Aluminium Tolerance Mechanisms in *Brachiaria sp.*

CATALINA ARROYAVE QUICENO

Universitat Autònoma de Barcelona

Tesis doctoral para obtener el grado de Doctor en Biología y Biotecnología Vegetal, dirigida por

Dra. Roser Tolrà Pérez

Dra Charlotte Poschenrieder Wiens

Departamento de Biología Animal, Biología Vegetal y Ecología

Universidad Autónoma de Barcelona

2012

Acknowledgements

This Ph.D Thesis was granted by a fellowship PIF 2008–2012 from Autonomous University of Barcelona and by a research stay award to Catalina Arroyave (travel fund fellowships for research outside Cataluña) from the Autonomous University of Barcelona, Spain 2010-2011 at the College of Agriculture Food and Natural Resources, Division of Plant Science, University of Missouri–Columbia. USA. Experimental work was financed by MICIN through the research project BFU2010-14873/subprogram BFI

Technical assistance from the Microscopy Service of UAB is gratefully acknowledged. Especially thank to Alejandro Sánchez Chardi.

Thanks to Sílvia Bronsoms a member of ProteoRed-ISCIII network.

Agradecimientos

Con el más sincero agradecimiento a Dra Charlotte Poschenrieder Wiens y a la Dra Roser Tolrà Pérez por todo su gran apoyo en la realización de este trabajo. Al Dr Juan Barceló Coll y su familia por todo su gran apoyo, no sólo en el trabajo sino además por todo su respaldo emocional. Por toda su colaboración a todos los miembros de la unidad de Fisiología Vegetal. A Rosa Padilla, Than Thuy y Jesús Goday por toda su contribución en la realización de este trabajo.

Index

Index of abbreviations used

Al Aluminium
Al^{3+} Aluminium ion
ALMT Al activated malate transporter

CHAPS 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

DAHP 3-deoxy-d-*arabino*-heptulosonate 7-phosphate
DAPI 4'-6-Diamidino-2-phenylindole
DFQA 1,3-di-O-trans-feruloylquinic acid
DIGE Difference gel electrophoresis
DIMBOA 2,4-dihydroxy-7-methoxy-l,4 - benzoxazin-3-one
2D DIGE Two-dimensional difference gel electrophoresis
DTT Dithiothreitol

GAX glucoarabinxilans

IEF Isoelectric focusing
ICP-OES Inductively coupled plasma atomic emission spectroscopy
IPG Immobilized pH gradient

MALDI-TOF MS Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MATE Mitogen activated toxin extrusion
MES 4-Morpholineethanesulfonic acid
MeOH methanol
Morin 2',3',4',5,7-pentahydroxyflavanone

NAD nicotinamide adenine dinucleotide
NADH nicotinamide adenine dinucleotide reduce form
NCBI Biotechnology Information non-redundant protein database
Nrat1 Nramp aluminium transporter 1

Lumogallion (3-[2,4 dihydroxyphenylazo]-2-hydroxy-5-chlorobenzene sulfonic acid)

OA organic acid

OsO_4 osmium tetroxide
PMF Peptide mass fingerprinting

R Receptor
ROS Reactive oxygen species

SEM Scanning electron microscopy

TFA trifluoroacetic acid
TCA trichloroacetic acid
TEM Transmission electron microscopy

XET xyloglucan endotransglucosylase

OBJECTIVES

Aluminium toxicity is among the main abiotic stress factors limiting crop productivity in acidic environments, which comprise up to 50% of the world's arable lands. The general objective of this Thesis is to elucidate the mechanisms underlying differences in aluminium tolerance, by physiological, biochemical and proteomic approaches in Brachiaria species, *B. decumbens, B. Brizantha*, and *B. ruziziensis*. First, we aim to get a general overview of the species differences in tolerance to the low nutrient supply and high Al^{3+} availability that typically occur in acid mineral soils of the tropics. For this purpose root elongation and mineral nutrition of Brachiaria species growing in low ionic strength nutrient solutions is studied. The possible relevance of differences in Al location in root tips, the Al-induced alterations in root architecture, and the production of phenolics compounds as potential Al binding substances is a second step for typifying species differences in Al tolerance. Once characterized the most Al tolerant species, the proteomic study is focused on this species. Proteomics is considered to bridge the gap between gene sequence and phenotype. In the case of Brachiaria genomic information is scarce. Under these circumstances the proteomic approach may help not only to better understand the metabolic events underlying Al-induced phenotype changes leading to the expression of Al tolerance in Brachiaria, but also to focus future investigations for identifying the responsible genes.

INTRODUCTION

General Introduction

1. *Brachiaria*

Brachiaria (Poaceae), a genus of forage grasses of African origin, comprises about 100 species. Currently, some of them are gaining considerable relevance as fodder crops in tropical regions worldwide. The economic interest in these grasses is greatest in tropical America. Cultivation of Brachiaria is of increasing importance as a pasture for livestock production, especially in South American savannas with 40±70 million hectares in Brazil alone. In this region extensive adoption of Brachiaria cultivation over the past three decades has had a revolutionary impact on the productivity of vast areas of previously underused or marginal soils (Boddey *et al.,* 1996; Wenzl *et al.,* 2000; Kochian *et al.,* 2004) The reasons for this fast expansion are good nutritional quality combined with withstanding to heavy grazing, high seed production, good feeding quality, and high resistance to multiple environmental stress factors (Miles *et al.,* 1998).

Acidic soils limit crop production in half the world's potentially arable land, mostly in developing countries in Africa, Asia and South America. Aluminium toxicity is considered among the most relevant abiotic stress factors limiting crop production on acid mineral soils in the tropics. In soils with a pH below 5 and especially if pH values fall below 4.5, toxic forms of Al are solubilised into the soil solution. Plant available Al ions inhibit root growth and functioning, and thus reducing yields of crops, such as wheat (*Triticum aestivum L.*), corn (*Zea mays L.*), or soybean (*Glycine max* (L.) Merr.). Besides Al toxicity, acid soils are characterized by having excess H^+ and Mn^{2+}, as well as deficiency of Ca^{2+}, Mg^{2+}, and PO_4^{3-} (Kochian *et al.,* 2004; Panda and Matsumoto, 2007). Certain Brachiaria species are performing well under the same or similar harsh conditions. The condition of being plants well-adapted to tropical climate with C4-type photosynthesis makes Brachiaria even more attractive and further breeding efforts.

Brachiaria species are a main basis for animal nutrition. Those plants are commercially exploited in tropical regions because they are best adapted to both the low-fertility and the aluminium toxicity typically found in these zones. *Brachiaria brizantha* (A. Rich) (palisadegrass) besides Al-tolerance also has resistance to spittlebug. *Brachiaria ruziziensis* (ruzigrass) is less adapted to acid soil conditions, but has better nutritional quality than other species of Brachiaria. *Brachiaria decumbens* (signalgrass) is one of the most widely sown forage grasses

in the tropics with 26.4 million ha in Brazil alone. Unlike most food crops, it is directly derived from a wild apomictic germplasm accession that deserves special attention because of its extreme Al tolerance and good adaptation to infertile acid soils (Wenzl *et al.,* 2001, 2002).

2. Aluminium: speciation

Aluminium is a major constituent of the mineral fraction of most soils. Aluminium usually is tightly bound in the silicate lattice; so this amphoterous element is sparingly soluble and mostly unavailable to plants. However, in soils with acid or highly alkaline pH the solubility of Al is enhanced. Aluminium has the electron configuration $[Ne]3s^2 3p^1$. At acid pH the formation and stability of the Al aqua complex $Al(H_2O)_6^{3+}$ is favoured, This trivalent ionic compound usually written as Al^{3+} seems to be the most phytotoxic Al species. Part of the difficulty of studying Al-related processes in plants can be attributed to the complex chemistry of Al. Depending on the conditions; Al can undergo hydrolysis reactions to several different mononuclear hydroxides or polymerization reactions to polycations (Casey, 2006). The most important variables in the aqueous chemistry of aluminium are the pH and soluble organic matter.

At low pH Al hydrolyzes in solution yielding the aluminium hexahydrate cation $[Al(OH_2)^6]^{3+}{}_{(aq)}$ This trivalent cationic Al species simply written as Al^{3+}, is the predominant form of the monomeric aluminium ion in the strongly acidic range ($pH \leq 4.$), whereas hydroxyl species such as $[AlOH]^{2+}$ and $[Al(OH)^2]^+$ form as the pH increases to values around 6. In the near neutral pH range ($5 < pH < 8$) aluminium trihydroxide ($Al(OH)_{3(s)}$) or gibbsite, occurs, whereas $Al(OH)_4^-$, or aluminates, dominate in alkaline conditions. Many of the monomeric Al cations bind to various organic and inorganic ligands such as PO_4^{3-}, SO_4^{2-}, F^-, organic acids, proteins, and lipids (Poschenrieder *et al.,* 2008; Casey, 2006; Delhaize and Ryan, 1995)

The phytotoxic Al^{3+} is the hardest Lewis acid among other trivalent metal cations such as La^{3+}, Cr^{3+} or Ga^{3+}. Hard Lewis acids are characterized by a low covalent and a high ionic index. Despite the fact that Al is the most abundant metal on earth, so far there is no recognized biological function for this element. This may be attributed to different factors such as slow ligand exchange rates, hampered membrane transport, and its interference with Mg^{2+} binding sites at phosphate esters (DNA/RNA, ATP, etc.) (Merkx and Averill, 1999; Poschenrieder and Barceló, 2004)

3. *Physiological mechanisms of aluminium toxicity*

3.1 *Phytotoxic Al species*

The aluminium hexahydrate $[Al(OH_2)_6]^{3+}$, which is solubilised into the soil solution under low pH, is considered the main toxic species for plants. It has six coordination sites and as class A metal preferentially binds to oxygen donor ligands. Multiple molecular targets in plant cells can act as potential ligands for Al^{3+} and both the toxicity mechanisms and the means for detoxification are based on the availability of potential binding sites (Barceló and Poschenrieder, 2002).

The target site of Al toxicity is the root apex. The primary symptom of Al-toxicity in plants is the inhibition of root growth. This process has been studied at the tissue and cellular levels. At tissue level, the distal transition zone has been identified as the most Al sensitive root zone (Horst *et al*., 2010). This region corresponds to the zone where cells leaving the meristem (cell division zone) are about to enter the elongation zone (cell expansion zone). Aluminium causes fast inhibition of both root tip cell division and cell elongation. Moreover, Al toxicity changes the entire root architecture by fast induction of lateral root development (Doncheva *et al*.,2005; Amenós *et al.,* 2009).

In sensitive species the distal transition accumulates more Al than the mature root tissues. Multiples studies have clearly shown that both apoplast and symplast are sites of aluminium location in plants (Vázquez *et al.,* 1999; Zheng and Yang, 2005; Horst *et al*., 2010)

Cell walls seem primary sites for Al binding and toxicity. In Al-sensitive plants Al causes changes in the amount of cell wall polysaccharides. It has been proposed that Al^{3+} is attracted to the negatively charged carboxyl groups of unmethylated pectins. Both a low degree of methylesterification of cell wall pectins and an Al-induced accumulation of β-1,3-glucan (callose) synthesis in the apoplast have been found to be related to Al sensitivity in different crop plants (Eticha *et al.,* 2005 and Yang *et al.,* 2008; Panda and Matsumoto, 2007). Also Yang *et al.*, (2008) found in resistant rice cultivars a relation between higher degree of methylesterification of cell wall polysaccharides and less Al-binding sites. Thus the degree of methylesterification of cell wall pectins could be a mechanism of Al resistance.

The plasma membrane is a further target for Al toxicity. Aluminium may damage the plasma membrane by inducing reactive oxygen species causing lipid

peroxidation and oxidative stress (Liu *et al*., 2008). Wheat roots treated with Al showed increased H^+ efflux near the root apex and root cap. Aluminium interfered with calmodulin-stimulated ATPase activity and proton transport in plasma-membrane (Miyasaka *et al*., 1989). Furthermore, Al^{3+} affects the mechanisms controlling the organization of microtubule cytoskeleton; dividing root-tip cells and the nucleus could be targets for injury (Frantzios *et al*., 2000; Kochian *et al*., 2004; Doncheva *et al*., 2005)

3.2 *Mechanisms of Al resistance*

3.2.1 *Mucilage and border cells*

Border cells have been described as a possible mechanism for Al-tolerance. The root border cells (RBCs) exude mucilage that may help to protect plants by inhibiting Al uptake into the root meristem. Studies in pea (*Pisum sativum*) also suggest a possible role of cell-wall pectins in the root border cells for immobilizing Al (Miyasaka *et al.,* 2001). Comparative studies in soybean cultivars Zhechun (Al-tolerant cultivar) and Huachun (Al-sensitive cultivar) revealed enhanced inhibition of root growth by Al when RBCs were removed from the root apex (Cai *et al.,* 2011.) Aluminium induced a thicker mucilage layer around detached border cells in two snapbean (*Phaseolus vulgaris*) cultivars, avoiding Al uptake into the roots (Yu *et al.,* 2009)

3.2.2 *Rhizosphere pH*

The most important factor for root Induced changes in rhizosphere pH is the uptake of nutrients (cation-anion balance). The rhizosphere pH, in turn, has a marked influence on the availability to plants of essential nutrients and toxic elements present in the soil solution. Increase of rhizosphere pH has been proposed as an important mechanism to avoid aluminium toxicity. This would be one of the most effective external Al resistance mechanisms of plants (Marschner, 2012). Several studies were directed toward the analysis of the possible correlation between changes in the rhizosphere pH and plant Al tolerance. In two varieties on the wheat (*Triticum aestivum L*.) under Al stress, the Al-tolerant variety showed a stronger capacity of enhancing rhizosphere pH than the Al-sensitive variety. Similar results are now available for other species (wheat, sorghum, and *Arabidopsis thaliana* mutant *talr-104*) that can change the rhizos-phere pH increased to tolerate Al toxicity (Degenhardt *et al.,* 1998; Yang *et al.,*

2011). Al-sensitive mutants (als3 and als5) exhibited lower rhizosphere pH values (Bose *et al*., 2010). Contrastingly, cultivation of Al hypertolerant plants, such as tea causes decrease of soil pH rather than an increase (Alekseeva *et al.,* 2011).

