPES GROWN HYDROPONICALY

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**Abstract**

Aluminum toxicity is a serious problem in Brazilian soils and selecting oat genotypes is an important strategy to produce this crop on these types of soils. Aluminum-sensitive, intermediate and tolerant oat genotype seedlings were grown in a nutrient solution (pH 4.0) with 0, 5, 10, 20 and 30 mg/L Al. After 10 days, Al concentration in both root and shoot systems of all genotypes increased with increasing Al levels. Sensitive roots have the highest values of aluminum accumulated and their growth was the most affected. This study showed that sensitive seedlings presented higher catalase (CAT - E.C. 1.11.1.6), ascorbate peroxidase (APX - E.C. 1.11.1.11), and superoxide dismutase (SOD - E.C.1.15.1.1) activities, however, their antioxidant system was unable to overcome toxicity resulting in negative effects such as lipid peroxidation and $H_2O_2$ content in this genotype. Intermediate and tolerant genotypes showed resistance to aluminum toxicity. The pH measurements in the boxes of tolerant genotypes showed an important increase in the pH values. These results suggest that tolerant genotypes have mechanisms of external detoxification and intermediate genotypes have mechanisms of internal detoxification. The different behavior of enzymatic and non-enzymatic antioxidants of the genotypes showed that aluminum resistance in tolerant and intermediate genotypes may be related to oxidative stress. The intermediate genotype accumulated the highest Al levels and presented tolerance to aluminum showing features of a hyperaccumulator plant.

**Keywords:** aluminum toxicity, antioxidative enzymes, growth, oxidative stress, Avena sativa

## 1. Introduction

Aluminum (Al) is a tin-white, malleable, odourless, ductile metal, with a melting point of 660ºC. It is soluble in alkalis, hydrochloric and sulphuric acid, and it is a ubiquitous element without a known, specific, and biological function. As a major constituent of mineral soils, it is present in the daily life of all organisms. However, the bioavailability and, in consequence, the toxicity of Al is mainly restricted to acid environments (Delhaize et al., 2009). Aluminum has been recognized as a toxic factor of acid soils for more than a century. Phytotoxic Al ion (mainly $Al^{3+}$) restricts crop production in acidic soils that cover almost 40% of world arable land (Chaffai et al., 2005, Silva et al., 2006). Micromolar concentrations of $Al^{3+}$ can inhibit root growth within minutes or hours in many agriculturally important plant species. The subsequent effects on nutrient and water acquisition result in poor growth and productivity (Zhang et al., 2007).

The phytotoxic effects of aluminum and the mechanisms of genetically-based Al tolerance have been widely investigated, as reported in several papers and reviews (Delhaize & Ryan, 1995; Ciamporová, 2002). The exclusion of Al from root tips, the most Al sensitive site, by exudation of organic ligands with high affinity for $Al^{3+}$ is considered a major factor for Al resistance (Ryan et al., 2001). Oat, an important upper Midwest small grain crop, is often the principle grain for young animals, poultry, and dairy cows. The high value of oat as feed has long been recognized. However, developmental changes during the growth of an oat plant are not well known. Understanding such basic developmental processes of oat is particularly important so that we can use our resources to obtain the best advantages (Reeves et al., 1986). However, investigations on several Al-sensitive and Al-resistant species demonstrate that Al phytotoxicity and mechanisms of Al-resistance are extremely complex phenomena. When comparing the physiology and biochemistry of genotypes that differ in Al tolerance, it is preferable that the

genetic backgrounds be similar so as to eliminate differences that are unrelated to Al tolerance (Ezaki et al., 2000).

In this study, we have used three different genotypes of oat (*Avena sativa* L) that differ in Al tolerance, namely UFRGS 17, UFRGS 930598, and UFRGS 280 (Federizzi et al., 2000). The identification of efficient parameters on the evaluation of toxic aluminum tolerance with the use of hydroponic culture can be of greatest use in oat breeding (Walton & Holly, 1990).

The fact that the *Avena* species possesses genetic variations for abiotic stresses is not only interesting for oat breeding but also as a model plant to study other aspects of physiological resistance. An appropriate approach to evaluate the Al stress response is a genotype evaluation in nutrient solution under controlled conditions (Boscolo et al., 2003). In spite of the importance of oat, there is no report in the literature on its antioxidant system under Al stress conditions. The antioxidant system is responsible for scavenging excess free radicals caused by environmental stresses. Studying the major components of the antioxidant system under Al stress, it is possible to ascertain whether Al induces oxidative stress, and whether it is involved in Al-tolerance mechanisms.

The objective of the present study was therefore to investigate and compare some physiological and oxidative stress responses of three oat genotypes exposed to Al in nutrient solution.

## 2. Material and Methods

### *2.1. Plant material and growth conditions:*

*Avena sativa* L. seeds of three genotypes (UFRGS 17 Al-tolerant; UFRGS 93605 Al-sensitive, UFRGS 280 Al-intermediate), commonly known as oat, provided by Programa de Melhoramento Genético de Aveia from the Universidade Federal do Rio Grande do Sul (UFRGS)

were germinated in plastic recipients (1000 mL) containing filter paper. No nutritive solution was added. The seedlings made use of the seed nutrition in the initial stage of development, and in a previous experiment, it was verified that up to the tenth day the plants did not suffer any nutrient deficiency (data not shown). Three-day-old plantlets from plastic recipients with filter paper were transferred into plastic boxes (10 000 mL) with polystyrene plates with holes that were used as a physical support for the plants; roots were submerged in aerated full nutrient solution of low ionic strength. The nutrient solution had the following composition (mg $l^{-1}$): 85.31 N; 11.54 S; 97.64 Ca; 23.68 Mg; 104.75 K; 176.76 Cl; 0.27 B; 0.05 Mo; 0.01 Ni; 0.13 Zn; 0.03 Cu; 0.11 Mn and 2.68 Fe. Evaporate and transpired water was continuously replaced with distilled water and the nutrient solution was completely renewed every week. Five different $Al_2(SO_4)_3$ treatments (0, 5, 10, 20 and 30 mg $L^{-1}$) were applied randomly. The medium pH was adjusted to 4.0 daily.

After 7 days of $Al^{3+}$ exposure, 24 plantlets per replicate (each treatment consisted of three replicates) were randomly harvested from hydroponic recipients and oat plantlets were carefully washed with distilled water and then divided into roots and shoot for growth and biochemical analysis.

*2.2. Metal determination*

Al content was determined in the roots and shoot of 10-day-old oat seedlings. Approximately 50 mg of roots and shoot were digested with 4 mL $HNO_3$ utilizing the following stages of heating: a) $50^0$C for 1 h; b) $80^0$C for 1 h; and $120^0$C for 1 h in a digester block (Velp, Italy). The samples were then diluted to 50 mL with high-purity water. Al concentrations were determined using a Model AAS 5 EA atomic absorption spectrometer (Analytic Jena, Germany) equipped with a transversely heated graphite furnace and an autosampler (MPE 5). The content absorbed was expressed in μg/g dry weight.

*2.3. Growth analysis*

To obtain the fresh mass, excess water was removed and the plants were weighed. To obtain dry mass, the plants were left at 65 ◦C for 5 days. Growth of oat plantlets was determined by measuring the roots and shoot (Tennant, 1975) length.

*2.4. pH Measurement*

Oat seedlings were transferred into plastic boxes (2 L) with polystyrene plates with holes that were used as a physical support for the plants; roots were submerged in aerated full nutrient solution of low ionic strength. The three genotypes were separated in different plastic boxes. The values of pH were anoted and adjusted. The solution pH was adjusted daily to 4.0 ± 0.1 by titration with HCl or NaOH solutions of 0.1 M.

*2.5. Antioxidant enzyme activities*

*2.5.1. Catalase*

For the catalase assay, the oat root and shoot seedlings were prepared by the homogenization of fresh tissue material in a solution containing 50mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 10g $L^{-1}$ PVP, 0.2mM EDTA and 10 mL $L^{-1}$ Triton X-100, at a ratio of 1:5 (w/v). After the homogenate was centrifuged at 12 000 x g at 4ºC for 20 min, the supernatant was used to determine catalase activity, which was assayed according to the modified method of Aebi (1984) by monitoring the disappearance of $H_2O_2$ by measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mM $H_2O_2$ in 50 mM $KPO_4$ buffer (pH 7.0) and 30 µL extract. Catalase activity was expressed as ΔE/min/mg protein.

*2.5.2. Ascorbate peroxidase*

For determination of APX activity, oat root and shoot were homogenized in a 50 mM potassium phosphate buffer containing 1mM EDTA and 2% PVP, pH 7.8, at a ratio of 1:3 (w/v). The homogenate was centrifuged at 13 000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity, which was assayed according to the modified method of Zhu (2004). The reaction mixture, at a total volume of 2 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0mM $H_2O_2$ and 100 µL extract. $H_2O_2$–dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm ($E$ = 2.8 $mM^{-1}$ $cm^{-1}$). Ascorbate peroxidase activity was expressed as µmol oxidized ascorbate /min/mg protein.

*2.5.3. Superoxide dismutase*

The activity of superoxide dismutase was assayed according to McCord and Fridovich (1969). About 200 mg fresh tissues were homogenized in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered and centrifuged at 22 000 x g for 10 min at 4ºC, and the supernatant was utilized for the assay. The assay mixture consisted of a total volume of 1 ml, containing glycine buffer (pH 10.5), 60 mM epinephrine and enzyme material. Epinephrine was the last component to be added. The adrenochrome formation over the next 4 min was recorded at 480 nm in a UV- Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. SOD activity was expressed as U SOD/mg protein. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at

480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. SOD has been found to inhibit this radical-mediated process.

*2.6 Determination of hydrogen peroxide*

The $H_2O_2$ contents of both control and treated seedlings were determined according to Loreto and Velikova (2001). Approximately 100 mg of root and shoot were homogenized at 4ºC in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000g for 15 min and 0.5 mL of 10 mM potassium phosphate buffer pH 7.0 and 1 mL of 1M KI. The $H_2O_2$ content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The $H_2O_2$ content was expressed as µmol/g fresh weight.

*2.7. Estimation of lipid peroxidation*

The levels of lipid peroxides in the seedling were determined by measuring malondialdehyde (MDA) content from the thiobarbituric acid (TBA) reaction as described by El-Moshaty (1993). The root and shoot were homogenized in 0.2 M citrate-phosphate buffer, pH 6.5, at a ratio of 1:20 (w/v). The homogenate was filtered through two layers of paper filter and then centrifuged at 20 000 x g at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. MDA values were expressed in nmol MDA/mg protein.

*2.8. Non-protein thiol content*

Non-protein thiol content in root and shoot (mg) was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). The reaction was read at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). Treated seedlings were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g at 4 ºC for 10 min, and supernatants were used for total thiol group determination. Non-protein thiol groups were determined in the fraction obtained after mixing 1 volume of supernatant with 1 volume of 4% trichloroacetic acid followed by centrifugation and neutralization (to pH 7.5) with 1 M Tris as described by Jacques-Silva et al. (2001). A standard curve using cysteine was used to calculate the content of thiol groups in samples, and was expressed as μmol $SH^{-1}$g fresh weight.

*2.9. Ascorbic acid content*

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Briefly, root and shoot were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g for 10 min and protein was removed by dilution with 1 volume of 4% trichloroacetic acid followed by centrifugation. An aliquot of the sample was incubated at 37ºC in a medium containing 4.5 mg/ml dinitrophenylhydrazine, 0.6 mg/ml thiourea, 0.075 mg/ml $CuSO_4$, and 0.675 mol/l $H_2SO_4$ (final volume 1 ml). After 3 h, 1 ml of 65% $H_2SO_4$ was added and samples were read at 520 nm and were expressed as μg ASA/g fresh weight. A standard curve was constructed using ascorbic acid.

*2.10 Delta-aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity*

Oat shoots were homogenized in 10 mM Tris-HCl buffer, pH 9.0, at a proportion of 1:1 (w/v). The homogenate was centrifuged at 12,000 g at 4°C for 10 min. The supernatant was pre-

treated with 0.1% Triton X-100 and 0.5 mM dithiotreithol (DTT). ALA-D activity was assayed as described by Morsch et al. (2002) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0 and 3.6 mM ALA. Incubation was started by adding 100 µl of the tissue preparation to a final volume of 400 µl and stopped by adding 350 µl of the mixture containing 10% trichloroacetic acid (TCA) and 10 mM $HgCl_2$. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of $6.1\times10^4$ l $mol^{-1}cm^{-1}$ (Sassa, 1982) for the Ehrlich-porphobilinogen salt.

*2.11. Chlorophyll contents*

Chlorophyll contents were determined following the method of Hiscox and Israeslstam (1979) and estimated with the help of Lichtenthaler's formula (Lichtenthaler 1987). Briefly, 0.1 g chopped fresh leaves sample was incubated at 65ºC in dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 645 and 663 nm in order to determine the contents of chlorophyll.

*2.12. Statistical analysis*

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± S.D. of at least three independent replicates. The mean differences were compared utilizing Tukey's range test.

## 3. Results

### *3.1 Metal determination*

Roots of sensitive and tolerant seedlings showed the highest concentration of aluminum. The highest values of aluminum content for the intermediate seedlings were in the shoots. Roots of the sensitive (UFRGS 930598) seedlings showed an increase of aluminum content at 5, 10, 20, and 30 mg $L^{-1}$ when compared to control (Table I). For the tolerant and intermediate seedling roots (UFRGS 17) there was also an increase at all concentrations, but this increase was lower than that observed in the sensitive seedling roots. There was no significant difference in the tolerant roots (UFRGS 17) when compared with the intermediate (UFRGS 280) seedling roots. The content of aluminum was significantly higher in all genotype shoots, but it was greater in the intermediate seedling shoots at 20 and 30 mg/L of aluminum. Tolerant seedlings accumulated lower amount of aluminum and no significant differences at 20 and 30 mg/L of aluminum were observed.

**Table I**

**Aluminum content in root and shoots of oat.**

| **Al concentration** | **Root (mg/Kg dry weight)** | **Shoot (mg/Kg dry weight)** |
|---|---|---|
| | **sensitive** | **sensitive** |
| 0 mg $L^{-1}$ | 5886 ± 160 | 3794 ± 200 |
| 5 mg $L^{-1}$ | $^{d}$7493± 240 | $^{d}$5703 ± 500 |
| 10 mg $L^{-1}$ | $^{c}$8991 ± 400 | $^{c}$7909 ± 450 |
| 20 mg $L^{-1}$ | $^{b}$10012 ± 450 | $^{b}$8470 ± 600 |
| 30 mg $L^{-1}$ | $^{a}$11126 ± 350 | $^{a}$9723 ± 500 |
| | **tolerant** | **tolerant** |
| 0 mg $L^{-1}$ | 5404 ± 200 | 3421 ± 290 |
| 5 mg $L^{-1}$ | $^{d}$6969 ± 390 | $^{c}$4533 ± 350 |
| 10 mg $L^{-1}$ | $^{c}$7992 ± 150 | $^{b}$5129 ± 380 |
| 20 mg $L^{-1}$ | $^{b}$8925 ± 250 | $^{a}$6091 ± 342 |
| 30 mg $L^{-1}$ | $^{a}$9910 ± 450 | $^{a}$6637 ± 300 |
| | **intermediate** | **intermediate** |
| 0 mg $L^{-1}$ | 5747 ± 220 | 3613 ± 180 |
| 5 mg $L^{-1}$ | $^{b}$7024 ± 350 | $^{d}$5918 ± 250 |
| 10 mg $L^{-1}$ | $^{b}$7908 ± 200 | $^{c}$8919 ± 240 |
| 20 mg $L^{-1}$ | $^{a}$8918 ± 390 | $^{b}$11520 ± 200 |
| 30 mg $L^{-1}$ | $^{a}$9541 ± 150 | $^{a}$13719 ± 200 |

*3.2 Growth analysis*

The growth of roots was inhibited in sensitive seedlings when compared to control. Sensitive (UFRGS 930598) seedlings showed an inhibition of length root higher than the other genotypes (Figure 1A). In the tolerant (UFRGS 17) and intermediate root (UFRGS 280)

seedlings no significant decrease of growth at 5 and 10 mg/L $Al_2(SO_4)_3$ was observed. Also, there was no significant difference in the growth roots of the tolerant (UFRGS 17) seedlings at 20 and 30 mg/L of aluminum. The same occurred for intermediate root (UFRGS 280) seedlings. However, more inhibition of growth occurred in the intermediate roots when compared with the tolerant ones.

The decreased growth of shoot was significant in sensitive seedlings at 5, 10, 20, and 30 mg/L of aluminum (Figure 1B). In tolerant and intermediate shoots no significant decrease was observed at 5 and 10 mg/L of aluminum, but the shoot increased at 20 and 30 mg/L of aluminum in intermediate seedlings. There was no significant difference in the growth shoots of the tolerant (UFRGS 17) seedlings at 20 and 30 mg/L of aluminum.

In the hydroponically grown plantlets, root dry weight in sensitive seedlings was reduced at $Al^{3+}$ levels exceeding 10mg/L (Figure 1E). In contrast, root dry weight in tolerant and intermediate seedlings was not altered. Shoot dry weight in sensitive seedlings decreased at 10, 20 and 30 mg/L $Al^{3+}$ (Figure 1F). On the other hand, in intermediate seedlings, shoot dry weight was increased at 20 and 30 mg/L, whereas in tolerant seedlings the shoot dry weight kept constant.

The results for fresh weight were similar to dry weight (Figure 1C and 1D).

*3.3. pH Measurement*

The pH was measured daily. The values of pH increased in nutrient solution of tolerant seedling boxes at 20 and 30 mg/L of aluminum from the day 4 of the experiment (Table II). The intermediate seedlings only increased the pH values on day 7 at 30 mg/L (data not showed). There was no significant difference in the values of pH in nutrient solution of sensitive seedlings boxes (data not showed).

**Table II pH values in nutrient solution of tolerant seedlings**

| pH values | 2º day | 3º day | 4º day | 5º day | 6º day | 7º day |
|---|---|---|---|---|---|---|
| | tolerant | tolerant | tolerant | tolerant | tolerant | tolerant |
| 0 mg $L^{-1}$ | 4.27**±0.15** | 4.22**±0.14** | 4.26**±0.14** | 4.21**±0.14** | 4.23**±0.1** | 4.29**±0.13** |
| 5 mg $L^{-1}$ | 4.16**±0.12** | 4.19**±0.15** | 4.19**±0.15** | 4.09**±0.15** | 4.12**±0.12** | 4.06**±0.11** |
| 10 mg $L^{-1}$ | 4.23**±0.05** | 4.26**±0.11** | 4.21**±0.11** | 4.12**±0.11** | 4.25**±0.1** | 4.18**±0.1** |
| 20 mg $L^{-1}$ | 4.19**±0.11** | 4.20**±0.1** | [b]4.46**±0.1** | [b]4.59**±0.16** | [a]4.67**±0.15** | [a]4.75**±0.16** |
| 30 mg $L^{-1}$ | 4.13**±0.05** | 4.15**±0.1** | [a]4.63**±0.1** | [a]4.76**±0.14** | [a]4.73**±0.1** | [a]4.79**±0.1** |

*3.4 Activities of antioxidant enzymes*

The activities of antioxidant enzymes, namely catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) were determined in 10-day-old roots and shoots of the sensitive, intermediate and resistant genotypes (Figure 2). As a result of Al stress, root and shoot CAT activities increased at all concentrations of aluminum in sensitive genotypes (Figures 2A and 2B), wherea shoot CAT activity of the tolerant seedling showed no significant difference when compared to control. There was no significant change in root and shoot CAT activity at 5 and 10 mg/L $Al^{3+}$ in the intermediate seedlings. However, root and shoot CAT activity was increased in the intermediate seedlings at 20 and 30 mg $L^{-1}$.