3.2.3 *Detoxification of Al by organic acids*

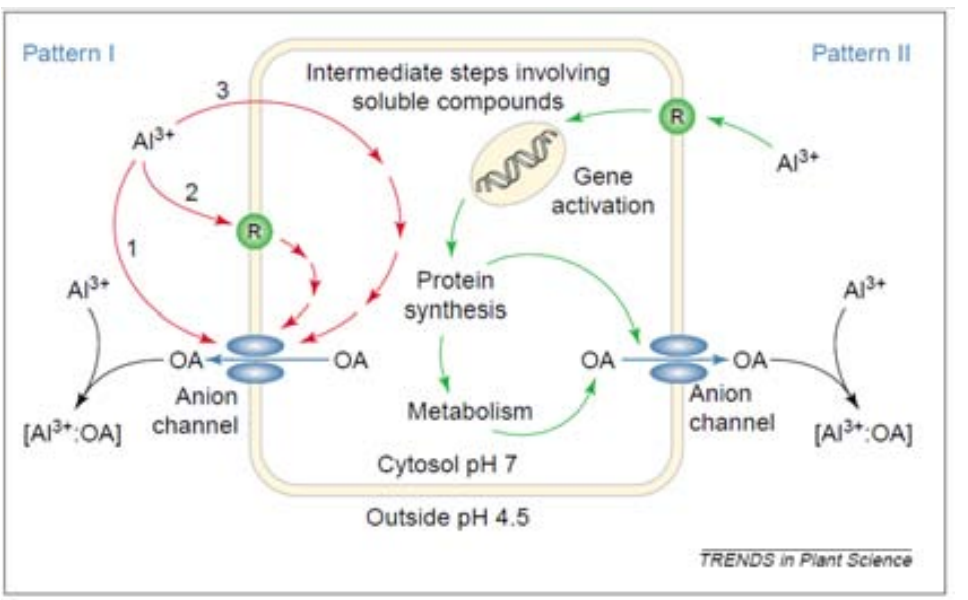


Fig1. Hypothetical models for the aluminium (Al)-stimulated secretion of organic acid anions (OA) from plant roots. Pattern I-type responses: Al^{3+} activates a constitutively expressed anion efflux channel that facilitates exudation of organic acids (OA) from root tips upon Al exposure. Al^{3+}-resistance genotypes show greater expression than Al^{3+}-sensitive ones. Activation of organic anion (OA) efflux by Al interacting directly with the pre-existing proteins in the plasma membrane (members of the ALMT and MATE families of proteins in wheat). In the Pattern II response, Al^{3+} first interacts with the cell, possibly involving a specific receptor (R) on the plasma membrane; this induces the activation of genes coding for the transport proteins. Organic acid anions form stable, non toxic complex with Al(Ma *et al.*, 2001; Delhaize *et al*., 2007)

Plants produce a range of mono-, di- and tri- carboxylates. Most of these organic acids, such as citrate, malate, and oxalate, form strong complexes with Al^{3+} as indicated by the high stability constants (Poschenrieder *et al.,* 2008). Many plant species can increase the efflux of these organic acids at the root level as a mechanism for Al tolerance. The kind of organic acids secreted, as well as secretion patterns differ among plant species (fig. 1 taken from Ma *et al.,* 2001). A fast Al–induced efflux of malate, following pattern 1, seems responsible for Al

tolerance in wheat (*Triticum aestivum L.*). In Al^{3+}-resistant *Arabidopsis thaliana* (Delhaize *et al*., 1993; Sasaki *et al*., 2004; Delhaize *et al*., 2007) exposure to Al nearly instantaneously activated a concentration-dependent citrate release. Citrate exudation from root tips has also been observed in Al-tolerant maize (*Zea mays*) and barley (*Hordeum vulgare L.*) (Piñeros *et al*., 2002; Furukawa *et al*., 2007). Aluminium-resistant buckwheat secretes oxalic acid from roots in response to aluminium stress (Ma *et al*., 1997), while radish (*Raphanus sativus*) and rye (*Secale cereale*) release two organic acids malate and citrate (Ma *et al*., 2001)

Recently there is molecular and genetic evidence showing that other properties of the root may contribute to Al resistance. In recent investigations Ma *et al*., 2009 were able to discover two genes *STAR1* and *STAR2* that are implicated in Al resistance in rice (*Oryza sativa L.*). *STAR1* interacts with *STAR2* to form a complex that functions as a unique ATP binding cassette ABC transporter. The complex works as a UDP-GLc efflux transporter, which is required for detoxification of aluminium. Recently a study localized an aluminium transporter (Nrat1) in plasma membrane of rice. This transporter is a member of the Nramp family (Xia *et al*., 2010). This finding is highly relevant because identification of Al- transporters helps to a better understanding of the mechanisms that are involved in Al-tolerance or Al-toxicity.

Despite the fact that much progress has been made in our understanding of Al toxicity and tolerance mechanisms in major crop plants with a relatively low tolerance level, such as wheat or bean, the mechanisms underlying Al hyperresistance as observed in Brachiaria are still not established.

In this investigation we have compared the Al resistance in three species of Brachiaria, *Brachiaria decumbens*, *Brachiaria brizantha* and *Brachiaria ruziziensis. B. decumbens* and *B, brizantha*, both species have a high tolerance to aluminium toxicity. However, *B. ruziziensis* is sensitive to high concentrations of aluminium. Previous investigations into the mechanisms of Al hyperresistance in Brachiaria evidenced that they are neither based on external detoxification of Al by organic acid exudation nor on apical root-induced alkalinisation (Wenzl *et al.,* 2001).

References

Amenós, M., Corrales, I., Poschenrieder, C., Illés, P., Baluska, F., Barceló, J. 2009. Different Effects of Aluminum on Actin Cytoskeleton and Brefeldin A-sensitive Vesicle Recycling in Root Apex Cells of Two Maize Varieties Differing in Root Elongation Rate and Al Tolerance. Plant Cell Physiol 50: 528-540

Barceló, J., Poschenrieder, C. (2002) Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium resistance and tolerance: a review. Environ Exp Bot 48: 75-92

Boddey, R., Rao, I. and Thomas R. 1996. Nutrient cycling and environmental impact of Brachiaria pastures. In: Miles J. Maass B and do Velle C. (eds). Brachiaria: Biology, Agronomy and improvement. Cali: CIAT and Brasilia: EMBRAPA. 72-86

Bose, J., Babourina, O., Shabala, S., Rengel, Z. 2010. Aluminium-induced ion transport in Arabidopsis: the relationship between Al tolerance and root ion flux. J Exp Bot 61: 11 3163–3175

Casey, W. 2006. Large Aqueous Aluminum Hydroxide Molecules. Chem Rev 106: 1 1–16

Cai, M., Wang, F., Li, R., Zhang, S., Wang, S., Xu, D. Response and tolerance of root border cells to aluminum toxicity in soybean seedlings. J Inorg Biochem 105: 966-971

Degenhardt, J., Larsen, P., Howell, S., Kochian, L. 1998. Aluminum Resistance in the Arabidopsis Mutant alr-104 Is Caused by an Aluminum-Induced Increase in Rhizosphere pH. Plant Physiol 117: 19–27

Delhaize, E., Gruber, B., Ryan PR. 2007. The roles of organic anion permeases in aluminium resistance and mineral nutrition. FEBS Letters 581:2255–2262

Delhaize, E., Ryan PR. 1995. Aluminum Toxicity and Tolerance in Plants. Plant Physiol 107: 31 5-321

Doncheva, S., Amenós, M., Poschenrieder, C., Barceló, J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. J Exp Bot 56: 414 1213–1220

Furukawa, J. Yamaji, N. Wang, H. Mitani, N. Murata, Y. Sato, K. Katsuhara, M. Takeda, K. Ma, J. 2007. An aluminum-activated citrate transporter in barley. Plant Cell Physiol 48:1081–1091

Frantzios, G., Galatis, B., Apostolackos, P. 2000. Aluminium effects on microtubule organization in dividing root-tip cells of Triticum turgidum. I. Mitotic cells. New Phytol 145: 211-224

Horst, W., Wang, Y., Eticha, D. 2010. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. Ann Bot 106: 1 185-197

Huang, C., Yamaji, N., Mitani, N., Yano, N., Nagamura, Y., Ma, J. 2009. A Bacterial-Type ABC Transporter Is Involved in Aluminum Tolerance in Rice W. Plant Cell 21: 655–667

Kochian, L., Hoekenga, O., Piñeros, M. 2004. How do Crop Plants Tolerate Acid Soils? Mechanisms of Aluminum Tolerance and Phosphorous Efficiency. Annu Rev Plant Biol 55:459-493

Ma, J. 2007. Syndrome of aluminum toxicity and diversity of aluminum resistance in higher plants. Int Rev Cytol 264: 225–252

Ma, J., Ryan, P., Delhaize, E. 2001. Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6: 6 273-278

Ma, J., Zheng, S., Hiradate, S., Matsumoto, H. 1997b. Detoxifying aluminum with buckwheat. Nature 390: 569–570

Marschner P. 2012. Third edition Marschner's Mineral Nutrition of Higher Plants. Elsevier Ltd. ISBN-978-0-12-384905-2. 353Merkx and Averill. 1999. Probing the Role of the Trivalent Metal in Phosphate Ester. J Am Chem Soc 121: 6683-6689

Miles J.W. Maass, L., Do Valle, C., Kumble, V (Eds.). 1998. Brachiaria: Biología, Agronomía y Mejoramineto. Centro Inter de Agricult Trop Publication number 154, Cali Colombia 1998

Miyasaka, S,. Hawes, M. 2001. Possible Role of Root Border Cells in Detection and Avoidance of Aluminum Toxicity. Plant Physiol 125: 1978–1987

Miyasaka,S,. Kochian, L., Shaff, J. Foy, C. 1989. Mechanisms of Aluminum Tolerance in Wheat. Plant Physiol 91: 1188-1196

Liu, Q., Yang, J., He, L., Li, Y. Zheng, S. 2008. Effect of Aluminum on Cell Wall, Plasma Membrane, Antioxidants and Root Elongation in Triticale. Biol Plant 52:1 87-92

Panda and Matsumoto. 2007. Molecular physiology of aluminum toxicity and tolerance in plants. Bot Rev 73: 4 326-347

Piñeros, M., Magalhaes J., Carvalho, V., Kochian, L. 2002. The Physiology and Biophysics of an Aluminum Tolerance Mechanism Based on Root Citrate Exudation in Maize. Plant Physiol 129: 1194–1206

Poschenrieder, C., Gunsé, B., Corrales, I., Barceló, J. 2008. A glance into aluminum toxicity and resistance in plants. Sci Total Environ 400: 356-368

Ryan,P., Tyerman, S., Sasaki,T., Furuichi,T., Yamamoto, Y., Zhang, W., Delhaize, E. 2011. The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. J Exp Bot 62: 1 9–20

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S., Ryan, P., Delhaize, E., Matsumoto, H.2004. A wheat gene encoding an aluminium-activated malate transporter. Plant J 37, 645–653

Tice, K., Parker, D., DeMason, D. 1992 Operationally Defined Apoplastic and Symplastic Aluminum Fractions in Root Tips of Aluminum-intoxicated Wheat. Plant Physiol 100: 309-318

Vázquez, M., Poschenrieder, Ch., Corrales, I., Barceló, J. 1999. Change in apoplastic aluminum during the initial growth response to aluminum by roots of a tolerant maize variety. Plant Physiol 119: 435-444

Wenzl, P., Chaves, A., Buitrago, M., Patiño, M., Meyer, J., Rao, I. 2002. Aluminium Stress Stimulates the Accumulation of organic acids in Root Apice of Brachiaria species. J Plant Nutr Soil Sci 165: 582-588

Wenzl, P., Chávez, L., Mayer, J., Rao, I. and Nair, M. 2000. Roots of nutrient-deprived Brachiaria species accumulate 1,3-di-0-trans-feruloylquinic acid. Phytoche 55: 389-395

Wenzl, P., Patiño, G., Chaves, A. 2001.The high level of aluminum resistance in signalgrass is not associated with known mechanisms of external aluminum detoxification in root apices. Plant Physiol 125: 3 1473-1484

Xia, J., Yamaji, N., Kasai, T., Ma, J. 2010. Plasma membrane-localized transporter for aluminum in rice. PNAS 107: 43 18381–18385

Yang, Y., Wang, Q., Geng, J., Guo1, Z., Zhao, Z. 2011. Rhizosphere pH difference regulated by plasma membrane H^+-ATPase is related to differential Al-tolerance of two wheat cultivars. Plant Soil Environ 57: 5 201–206

Yang, J., Li, Y., Zhang, Y., Zhang, S., Wu, Y., Wu, P., Zheng, S. 2008. Cell Wall Polysaccharides Are Specifically Involved in the Exclusion of Aluminum from the Rice Root Apex. Plant Physiol 146: 602–611

Yu, M., Shen, R., Liu, J., Chen, R., Xu, M., Yang, Y., Xiao, H., Wang, H., Wang, H., Wang, C. 2009. The role of root border cells in aluminum resistance of pea (*Pisum sativum*) grown in mist culture. J Plant Nutr Soil Sci 172: 528–534

Zheng and Yang. 2005. Target sites of aluminum phytotoxicity. Biol Plant 49: 3 321-331

Chapter 1

Evaluation of Aluminium Tolerance in Brachiaria Species

Chapter 1

1. Introduction

1.1. *Influence of Al on growth and mineral nutrition of Brachiaria species*

The acquisition of water and nutrients from soil are the main functions of plant roots. The ability of species to develop on soils with low nutrient availability depends on both nutrient uptake efficiency and nutrient use efficiency. High uptake efficiency implies powerful mechanisms for mobilization of essential nutrients from the rhizosphere and high affinity uptake and transport mechanisms. Nutrient use efficiency, in turn, depends on low internal requirements for optimal growth (Rengel *et al*., 2009; Hinsinger *et al.,* 2011)

In the case of acid soils, the extremely low ionic strength of the soil solution is associated with deficiency of essential nutrients and complex other stress factors including aluminium (Al) and manganese (Mn) toxicities. At lower pH, H^+ toxicity may also affect plant growth disturbing the plant nutrient balance, especially affecting tissue concentrations of Ca, Mg, K and B (Poschenrieder *et al.*, 1995). Aluminium exposure can inhibit root elongation and specific nutrient absorption , decreasing uptake of Ca, Mg, K, P and B. Aluminium-induced alteration of Ca^{2+} and K^+ homeostasis have frequently been observed. For example, Piñeros and Kochian (2001) found that Al^{3+} interact with plasma-membrane channel proteins, blocking the uptake of K^+ and Ca^{2+}. Other experiments suggest that the Al-induced callose synthesis depends on Al-induced disturbance of Ca homeostasis (Llugany *et al.*, 1994; Massot *et al.,* 1999; Rengel and Zhang, 2003).

Furthermore, Mg^{2+} is influenced by acidity and presence of Al^{3+}, since its uptake by plants decrease with the pH in the solutions. Whenever an increase in the aluminium concentration is observed, magnesium availability is affected (Kamprath and Foy, 1985). Maize varieties exposed to acid solutions containing Al showed alterations of nutrient uptake. This was due to both H^+ toxicity and short-term exposure to Al. Aluminium induced changes in the B uptake rate and decreased vacuolar K^+, and phosphate concentrations (Poschenrieder *et al.,* 1995; Garzón *et al.,* 2011)

High acid soil tolerance is a common feature of Brachiaria species such as *Brachiaria decumbens* and *Brachiaria brizantha*. These species must have high tolerance to Al^{3+} and H^+ toxicity. However, the absolute importance of different

soil nutrients influencing growth and productivity of Al and proton tolerant plants also depends on their physiological adaptation to the low availability of essential nutrient in the acid soils (Wenzl *et al.,* 2001, 2003)

The aim of this study was to investigate the influence of different high Al concentrations in low ionic strength nutrient solution on growth and mineral nutrition of three Brachiaria species in order to further clarify the role of the ability to maintain nutrient homeostasis in acid soil tolerance of Brachiaria. For this purpose the hydroponic solution used here was based on the modified solution from Wenzl *et al.,* 2001, which was designed for being a real approximation to the chemical soil properties that limit forage productivity on acid mineral soils. This is a highly relevant aspect because practically useful conclusions only can be drawn if experimental conditions simulate natural environments and closely resemble those that would be observed under natural conditions.

1.2. Materials and methods

1.2.1. *Plant materials and growth conditions*

1.2.1.1. *Cultivating plants in low-ionic-strength nutrient solutions*

Seeds of different Brachiaria species, signalgrass *B. decumbens* (Stapf), palisade grass *B. brizantha* (Hochst. Ex A. Rich.) Stapf, and Congo grass *B. ruziziensis* (R.Germ. and C.M.Evrard) Unipasto Marangatu, Brasil and Agrosemillas, Medellín, Colombia) were germinated on floating treys on distilled water. Emerged seedlings were transferred to continuously aerate nutrient solution (plastic vessels of 5 L capacity; 10 plants per beaker). The solution contained low nutrient concentrations. The composition was (in µM): 106 $(NH_4)_2SO_4$; 100 KNO_3; 20 K_2SO_4; 200 $Ca(NO_3)_2$; 1 $NaH_2PO_4.H_2O$; 169 $CaCl_2$; 120 $MgSO_4.7H_2O$; 10 Fe-EDTA; 1 $ZnSO_4.7H_2O$; 0.2 $CuSO_4.5H_2O$; 1 $MnSO_4.H2O$; 0.004 $(NH_4)_2SO_4$; 20 $SiCl_4$; 0,001 $(NH_4)_6MoO_{24}.4H_2O$; 6 H_3BO_3. Nutrient solution's pH was checked daily and adjusted to pH 4.2±0.1. The solutions were renewed every two days. After 15 days in control nutrient solution, plants were transferred to treatment solutions supplemented or not (controls) with 200 µM Al as $AlCl_3$. The activity of Al^{3+} in the solution was 31.7 µM (GEOCHEM-EZ, Cornell University, USA). Plants were grown in an environmental-controlled growth chamber (Conviron® SH10) under the

following conditions: 12/12 h photoperiod, photon fluence rate 600 $\mu Em^{-2}\ s^{-1}$; day/night temperature 27/25 °C, and relative humidity 60%.

1.2.2. *Mineral nutrition and growth analysis*

1.2.2.1. Growth and Mineral nutrition

Length of the main root was measured before Al supply and every 24h until 96h after start of the treatments (time samples 24, 48, 72 and 96h). Nutrition assays were performed in triplicate (plastic vessels of 6 L capacity; 21 plants per beaker). Plants pre-cultured for 15 days in control nutrient solution were used for the 72h Al uptake and mineral nutrition experiments. For this purpose plants were divided into 4 groups. One group was maintained in control nutrient solution for the following 72h (control), the others were transferred to solutions containing 200 µM Al either immediately (total Al exposure time 72h) or after a further 24h or 48h period in control solution. So these last two groups received Al for 48 and 24h, respectively. This experimental design allowed using the same control for all Al exposure treatments (all plants were of the same age when harvested). Before sample processing the root samples collected from control and Al treatment plants were desorbed with 1 mM citrate for 30 minutes followed by distilled water for 15 minutes.

Root dry weight was measured on 42 plants of each species and treatment after oven drying (105°C). Dried samples were ground to fine powder. The analysis of nutrients was done after digestion of plant material with acid mixture in an open hotblock digestion system (Item No.: SC154- 54-Well HotBlock™, United Kingdom). The method prepares plant tissue for the quantitative determination of the concentration of boron (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), zinc (Zn) and aluminium (Al) using 5 ml nitric acid 69 % and 2 ml hydrogen peroxide 30 % ($HN0_3/H_20_2$) and 25 mL miliQ water. Concentrations of these elements were determined in three samples per species and treatment. The concentrations of nutrients were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Jarrell-Ash, model 61E Polyscan, England).

1.2.3 *Statistical analysis*

1.2.3.1. *Statistical analysis*

The experiment considers nutrient uptake kinetics by determining element concentrations in the Brachiaria species after three different exposure times 24, 48 and 72h under aluminium treatment and plants without aluminium (controls). Three replicates per species and treatment (21 plants for each replicate n = 3) were analysed. Variance-stabilizing transformations were undertaken, where necessary, to conform to the assumptions of ANOVA. Subsequently, data were analysed by ANOVA followed by Tukey HSD to identify significant differences between Brachiaria species for each time. Test of Dunnet HSD was applied in order to compare upon time the nutrient uptake for each species with its control.

1.3. Results

1.3.1. *Effect of Al on root growth*

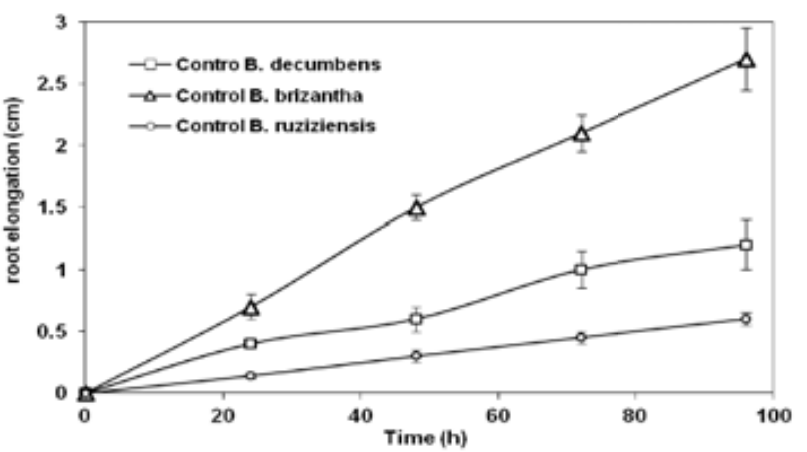


Fig. 2 Elongation of main root of three Brachiaria species exposed to Control (no Al supply) nutrient solutions. Values are means ± SE from n = 4 plants per time sample in the case of *B. decumbens* and *B. brizantha*, n=21 for *B. ruziziensis*

Under control conditions the three species maintained constant root elongation. In *B. brizantha* root elongation rate was approximately 0.7 cm /day while the mean elongation rate in *B. decumbens* was around 0.3 cm day^{-1}, and nearby 0.15 cm day^{-1} for *B. ruziziensis* (Fig 2). Roots elongation rates in *B. decumbens* and *B.*

ruziziensis were about 56 % and 78 %, respectively, lower than the rate observed in *B. brizantha*.

The Brachiaria species where exposed to 200 µM Al (Fig 3). In this case, roots of *B. brizantha* maintained a constant elongation rate of 0.4 cm day^{-1}. But after 96h exposure to Al the relative root length was 41% that of control plants. Aluminium supply had a time-dependent effect on the root elongation rate in *B. decumbens*. A strong initial reduction on root elongation of 75% was observed after 24h exposure to Al. However, the root elongation rate recovered upon time. After 96h a reduction of only 17% in comparison to control in the root elongation rate was observed (Fig3). In the case of *B. ruziziensis*, after 24 h a strong initial reduction of 68 % was observed. No recovering of the root elongation rate occurred during the experimental period of 96h when a reduction rate of 67% was found in the Al treated plants (Fig. 3). Species differences in root elongation rates were statistically significant according to ANOVA ($p < 0.05$).

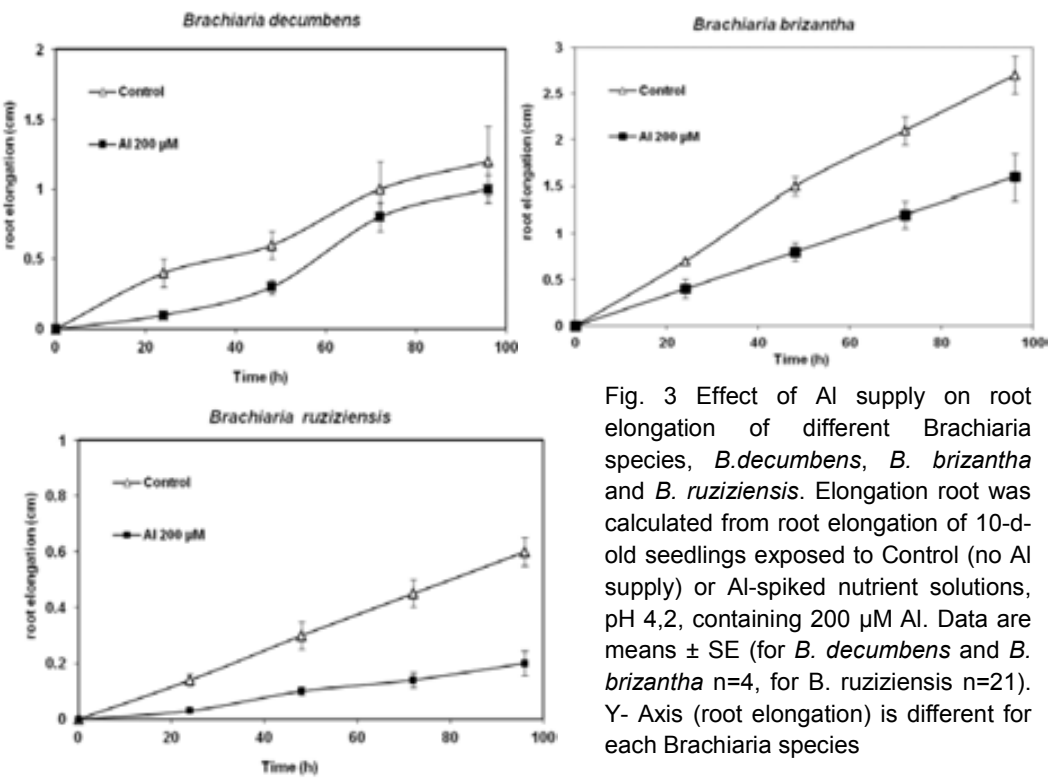


Fig. 3 Effect of Al supply on root elongation of different Brachiaria species, *B.decumbens*, *B. brizantha* and *B. ruziziensis*. Elongation root was calculated from root elongation of 10-d-old seedlings exposed to Control (no Al supply) or Al-spiked nutrient solutions, pH 4,2, containing 200 µM Al. Data are means ± SE (for *B. decumbens* and *B. brizantha* n=4, for B. ruziziensis n=21). Y- Axis (root elongation) is different for each Brachiaria species