Root APX activity was increased at all concentrations in root and shoot sensitive seedlings (Figures 2C and 2D). Nonetheless, root APX activity was decreased in the sensitive seedling at 30mg/L $Al^{3+}$ (Figure 2C). The maximum APX activity was 7.20 µmol ascorbate oxidized/min/mg protein at 20 mg $L^{-1}$ $Al_2(SO_4)_3$ in sensitive root (Figure 2C) and 8.23 µmol ascorbate oxidized/min/mg protein at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in sensitive shoot (Figure 2D).

Figure 2E and 2F shows the SOD activity of oat seedlings. Root SOD activity was increased at 10 and 20 mg $L^{-1}$ $Al_2(SO_4)_3$ in the sensitive seedlings (Figure 2E) and decreased at 30 mg $L^{-1}$ $Al_2(SO_4)_3$. Shoot SOD activity increased at all concentrations in sensitive seedling

(Figure 2E). There was no significant change in the root and shoot SOD activity in tolerant seedlings. Nonetheless, root and shoot SOD activity in the intermediate seedlings was increased at 20 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$ when compared with the shoot of the tolerant ones. The maximum SOD activity was 5000 U SOD/mg protein at 20 mg $L^{-1}$ $Al_2(SO_4)_3$ in sensitive roots (Figure 2E) and 6100 at U SOD/mg protein at 30 mg $L^{-1}$ $Al_2(SO_4)_3$ in intermediate shoots (Figure 2F).

*3.5 Lipid peroxidation and hydrogen peroxide levels*

Al showed increased root MDA content at all concentrations in sensitive seedlings when compared to control (Figure 3C). The root MDA content increased to a greater extent in sensitive seedlings when compared to the resistant and intermediate seedlings during Al stress. However, shoot MDA content of the resistant and intermediate seedlings did not change (Figure 3D), while shoot MDA content showed significant increase in the sensitive seedlings.

The effect of $Al_2(SO_4)_3$ on $H_2O_2$ content is shown in Figure 3A and 3B. Root endogenous $H_2O_2$ increased at about 50% when compared to control in sensitive seedlings (Figure 3A). Moreover, shoot $H_2O_2$ content increased in sensitive seedlings, but no change was observed in the resistant seedlings at any Al concentration (Figure 3B). Nonetheless, root and shoot endogenous $H_2O_2$ increased in the intermediate seedlings at 20 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$ when compared with the resistant seedlings.

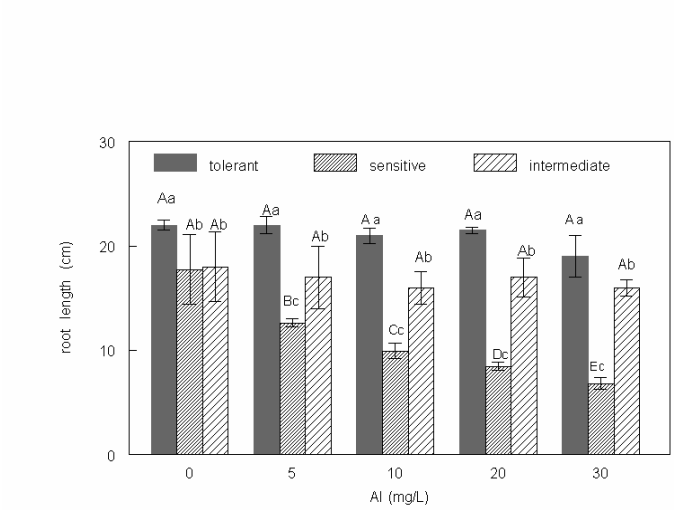
*3.6. Ascorbic acid (AsA) and non-protein thiol group (NPSH) concentrations*

NPSH content increased upon Al exposure at the highest Al concentrations (20 and 30 mg L), and root NPSH content was increased in the resistant seedling (Figure 4A). Al effects on shoot NPSH content are shown in Figure 4B. Shoot NPSH content showed an increase in the

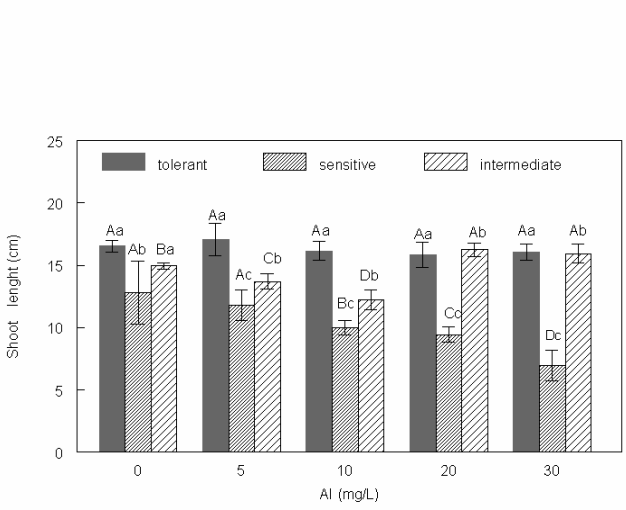
resistant seedlings at 20 and 30 mg $L^{-1}$ $Al_2(SO_4)_3$. NPSH content increased upon Al exposure at all concentrations in root and shoot intermediate seedlings. There was no significant change at 5 and 10 mg/L of $Al_2(SO_4)_3$ in shoot sensitive seedlings, however, there was an increase of NPSH content at 20 and 30 mg/L of $Al_2(SO_4)_3$.

ASA content increased as a function of Al concentration in roots and shoots of all seedlings (Figure 4C and 4D). ASA content increased to a greater extent in shoots than in roots during Al stress. The maximum accumulation of ASA was 385.83 µg $ASA^{-1}$g fresh weight in intermediate seedling shoots treated with 20 mg $L^{-1}$ $Al_2(SO_4)_3$ and 745.63 µg $ASA^{-1}$ g fresh weight in tolerant seedling roots treated with 30 mg $L^{-1}$ $Al_2(SO_4)_3$. There was no significant change in root and shoot sensitive seedlings.

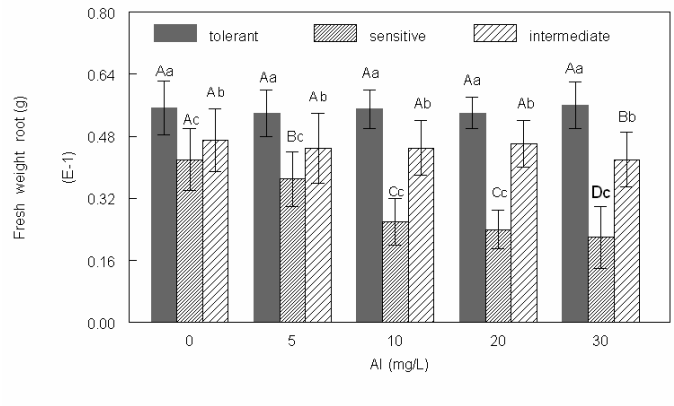
**(A)**


tolerant
sensitive
intermediate
root length (cm)
Al (mg/L)
0
5
10
20
30
Aa
Ab
Ab
Aa
Bc
Ab
Aa
Cc
Ab
Aa
Dc
Ab
Aa
Ec
Ab

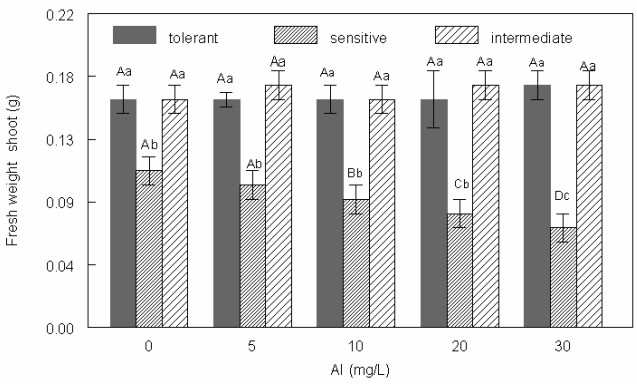
**(B)**


tolerant
sensitive
intermediate
Shoot lenght (cm)
Al (mg/L)
0
5
10
20
30
Aa
Ab
Ba
Aa
Ac
Cb
Aa
Bc
Db
Aa
Cc
Ab
Aa
Dc
Ab

**(C)**


tolerant
sensitive
intermediate
Fresh weight root (g)
(E-1)
Al (mg/L)
0
5
10
20
30
Aa
Ac
Ab
Aa
Bc
Ab
Aa
Cc
Ab
Aa
Cc
Ab
Aa
Dc
Bb

**(D)**


tolerant
sensitive
intermediate
Fresh weight shoot (g)
Al (mg/L)
0
5
10
20
30
Aa
Ab
Aa
Aa
Ab
Aa
Aa
Bb
Aa
Aa
Cb
Aa
Aa
Dc
Aa

**(E)** **(F)**

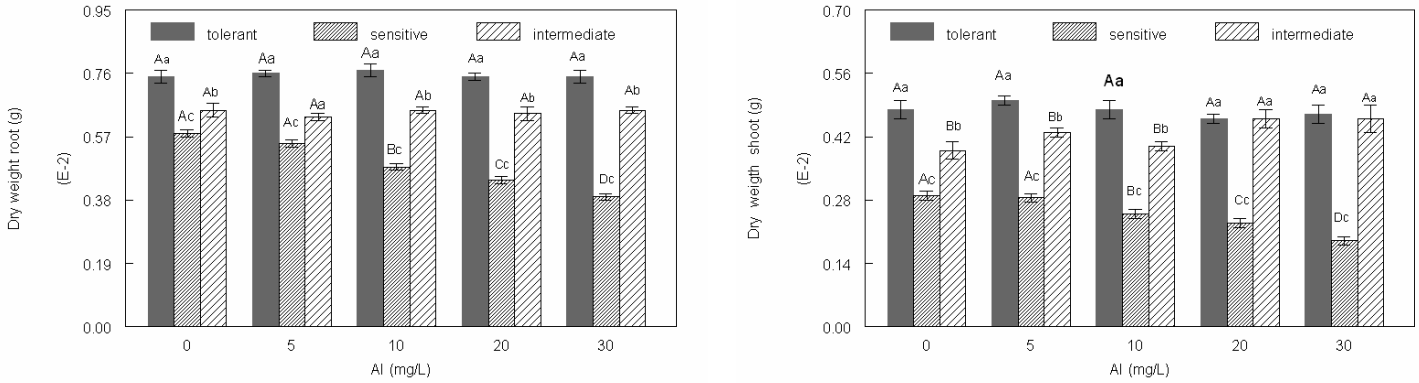



**Figure 1.** Effect of increasing concentration of $Al_2(SO_4)_3$ on root length (**A**), fresh weight of root (B), shoot growth (C), fresh weight of shoot (D), dry weight of root (E) and dry weight of shoot (F) in 10 day-old oat seedlings. Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in concentrations (0, 5, 10, 20, and 30) and a=difference between genotypes at the same concentration.

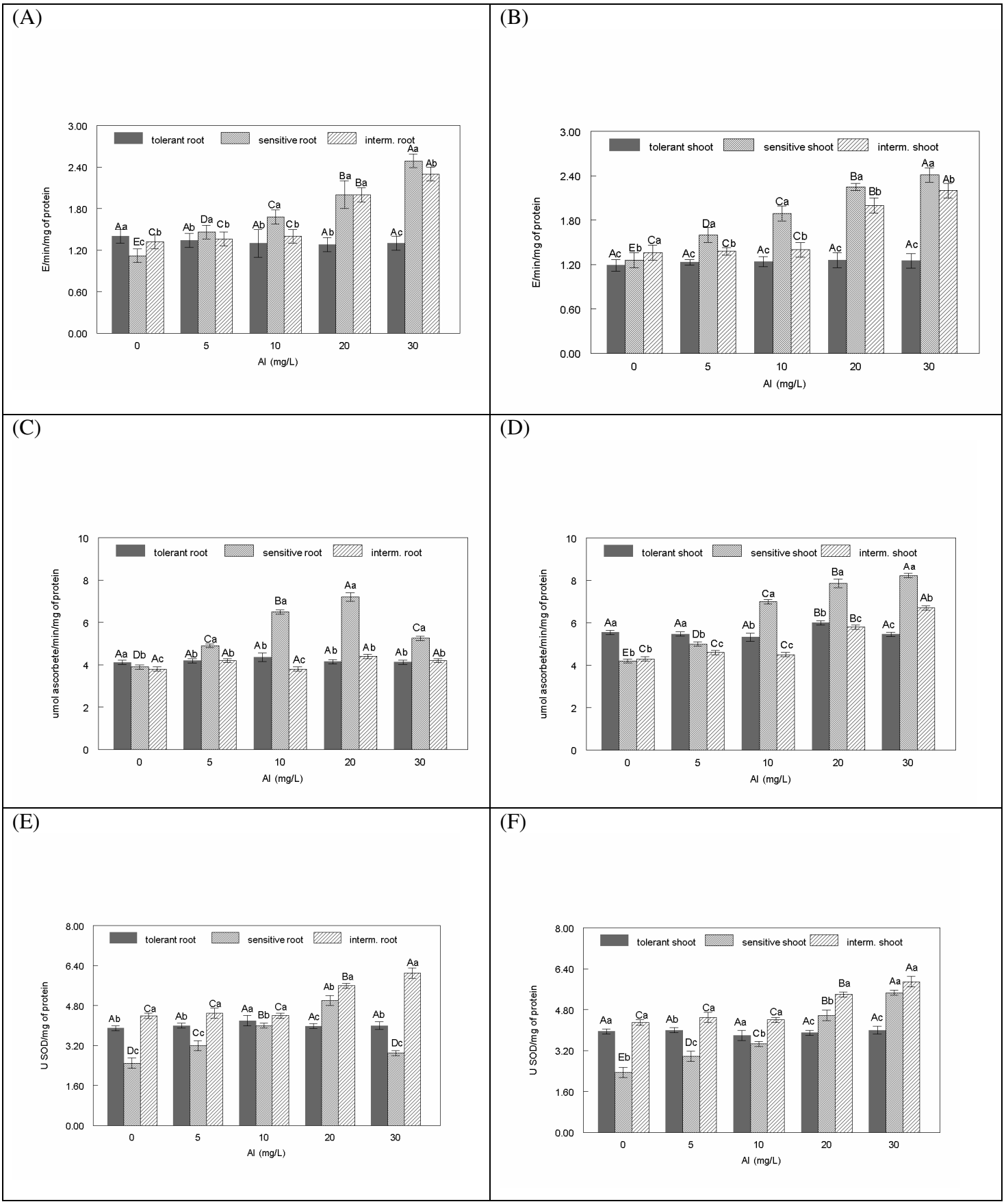



**Figure 2.** Effect of increasing concentration of $Al_2(SO_4)_3$ on CAT activity in roots (**A**) and shoots (**B**), on APX activity, in roots (C) and shoots (D), on SOD activity, in roots (E) and shoots (F) in

10 day-old oat seedlings. Data represent the mean ± SD of three different experiments. Different from control at p<0.05. A=difference into concentrations (0, 5, 10, 20, and 30) and a=difference between genotypes at the same concentration.

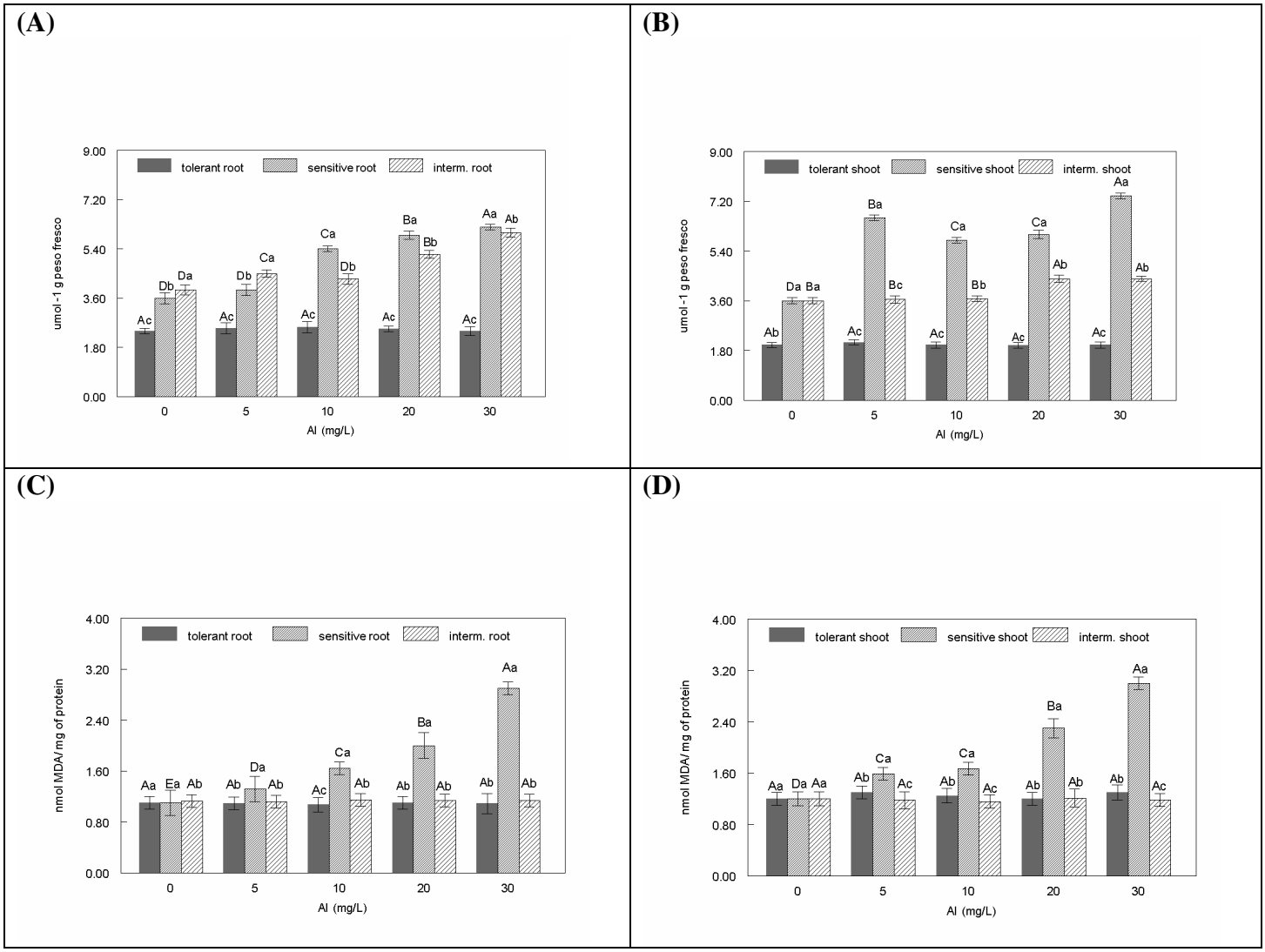



**Figure 3.** Effect of increasing concentration of $Al_2(SO_4)_3$ on $H_2O_2$ content, in root (**A**) and shoot (**B**) and effect of increasing concentration of $Al_2(SO_4)_3$ on level of lipid peroxides in root (C) and shoot (D). Data represent the mean ± SD of three different experiments. Different from control at p<0.05. A=difference in concentrations (0, 5, 10, 20, and 30) and a=difference in genotypes at the same concentration.