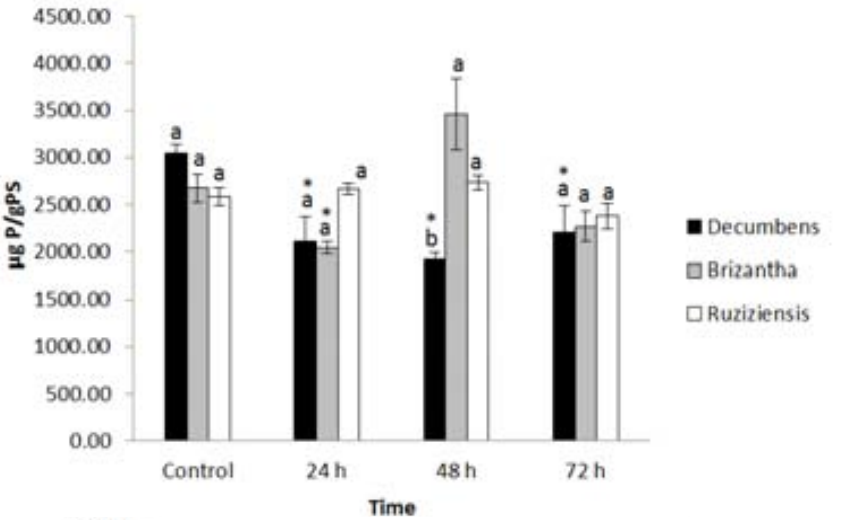
1.3.2. *Mineral nutrition*

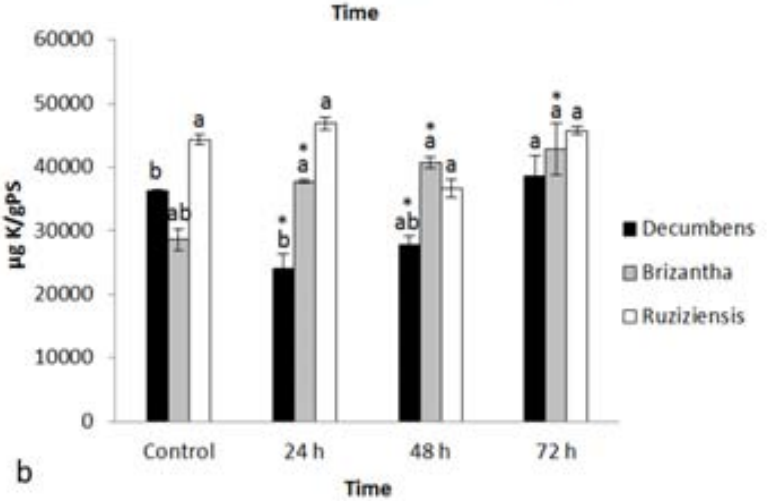
1.3.2.1. *Effects of aluminium exposure on nutrient uptake by Brachiaria species*

Under control conditions *B. decumbens*, *B. brizantha,* and *B. ruziziensis* showed significant differences among their root concentrations of several essential nutrients. (Fig. 4 b, c, d, e, g, h and i). The root concentrations of essential alkaline and alkaline earth elements were lower in *B. decumbens* (K, Ca, and Mg) and *B. brizantha* (K and Ca) than in *B. ruziziensis*. Excepting Fe, concentrations of essential transition metals were also lower in roots of *B. decumbens* (Cu, Zn) and *B. brizantha* (Mn, Zn, Cu) than in those of *B. ruziziensis*. Root boron concentrations were substantially lower in *B. decumbens* and *B. brizantha* than in *B. ruziziensis.* Under control conditions no statistically significant differences in root P concentrations among the Brachiaria species were observed. *Brachiaria brizantha* had significantly lower root sulphur concentrations than the other Brachiaria species.

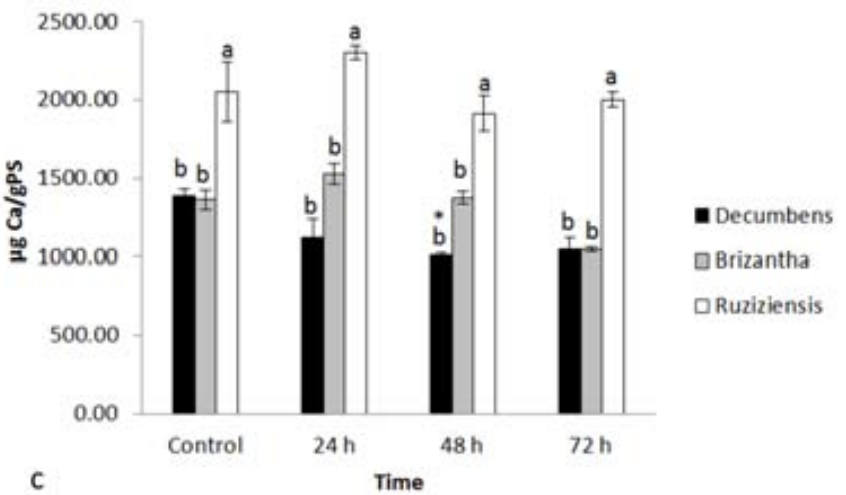
Although all species had low Al root concentrations under control conditions, the mean value for *B. ruziziensis* was slightly higher than in the other species. When exposed to Al the root concentrations of this element steadily increased with exposure time in all species. Roots of *B. ruziziensis* accumulated considerably higher concentrations than those of *B. decumbens* and *B. brizantha*.

Aluminium supply had a marked, time and species dependent influence on the root concentrations of mineral nutrients. In *B. decumbens* root concentrations of P and K exhibited a U shape behaviour in response to Al exposure time with lowest values for short (24 and /or 48h) and recovering after 72h exposure. Root concentrations of P and K of the other Brachiaria species were little affected by Al supply. Aluminium exposure considerably decreased root Mg and Mn concentrations, while Cu and Fe concentrations rather tended to increase, especially Fe in roots of *B. ruziziensis* and Cu in those of *B. brizantha* and *B. decumbens*. The most notable species differences in Al-induced changes were observed for boron. Exposure for 24h to Al caused a 2-3 fold increase of root B concentrations in *B. decumbens* and *B. brizantha*, but did not affect the considerably higher B concentration in *B. ruziziensis*. Longer Al exposure however decreased root B levels in *B. ruziziensis* below detection limit (< 1 µg/g d.w.), while B concentrations *in B. decumbens* and *B. ruziziensis* returned to values after 72h of Al exposure.

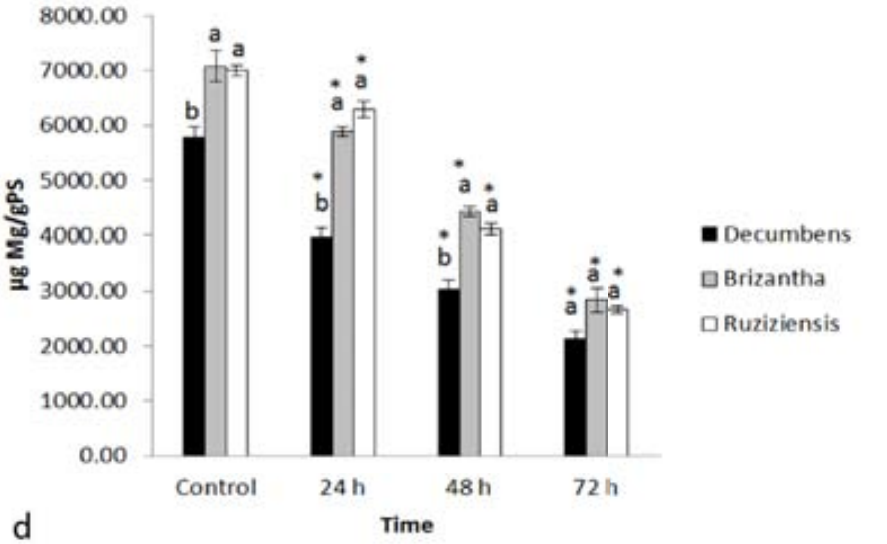

4500.00
4000.00
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
µg P/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis


60000
50000
40000
30000
20000
10000
0
µg K/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

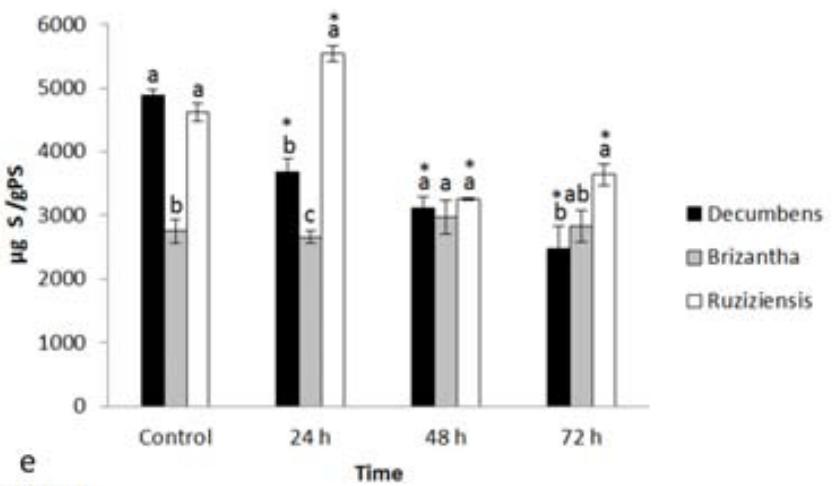
b


2500.00
2000.00
1500.00
1000.00
500.00
0.00
µg Ca/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

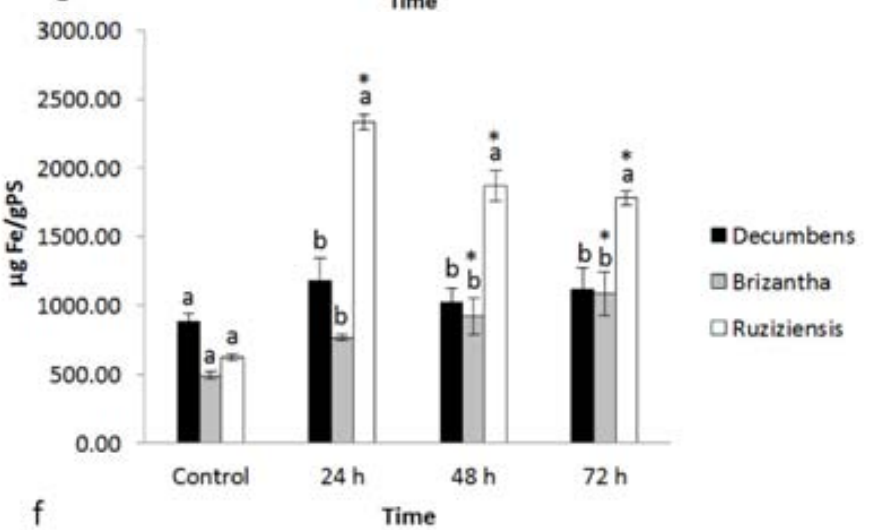
c


8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
µg Mg/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

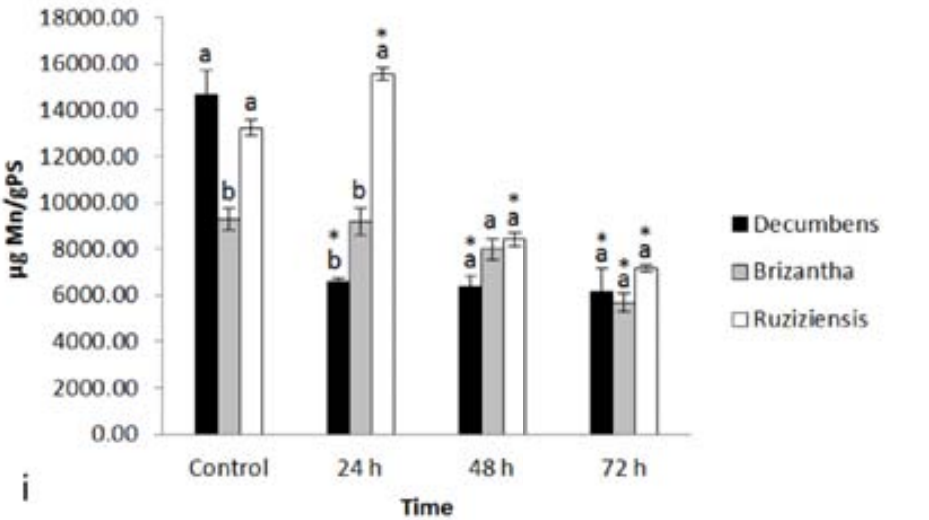
d


6000
5000
4000
3000
2000
1000
0
µg S /gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