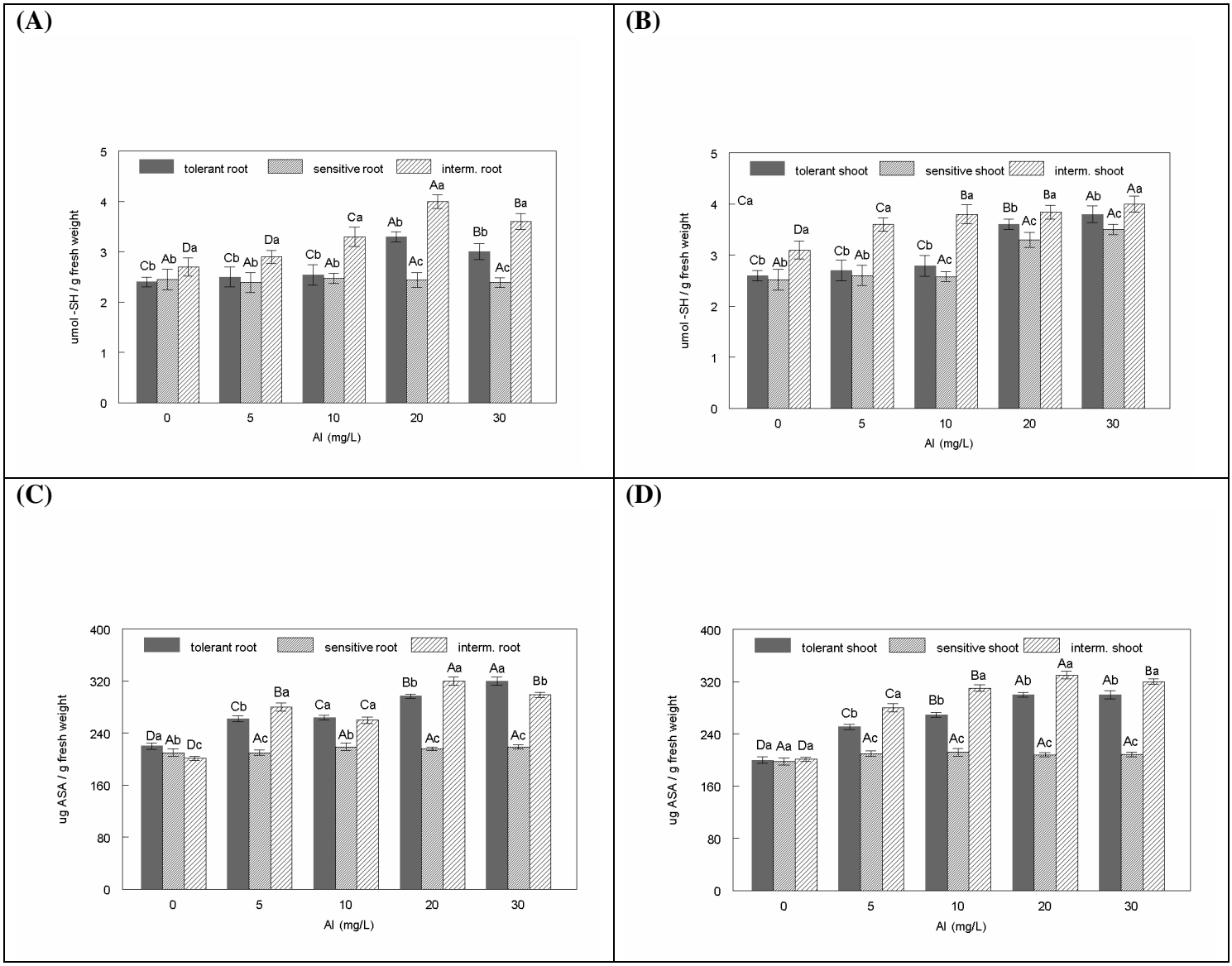



**Figure 4.** Effect of increasing concentration of $Al_2(SO_4)_3$ on non-protein thiol content in root (**A**) and shoot (**B**), and the effect of increasing concentration of $Al_2(SO_4)_3$ on ascorbic acid content in root (C) and shoot (D). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in concentrations (0, 5, 10, 20, and 30) and a=difference in genotypes at the same concentration.

*3.7 Photosynthetic pigments and ALA-D activity*

Chlorophyll content in tolerant and intermediate seedling was not affected by increasing $Al^{3+}$ level, but in sensitive seedlings it showed a decrease at the two highest $Al^{3+}$ levels (Figure 5A). ALA-D activity in sensitive seedlings showed a continuous inhibition with increasing $Al^{3+}$

level, whereas in intermediate seedlings its activity increased only upon addition of $Al^{3+}$ level exceeding 20 mg/L $Al_2(SO_4)_3$ (Figure 5B). The ALA-D activity in tolerant seedlings was not affected by increasing $Al^{3+}$ level. Moreover, tolerant and intermediate seedlings showed higher ALA-D activity than sensitive seedlings (Figure 5B).

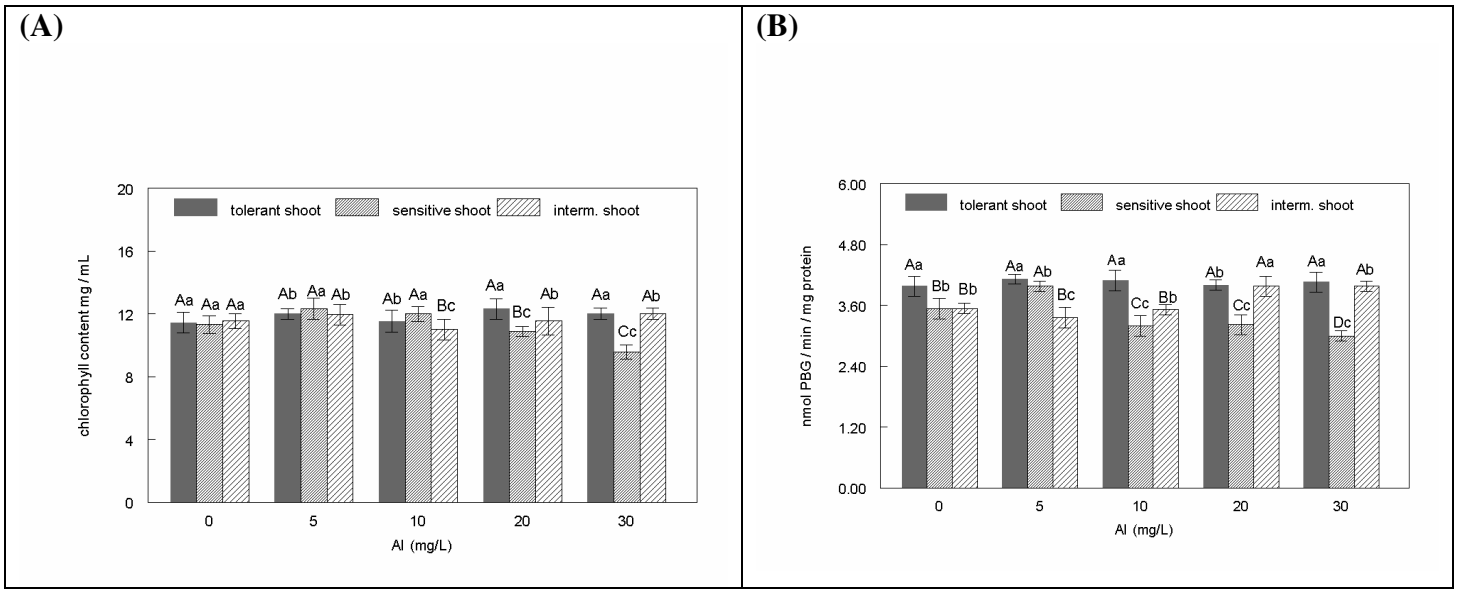



**Figure 5.** Effect of increasing concentrations of $Al_2(SO_4)_3$ on chlorophyll content (**A**) and ALA-D activity (**B**). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in the concentrations (0, 5, 10, 20, and 30) and a=difference in genotypes at the same concentration.

**4. Discussion**

In the present study the sensitive, intermediate, and tolerant seedling genotypes showed a continuous increase in the concentration of $Al^{3+}$ in roots and shoots with increasing $Al_2(SO_4)_3$ external concentration and accumulated significantly higher $Al^{3+}$ concentration in roots. This finding has been reported by other researchers working with distinct plant species exposed to varied $Al^{3+}$ concentrations (Yamamoto et al., 2003; Poschenrieder et al., 2008). This $Al^{3+}$ accumulation in the root system may indicate that roots serve as a partial barrier to transport to the shoots. The root sensitive seedlings have the highest values of aluminum accumulated and their growth was the most affected.

In the shoots of intermediate seedlings there was a higher $Al^{3+}$ concentration when compared with the shoots of tolerant and sensitive seedlings. These data suggest a genotypic difference among oat genotypes concerning the partitioning of $Al^{3+}$. In fact, differences in metal accumulation among the genotypes of the same species have been observed in different plants (Tamás et al. 2006; Tria et al. 2007).

According to Poschenrieder et al. (2008), the inhibition of seedling growth is a common effect of many metals and it is used as a parameter to characterize phytotoxicity. The growth analyses of oat showed that intermediate and tolerant seedlings grow normally in presence of the aluminum concentration and the root intermediate seedlings was not decreased and the shoot growth was activated, different from the sensitive seedlings. This effect may be due to adaptations in the intermediate genotype which grow in high aluminum concentrations. The growth of the tolerant genotype was not affected by aluminum concentrations either. In addition, it was observed a decrease in the fresh and dry weight of sensitive seedlings. In contrast, the fresh and dry weight was increased in intermediate seedlings. The majority of Al in root cells is found in the apoplast (Delhaize 1993) and may represent up to 99.9% of the total Al (Rengel & Reid, 1997). The accumulation of Al in the appoplast occurs under acidic conditions where the trivalent $Al^{3+}$ ion is the dominant species in the solution (kinraide, 1991) and strongly binds to negative

charges of the cell wall and the outer face of plasma-membrane (Zhang & Taylor, 1990; Masion & Bertsch, 1997). The sensitive genotype showed a significant decrease in the amount of organic matter indicating a decrease in photosynthesis. According to Kumari (2007), aluminum directly affects the synthesis of enzymes, pigments, and essential cofactors for the process. The level of chlorophyll was postulated as a simple and reliable indicator of heavy metal toxicity for higher plants (Gratão et al., 2005). Results of the present study showed a decrease of chlorophyll content only in sensitive seedlings at the two highest $Al^{3+}$ levels. This genotypic variation has been reported for other species (Drazkiewicz and Baszynski 2005; Mishra et al. 2006; Singh et al. 2006) and suggests that the decrease of chlorophyll content provokes a perturbation of cell redox homeostasis. As a consequence, an enhanced production of ROS is observed since the chlorophyll molecule is an electron acceptor (Mitler, 2002). Interestingly, the sensitive genotype had higher $H_2O_2$ content and lipid peroxidation. The decrease in chlorophyll content may be attributed to the inhibition of chlorophyll biosynthesis.

The reaction catalyzed by ALA-D is common to tetrapyrrol biosynthesis, including chlorophyll molecules, and it is essential for cellular life (Pereira et al., 2006). Al-exposure caused a severe inhibition of ALA-D activity in sensitive seedlings. Thus, the reduced chlorophyll content observed in the present study may be attributed to reduced chlorophyll synthesis because $Al^{3+}$ inhibits the ALA-D activity. $Al^{3+}$ may also be interfering with heme biosynthesis and chlorophyll formation by interacting with functional –SH groups of sulfhydryl-requiring enzymes like ALA-D (Morsch et al., 2002; Rocha et al., 2004). Indeed, the excess of the reaction substrate, ALA, catalyzed by ALA – D enzyme can yield superoxide radical, hydrogen peroxide, and hydroxyl radical. This way, ALA-D inhibition could have led to an ALA accumulation that in cell may endogenously contribute to enhanced levels of ROS (Noriega et al. 2006).

A common feature of stress, such as Al toxicity, is a perturbation of cell redox homeostasis and, as a consequence, enhanced production of ROS (Mitler, 2002). During stress,

the impairment of electron transport chains on injured membranes leads to the formation of ROS and to the subsequent activation of the antioxidant defense system. APX, CAT and SOD are major ROS-scavenging mechanisms in plants. The balance between these enzymes is crucial for determining the steady-state level of ROS excess (Tamás et al., 2006).

This study showed that sensitive seedlings showed higher CAT, APX and SOD activities than the other seedlings (Figure 2A and 2B). However, these results suggest that the antioxidant system was not more efficient in the sensitive seedlings because their growth was inhibited and a high level of lipid peroxidation and $H_2O_2$ content of this genotype occurred. In intermediate seedlings the antioxidant system was more efficient than in the sensitive seedlings. The high aluminum concentration in root and shoot tissues of intermediate seedlings caused increase in the $H_2O_2$ content. However, the enzymes were efficient in the removal of $H_2O_2$ content formed at 20 and 30 mg/L of $Al^{3+}$ because this genotype presented no significant damage in its growth and the levels of lipid peroxidation were not altered.

However, in the tolerant seedlings the activities of CAT, APX and SOD were not signicantly activated, and tolerant seedlings had low level of lipid peroxidation and $H_2O_2$ content. Plant- induced modification of rhizosphere pH has been proposed as a mechanism of Al exclusion (Wherrett et al., 2005). This tolerance mechanism has been established based on the fact that Al solubility is pH-dependent (Kinraide, 1991). As the pH solution increases the solubility of $Al^{3+}$, one the most toxic Al species, its toxicity is alleviated. Thus, plants that maintain a relatively high pH in the rhizosphere or apoplasm are, in fact, exposed to a lower $Al^{3+}$activity. The present study shows that the tolerant seedlings have an increase on pH values in nutrient solutions. The sensitive and intermediate seedlings showed no significant changes in the pH values in nutrient solutions. Several earlier studies have observed a positive relationship between the degree of Al resistance and the ability of plants to maintain a relatively high pH in the growth medium (Matsumoto, 2000). Al resistance can be divided into mechanisms facilitating Al exclusion from the root apex (Al exclusion) and mechanisms conferring the ability of plants to

tolerate Al in the plant symplasm (Al tolerance). Besides, chelating Al at cytoplasmic pH organic acids also have a net negative charge that can be protoned upon their exudation to the external solution, resulting in an increase in the pH of the solution surrounding the roots and thereby reducing Al activity (Matsumoto, 2000).

ASA is found in millimolar concentrations in leaves and plays an important role in plant tolerance to stress as a component of the antioxidant system (Noctor & Foyer, 1998). In line with this, our results show that the tolerant seedlings accumulated more ASA in shoot and root than the sensitive seedlings. Antioxidants such as ascorbic acid and glutathione, which are found at higher concentrations in chloroplasts and other cellular compartments (5-20 mM ascorbic acid and 1-5 mM glutathione), are crucial for plant defense against oxidative stress (Noctor & Foyer, 1998).

Intermediate and tolerant seedlings of oat are two genotypes that show resistance mechanisms to decrease the effects toxics of aluminum. Through our measurements of Al effects on seedlings, it can be suggested that UFRGS 17 (tolerant seedlings) shows mechanisms that facilitate Al exclusion from the root apex while UFRGS 280 shows mechanisms conferring the ability to plants to tolerate Al in the plant symplasm. However, UFSGS 930598 (sensitive seedling) shows no mechanisms that prevent the aluminum toxicity.

Citrate, oxalate, and malate are some of the commonly released organic acid anions that can form sufficiently strong complexes with $Al^{3+}$ to protect plant roots. Oat roots release both malate and citrate (Delhaize et al., 2001). For organic acids to detoxify Al in the rhizosphere, they must be transported from cytosol to apoplasm. At the near-neutral pH of the cytoplasm, organic acids are almost entirely dissociated from their protons and exist as organic acid anions. These organic anions are likely to be transported out of the root cells. The tolerant seedlings can release malate and citrate from apices roots to the solutions and increase the pH values (Silva et al., 2002).

In conclusion, intermediate seedlings accumulated high concentrations of Al in the shoot without showing symptoms of Al toxicity and their growth was stimulated by Al. The following hypotheses have been suggested for internal detoxification of aluminum after it has entered the cytoplasm: formation of aluminum chelates by organic acids, proteins or other organic ligands; compartmentalization of aluminum in the vacuole; the synthesis of aluminum tolerant proteins (Jansen et al., 2002). Intermediate seedlings show characters of a hyperaccumulator plant.

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**MANUSCRITO III -** DIFFERENTIAL SPEED OF ACTIVATION IN ANTIOXIDANT SYSTEM IN THREE OAT GENOTYPES

# DIFFERENTIAL SPEED OF ACTIVATION IN ANTIOXIDANT SYSTEM IN THREE OAT GENOTYPES

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**ABSTRACT**

The objective of this study was to evaluate whether the oxidative stress caused by aluminum (Al) toxicity is an early symptom that can trigger root growth inhibition in tolerant, sensitive, and intermediate oat genotype seedlings. Oat seedlings were grown in a nutrient solution (pH 4.0) with 0 and 20 mg/L Al. At 12, 24, and 36 h after Al addition, growth (root length) and biochemical parameters (catalase (CAT - E.C. 1.11.1.6), ascorbate peroxidase (APX - E.C. 1.11.1.11) and superoxide dismutase (SOD - E.C.1.15.1.1) activities, lipid peroxidation, ascorbic acid (AsA) and non-protein thiol group (NPSH) concentration) were determined. Regardless the exposure time, root length of the tolerant genotype did not decrease with any Al treatments. Al supply caused lipid peroxidation only in the Al-sensitive genotype in roots and shoots (at 12, 24, and 36 h). In sensitive genotype seedlings, CAT, APX, and SOD were activated only at 24 or 36 h. In tolerant and intermediate genotypes, CAT, APX, and SOD were activated at 12, 24, and 36 h. Data for root growth and lipid peroxidation suggested that lipid peroxidation in the sensitive genotype may be a direct effect of Al toxicity on root growth. In tolerant and intermediate seedlings, Al presence at 12 h provoked an increase only in AsA concentration. This increase may have contributed to free radicals and ROS detoxification and suggests their active participation in Al detoxification, since root growth inhibition and lipid peroxidation were not observed in these genotypes. Therefore, the tolerant, intermediate, and sensitive genotypes differ in the expression of the amount, type of antioxidants, and speed of activation of antioxidant system, suggesting a varying capacity of these genotypes to deal with oxidative stress, which resulted in varying sensitivity and tolerance to Al.

**Keywords:** *Avena sativa*, aluminum, genotypes, lipid peroxidation, antioxidant system.

## 1. Introduction

Al ions have a toxic effect on both plant and animal cells (Kochian, 1995). It has been suggested that Al ions enhance the peroxidation of phospholipids and proteins in cell membranes (Cakmak and Horst, 1991; Yamamoto et al., 2001).

The primary effect of Al toxicity is the inhibition of root elongation; however, the molecular mechanisms involved in this toxicity are unknown (Matsumoto, 2000). Micromolar concentrations of $Al^{3+}$ can inhibit root growth within minutes or hours in many agriculturally important plant species (Delhaize et al., 2009)

A common feature of several stresses including Al toxicity is the enhanced production of reactive oxygen species (ROS), which are generally considered harmful to plant cells (Tamás et al., 2004). All cells possess a defensive system, consisting of various enzymes such as catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) that are activated to reduce ROS in cells (Buckner et al., 2000; Jones, 2000). Oxidative stress is an important component of the plant reaction to Al toxicity (Jones et al., 2006). There are several proposed mechanisms of Al tolerance in plants that involve external avoidance or internal tolerance (Kochian, 1995). Several works on the relation between tolerance aluminum stress and oxidative stress in plants have recently been obtained with transgenic Arabidopsis plants (Ezaki et al., 2000; Poschenrieder et al., 2009). Differential uptake of Al in roots could account for differences in tolerance between genotypes of crop plants (Delhaize et al., 2009). Aluminum tolerance is genetically controlled and the genetic variability within and among species are often due to a few major genes. Research reports on oat are scarce. Genetic studies made in Brazil on oat indicate that aluminum tolerance is regulated by one or two dominant genes (Sánchez-Chacón et al., 2000).