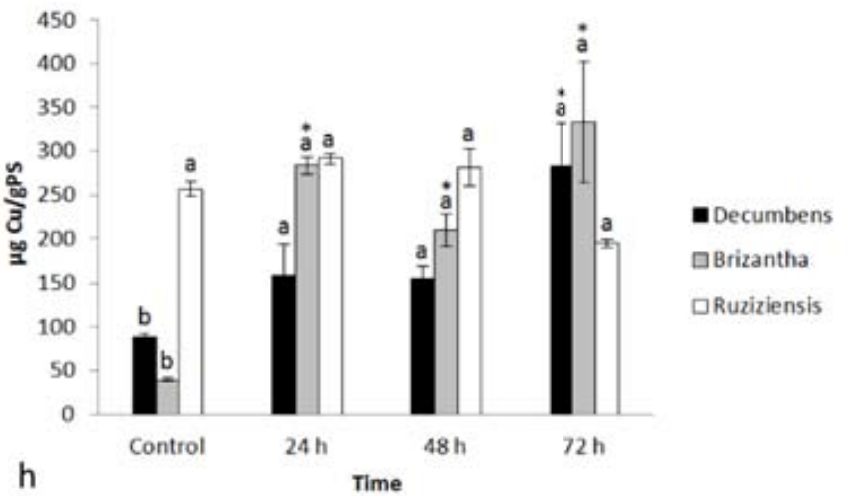
e


3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
µg Fe/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

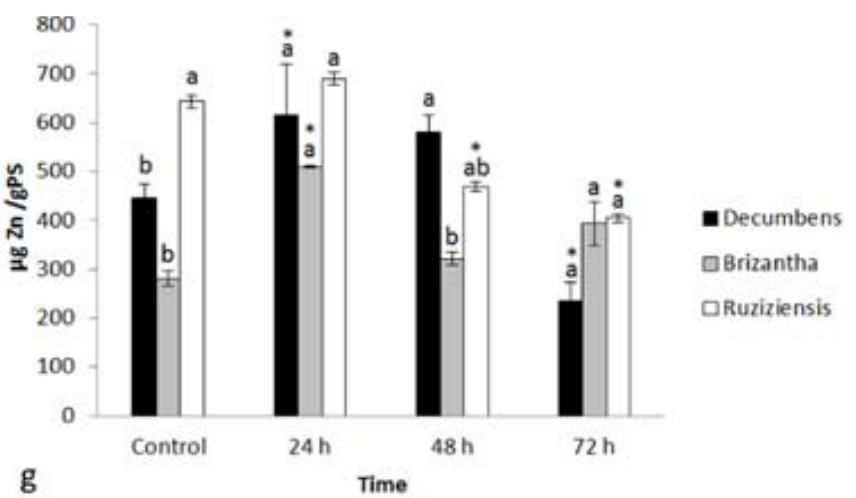
f


18000.00
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
µg Mn/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

i


450
400
350
300
250
200
150
100
50
0
µg Cu/gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

h


800
700
600
500
400
300
200
100
0
µg Zn /gPS
Control
24 h
48 h
72 h
Time
Decumbens
Brizantha
Ruziziensis

g

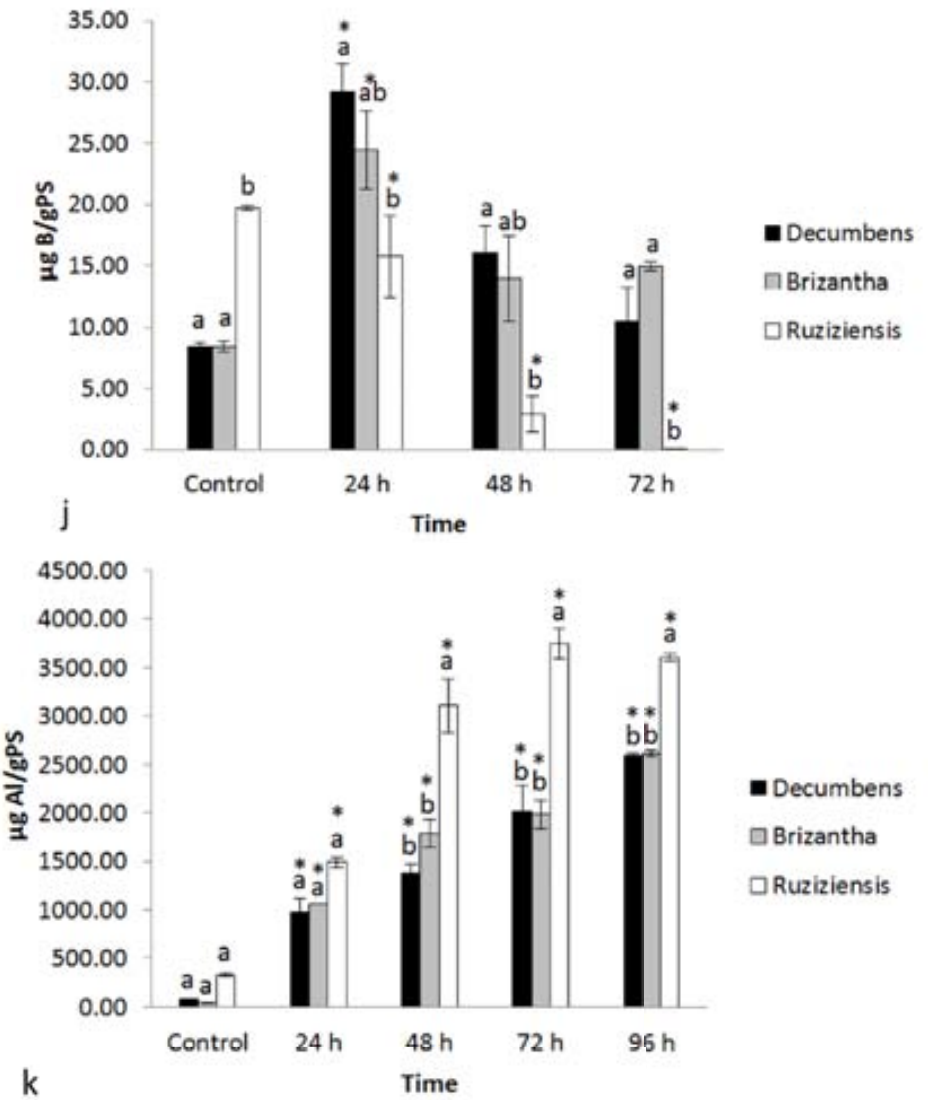


Fig. 4 Root nutrient uptake kinetics for Brachiaria species. Kinetics was evaluated at three times 24, 48 and 72h, under aluminium-treatment 200 µM $AlCl_3$ and control without aluminium. Mean±SE is shown. ANOVA-Tukey HSD **a**. Concentration of Phosphorus (P: µg g^{-1} PS^{-1}). **b**. Concentration of potassium (K: µg g^{-1} PS^{-1}).**c**. Concentration of calcium (Ca: µg g^{-1} PS^{-1}). **d.** Concentration of magnesium (Mg: µg g^{-1} PS^{-1}) **e**. Concentration of sulphur (S: µg g^{-1} PS^{-1}). **f.** Concentration of iron (Fe: µg g^{-1} PS^{-1}). **g**. Concentration of zinc (Zn: µg g^{-1} PS^{-1}). **h**. Concentration or copper (Cu: µg g^{-1} PS^{-1}). **i**. Concentration of manganese (Mn: µg g^{-1} PS^{-1}). **j**. Concentration of Boron (B: µg g^{-1} PS^{-1}). **k**. Concentration of aluminium (Al: µg g^{-1} PS^{-1}). Test Dunnet HSD for species *B. decumbens*, *B. brizantha* and *B. ruziziensis* regarding their respective controls (*)

1.4. Discussion

Considerable species differences in root elongation rates and root mineral nutrient concentrations were observed in control plants growing in acid; low ionic strength nutrient solution without Al. *Brachiaria ruzizie*nsis exhibited the lowest root elongation rate in control solutions. Moreover, this species had by far the highest root Ca and B concentrations under control conditions. In general, plant species largely differ in their Ca and B requirements. Monocots and more specifically grasses have low requirements. But considerable differences in Ca and B accumulation within grasses have been observed. As an example *Agrostis setacea* from acid soils accumulate less Ca than *A. stolonifera* adapted to calcareous soils (Clarkson 1965). Both Ca and B are typically required for cell wall stability (Bolaños et al., 2004; Marschner, 2012) and the higher concentrations of these elements in roots of *B. ruziziensis* suggest higher Ca and B requirements that may reflect differences in cell wall composition. Those, in turn, may be related to the considerably higher Al sensitivity in this species (see below) in comparison to *B. brizantha* and *B. decumbens*. Taken together these results suggest that under our control conditions the root elongation rate of *B. ruziziensis* may have been limited by the acid pH and the low ionic strength of the nutrient solution. The use of this nutrient solution is justified, however, because it represents a realistic approximation to chemical acid soil properties (Wenzl *et al*., 2003).

The marked growth difference between the three Brachiaria species under simultaneously Al-treatment, low pH and by low ionic strength nutrient solution conditions probably was the result of an interaction between the effects of the three stress factors (Poschenrieder *et al*., 1995; Wenzl *et al*., 2003; Schroth *et al*., 2003). Here only the influence of Al-toxicity was evaluated in detail, but comparative study of the mineral nutrients in the plants revealed clear differences in both the quantitative requirements under control conditions and the ability to maintain nutrient homeostasis under Al supply.

According to relative root elongation rates under Al supply, the Al tolerance of Brachiaria species decreased in the order of *B. decumbens >B. brizantha > B. ruziziensis.* In most crop species root growth is reduced by about 50% when exposed to Al^{3+} activities between < 1 to 5 µM. The inhibitory effect can be fast and may occur within 30 to 120 minutes after the exposure to Al (Barceló and Poschenrieder 2002, Doncheva *et al*. 2005; Llugany *et al.,* 2005; Poschenrieder *et*

al., 2008). In this work, the roots of *B. decumbens* and *B. brizantha* continued to elongate in solutions containing 200 µM total Al corresponding to 32 µM Al^{3+} activity, for the entire period of evaluation. After 96 h Al reduced root elongation in *B. brizantha* by 41% while in *B. decumbens* the reduction was only 17%. By contrast, *B. ruziziensis* suffered a 67% inhibition of elongation after this exposure period (96 h). The response to Al in *B. ruziziensis* found here is in line with the observation by Wenzl *et al*., 2001 who reported a 50% inhibition in root elongation for *B. ruziziensis* growing in solution with 115 µM total Al. Our results clearly reveal species differences in Al tolerance within the genus Brachiaria. Aluminium tolerance in the three species considered here followed the order *B. decumbens* > *B. brizantha* > *B. ruziziensis*.

The comparative use of relative root elongation under ion stress conditions as a tolerance index can be influenced by differences among species in root elongation rates under control conditions. A low root elongation rate is a common characteristic in many Al-resistant species or varieties (Amenós *et al*., 2009). This is also the case in the Brachiaria species considered here. The root elongation rate of *B. decumbens* is characteristically lower than that of *B. brizantha*. This lower growth rate can contribute to a certain overestimation of Al resistance, at least in short-term evaluations. However, the observation that *B. decumbens* exposed to Al recovered a root elongation rate close to control values after a lag time of 48 to 72 hours not only confirms the Al hyperresistance of this species, but also suggest that at least part of the Al tolerance mechanism is Al-inducible. In this sense *B. decumbens* behaves similar to some Al tolerant maize varieties were also a lag time of several hours was required for full expression of Al tolerance (Kidd *et al.*, 2001).

Although *B. ruziziensis* is the most Al sensitive species among those analysed here, its ability to maintain a low but still significant root elongation at 32 µM Al^{3+} activity makes it clearly more tolerant than most other fodder grasses. In this sense *B. ruziziensis* can be classified as a species with low Al sensitivity, while *B. brizantha* and *B. decumbens* can be classified as moderately tolerant and hypertolerant, respectively.

The higher Al tolerance in *B. decumbens* and *B. brizantha* than in *B. ruziziensis* was clearly related to better Al exclusion from the roots as indicated by the lower root Al concentrations in these species (Fig 4 k). Besides Al tolerance, tolerance to H^+,

and Mn toxicity, and Mg, Ca, K, P and Mo deficiency seem important characteristics for performance in acid tropical soils (Kamprath and Foy, 1985; Rao, 2001; Poschenrieder *et al*., 1995, 2008). Brachiaria species (*B. decumbens*, *B. brizantha* and *B. ruziziensis*) showed differences in their ability to maintain nutrient homeostasis under Al-treatment upon time.

Exposure to Al can inhibit the uptake of many cations including Ca^{2+}, Mg^{2+}, K^{+}, and NH^{+}_{4}. Al^{3+}may interact directly with several different plasma-membrane channel proteins blocking the uptake of ions such as K^{+} and Ca^{2+} (Kochian *et al*., 2005). All three Brachiaria species analysed here maintained low but constant root Ca^{2+} levels under Al supply, while Mg concentrations were substantially decreased in all of them. In rice and bean it has been demonstrated that Mg can alleviate Al toxicity. This, ameliorative effect of Mg might be related to greater citrate efflux (Yang *et al*., 2007). While reduction of Mg concentrations has frequently been described as a sign of Al toxicity, better Al tolerance in wheat or maize varieties were not accompanied by the ability to avoid this Al-induced decrease of Mg tissue levels (Poschenrieder *et al*., 1995; Silva *et al.*, 2010). This is in line with the findings in Brachiaria reported here and also by Wenzl et al. (2003) in signalgrass and ruzigrass. Also root Mn concentrations decrease with Al supply in all three species. Nonetheless, concentrations remained higher than root Fe concentrations. The considerably high root Mn concentrations may reflect the high Mn tolerance reported for these Brachiaria species by Rao (2001).

Potassium is extremely important for osmotic balances, and therefore for cell extension (Silvia *et al.*, 2010). Root K^{+} concentrations were hardly affected *in B. briza*ntha and *B. ruziziensis*. Surprisingly, in the most Al tolerant *B. decumbens* K^{+} was significantly reduced by exposure to Al for 24 h, but recovered after 48 to 72h. This decrease is coincident with the transient Al induced inhibition of root elongation in *B. decumbens*.

In many studies it has been proposed that the Al resistance may be associated with an immobilization of Al by P in the root tissues. Zheng *et al*., 2005 found that the concentration of Al and P in an Al-resistant cultivar was significantly higher than that of a sensitive cultivar of *Fagopyrum esculentum*. They suggested that immobilization of Al with P within cell walls was involved in the high Al resistance of buckwheat. Similar results have also been found in maize (Vázquez *et al*., 1999) and *Avena sativa* (Marienfeld and Stelzer, 1999). Root P concentrations observed

here are not in line with this view. Phosphorus and S concentrations were more affected (in the kinetic) by Al in *B. decumbens* than in *B. brizantha and B. ruziziensis*. The levels for all Brachiaria species were below the usual levels reported for grasses (Bergman, 1988).

Many soils affected by metal toxicity have lower-than-optimal concentrations of available essential nutrients, low pH, and combinations of toxic metals, thus intensifying the effect of individual toxic metals other than aluminium (Rao, 2001; Wenzl *et al.,* 2003). Furthermore, as Al availability increases under those conditions, the accumulation of other elements such as Cu or Fe also may increase and eventually may reach toxic levels in Al sensitive genotypes (Silvia *et al*., 2010). In fact, the most Al sensitive *B. ruziziensis* exhibited a strong Al-induced increase in roots Fe concentrations (Fig. 4 f). Contrastingly, *B. ruziziensis* maintained Cu concentrations along the Al exposure period while an Al-induced increase of Cu levels in the Al tolerant *B. brizantha* was found (Fig. 4 h).

The most conspicuous differences among species were found for Al–induced changes in root boron levels. While in *B. decumbens* a sharp but transient increase of B levels was observed after 24h exposure to Al, in the Al sensitive *B. ru*ziziensis Al decreased B concentrations (Fig. 4 j). After 72h Al exposure the B concentrations in roots of *B. ruziziensis* felt below detection limit (< 1µg g^{-1} dry weight). Previous investigations revealed better maintenance of B homeostasis in Al tolerant than in Al sensitive maize varieties (Poschenrieder *et al*., 1995). B-deficiency exacerbates Al toxicity in both dicots and monocots (Corrales *et al*., 2008). Boron deficiency has been reported to increase the proportion of unmethylated pectins in root tip cell wall. In primary cell walls (PCW) rhamnogalacturonans II (RG-II) predominantly exist as dimer (dRG-II) that is covalently cross-linked by borate diester. So, cell wall structure and function largely depends on the interaction between borate and this type of pectic polysaccharide. Pectins are required for the formation of the pectin network in cell walls and contribute to the mechanical strength and physical properties of the PCW and are essential to normal plant growth and development (Yapo, 2011). When the pectins are not methylated, the pectic matrix may strongly bind Al to the cell wall. In this sense the degree of pectin methylation has been proposed as a main factor for Al resistance of roots (Horst *et al.,* 2010).

In accordance to this, the strong Al-induced reduction of root B concentrations in *B. ruziziensis* may decrease pectin methylation and enhance Al binding, thus causing severe Al toxicity in the form of inhibition of root elongation. In addition, root growth in *B. ruziziensis* was probably further inhibited due to acute B deficiency induced by Al in combination with poor adaptation to deficiency of other mineral nutrients (Wenzl *et al.,* 2003).

Otherwise, the Al resistant *B. decumbens* and *B. brizantha* species exhibited a strong but transient increase of root B 24h after Al exposure. In *B. decumbens* this increase was coincident in time with Al induced inhibition of root elongation and substantial alterations of cell wall structure. If this jump in cell wall B concentrations is related or not to the activation of Al tolerance mechanisms in *Brachiaria decumbens* clearly deserves further investigations (see also chapter 5).

1.5. References chapter 1

Amenós M., Corrales, I., Poschenrieder, C., Illes, P., Baluska, F., Barceló J. 2009. Different effects of aluminum on the actin cytoskeleton and brefeldin A-sensitive vesicle recycling in root apex cells of two maize varieties differing in root elongation rate and aluminum tolerance. Plant Cell Physiol 50: 528-540

Bolaños L., Lukaszewski K, Bonilla I, Blevins D., 2004. Why boron? Plant Physiol Biochem 42: 907-912

Clarkson, DT., 1965 Calcium uptake by calcicole and calcifuges species in the genus Agrostis L. J Ecol 53: 427-435

Fukuda, T., Saito, A., Wasaki, J., Shinano, T., Osaki, M. 2007. Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. Plant Sci 172: 1157–1165

Garzón, T., Gunsé, B., Rodrigo, A., Tomos, D., Barceló, J., Poschenrieder, C. 2011. Aluminium-induced alteration of ion homeostasis in root tip vacuoles of two maize varieties differing in Al tolerance. Plant Sci 180: 709–715

Hinsinger, P., Brauman, A., Devau, N., Gerard, F., Jourdan, C., Laclau, JP., Le Cadre., E., Jaillard, B., Plassard, C. Acquisition of phosphorus and other poorly mobile nutrients by roots. Where do plant nutrition models fail? Plant Soil 348: 29-61

Horst, W., Wang, Y., Eticha, D. 2010. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. Ann Bot 106: 1 185-197

Kamprath E. and Foy, C. 1985. Lime fertiliser plant interactions in acid soils. In: O. Engelstad. (Ed.) Fertiliser Plant technology and use. Soil Sci Soc Amer Madison, Wisconsin 91-151

Kidd, P., Llugany, M., Poschenrieder, C., Gunsé, B., Barceló, J. 2001. The Role of exudates in aluminum resistance and silicon–induced amelioration of aluminum toxicity in three varieties of maize (*Zea mays* L.). J Exp Bot 52: 1339 – 1352

Kochian, L., Piñeros, M., Hoekenga, A. 2005. The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. Plant Soil 274: 175–195

Llugany, M., Massot, N., Wissemeier, AH., Poschenieder, C., Horst WJ., Barceló, J. 1994. Aluminum tolerance of maize cultivars as assessed by callose production and root elongation. Z Pflzernähr Bodenk 157: 447-451

Marienfeld ,S., Stelzer, R. 1993. X-ray microanalyses in roots of Al-treated *Avena sativa* plants. J Plant Physiol 141: 569–573

Marschner, P. 2012. MARSCHNER'S Mineral Nutrition of Higher Plants. 3rd edition Academic Press, Amsterdam

Massot, N., Llugany, M., Poschenrieder, C., Barceló, J. 1999. Callose production as indicator of aluminium toxicity in bean cultivars. J Plant Nutr 22: 1-10

Piñeros, A., Kochian, L. 2001. A patch-clamp study on the physiology of aluminum toxicity and aluminum tolerance in maize. Identification and characterization of Al (3+)-induced anion channels. Plant Physiol 125: 292–305

Poschenrieder, C., Gunsé , B., Corrales, I., Barceló, J. 2008. A glance into aluminum toxicity and resistance in plants. Sci Total Environ 400: 356–368

Poschenrieder, C., Llugany, M., Barceló, J. 1995. Short-term effect of pH and aluminium on mineral nutrition in maize varieties differing in proton and aluminium tolerance. J Plant Nutr 18: 7, 1495-1507

Louw-Gaume, A., Rao, M., Gaume, A., Frossard, E. 2010. A comparative study on plant growth and root lasticity responses of two Brachiaria forage grasses grown in nutrient solution at low and high phosphorus supply. Plant Soil 328:155–164

Rao, I. 2001. Adapting tropical forages to low-fertility soils. In: J. A. Gomide, W. R. S. Mattos and S. C. da Silva (Eds.) Proceedings of the XIX International Grassland Congress. Brazilian Society of Animal Husbandry, Piracicaba, Brazil 247-254

Rengel, Z. Damon, PM. 2009. Crops and genotypes differ in efficiency of potassium uptake and use. Physiol Plant 133: 624-636

Rengel, Z., Zhang, WH. 2003. Role of dynamics of intracellular calcium in aluminium-toxicity syndrome. New Phytol 159: 295-314

Schroth, G., Lehmann, J., Barrios, E., 2003. Soil nutrient availability and acidity. In: Schrith, Sinclair (Eds.), Trees, Crops and Soil Fertility. CAB International 93–130

Vázquez, M., Poschenrieder, C., Corrales, I., Barceló J. 1999. Changes in apoplastic aluminum during the initial growth response to aluminum by roots of tolerant maize variety. Plant Physiol 119: 435–444

Wenzl, P., Chaves, A., Patiño, M., Meyer, J., Rao, I. 2001. The High Level of Aluminum Resistance in Signalgrass Is Not Associated with Known Mechanisms of External Aluminum Detoxification in Root Apices. Plant Physiol 125: 1473–1484

Wenzl, P., Chaves, A., Buitrago, M., Patiño, M., Meyer, J., Rao, I. 2002. Aluminium Stress Stimulates the Accumulation of organic acids in Root Apice of Brachiaria species. J Plant Nutr Soil Sci 165: 582-588

Wenzl, P., Mancilla, L., Mayer, J., Albert, R., Rao, I. 2003. Simulating Infertile Acid Soils with Nutrient Solutions: The Effects on Brachiaria Species. Soil Sci Soc Am J 67:1457–1469

Wenzl, P., Arango, A., Chaves, A., Buitrago, M., Patiño, M., Miles, J. Rao, I. 2006. A Greenhouse Method to Screen Brachiariagrass Genotypes for Aluminum Resistance and Root Vigor. Crop Sci 46:968-973

Yapo, B. 2011. Pectin Rhamnogalacturonan II: On the “Small Stem with Four Branches” in the Primary Cell Walls of Plants. Int J of Carb Chem ID 964521. 2011:10.1155- 964521

Yang, J., Jiang, F., Ya, Y., Ping, W., Shao, J. 2007. Magnesium enhances aluminum-induced citrate secretion in rice bean roots (*Vigna umbellata*) by restoring plasma membrane H+-ATPase activity. Plant Cell Physiol 48: 66–73

Chapter 2

Toxic Effects and Distribution of Aluminium in the Root Apex of Brachiaria Species

Chapter 2

2.1. Introduction

Root tips have been identified as the primary site of Al accumulation and Al toxicity effects in sensitive plants. Target sites for aluminium attack in plant root tips are located in the apoplasm (cell walls), at the plasma membrane, and in the symplasm (cytosol). Cell walls are primary sites for Al binding. The chemical and mechanical properties of the cell wall can be modifying by aluminium. Cell wall pectins containing negatively charged carboxyl groups may attract the trivalent Al^{3+}, causing cross linking of cell wall components and reduce cell wall extensibility. Al has a higher binding affinity to carboxylic groups than Ca^{2+}, thus resulting in a displacement of cell wall Ca^{2+} by Al. Al stress often results in the deficiency of calcium. In Al-sensitive wheat it was found that aluminium modifies the metabolism of cell-wall components and thus makes the cell wall thick and rigid, increased the weight-average molecular mass of hemicellulosic polysaccharides. Al^{3+} modifies Ca^{2+} homeostasis, inducing perturbations in cellular Ca^{2+}metabolism. Exposure to aluminium may induce alterations of the structure of calcium receptors that regulate the biological activities of a large variety of proteins. The binding of Al ions to the plasma membrane proteins can accelerate the efflux of K^+ and inhibit the influx of K^+. Aluminium exposure caused rapid depolarization of the plasma membrane and long-term may cause peroxidation of membrane lipids causes' loss of membrane integrity (Ishikawa and Wagatsuma, 1998; Piñeros *et al.,* 2001; Basset and Matsumoto, 2008). Aluminium affects the hydraulic conductivity of root cortical cells and of the entire root system (Gunsé *et al*., 1997; Tabuchia and Matsumoto, 2001; Yang *et al.,* 2008)

The objectives of the present study were to investigate location of Al in roots of Brachiaria species differing in AI tolerance in relation to toxic effects visualized by membrane damage.