There are several indications that oxidative stress is involved in the plant tolerance responses to Al stress. Therefore, the objective of this study was to evaluate whether the oxidative stress caused by Al toxicity is an early symptom that can trigger root growth inhibition in three oat genotypes that differ in Al tolerance.

## 2. Material and Methods

### *2.1. Plant material and growth conditions:*

*Avena sativa* L. seeds of three genotypes (UFRGS 17 Al-tolerant; UFRGS 93605 Al-sensitive, UFRGS 280 Al-intermediate), commonly known as oat, provided by Programa de Melhoramento Genético de Aveia from the Universidade Federal do Rio Grande do Sul (UFRGS) were germinated in plastic recipients (1000 mL) containing filter paper. No nutritive solution was added. The seedlings made use of the seed nutrition in the initial stage of development, and in a previous experiment, it was verified that up to the tenth day the plants did not suffer any nutrient deficiency (data not shown). Three-day-old plantlets from plastic recipients with filter paper were transferred into plastic boxes (10 000 mL) with polystyrene plates with holes that were used as a physical support for the plants; roots were submerged in aerated full nutrient solution of low ionic strength. The nutrient solution had the following composition (mg $l^{-1}$): 85.31 N; 11.54 S; 97.64 Ca; 23.68 Mg; 104.75 K; 176.76 Cl; 0.27 B; 0.05 Mo; 0.01 Ni; 0.13 Zn; 0.03 Cu; 0.11 Mn and 2.68 Fe. Evaporate and transpired water was continuously replaced with distilled water and the nutrient solution was completely renewed every week. Two different $Al_2(SO_4)_3$ treatments (0, 20 mg $L^{-1}$) were applied randomly. The medium pH was adjusted to 4.0 daily.

After 12, 24 and 36 hours of $Al^{3+}$ exposure, 24 plantlets per replicate (each treatment consisted of three replicates) were randomly harvested from hydroponic recipients and

oat plantlets were carefully washed with distilled water and then divided into roots and shoot for growth and biochemical analysis.

*2.2. Root Growth analysis*

Growth of oat root was determined by measuring the roots (Tennant, 1975) length. The roots were harvest in 0, 12, 24 and 36 hours.

*2.3. Antioxidant enzyme activities*

*2.3.1. Catalase:*

For the catalase assay, the oat root and shoot seedlings were prepared after the harvest in 0, 12, 24 and 36 hours of the exposure at aluminum. For the homogenization of fresh tissue material in a solution containing 50mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 10g $L^{-1}$ PVP, 0.2mM EDTA and 10 mL $L^{-1}$ Triton X-100, at a ratio of 1:5 (w/v). After the homogenate was centrifuged at 12 000 x g at 4ºC for 20 min, the supernatant was used to determine catalase activity, which was assayed according to the modified method of Aebi (1984) by monitoring the disappearance of $H_2O_2$ by measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mM $H_2O_2$ in 50 mM $KPO_4$ buffer (pH 7.0) and 30 µL extract. Catalase activity was expressed as ΔE/min/mg protein.

*2.3.2. Ascorbate peroxidase*

For determination of APX activity, oat root and shoot were homogenized in a 50 mM potassium phosphate buffer containing 1mM EDTA and 2% PVP, pH 7.8, at a ratio of 1:3 (w/v). The homogenate was centrifuged at 13 000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity, which was assayed according to the modified method of Zhu (2004). The

reaction mixture, at a total volume of 2 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0mM $H_2O_2$ and 100 µL extract. $H_2O_2$–dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm ($E$ = 2.8 $mM^{-1}$ $cm^{-1}$). Ascorbate peroxidase activity was expressed as µmol oxidized ascorbate /min/mg protein.

*2.3.3. Superoxide dismutase*

The activity of superoxide dismutase was assayed according to McCord and Fridovich (1969). About 200 mg fresh tissues were homogenized in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered and centrifuged at 22 000 x g for 10 min at 4ºC, and the supernatant was utilized for the assay. The assay mixture consisted of a total volume of 1 ml, containing glycine buffer (pH 10.5), 60 mM epinephrine and enzyme material. Epinephrine was the last component to be added. The adrenochrome formation over the next 4 min was recorded at 480 nm in a UV- Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. SOD activity was expressed as U SOD/mg protein. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. SOD has been found to inhibit this radical-mediated process.

*2.4. Estimation of lipid peroxidation*

The levels of lipid peroxides in the seedling were determined by measuring malondialdehyde (MDA) content from the thiobarbituric acid (TBA) reaction as described by El-

Moshaty (1993) in 0, 12, 24, and 36 hours of exposure at aluminum. The root and shoot were homogenized in 0.2 M citrate-phosphate buffer, pH 6.5, at a ratio of 1:20 (w/v). The homogenate was filtered through two layers of paper filter and then centrifuged at 20 000 x g at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. MDA values were expressed in nmol MDA/mg protein.

*2.5. Non-protein thiol content (NPSH)*

Non-protein thiol content in root and shoot (mg) was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). The reaction was read at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). Treated seedlings were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g at 4 ºC for 10 min, and supernatants were used for total thiol group determination. Non-protein thiol groups were determined in the fraction obtained after mixing 1 volume of supernatant with 1 volume of 4% trichloroacetic acid followed by centrifugation and neutralization (to pH 7.5) with 1 M Tris as described by Jacques-Silva et al. (2001). A standard curve using cysteine was used to calculate the content of thiol groups in samples, and was expressed as µmol $SH^{-1}$g fresh weight.

*2.6. Ascorbic acid content (AsA)*

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Briefly, root and shoot were homogenized in 10 mM Tris/HCl, pH 7.4, centrifuged at 4000 x g for 10 min and protein was removed by dilution with 1 volume of 4% trichloroacetic acid

followed by centrifugation. An aliquot of the sample was incubated at 37ºC in a medium containing 4.5 mg/ml dinitrophenylhydrazine, 0.6 mg/ml thiourea, 0.075 mg/ml $CuSO_4$, and 0.675 mol/l $H_2SO_4$ (final volume 1 ml). After 3 h, 1 ml of 65% $H_2SO_4$ was added and samples were read at 520 nm and were expressed as µg ASA/g fresh weight. A standard curve was constructed using ascorbic acid.

*2.7. Statistical analysis*

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± S.D. of at least three independent replicates. The mean differences were compared utilizing Tukey's range test.

## 3. Results

*3.1 Growth analysis*

The growth of roots was inhibited in sensitive seedlings when compared to control. Sensitive (UFRGS 93605) seedlings showed an inhibition of length root higher than other genotypes (Figure 1A). The growth of root was paralyzed after 12 h of exposure at aluminum. In the tolerant root (UFRGS 17–) and intermediate root (UFRGS 108) seedlings, no significant decrease of root growth was observed (Figure 1B and 1C). However, the growth of root intermediate was activated in 24 h when compared with control (Figure 1C).

| (A) | (B) |
|---|---|

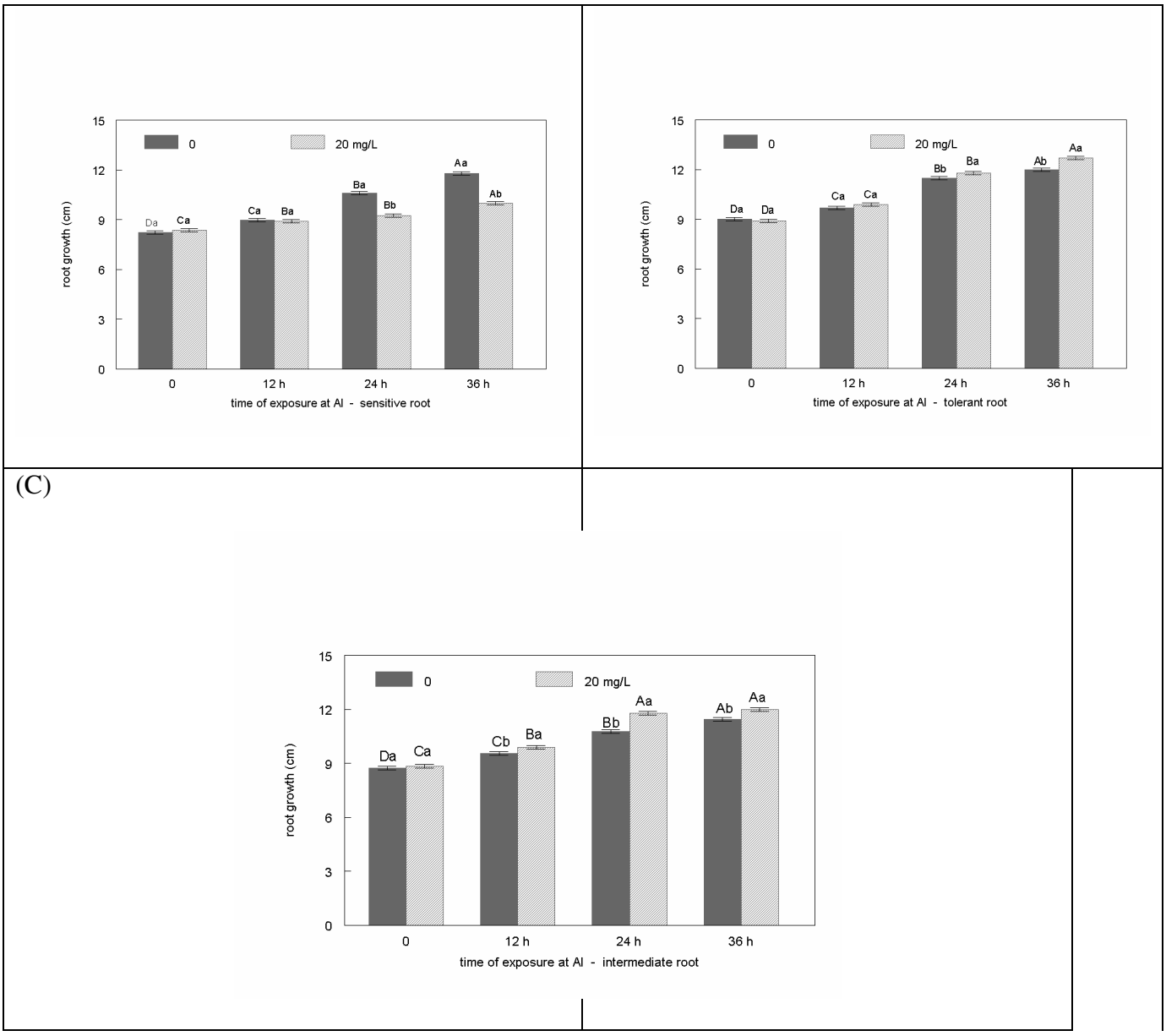


**Figure 1.** Effect of 20 mg/L $Al_2(SO_4)_3$ on root growth in different times 0, 12, 24, and 36 h. Sensitive root (**A**), tolerant root (B), and intermediate root (C). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

*3.2 Activities of antioxidant enzymes*

The activities of antioxidant enzymes, namely catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) were determined after 0, 12, 24, and 36 h for roots and shoots of sensitive, resistant, and intermediate genotypes (Figures 2 - 10). As a result of Al stress, root CAT activity increased after 12h in tolerant genotypes and the same occurred in the root and shoot intermediate genotypes (Figures 3A, 4A, and 4B). On the other hand, root and shoot CAT activity of the sensitive genotype showed no significant difference when compared to control after 12h (Figure 2A and 2B). The CAT activity was activated only after 24h of the exposure at aluminum in sensitive seedlings (Figure 2A and 2B). By contrast, shoot CAT activity was activated after 36h of exposure to aluminum in tolerant seedlings (Figure 3B).

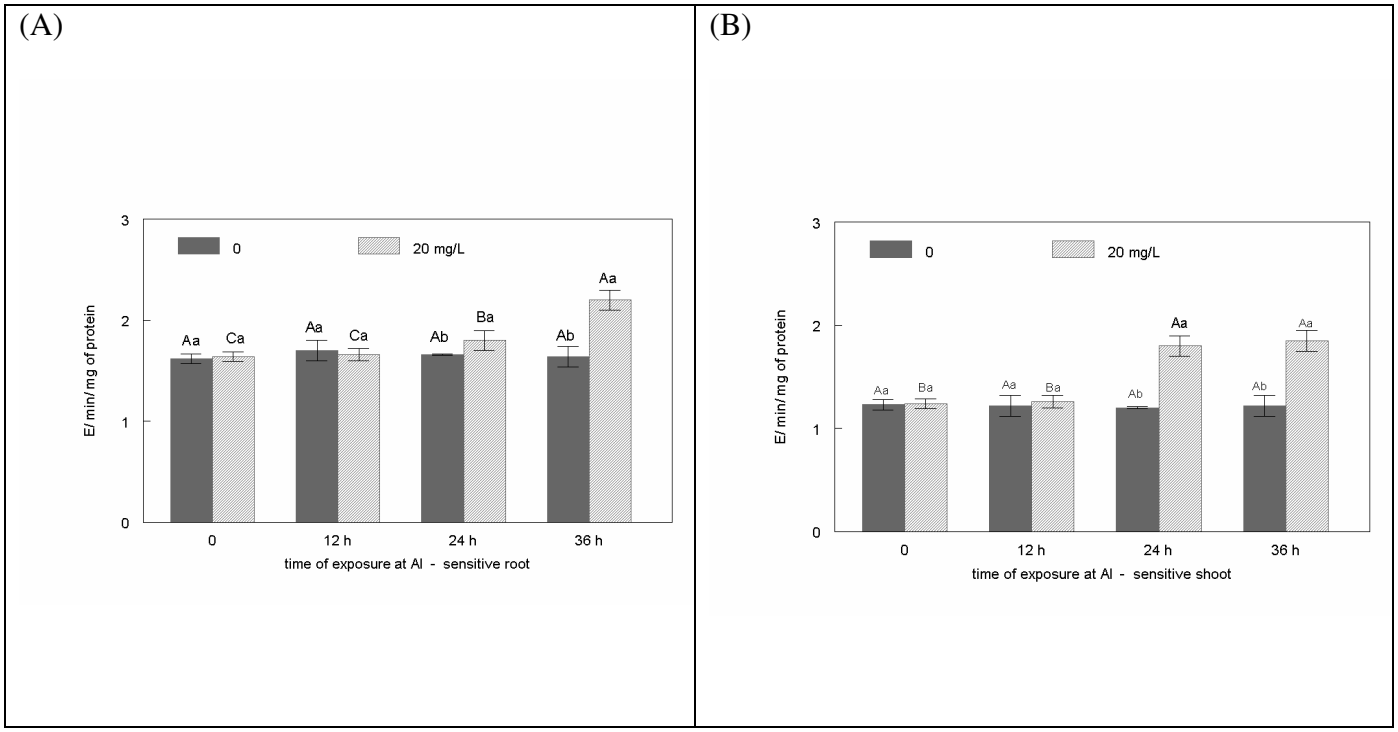


**Figure 2.** Effect of 20 mg/L $Al_2(SO_4)_3$ on Catalase (CAT) activity in different times 0, 12, 24, and 36 h. Sensitive root (**A**) and sensitive shoot (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

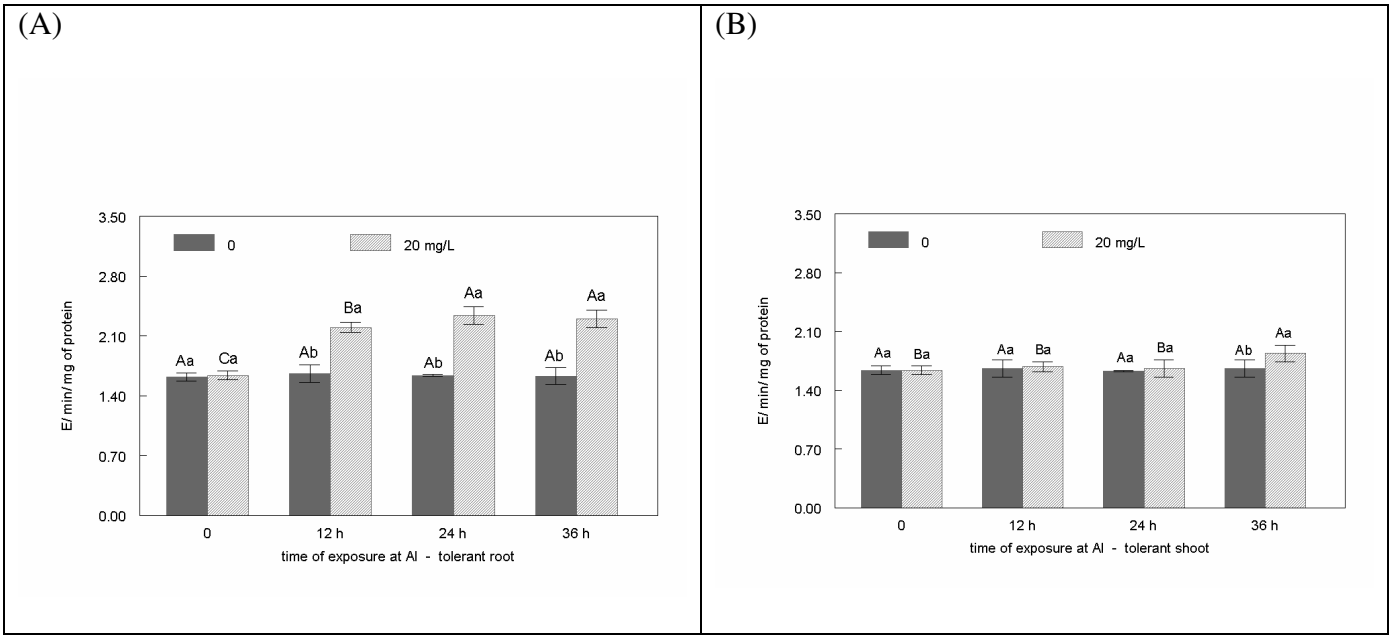


**Figure 3.** Effect of 20 mg/L $Al_2(SO_4)_3$ on CAT activity in different times 0, 12, 24, and 36 h. Tolerant root (**A**) and tolerant shoot (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24 and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

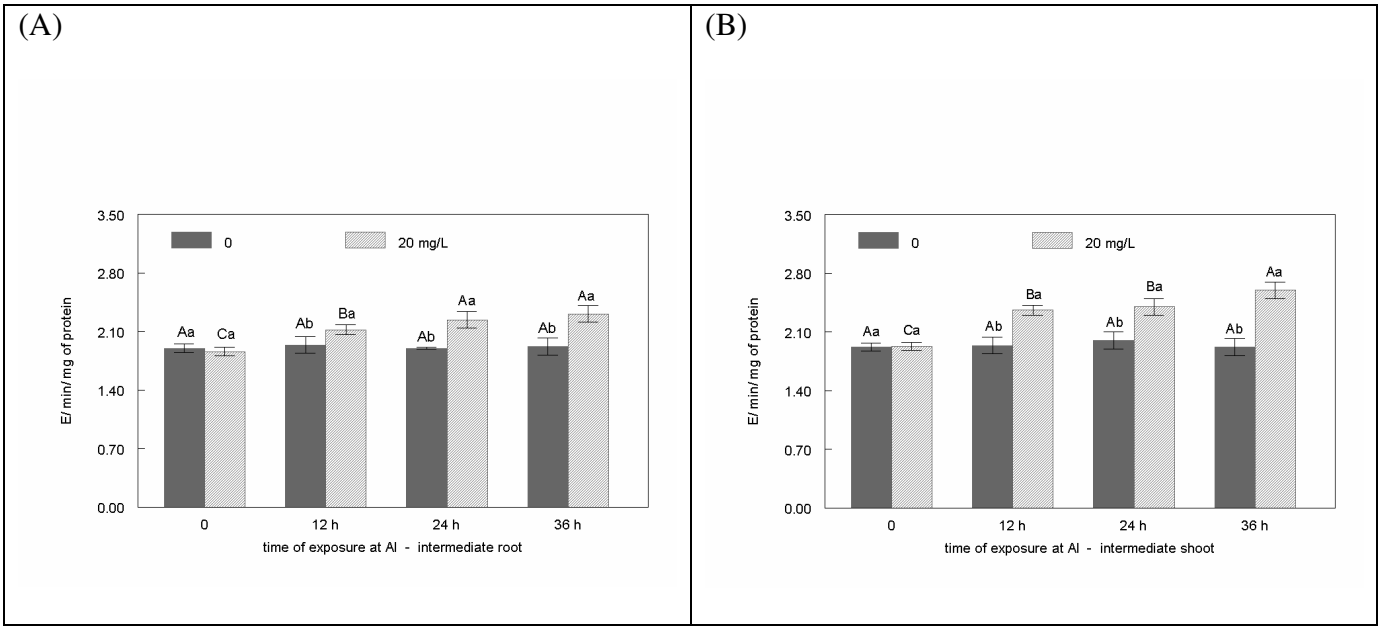


**Figure 4.** Effect of 20 mg/L $Al_2(SO_4)_3$ on CAT activity in different times 0, 12, 24, and 36 h. Intermediate root (**A**) and intermediate shoot (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

Root and shoot APX activities were increased after 24 h in the sensitive genotype (Figure 5A and 5B). Nonetheless, root and shoot APX activities were activated in tolerant and intermediate seedlings after 12 h (Figures 6A, 6B, 7A, and 7B). The maximum APX activity was 5.00 µmol ascorbate oxidized/min/mg protein after 36 h in intermediate shoots. In tolerant shoots the APX activity returned to basal levels after 36 h (Figure 6B).