2.2. Materials and methods

2.2.1. *Plasma membrane integrity*

The loss of plasma membrane integrity was evaluated by a spectrophotometric assay using Evans blue stain. Roots tips were stained in 0.25% (w/v) aqueous solution of Evans blue for 15 min, washed three times with distilled water, for 10 min each, according Baker and Mock (1994) and photographed. After staining with

Evans blue, 1 cm root tips of 15 roots per species and treatment were incubated for quantification of membrane damage with 1 mL N, N dimethylformamide. Optical density was measured spectrophotometrically at 600 nm, according to Kikui *et al*., (2005)

2.2.2. *Hematoxylin staining of roots*

After 24-h exposure to 200 µM Al, seedlings were stained for visual detection of aluminium using the method proposed by Polle *et al*., (1978)

2.2.3. *Aluminium detection with morin staining*

Morin is histochemical stain with high specificity for Al. For morin staining, roots of seedlings grown in solution culture were exposed for 24 h in aluminium to 200 µM; staining with morin was performed according to the method described by Larsen et al., (1996). Visualization was done with a fluorescence microscope equipped with a lens Nikon H55OS, intralux 5100 and Nikon digital sight DS-V2-NIS-Elements F2.30 program.

2.2.4. *Fixation, embedding and sectioning for microscopy*

Root tip segments (5 mm) of primary root apices from control and Al treatments were excised and fixed, according to Amenós *et al.*, (2007, 2009). Sample thickness after microtome sectioning was 14 µm.

2.2.5. *Aluminium detection with lumogallion-DAPI stain*

Roots from both plants exposed and not (control) to Al were stained with lumogallion to visualize Al distribution in the root tip of the Brachiaria species. Lumogallion is an Al-specific stain that shows green fluorescence due to Al-lumogallion complex. DAPI counter-staining was used to visualize the nuclei.

The samples were fixed in 3.7% formaldehyde in saline phosphate-buffer (PBS), dehydrated in a graded alcohol series (alcohol 30, 50, 70, 90 and 97 %) according to Amenós et al., 2009 but with a modification in the dehydration time (forty minutes for each series of alcohol). Samples were embedded in low melting point Steedman's WAX. Embedded samples were sectioned at a thickness of 15 µm on a Cambridge rotary rocking Reichert microtome. The samples were rehydrated in a decreasing concentration series of alcohol and stained with lumogallion (10 µM acetate buffer at pH 5.2 for 60´at 50 º C) and with DAPI (10 µM acetate buffer, pH

5.2). Sections were mounted on slides and sealed with 90% glicerol. The fluorescence emitted from Al-lumogallion complex was observed under a confocal laser optic microscope (TCS SP2 AOBS TCS SP2 AOBS; Leica microscopy Systems, LTD, Heidelberg) using the 488 nm excitation line from the argon laser.

2.3. Results

2.3.1. *Comparison of Al-resistance between three Brachiaria species*

The influence of Al supply on plasma membrane integrity was followed using Evans blue staining. The technique not only allows visualizing the membrane damage, but also estimating it quantitatively (Fig. 6). Roots of *B. decumbens* seedlings exposed to aluminium for different time periods showed a shallow staining mainly after time 24 and 96 h. For *B. brizantha* there was intense staining with Evans blue. Intense colouration was mainly observed in the meristematic zone. In this species the colouration was constantly high after 48, 72 and 96 h. Root tip membrane integrity was most intensively affected by Al in *B. ruziziensis* (Fig. 6). *Brachiaria ruziziensis* showed symptoms of Al injury, stubby appearance, cracks and deformed root apices. The staining with Evans blue was most intense after 48 and 72 h of Al-treatment, while after 96 h of treatment staining intensity decreased, coincident with the appearance of new lateral roots close to the tip..

Seedlings differences in Al-tolerance could also be shown by staining with haematoxylin after 24 h exposures to nutrient solutions that contained 200 µM Al (Fig. 5). Root tips of the Al-sensitive *of B. ruziziensis* seedlings stained with haematoxylin exhibiting intense purple colour. This staining indicated the presence of Al in the root tip, in the meristematic zone, the transition zone and the mature root zone. In root tips of *B. brizantha* haematoxylin stain indicated presence of Al, in the meristematic zone and the transition zone; but staining was less intense than in *B. ruziziensis* (Fig. 5). In roots of *B. decumbens* the root remained unstained excepting numerous small spots with intense stain accumulation scattered all over the entire root from cap to the mature root zone. (Fig. 5B)

2.3.2. *Localization of aluminium by lumogallion-Al, and morin-Al complex fluorescence in roots of B. decumbens and B. brizantha*

Morin is an Al-specific fluorochrome and is widely used to localize Al in plant tissues (Eticha *et al*., 2005). Roots tips of Brachiaria species stained with morin exhibiting similar distribution as observed for haematoxylin. *Brachiaria brizantha* showed fluorescent signal intensity much higher than that of *B. decumbens* (Fig. 7). In *B. decumbens* the green fluorescence was concentrated in structures (spots) looking like root hairs and border cells (Fig. 7).

Comparisons of fluorescent and white light microscopy pictures of morin-stained root tips of *B. decumbens* (Fig. 8) revealed that the abundant border cells detaching from the root cap did not stain for Al (Fig. 8 A, B). As an example, the head of the red arrow in picture 8 A points exactly to the same border cell as that seen in (Fig. 8 B) and in Fig. 8 D and E at higher magnification. Morin-stainable structures were only found in close contact with the root surface (Fig. 8 A, D). Fig. 8F shows a white light microscopy picture of a root tip from *B. decumbens* exposed to Al for 72 h. Small root hairs emerging from the epidermal cells are clearly visible at a distance between 300 and 500 µm from the tip. Fluorescence image of morin-stained tips at this distance revealed Al accumulation in these young root hairs (Fig. 8 G). Also the older hairs at larger distance from the tips intensively stained for Al with morin (Fig. 8 C). Aluminium apparently did not restrict root hair formation in *B. brizantha* (not shown).

When longitudinal-section were stained with lumogallion (green) and the nuclei visualized by DAPI (blue), intense fluorescence was evident in the root cell wall of *B. brizantha* (Fig.7 d). While in longitudinal-section of roots apex of *B. decumbens* lumogallion stain remained in the root cap and in the cell walls of the detaching outermost cell layer of the root tip (Fig. 7c)

2.4. Discussion

2.4.1. *The integrity of the plasma membrane*

The plasma membrane is considered a primary target for Al toxicity. Uptake of Evans blue into the cells is as an indicator of plasma membrane of damage and of cell death (Baker and Mock 1994).

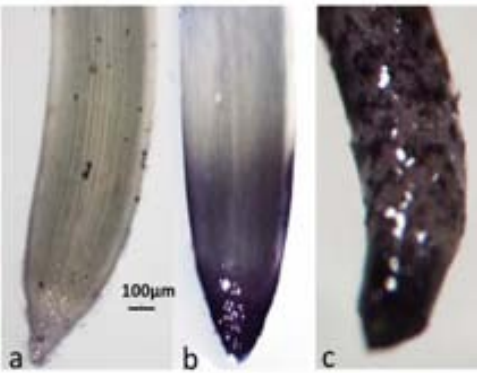


Fig.5. Haematoxylin stained root tips of Brachiaria species. a) *B. decumbens*, b). *B. brizantha* and c) *B. ruziziensis* roots were exposed for 24 h to 200 µM $AlCl_3$ in (32 µM Al^{3+} activity) in low ionic strength nutrient solution, at pH 4.2.

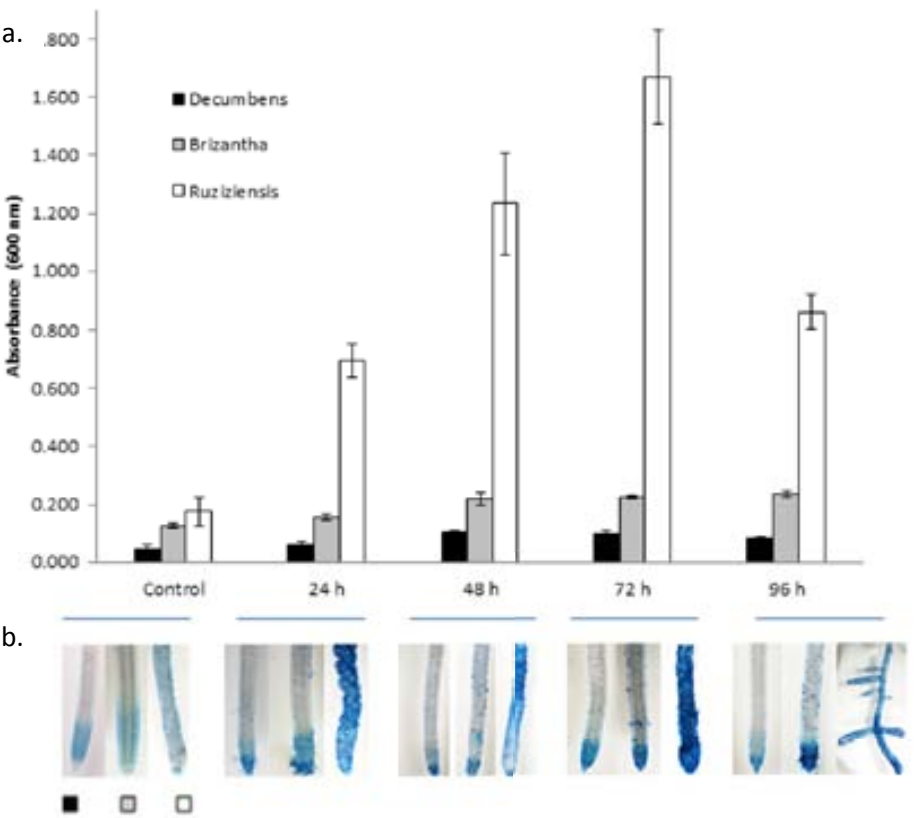


Fig.6. Histochemical detection showed: Aluminium effect on plasma membrane integrity of Brachiaria species roots apex. After treatment of seedlings with or without 200 µM $AlCl_3$ for 4 times 24, 48, 72 and 96 h. a) the quantitative determination of Evans blue stain retained in a 10-mm section from root tip was performed. b) The integrity of plasma membrane of roots was examined by the degree of Evans blue uptake. All values are means ± SD (n =15)

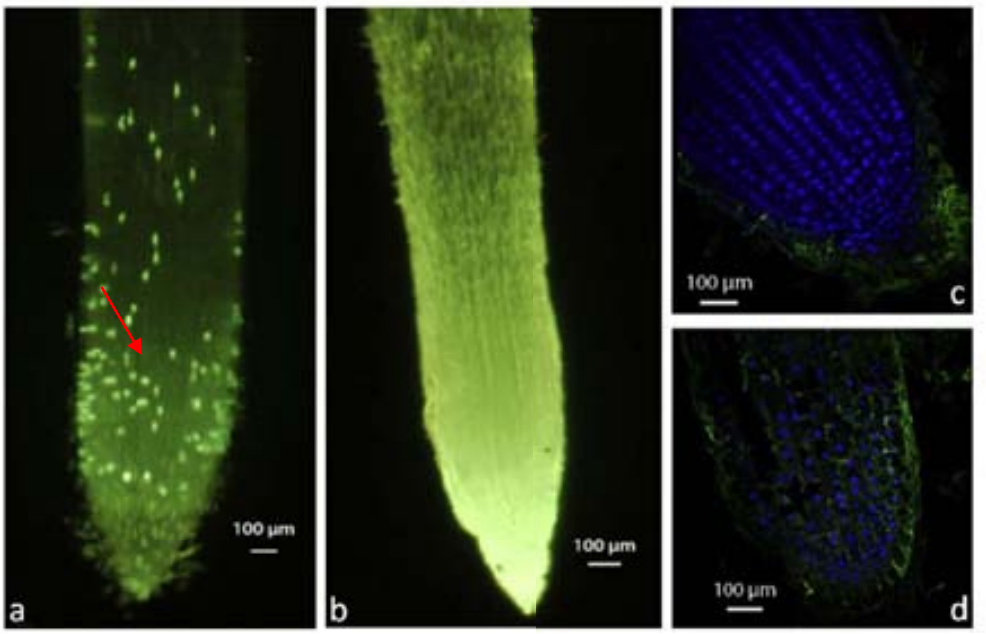


Fig.7 . Morin-stained whole root tips of a) and *B. decumbens*. b) *B. brizantha* plants exposed for 24 h to 200 µM Al (32 µM Al^{3+} activity) in low ionic strength nutrient solution. (Green fluorescence morin-Al). Longitudinal sections of root tips stained with lumogallion-DAPI. The roots were exposed for 24 h to 200 µM Al (32 µM Al^{3+} activity). From the root tip c) and *B. decumbens* d) *B. brizantha* for better visualization of cells root tip nuclei were stained with DAPI (blue) and lumogallion-Al (green)

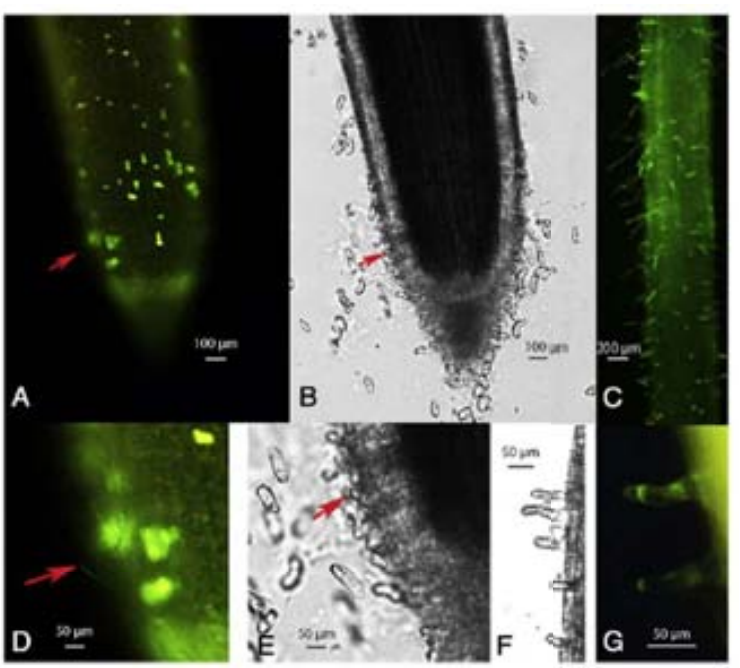


Fig.8. Root tips of *B. decumbens* exposed to Al for 4 h (A, B, D, E) or 72 h (C, F, G) and stained with morin. Abundant border cells either adhering to (arrow) or scattered around the root are seen in white light microscopy images (B, E), but poorly visible in fluorescent microscopy (A, D). Root hairs developing close (300–500 µm) to the tip (F, G) and in upper root zones (500 to 1500 µm from tip) (C) stained with morin.