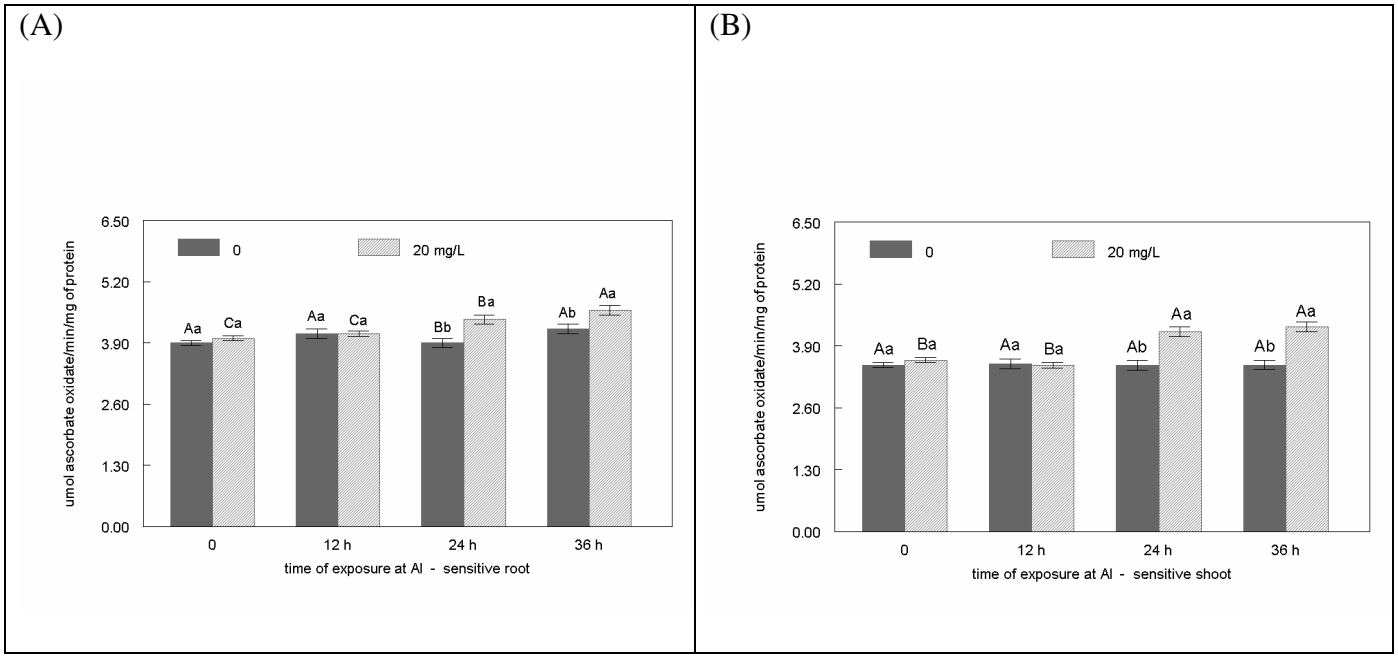


**Figure 5.** Effect of 20 mg/L $Al_2(SO_4)_3$ on Ascorate peroxidase (APX) activity in different times 0, 12, 24, and 36 h. Sensitive root (**A**) and sensitive shoot (B). Data represent the mean ± SD of three different experiments. Different from control, $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

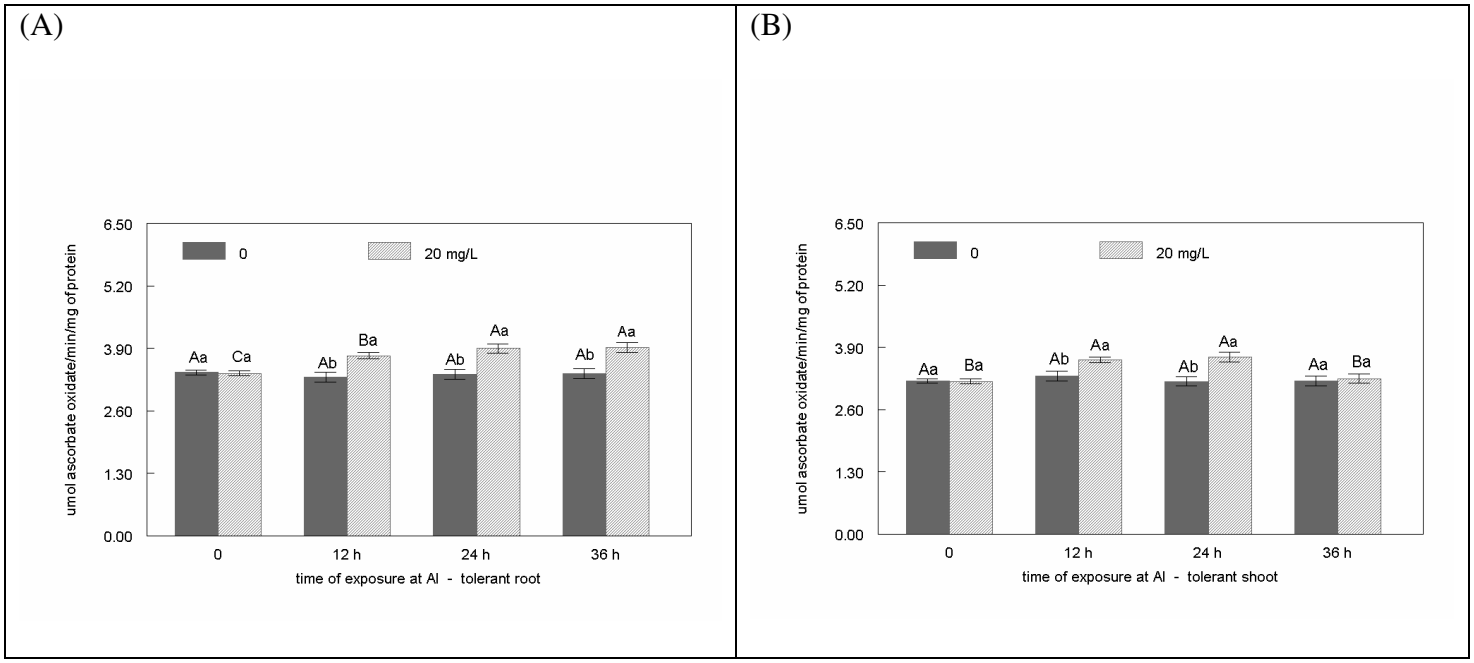


**Figure 6.** Effect of 20 mg/L $Al_2(SO_4)_3$ on APX activity in different times 0, 12, 24, and 36 h in tolerant roots (**A**) and tolerant shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

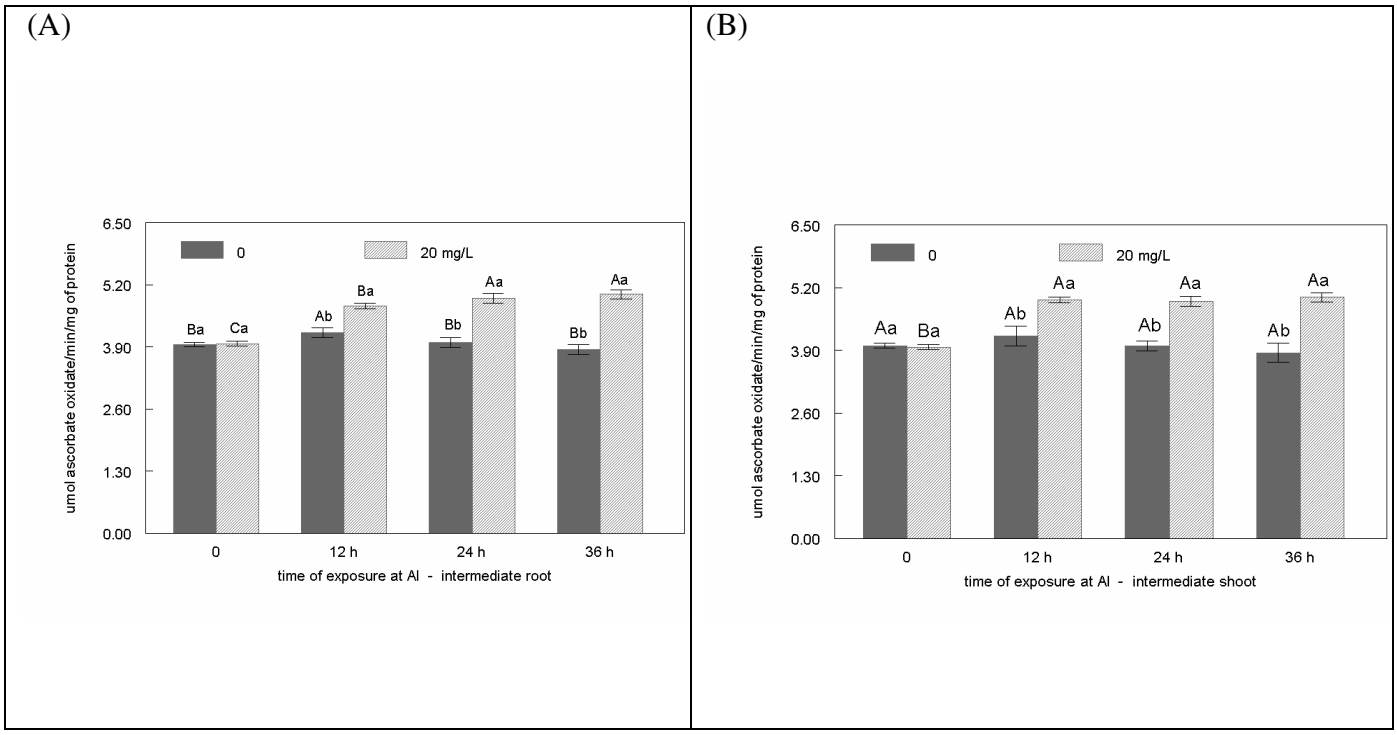


**Figure 7.** Effect of 20 mg/L $Al_2(SO_4)_3$ on APX activity in different times 0, 12, 24, and 36 h in intermediate roots (**A**) and intermediate shoots (B). Data represent the mean ± SD of three different experiments. Different from control

at p<0.05. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

Figures 8, 9, and 10 show the SOD activity of oat seedlings. Root and shoot SOD activities were increased after 36 h in the sensitive seedlings (Figure 8A and 8B). Root SOD activity increased in tolerant and intermediate genotypes after 12 h of exposure of aluminum (Figure9A and 10A). There was no significant change in the shoot SOD activity in tolerant seedlings (Figure 9B). Nonetheless, shoot SOD activity in the intermediate seedlings was increased after 36 h (Figure 10B). The maximum SOD activity was 3900 U SOD/mg protein after 36 h in the shoots of the intermediate genotype.

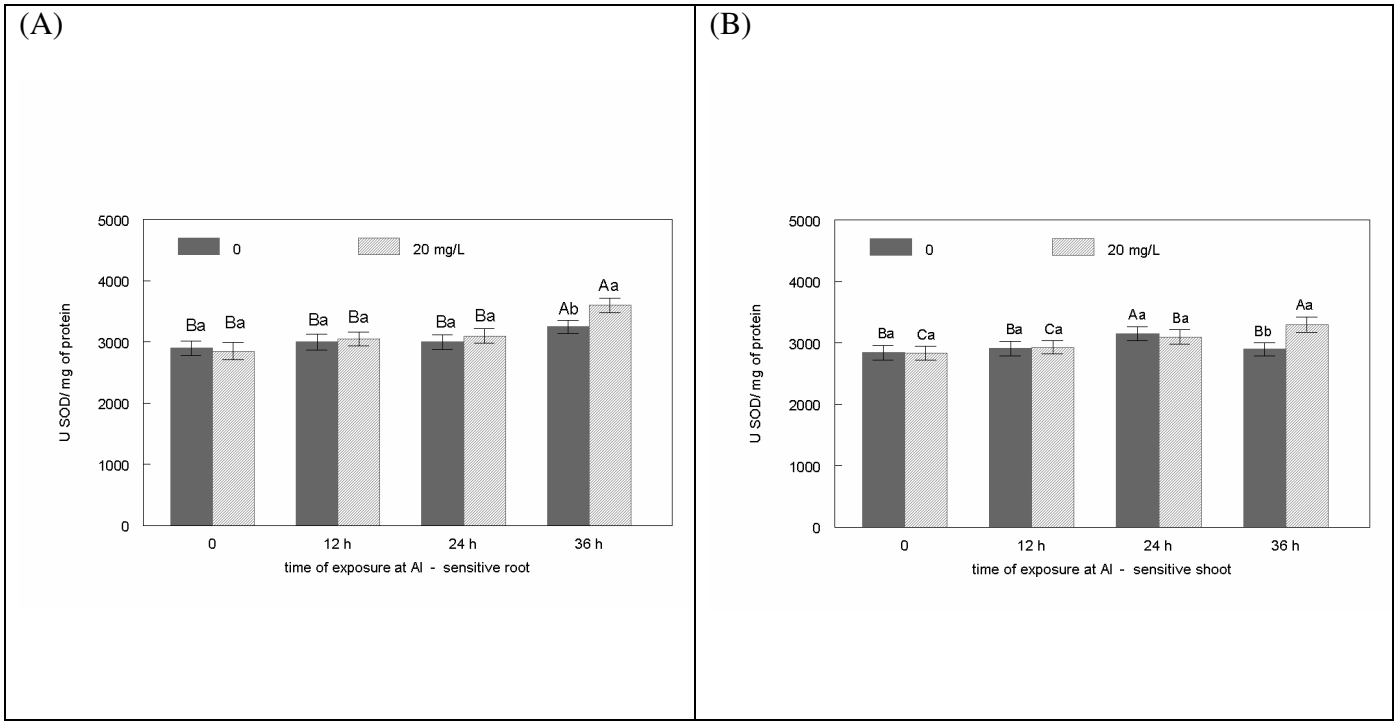


**Figure 8.** Effect of 20 mg/L $Al_2(SO_4)_3$ on Superoxide dismutase (SOD) activity in different times 0, 12, 24, and 36 h in sensitive roots (**A**) and sensitive shoots (B). Data represent the mean ± SD of three different experiments. Different from control at p<0.05. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

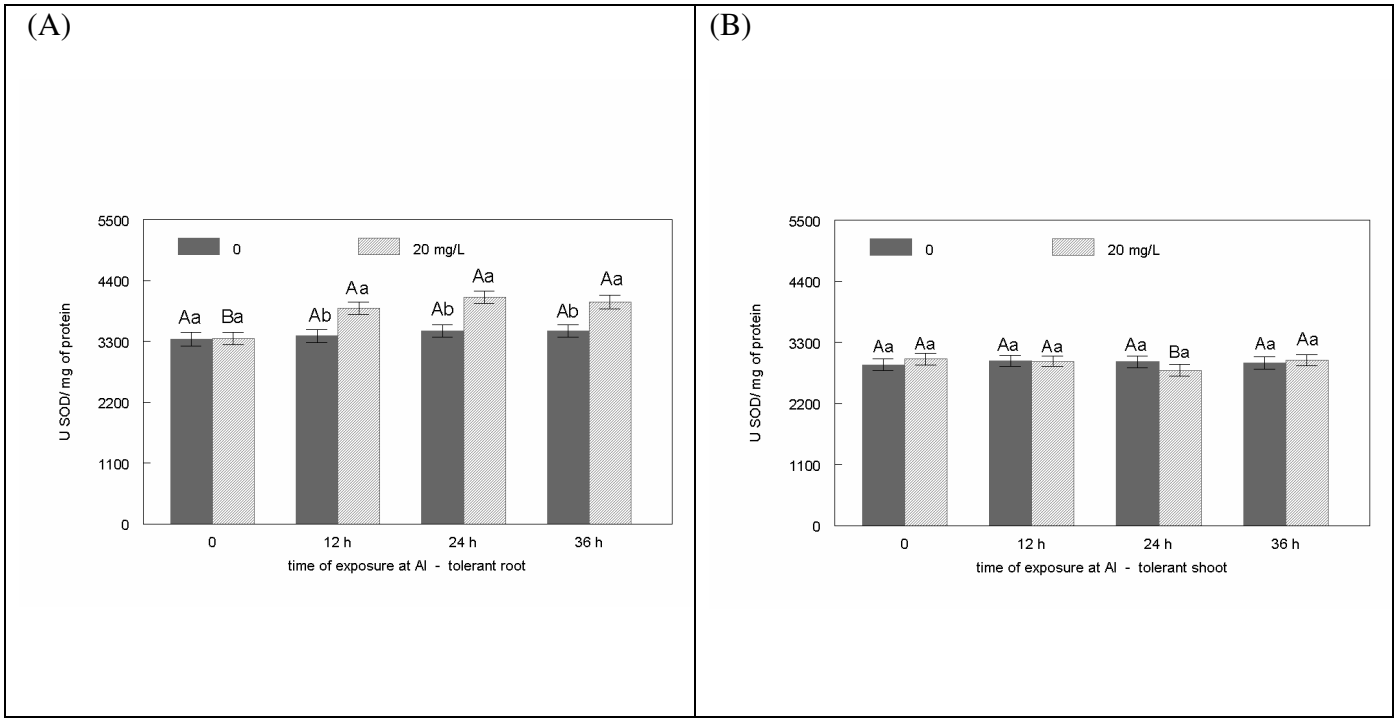


**Figure 9.** Effect of 20 mg/L $Al_2(SO_4)_3$ on SOD activity in different times 0, 12, 24, and 36 h in tolerant roots (**A**) and tolerant shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

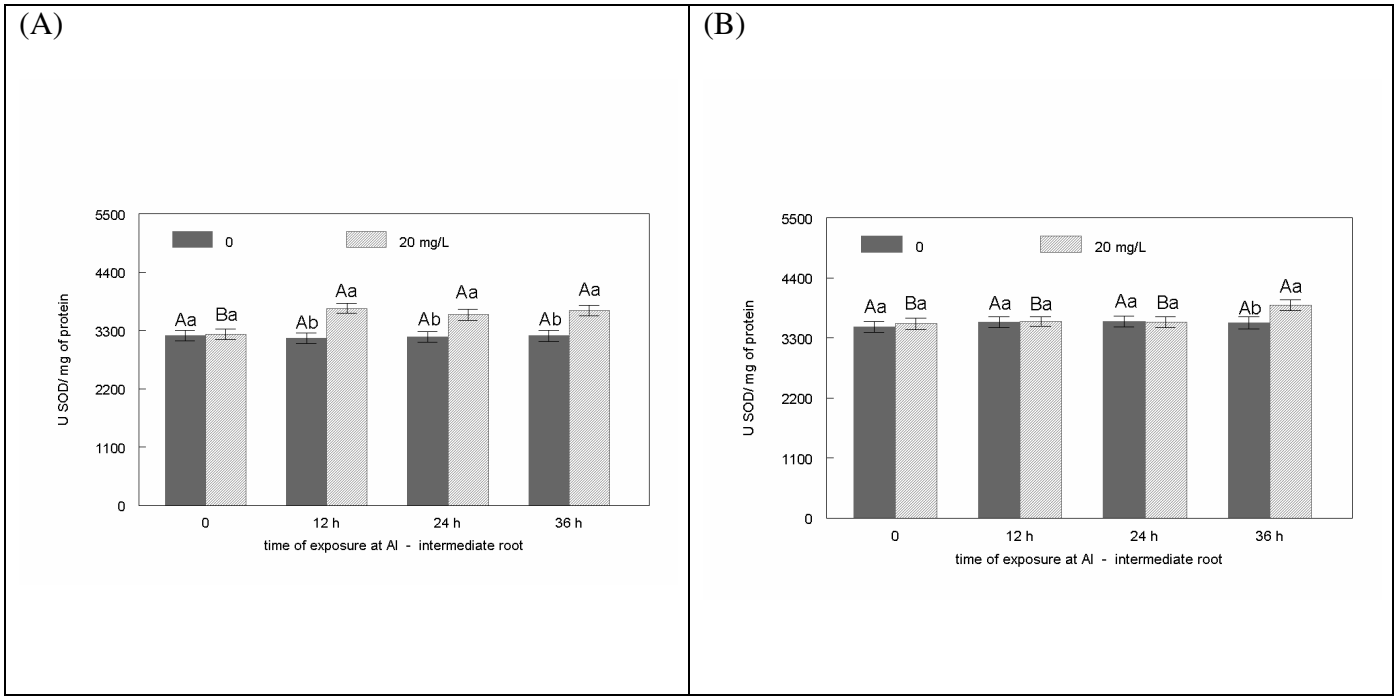

**Figure 10.** Effect of 20 mg/L $Al_2(SO_4)_3$ on SOD activity in different times 0, 12, 24, and 36 h in intermediate roots (**A**) and intermediate shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

*3.3 Lipid peroxidation*

Al showed an increase in the root and shoot MDA content in sensitive seedlings after 12 h of exposure to aluminum when compared to control (Figure 11A and 11B). There was no significant change at MDA content in tolerant and intermediate shoots and roots (data not showed)

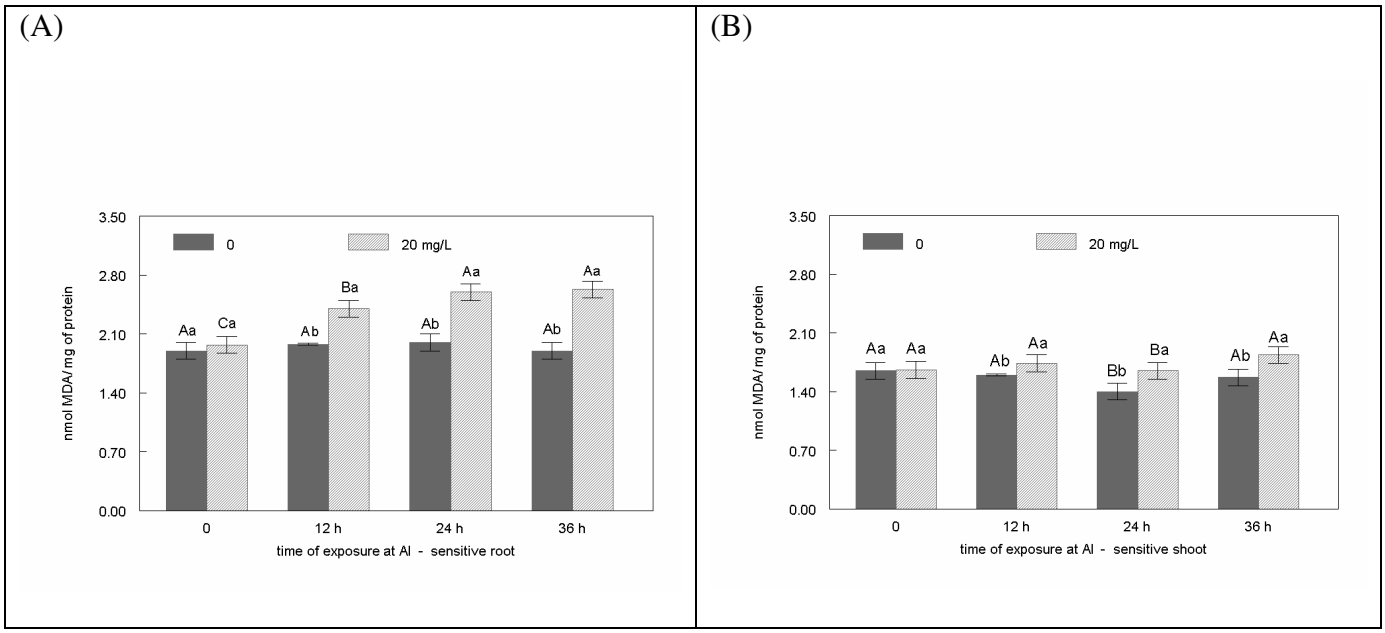


**Figure 11.** Effect of 20 mg/L $Al_2(SO_4)_3$ on levels of lipid peroxidation in different times 0, 12, 24 and 36 h in sensitive roots (**A**) and sensitive shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

*3.4. Ascorbic acid (AsA) and non-protein thiol group (NPSH) concentrations*

NPSH content increased after 12 h of Al exposure in tolerant and intermediate roots (Figure 12A and 12B). There was no significant change in NPSH content in the shoots of tolerant and intermediate genotypes. Besides, there was no significant change in NPSH content in the roots of sensitive seedlings (data not showed).

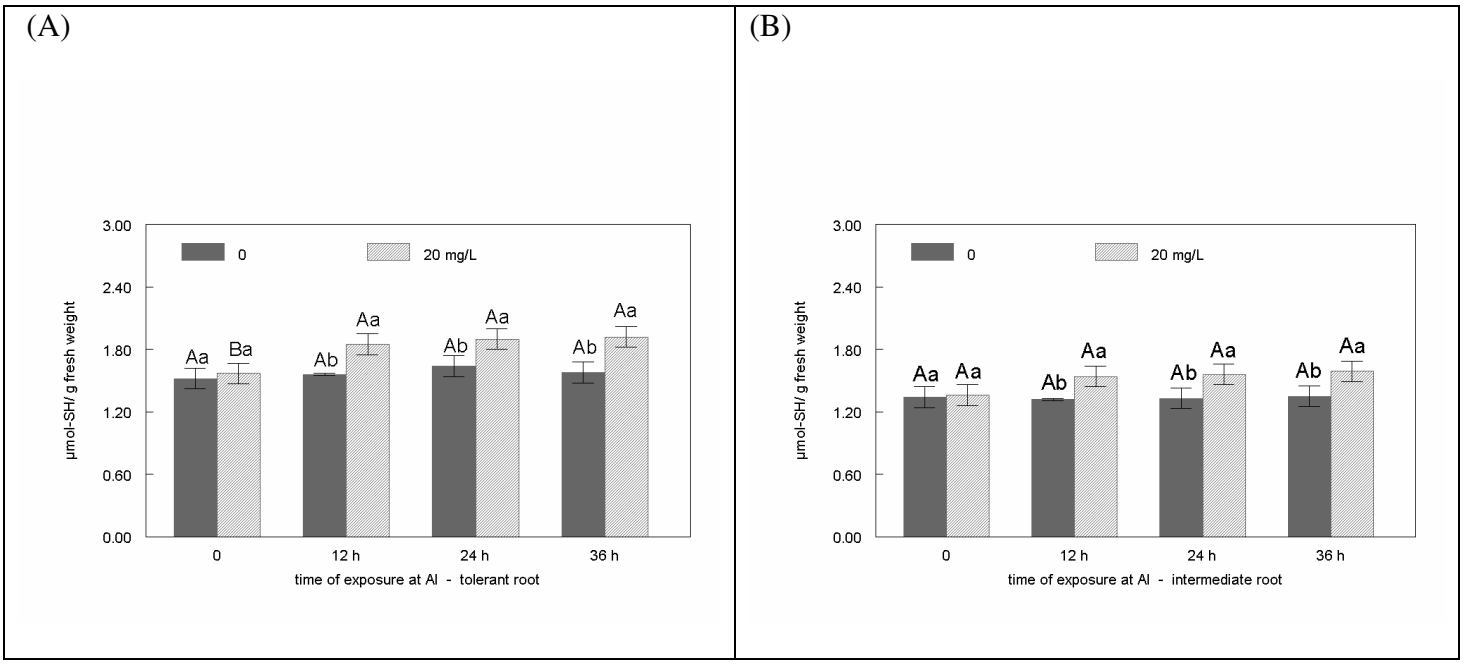


**Figure 12.** Effect of 20 mg/L $Al_2(SO_4)_3$ on non-protein thiol group (NPSH) concentrations in different times 0, 12, 24, and 36 hin tolerant roots (**A**) and intermediate roots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

AsA content increased after 12 h of Al exposure in tolerant and intermediate roots and shoots (Figures 13A, 13B, 14A, and 14B). ASA content increased to a greater extent in shoots than in roots during Al stress in intermediate seedlings (Figure 14B). There was no significant change in roots and shoots of sensitive seedlings (data not showed).

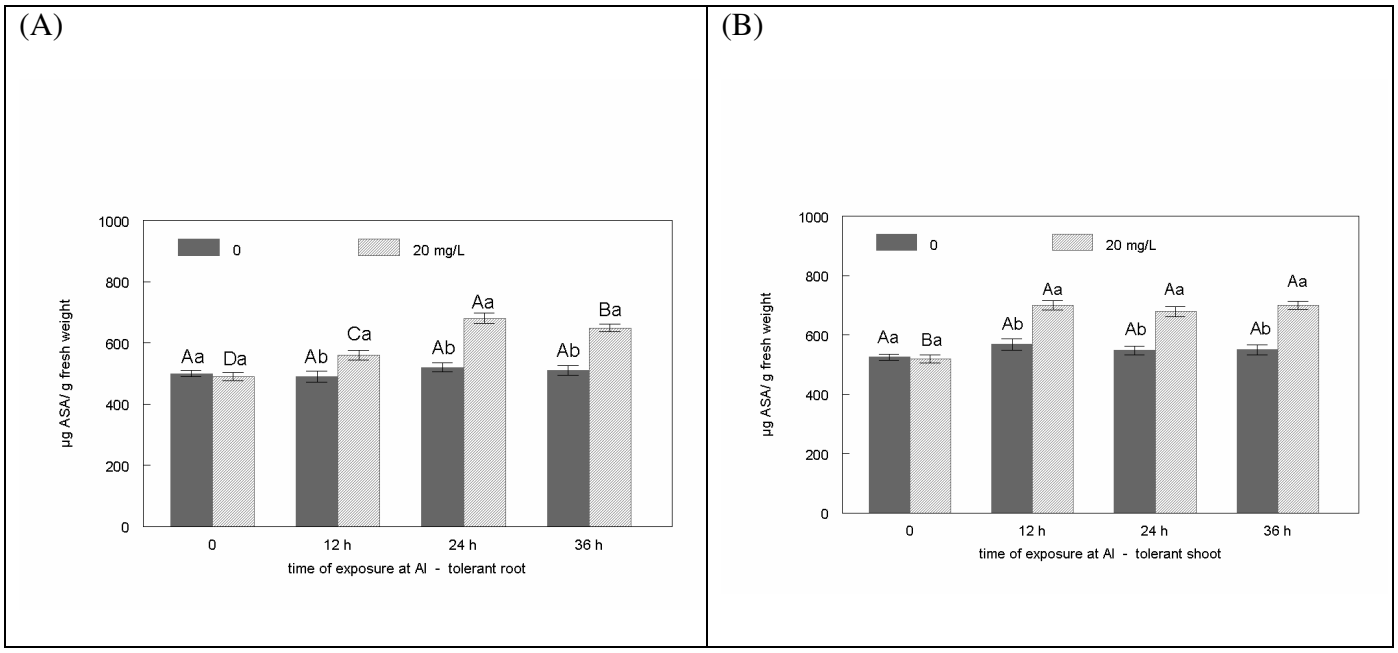


**Figure 13.** Effect of 20 mg/L $Al_2(SO_4)_3$ on Ascorbic acid (AsA) levels in different times 0, 12, 24, and 36 hin tolerant roots (**A**) and tolerant shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

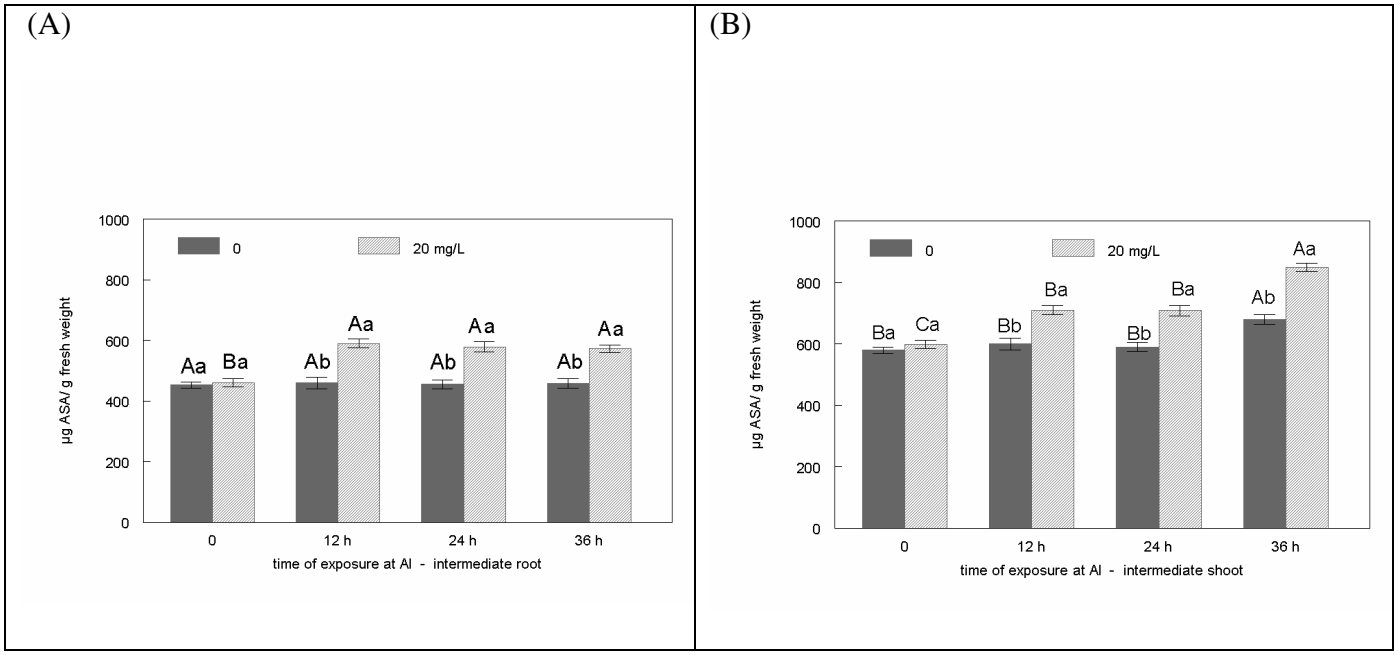

**Figure 14.** Effect of 20 mg/L $Al_2(SO_4)_3$ on Ascorbic acid (AsA) levels in different times 0, 12, 24, and 36 hin intermediate roots (**A**) and intermediate shoots (B). Data represent the mean ± SD of three different experiments. Different from control at $p<0.05$. A=difference in different times (0, 12, 24, and 36 h) and a=difference at concentrations (0 and 20 mg/L) in the same time.

## 4. Discussion

Our study revealed the importance of the effects of oxidative stress and the antioxidant defense mechanism after Al exposure in three genotypes of oats. It is clear that Al toxicity depends not only on the concentration and exposure time but also on the genotype used.

Inhibition of root elongation is the primary Al toxicity symptom (Boscolo et al., 2003; Dipierro et al., 2005, Rossielo et al., 2006) and has been used as suitable parameter for assigning genotypic differences in Al tolerance (Barceló, 2009). The presence of 20 mg/L of Al in solution inhibited significantly root growth only in the sensitive genotype (Figure 1), indicating a different genetic sensitivity to Al supply. This effect firstly occurred at 12 h of Al exposure and was observed throughout the experiment. Root growth reductions may, therefore, be the result of elevated Al concentrations in the medium, leading to the disruption of cell functioning as proposed by Tamás et al. (2006) and Giannakoula et al. (2008), including apoplastic lesions, interactions within the cell wall, the plasma membrane, or the root symplasm.

The most prominent indicator of plasma membrane damage is Al-stimulated lipid peroxidation (Silva et al., 2002). In the present study data for root growth (Figure 1) and lipid peroxidation (Figure 11) suggest that lipid peroxidation in the sensitive genotype may be a direct effect of Al toxicity on root growth, indicating that Al induced oxidative stress and, as a result, irreversible damage to tissue development functions. Similar results were obtained with peas (Yamamoto et al., 2001), soybeans (Cakmak & Horst 1991), and maize (Boscolo et al., 2003). In the present study, lipid peroxidation in Al-sensitive genotype seems to be an early symptom induced by Al toxicity, indicating that lipids are the primary cellular target of the oxidative stress.

In the tolerant and intermediate genotypes, Al treatment provoked lipid peroxidation neither in the roots nor in the shoots at any time of exposure (data not showed). These results indicate that the antioxidant system in this genotype may be more efficient to protect membrane lipids of reactive oxygen species (ROS). Plants respond to metal stress by physiological and biochemical strategies. Anti-oxidation mechanisms of the cell include the enzymatic ROS-scavenging and non-enzymatic antioxidants (ascorbic acid, ASA and non-protein thiol groups (NPSH)), which function in order to interrupt the cascades of uncontrolled oxidation in each organelle. In tolerant and intermediate seedlings the antioxidant system was activated after 12 h of exposure to aluminum while in sensitive seedlings the antioxidant system was activated after 24 or 36 h of exposure. The activation speed of the antioxidant system can be relevant to prevent the aluminum toxicity. Glutathione is the predominat NPSH, redox-buffer, phytochelatin precursor and substrate for keeping ascorbate in its reduced form in the ascorbate-gluthathione pathway (Smirnoff, 2000). In sensitive seedlings the levels of ASA and NPSH were absent or in minor extent when compared to the levels found in tolerant and intermediate seedlings. These alterations occurred concomitantly with the inhibition of root elongation and the increase of lipid peroxidation. This may indicate the reductions in the concentration and in the activity of antioxidants were contributing to enhance damage provoked by Al treatment.

Antioxidants are present in plant tissue in milimolar concentrations and under stress conditions their levels increase (Mitler, 2002) as an attempt to defend the plant from this stress.

Plants can also tolerate Al toxicity by inducing antioxidant defense systems, and genes that activate antioxidant system may have relation with genes that provide tolerance to aluminum (Morita et al., 2008). In tolerant and intermediate seedlings, Al presence for 12 h provoked an increase only in ASA concentration. This increase may have contributed to free radicals and ROS detoxification and suggests their active participation in Al detoxification, since root growth

inhibition and lipid peroxidation were not observed in these genotypes. Therefore, the tolerant, intermediate, and sensitive genotypes differed in the expression of the amount, type of antioxidants and speed of activation of the antioxidant system, suggesting a varying capacity of these genotypes to deal with oxidative stress, which resulted in varying sensitivity and tolerance to Al.

These results show that the cellular redox status of oat genotypes seems to be affected by Al, and oxidative stress may be an important mechanism involved in Al tolerance. Al causes oxidative stress probably through indirect mechanisms such as interaction with the antioxidant defense, disruption of the electron transport chain, and induction of lipid peroxidation. Further researches on the indirect mechanisms of Al-induced oxidative stress are required to reveal the underlying molecular and biochemical events involved.

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**Manuscrito IV -** Physiological and oxidative stress responses of two *Avena sativa* genotypes and *Cucumis sativus* seedlings to aluminum in nutrient solution

# Physiological and oxidative stress responses of two *Avena sativa* genotypes and *Cucumis sativus* seedlings to aluminum in nutrient solution

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**ABSTRACT**

Oat and cucumber seedlings were grown in a nutrient solution (pH 4.0) with 0 and 20 mg/L Al. At 12, 24, and 36 h after aluminum (Al) addition, growth (root length), the biochemical parameters catalase (CAT - E.C. 1.11.1.6), ascorbate peroxidase (APX - E.C. 1.11.1.11), and lipid peroxidation were determined. Regardless of the exposure time, the root length of the tolerant genotype did not decrease with any Al treatments. The root length of the sensitive genotype and cucumber seedlings were decreased when compared with controls. Al supply caused lipid peroxidation only in the Al-sensitive genotype and cucumber root (at 12, 24, and 36 h). In the sensitive genotype and cucumber seedlings CAT was activated only at 36 h, however, the CAT activity was compensated by the increased activity of APX at 24 and 36 h. In the tolerant genotype CAT and APX were activated at 12, 24, and 36 h. Data for root growth suggested that lipid peroxidation in the sensitive genotype of oat and cucumber root may be an effect of Al toxicity and the increased production of reactive oxygen species (ROS). The antioxidant enzymes were not efficient in the removal of the excess of ROS. Tolerant and sensitive genotypes differ in the expression of the amount and type of antioxidants, as well as in the speed of activation of the antioxidant system, suggesting a varying capacity of these genotypes to deal with oxidative stress, which resulted in varying sensitivity and tolerance to Al. In addition, the cucumber root showed results similar to sensitive oat and may be classified as sensitive to Al, since it did not provide mechanisms to tolerate aluminum toxicity. Further research on the mechanisms of tolerance to Al are required to reveal the underlying molecular and biochemical events involved.

**Keywords:** *Avena sativa*, aluminum, *Cucumis sativus*, lipid peroxidation, CAT, APX, root growth.

## 1. Introduction

Oat, an important Upper Midwest small grain crop, is often the main grain for young animals, poultry, and dairy cows. The high nutritional value of oat as feed has long been recognized (David et al., 2008; Shimabukuro et al., 2001). The widely cultivated species of oat, *Avena sativa* L*,* is very sensitive to abiotic stress and studies have indicated that the Avena species possesses genetic diversity to abiotic stress (Federizzi et al., 2000). On a global scale, acid soils comprise a surface estimated at 37.8 million $Km^2$ (Eswaran et al., 1997). Moreover, soil acidification has been increased around the world as a result of human activities, atmospheric liberation of industrial contaminants, and continuous use of ammonia – and amide – containing fertilizers (Rengel & Zhang, 2003). Such soils are a major constraint to agricultural production in Latin America. They tend to contain low levels of essential cations, nitrogen, and plant- available phosphate in combination with high levels of phytotoxic aluminum (Al) (Rao et al., 1993), which in this study is $Al^{3+}$. As soil pH decreases, active Al in the soil shows a great increase, resulting in toxicity to plants. In Brazil, roughly 60% of soils with potential for agricultural activity present Al toxicity (Abreu et al., 2003).

The $Al^{3+}$ cation is toxic to many plants at micromolar concentrations, affecting primarily the normal functioning of roots, with a rapid inhibition of growth (Ryan et al., 1993), which may occur within minutes or hours of exposure to Al. Severe Al phytotoxicity reduces and damages the root system, which results in poor nutrient and water acquisition and transport, leading, consequently, to nutrient deficiency and crop yield reduction (Kochian, 1995).

During evolution, plants have developed numerous mechanisms that allow for survival in acid soils with higher availability of Al (Kochian, 1995). As a result of a selection pressure, inter– and intra species differences in response to Al are widely observed in the plant kingdom. Moreover, great differences in the tolerance to Al have been reported among genotypes of the same species. Over the last several years, a great diversity of results obtained in physiologic and molecular mapping studies have shown that plant tolerance to Al toxicity is a complex multigenic characteristic that can involve several mechanisms of tolerance (Kochian, 1995; Barceló & Poschenrieder, 2002; Kochian et al., 2004). These induce external (or exclusion) and internal detoxification mechanisms, such as the immobilization of Al by cell wall components (Zhang et al., 1997), the exudation of organic acids for the detoxication of Al in the apoplast (Ryan et al., 2001), and compartment in the vacuole. In this context, the selection of varieties that are productive and tolerant to Al toxicity should be considered a very important component of strategies for dealing with acid soils.

A wide range of cellular responses occur when plants are exposed to a variety of environmental stresses such as freezing, drought, salinity, and metal toxicity. It has been suggested that $Al^{3+}$, the most toxic of the soluble forms of Al, induces oxidative stress, since this ion is related to an increased ascorbate peroxidase activity, an increased level of ascorbic acid, and $H_2O_2$ concentration (Dipierro et al., 2005).

Oxidative stress is characterized by the production of ROS that is able to initiate a free radical chain reaction. ROS can cause oxidative damage to biomolecules such as lipid, protein hydrolysis, and even DNA strand breakage (Schüntzendübel & Polle, 2002). The antioxidant system is responsible for scavenging excess free radicals caused by environmental stresses. Studying the major components of the antioxidant system under Al stress, it is possible to

ascertain whether Al induces oxidative stress, and whether it is involved in Al-tolerance mechanisms.

The objective of the present study was therefore to investigate and compare some physiological and oxidative stress responses of two oat seedling genotypes and cucumber seedlings exposed to Al in nutrient solution as well as to evaluate the existence of tolerance mechanisms in cucumber when compared with oat genotypes.

## 2. Material and Methods

### 2.1. Plant material and growth conditions:

*Avena sativa* L. seeds of two genotypes (UFRGS 17 Al-tolerant; UFRGS 930598 Al-sensitive), commonly known as oat, provided by Programa de Melhoramento Genético de Aveia from the Universidade Federal do Rio Grande do Sul (UFRGS) and *Cucumis sativus* L. seeds (cv. Aodai), commonly known as cucumber, provided by Feltrin Ltd. (Santa Maria, RS) were germinated in plastic recipients (1000 mL) containing filter paper. No nutritive solution was added. The seedlings made use of the seed nutrition in the initial stage of development, and in a previous experiment, it was verified that up to the tenth day the plants did not suffer any nutrient deficiency (data not shown). Three-day-old plantlets from plastic recipients with filter paper were transferred into plastic boxes (10 000 mL) with polystyrene plates with holes that were used as a physical support for the plants; roots were submerged in aerated full nutrient solution of low ionic strength. The nutrient solution had the following composition (mg $l^{-1}$): 85.31 N; 11.54 S; 97.64 Ca; 23.68 Mg; 104.75 K; 176.76 Cl; 0.27 B; 0.05 Mo; 0.01 Ni; 0.13 Zn; 0.03 Cu; 0.11 Mn and 2.68 Fe. Evaporate and transpired water was continuously replaced with distilled water and the nutrient solution was completely renewed every week. Two different $Al_2(SO_4)_3$ treatments (0, 20 mg $L^{-1}$) were applied randomly. The medium pH was adjusted to 4.0 daily.

After 12, 24, and 36 hours of $Al^{3+}$ exposure, 24 plantlets per replicate (each treatment consisted of three replicates) were randomly harvested from hydroponic recipients. Oat and cucumber plantlets were carefully washed with distilled water and then roots were removed for growth and biochemical analysis.

### 2.2. Root growth analysis

The growth of oat and cucumber roots was determined by measuring the root (Tennant, 1975) length. Roots were harvest at 0, 12, 24, and 36 h.

### 2.3. Antioxidant enzyme activities

#### 2.3.1. Catalase

For the catalase assay, oat root and cucumber root seedlings were prepared after the harvest at 0, 12, 24, and 36 h of exposure to Al. For the homogenization of fresh tissue material in a solution containing 50mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 10g $L^{-1}$ PVP, 0.2mM EDTA, and 10 mL $L^{-1}$ Triton X-100, at a ratio of 1:5 (w/v) was used. After the homogenate was centrifuged at 12 000 x g at 4ºC for 20 min, the supernatant was used to determine catalase activity, which was assayed according to the modified method of Aebi (1984) by monitoring the disappearance of $H_2O_2$ measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mM $H_2O_2$ in 50 mM $KPO_4$ buffer (pH 7.0) and 30 μL extract. Catalase activity was expressed as ΔE/min/mg protein.

### 2.3.2. Ascorbate peroxidase

For the determination of the ascorbate peroxidase (APX) activity, oat and cucumber roots were homogenized in a 50 mM potassium phosphate buffer containing 1mM EDTA and 2% PVP, pH 7.8, at a ratio of 1:3 (w/v). The homogenate was centrifuged at 13 000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity, which was assayed according to the modified method of Zhu (2004). The reaction mixture, at a total volume of 2 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0mM $H_2O_2$, and 100 µL extract. $H_2O_2$–dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm ($E$ = 2.8 $mM^{-1}$ $cm^{-1}$). APX activity was expressed as µmol oxidized ascorbate /min/mg protein.

### 2.4. Estimation of lipid peroxidation

The levels of lipid peroxides in the seedlings were determined by measuring malondialdehyde (MDA) content from the thiobarbituric acid (TBA) reaction as described by El-Moshaty (1993) at 0, 12, 24, and 36 h of exposure to Al. Oat and cucumber roots were homogenized in 0.2 M citrate-phosphate buffer, pH 6.5, at a ratio of 1:20 (w/v). The homogenate was filtered through two layers of paper filter and then centrifuged at 20 000 x g at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. MDA values were expressed in nmol MDA/mg protein.

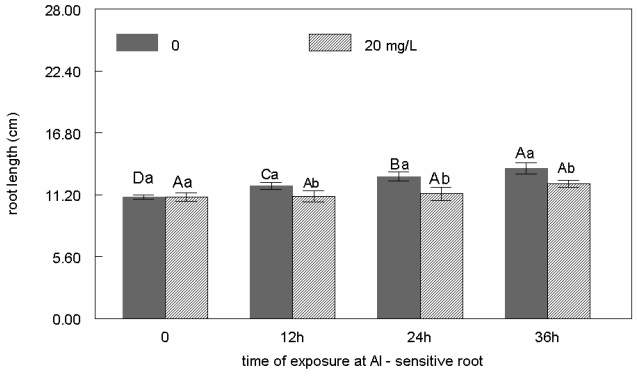
### 2.7. Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. Results are the means ± S.D. of at least three independent replicates. Mean differences were compared utilizing Tukey's range test.

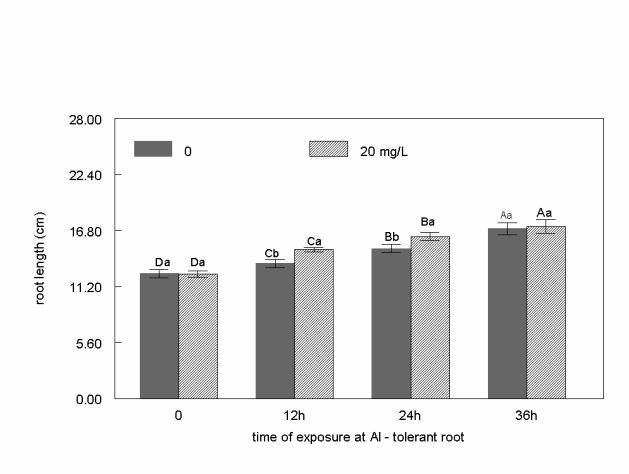
## 3. Results

### 3.1. Aluminum effects on growth

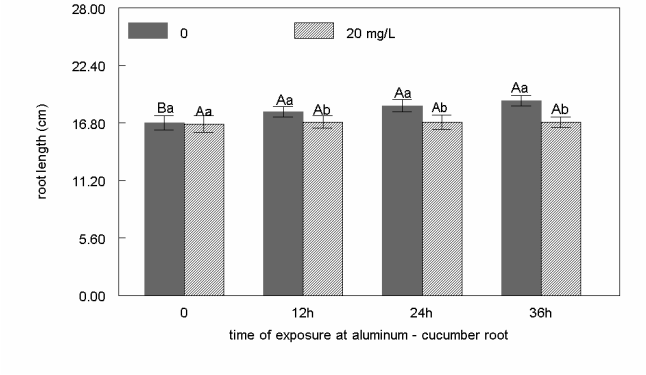
The effects of Al on the root growth of oat genotypes and cucumber roots are showed in Figure 1. Significant differences were observed in the root length among the oat genotypes. The cucumber root was inhibited after 12, 24, and 36 h of exposure to Al (Fig. 1C). Sensitive oat seedlings also had their root inhibited after 12, 24, and 36 h of exposure to Al (Fig. 1A). However, the tolerant genotype seedlings had an increase of the root growth after 12 and 24 h (Fig. 1B).




(A)




(B)




(C)

**Figure 1** – Time-course of Al inhibition of root length (Oat – sensitive genotype (A) and tolerant genotype (B), and cucumber (C) exposed to different concentrations of Al in nutrient solution. Data are the mean ± S.D. of three different replicates.

### 3.2 Al effect on lipid peroxidation

A significant increase in MDA content in both cucumber and sensitive oat roots was observed (Fig. 2A and 2B). The highest increase in MDA content was seen in roots of cucumber after the first 12 h, 4.8 nmol MDA/ mg of protein (Fig. 2B). Such effect was also observed at later times. At 24 and 36h an increase in MDA content occurred at 20 mg/L Al, indicating that, after a prolonged incubation at these highly toxic Al levels, the toxicity remained severe. In the

tolerant root, Al treatment did not provoke lipid peroxidation at any time of exposure (data not show).

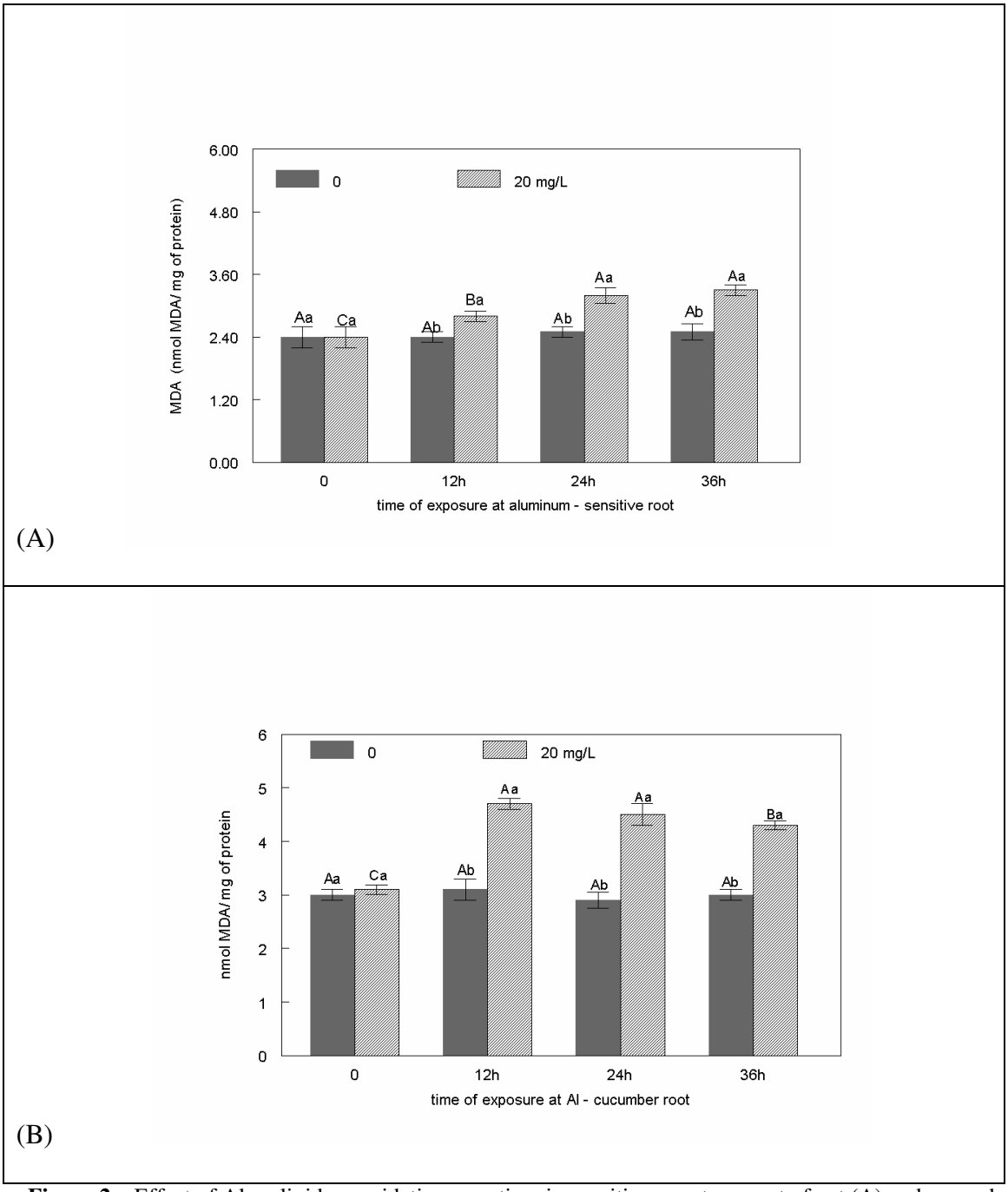
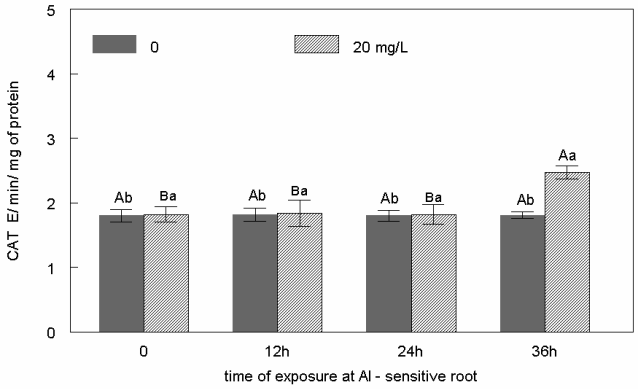



**Figure 2** – Effect of Al on lipid peroxidation over time in sensitive genotype root of oat (A) and cucumber root (B). Data are the mean ± S.D. of three different replicates.

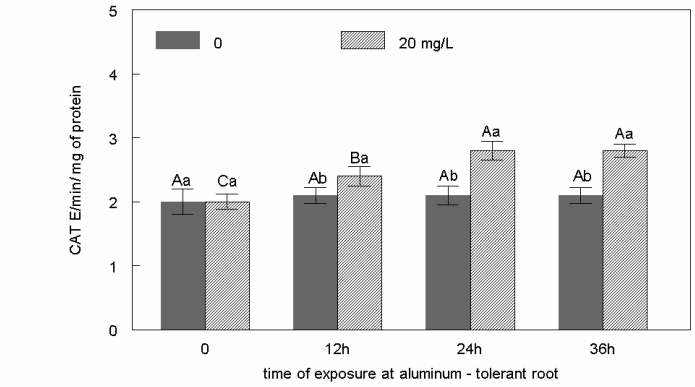
### 3.3. Effects of antioxidant enzymes

Among the enzymatic antioxidants in plants, catalase (CAT) may transform peroxides into non-reactive species (Fig 3). The CAT activity was greater for the Al- tolerant genotype (Fig 3B). Moreover, in both sensitive genotype and cucumber root, CAT activity was increased only after 36 h (Fig 3A and 3C). At 12 and 24 h after Al treatment, CAT activity remained unchanged in both sensitive oat roots and cucumber roots.

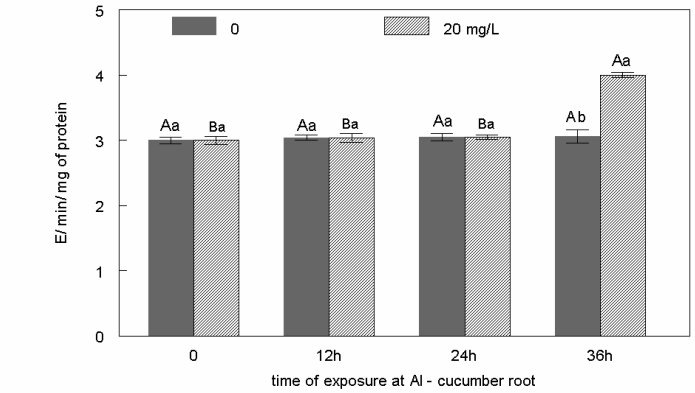
Ascorbate peroxidase (APX) activity (Fig. 4) was greater in tolerant roots. The APX activity was increased after the first 12 h of exposure to Al (Fig 4A). However, in roots of cucumber and sensitive oat the APX activity was increased after 24 and 36 h (Fig. 4B and 4C)




(A)

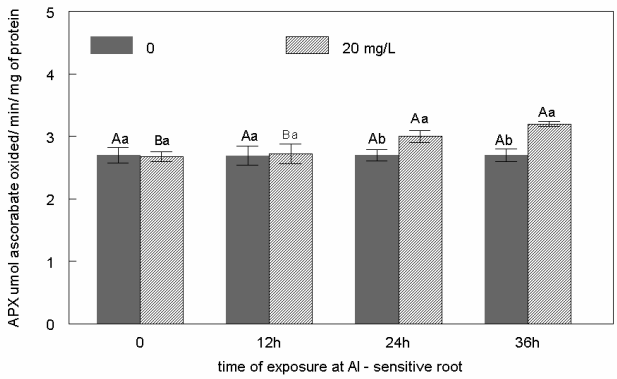



(B)




(C)

**Figure 3** – Effect of Al on the catalase (CAT) activity over time in sensitive and tolerant oat roots and cucumber roots. CAT activity was expressed as ΔE/ min/ mg of protein. Data are the mean ± S.D. of three different replicates.


APX umol ascorabate oxided/ min/ mg of protein
0
20 mg/L
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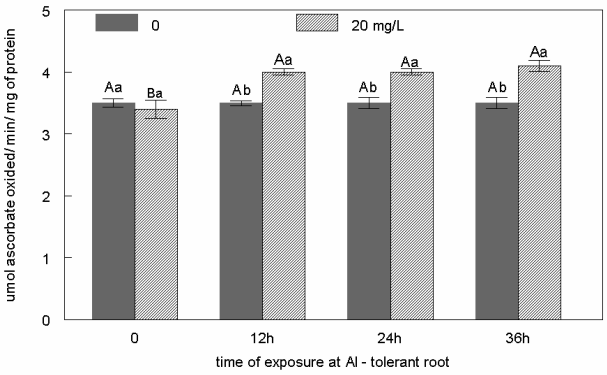
0
12h
24h
36h
time of exposure at Al - sensitive root

(A)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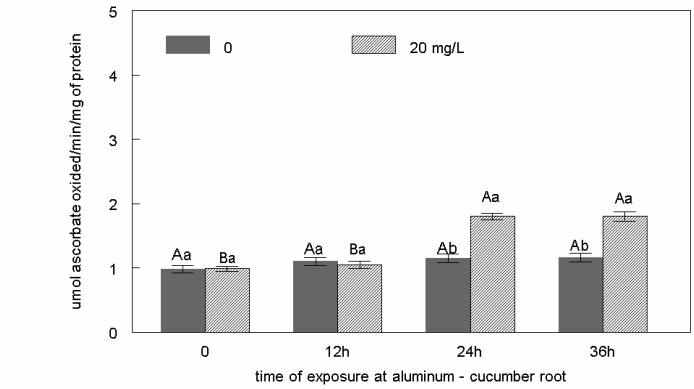
time of exposure at Al - tolerant root

(B)




(C)

**Figure 4** – Effect of Al on the ascorbate peroxidase (APX) activity over time in sensitive and tolerant oat roots and cucumber roots. APX activity was expressed as µmol ascorbate oxidized/ min/mg of protein. Data are the mean ± S.D. of three different replicates.

## 4. Discussion

The inhibition of root elongation is the primary Al toxicity signal (Dipierro et al., 2005) and has been used as a suitable parameter for assessing genotypic differences in Al tolerance (Collet et al., 2002; Jemo et al., 2007). The presence of 20 mg/L of Al in the solution inhibited significantly the root growth only in sensitive oat roots and cucumber roots (Fig 1A e 1C) indicating a distinct genetic sensitivity to Al supply when compared with tolerant roots of oat. This effect was observed at the first point of time after Al exposure (12h) and was observed throughout the experiment. Thus, these two oat genotypes were classified as Al-sensitive (UFRSGS 930598) and Al-tolerant (UFRRGS 17), confirming previous results (Federizzi et al., 2000). The cucumber presented inhibition in root growth, and this result was similar to the root sensitive genotype growth. Root growth reductions may therefore be the result of elevated Al concentrations in the rooting medium, leading to disruptions of cell functioning as proposed by Kochian (1995). This is likely caused by a number of different mechanisms, including apoplastic lesion, interactions within the cell wall, the plasma membrane, or the root symplasm (Marchner,

1995), resulting in mineral nutrient and water acquisition deficiency, and, consequently, leading to shoot nutrient deficiencies and poor crop yields.

The imposition of several abiotic stresses, including Al presence, can give rise to excess concentrations of ROS in plant cells (Lin & Kao, 2000), which are potentially harmful since they initiate the peroxidation and destruction of lipids, nucleic acids, and proteins (Yamaguchi-Shinozaki, 2006). The most prominent indicator of plasma membrane damage is Al-stimulated lipid peroxidation (Silva et al., 2002). In the present study, data for root growth (Fig. 1) and lipid peroxidatin (Fig. 2) suggested that peroxidation in the sensitive genotype of oat and cucumber may be a direct effect of Al toxicity on root growth, indicating Al induced oxidative stress and, as result, irreversible damage to tissue development and function. Thus, similar to results obtained with peas (Yamamoto et al., 2001), soybeans (Cakmak & Horst, 1991), and maize (Boscolo et al., 2003), lipid peroxidation in the Al-sensitive oat genotype and cucumber seems to be an early symptom induced by Al toxicity, indicating that lipids are the primary cellular target of oxidative stress. In the tolerant genotype (UFRGS 17), Al treatment did not provoke lipid peroxidation in either the roots at any time of exposure (data not showed). This indicates that the antioxidant system in this genotype may be more efficient to protect membrane lipids of ROS.

Plants respond to metal stress by physiological and biochemical strategies. Anti-oxidation mechanisms of the cell include the enzymatic ROS-scavenging system, which functions to interrupt the cascades of uncontrolled oxidation in each organelle. Among these enzymatic systems, CAT and APX transform peroxides into non-reactive species, but APX has a very high affinity for $H_2O_2$ as compared with CAT (Graham & Patterson, 1982).

At the first point of time after Al exposure (12 h), both CAT and APX activities were increased in the tolerant roots. These alterations occurred concomitantly with the normal growth of root and no formation of lipid peroxidation of this genotype, whereas contrary results were

observed in sensitive root and cucumber. These seedlings presented activation in antioxidant enzymes only 36 h and high levels of lipid peroxidation. This may indicate that, the lateness in the activation of antioxidant enzymes of sensitive oat roots and cucumber roots were contributing to enhance the damage provoked by Al treatment.

In the roots of the Al- sensitive genotype and cucumber, the CAT activity to Al was increased only at 36 h. The CAT activity was compensated by the increased activity of APX at 24 and 36 h. Even with these variations, the antioxidant system was not efficient, causing lipid peroxidation in the membrane. In our previous studies cucumber seedlings showed to be increased in the enzymes of the antioxidant system but they were not efficient in the protection of ROS.

In conclusion, these results show that the cellular redox status of oat and cucumber seems to be affected by Al, and the oxidative stress may be an important mechanism involved in Al toxicity, mainly in the sensitive genotype and cucumber root. Biochemical (lipid peroxidation) and morphological (root growth) alterations were observed in the first hours of Al exposure. Al causes oxidative stress probably through indirect mechanisms such as interaction with the antioxidant defense, disruption of electron transport chain, and induction of lipid peroxidation. The results for the cucumber root showed the absence of tolerance mechanisms to Al when compared with tolerance root of oat. Cucumber may be sensitive to Al. Therefore, further researches on the mechanisms of tolerance to Al are required to reveal the underlying molecular and biochemical events involved.

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# 4. DISCUSSÃO

O pepino (*Cucumis sativus*) quando avaliado em meio de crescimento semi – sólido (com ágar), após 10 dias de exposição ao alumínio (Al) mostrou ativação das enzimas do sistema antioxidante nas diferentes concentrações do metal. Os níveis de peróxido de hidrogênio, proteína carbonil e peroxidação de lipídeos foram significativamente aumentados o que pode explicar a inibição do crescimento da planta. Assim, o sistema antioxidante em pepino, mesmo sendo ativado não foi eficiente na remoção das espécies reativas de oxigênio (EROs).

Os genótipos de aveia (*Avena sativa*) estudados UFRGS 17, UFRGS 930598 e UFRGS 280 foram classificados previamente como tolerantes, sensíveis e intermediário ao alumínio através de análises morfológicas segundo FEDERIZZI et al. (2001). De acordo com POSCHENRIEDER et al. (2008) a inibição do crescimento da planta é uma característica comum a muitos metais e é utilizada como parâmetro para caracterizar a fitotoxicidade. As concentrações de alumínio aplicadas nos meios de crescimento dos três genótipos mostraram um efeito diferente para cada genótipo, tanto nos parâmetros bioquímicos, quanto nas avaliações fisiológicas do crescimento da planta, confirmando que UFRGS 930598 é o genótipo mais sensível ao alumínio. O alumínio diminuiu o crescimento da raiz e da parte aérea do genótipo 930598 em todos os experimentos. Além disso, no genótipo sensível ocorreu uma importante diminuição na quantidade de matéria orgânica, indicando uma diminuição na fotossíntese, conforme KUMARI (2007), o alumínio altera diretamente a síntese de enzimas, pigmentos e cofatores essenciais para o processo. Segundo GRATÃO et al. (2005) o conteúdo de clorofila é um indicador da toxicidade causada por metais para a maioria das plantas e os resultados do presente estudo mostraram uma diminuição do conteúdo de clorofila para pepino e para o genótipo sensível de aveia nas concentrações mais altas de Al. A diminuição do conteúdo de clorofila provoca uma perturbação na homeostase celular redox, visto que esta molécula desempenha a função de aceptor de elétrons, aumentando assim a produção de EROs (MITTLER, 2002). A diminuição do conteúdo de clorofila pode ser atribuída a inibição da síntese de clorofila.

A reação catalizada pela ð - ALA-D é o segundo passo de formação dos compostos tetrapirrólicos, incluindo as moléculas de clorofila que são essenciais para a

vida da planta. A exposição das plantas ao Al causou uma severa inibição da atividade da δ - ALA-D em plântulas do genótipo sensível de aveia e em plântulas de pepino. O reduzido conteúdo de clorofila pode ser atribuído a reduzida síntese de clorofila porque o Al inibe a atividade da δ - ALA-D que apresenta grupos sulfidrila. Sabe-se que esses grupos funcionais da enzima são inativados por metais (MORSCH et al., 2002; ROCHA et al., 2004).

Após 10 dias de exposição ao alumínio em solução hidropônica as enzimas antioxidantes do genótipo sensível foram ativadas, entretanto a ação das enzimas antioxidantes não foi eficiente para impedir a formação de altos níveis de espécies reativas de oxigênio (EROS), conteúdo de peróxido de hidrogênio e peroxidação lipídica. Antioxidantes como a glutationa e o ácido ascórbico (ASA) são encontrados em maior concentração nos cloroplastos. O ASA está envolvido na regulação da fotossíntese, expansão celular, crescimento da raiz e no transporte de elétrons através da membrana (SMIRNOFF, 2000). Ele é encontrado em concentrações milimolares nas folhas e desempenha uma função importante na tolerância das plantas ao estresse (NOCTOR & FOYER, 1998). Os resultados deste estudo demonstraram que o genótipo tolerante apresentou os maiores níveis de ASA, enquanto que o sensível teve uma concentração maior de glutationa na parte aérea nas maiores concentrações de alumínio.

A causa provável da falta de eficiência do sistema antioxidante no genótipo sensível é a velocidade em que ele é ativado para combater a produção de EROs. No genótipo sensível as enzimas tiveram suas atividades aumentadas depois de 24 ou 36 h de exposição ao alumínio, enquanto os genótipos UFRGS 17 (tolerante) e UFRGS 280 (intermediário) tiveram suas atividades aumentadas após 12 h de exposição ao alumínio.

O genótipo tolerante (UFRGS 17) e o genótipo intermediário (UFRGS 280) foram resistentes ao alumínio porque tiveram pouca ou nenhuma inibição do crescimento, embora tenham acumulado altos níveis de alumínio na raiz e parte aérea respectivamente. Os níveis de peróxido de hidrogênio e de peroxidação lipídica, encontrados nestes genótipos não foram suficientes para diminuir o crescimento da raiz e da parte aérea destas plantas, ao contrário, o alumínio ativou o crescimento após 12, 24 e 36 h de exposição, o que pode ser caracterizado como uma resposta hormética

que está relacionada a pequenas concentrações de metal capazes de ativar o desenvolvimento da planta (YOKEL, 2002). Porém, neste caso esta resposta está relacionada à rápida exposição ao alumínio, que coincide com a fase inicial de desenvolvimento da planta.

O genótipo intermediário mostrou características de espécies acumuladoras de alumínio porque apresentou altos níveis de alumínio na parte aérea sem causar nenhum dano ao crescimento da planta, enquanto que somente o genótipo tolerante apresentou um aumento do pH na solução hidropônica. A modificação do pH da rizosfera pelas plantas tem sido proposto como um mecanismo de exclusão de Al pela raiz de plantas resistentes ao Al (MATSUMOTO, 2000). A solubilidade do Al é dependente do pH da solução (KINRAIDE, 1991), como o pH da solução aumenta, a solubilidade do $Al^{3+}$ diminui, diminuído assim sua toxicidade. É provável que o genótipo tolerante apresente mecanismos de resistência ao Al relacionados com a exclusão do metal pelas raízes. O genótipo UFRGS 280, considerado como intermediário por ser resultado do cruzamento entre UFRGS 930598 e UFRGS 17 mostrou-se resistente ao alumínio e é provável que tenha mecanismos de destoxificação interna do metal.

O pepino (*Cucumis sativus*) quando colocado em solução hidropônica exposto a 20 mg/L de alumínio juntamente com o genótipo tolerante (UFRGS 17) e o genótipo sensível (UFRGS 930598), apresentou semelhanças com o genótipo sensível tais como: a atividade da enzima catalase foi aumentada após 36 h de exposição ao alumínio. Os níveis de peroxidação lipídica foram elevados após 12, 24 e 36h de exposição ao alumínio e como conseqüência a raiz teve uma diminuição do crescimento.

Através da avaliação conjunta, com os genótipos de aveia tolerante, UFRGS 17, e sensível, UFRGS 930598, o pepino (*Cucumis sativus*) pode ser considerado uma espécie sensível ao alumínio. Essa semelhança com o genótipo sensível da aveia não está ligada a fatores genéticos já que são duas espécies com características diferentes pertencentes às classes diferentes, que são as monocotiledôneas e as dicotiledôneas, respectivamente. Porém, essas plantas estão expostas a milhões de anos aos mesmos metais tóxicos presentes no solo, como o Al, e esta exposição possibilita a capacidade de adaptarem-se a esta toxicidade, cada espécie apresentando os mais variados mecanismos de adaptação.

# 5. CONCLUSÕES

- O Al inibe o crescimento das plântulas de pepino (*Cucumis sativus*) e das plântulas do genótipo de aveia, UFRGS 930598 – sensível ao Al.
- O Al não inibe o crescimento das plântulas pertencentes aos genótipos UFRGS 17 – tolerante ao Al e UFRGS 280 – intermediário ao Al nas doses e tempos de exposição testados.
- Os níveis de peroxidação lipídica e peróxido de hidrogênio, encontrados no genótipo tolerante e intermediário, foram baixos, ao contrário dos resultados encontrados para o genótipo sensível de aveia e para as plântulas de pepino que apresentaram altos níveis de peroxidação lipídica e de peróxido de hidrogênio.
- O Al teve um efeito diferente nas enzimas do sistema antioxidante CAT, APX e SOD de cada planta estudada.
- O sistema de defesa antioxidante foi mais eficiente nas plântulas pertencentes ao genótipo tolerante e intermediário de aveia do que as plântulas de pepino e as pertencentes ao genótipo sensível.
- Os genótipos tolerante e intermediário são resistentes ao alumínio e apresentam mecanismos de tolerância a este metal.
- A falta de eficiência do sistema antioxidante na remoção das EROs está ligada ao tempo que este sistema demora para ser ativado nas plântulas do genótipo sensível e na plântulas de pepino.
- A inibição do crescimento da raiz é conseqüência do estresse oxidativo, e os mecanismos de tolerância das plantas ao Al estão relacionados com a ativação das enzimas do sistema antioxidante.
- O pepino (*Cucumis sativus*) é uma espécie sensível ao Al, quando comparada a aveia - genótipo sensível e tolerante, uma vez que apresentou resultados semelhantes aos do genótipo sensível de aveia, como a inibição do crescimento da raiz e atraso na ativação das enzimas do sistema antioxidante.

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**FIGURA 3 – Cultivo em meio hidropônico de genótipos de aveia (*Avena sativa*) em câmara climatizada.**