Therefore, Evan's blue staining seems a reliable indicator for genotype differences in Al tolerance (Kikui *et al*., 2005). Here we observed clear differences in Evan's blue staining among the Brachiaria species. A slight and constant Evan's blue staining over the experimental Al exposure up to 96 h in *B. decumbens* root tips indicate rather limited plasma membrane damage in this species. *B. brizantha* exhibited a slight increase of Evans blue staining with the Al exposure time. This contrasts with the intense membrane damage in roots of *B. ruziziensis* where staining considerably increased with the Al exposure time, excepting after 96 h were lower absorbance was observed. This lower Evans blue staining after 96 h Al-treatment was coincident, however, with a considerable change in root architecture with abundant laterals roots emerging close to in the root tips. As the new laterals initially stained less this decrease of the absorbance after 96 h of Al-treatment is not a sign of stress recovery. On the contrary, the Al–induced enhancement of lateral root initiation is a sign of Al toxicity to the tip of the main root as found by Doncheva *et al*., 2005 in Al sensitive maize exposed to 50 µM Al for 180 min.

The staining results confirm the better Al tolerance of *B. decumbens*, followed by *B. brizantha*, being *B. ruziziensis* the most Al sensitive species. Results from Evans blue indicated that the integrity of the plasma membrane of roots was also slightly affected in control solutions (Fig. 6 a). This probably was due to the low ionic strength of the nutrient solution used here with considerably low Ca concentrations. Also under these control conditions *B. decumbens* stained less with Evan's blue than the other Brachiaria species indicating that *B. decumbens* is also better adapted than other Brachiaria species to the low ionic strength of the solutions that are typical for acid mineral soils in the tropics.

2.4.2. *Differential aluminium tolerance between two Brachiaria species*

Haematoxylin has widely been used by several researchers in different crop species, such as wheat (*Triticum aestivum L*.), rice mutants and Brachiaria species exposed to aluminium at lower concentration than those used here (Polle *et al*., 1978; Delhaize *et al.,* 1993; Wenzl *et al*., 2001; Kikui *et al*., 2005; Poschenrieder *et al.*, 2008) Haematoxylin staining can detect aluminium accumulation by the formation of an intense purple coloration in the root tips of sensitive species. The three species of the genus Brachiaria studied behaved differently in the presence

of aluminium in low ionic strength nutrient solution. *B. decumbens* accumulated less haematoxylin stainable Al than *B. brizantha* and *B ruziziensis*.

Both staining haematoxylin and morin revealed hot spots of Al concentrations scattered all over the root tip surface, while the rest of the root tip tissue remained unstained. These deposits (2–5 µm) (Fig. 13 D chapter 3) were much smaller and less abundant than the hot spots of Al accumulation with a size of (30–50 µm) observed by morin (Fig. 7 a) or haematoxylin (Fig. 2B) staining. These bigger Al-rich structures correspond to root hair initials that developed close to the apex (Fig.13 B, C; Fig. 8G chapter 3). Border cells either adhering to the tips or already detached (Fig. 13 B, E chapter 3), did not stain with morin (Fig. 8 A, D). As morin cannot stain all forms of Al, we cannot exclude the possibility that border cells of *B. decumbens* accumulate Al. Production of mucilage or of antioxidant enzymes by border cells has been suggested as protective mechanisms against Al toxicity in snap beans, wheat, and rice (Miyasaka and Howes, 2001; Cai *et al*., 2011)

The root meristem and transition zone have been found to be the primary site of Al entry (Illés *et al*., 2006) in Al-sensitive plants. In these zones usually no root hairs are formed. Root hairs that are important for the uptake of essential mineral nutrients are usually formed at much larger distance from the apex. In the Al-resistant Brachiaria, however some root hairs developed closer to the tip, even within the 100 to 500 µm root apex zone (Fig. 13 F, G chapter 3 and 12. a,b,c,d chapter 3). This formation of root hairs in unusual position indicates that Al causes a change in epidermal cell patterning in *B. decumbens*. Root hair initiation is dependent on auxin and ethylene signalling (Schiefelbein *et al*., 2009; Narukawa *et al*., 2009). Aluminium seems to be an efficient inhibitor of polar auxin transport in Al sensitive roots (Amenós et al., 2009).

Scanning Electron Microscope/Energy Dispersive Spectrometry, confocal fluorescence microscopy and optical microscopy of lumogallion or morin-stained roots was performed. Lumogallion and morin are fluorescent dyes with a high sensitivity for Al. (Kataoka *et al*., 2001; Tanoi *et al.,* 2001; Amenós *et al*., 2009) roots of *B. decumbens* accumulated less Al than those of *B. brizantha*. Moreover, location and Al form seemed different. In *B. decumbens* Al accumulation was localized in hot spots significant increase in morin staining was observed (Fig.7

arrow), whereas *B. brizantha* exhibited more lumogallion-stainable Al in both cell walls of the longitudinal-cross of root tip.

In conclusion, different Al specific staining techniques reveal clear differences in Al accumulation and distribution among the Brachiaria species. Highest Al tolerance in *B. decumbens* is accompanied by relatively low stainable Al and a concentration of this stainable Al in root hairs.

2.5. References chapter 2

Amenós, M., Corrales, I., Poschenrieder, C., Illés, P., Baluska, F., Barceló, J. 2009. Different Effects of Aluminum on Actin Cytoskeleton and Brefeldin A-sensitive Vesicle Recycling in Root Apex Cells of Two Maize Varieties Differing in Root Elongation Rate and Al Tolerance. Plant Cell Physiol 50: 528-540

Amenós, M. 2007. Respuestas Primarias a la Toxicidad por Aluminio en raíces de plantas de maíz con diferente resistencia. Tesis Doctoral, Universidad Autónoma de Barcelona

Baker, CJ., Mock NM. 1994. An improved method for monitoring 402 cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell Tiss Org 39: 7–12

Basset, R and Matsumoto, H. 2008. Aluminum toxicity and Ca depletion may enhance cell death of tobacco cells via similar syndrome. Plant Signaling & Behavior 3: 290-295

Cai, M., Zhang, S., Xing, C., Wang, F., Ning, W., Lei, Z. 2011. Developmental characteristics and aluminum resistance of root border cells in rice seedlings. Plant Sci 180 5: 702-708

Delhaize, E., Craig, S., Beaton, C., Bennet, R., Jagadish, V., Randall, P. 1993. Aluminum Tolerance in Wheat (Triticum aestivum 1.). 1. Uptake and Distribution of Aluminum in Root Apices. Plant Physiol 103: 685-693

Doncheva, S., Amenós, M., Poschenrieder, C., Barceló, J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. J of Exp Bot 56: 414 1213–1220

Eticha,D., Staß, A., Horst, W. 2005. Localization of aluminium in the maize root apex: can morin detect cell wall-bound aluminium?. J of Exp Bot 56: 415 1351–1357

Gunsé, B., Poschenrieder, C., Barceló, J. 1997. Water transport properties of roots and root cortical cells in proton- and Al-stressed maize varieties. Plant Physiol 113, 595–602

Illés, P., Schlicht, M., Pavlovkin, J., Lichtscheidl, I., Baluska, F., Ovecka, M. 2006. Aluminium toxicity in plants: internalization of aluminium into cells of the transition zone in Arabidopsis root apices related to changes in plasma membrane potential, endosomal behaviour, and nitric oxide production. J Exp Bot 57: 4201–4213

Ishikawa, S and Wagatsuma, T. 1998. Plasma Membrane Permeability of Root-Tip Cells Following Temporary Exposure to Al Ions Is a Rapid Measure of Al Tolerance among Plant Species. Plant Cell Physiol 39: 516-525

Kataoka, T and Nakanishi T. 2001. Aluminium distribution in soybean root tip for a short time Al treatment. J Plant Physiol 158: 731–736

Kikui, S., Sasaki, T., Maekawa, M., Miyao, A., Hirochika, H., Matsumoto, H., Yamamoto, Y. 2005. Physiological and genetic analyses of aluminium tolerance in rice, focusing on root growth during germination. J Inorg Biochem 99: 1837–1844

Miyasaka, S. and Hawes, M. 2001. Possible Role of Root Border Cells in Detection and Avoidance of Aluminum Toxicity. Plant Physiol 125: 1978–1987

Narukawa, M., Kanbara, K., Tominaga, Y., Aitani, Y., Fukuda, K., Kodama, T., Murayama, N., Nara, Y., Arai, T., Konno, M., Kamisuki, S., Sugawara, F., Iwai, M. Inoue, Y. Chlorogenic acid facilitates root hair formation in lettuce seedlings. Plant Cell Physiol 50 : 504–514

Larsen, P., Tai, C., Kochian, L., Howell, S. 1996. Arabidopsis Mutants with increased Sensitivity to Aluminum. Plant Physiol 110: 743-751

Lin, C,. Lin, CY., Chang, C., Lee, R., Tsai, T., Chen, Y., Chi, W., Huang, H. 2009. Early signalling pathways in rice roots under vanadate stress. Plant Physiol and Biochem 47: 369–376

Piñeros, A and Kochian, L. 2001. A patch-clamp study on the physiology of aluminum toxicity and aluminum tolerance in maize. Identification and characterization of Al (3+)-induced anion channels. Plant Physiol 125: 292–305

Polle, E., Konzak, C., Kittrick, J. 1978. Visual detection of aluminium tolerance levels in wheat by hematoxylin staining of seedling roots. Crop Sci 18: 823-827

Poschenrieder, C., Gunsé , B., Corrales, I., Barceló, J. 2008. Sci. A glance into aluminum toxicity and resistance in plants. Total Environ 400: 356–368

Schiefelbein, J., Kwak S., Wieckowski, Y., Barron, C., Bruex A. 2009. The gene regulatory network for root epidermal cell-type pattern formation in Arabidopsis. J Exp Bot 60: 5 1515-21

Silva , I. , Smyth , T., Moxley , D. , Carter , T. , Allen , A. and Rufty , T. 2000. Aluminium accumulaion at nuclei of cells in the root tip. Fluorescence detection using lumogallion and confocal laser scanning microscopy. Plant Physiol 123 : 543 – 552

Tabuchia, A and Matsumoto, H. 2001. Changes in cell-wall properties of wheat (*Triticum aestium*) roots. Physiol Plant 112: 353–358

Tanoi, K., Hayashi, Y., Iikura, H., Nakanishi, M. 2001. Aluminum Detection by Lumogallion Staining Method in Plants. Anal Sci 17 Supplement

Wenzl, P., Chaves, A., Patiño, M., Meyer, J. Rao, I. 2001. The High Level of Aluminum Resistance in Signalgrass Is Not Associated with Known Mechanisms of External Aluminum Detoxification in Root Apices. Plant Physiol 125: 1473–1484

Yamamoto, Y., Kobayashi, Y., Matsumoto, H. 2001. Lipid Peroxidation Is an Early Symptom Triggered by Aluminum, But Not the Primary Cause of Elongation Inhibition in Pea Roots. Plant Physiol 125: 199–208

Yang, J., Li, Y., Zhang, Y., Zhang, S., Wu, Y., Wu, P., Zheng, S. 2008. Cell Wall Polysaccharides Are Specifically Involved in the Exclusion of Aluminum from the Rice Root Apex.Plant Physiol 146: 602–611

Chapter 3

Changes in Root-Morphology of Brachiaria Species

(SEM and TEM observations)

Chapter 3

3.1. Introduction

3.1.1. *Changes in root-morphology of Brachiaria Species*

As already exposed in the previous chapters a primary response of plants to Al-toxicity is the inhibition of root elongation, but also fast alterations in root morphology can be observed. Aluminium toxicity characteristically changes the entire root architecture (Kochian *et al*., 1995; Doncheva *et al*., 2005). Transmission electron microscopes and scanning electron microscopes (SEM) alone or in combination with energy-dispersive X-ray analyses have being used in different species to identify responses to Al at the root surface and the ultrastructural level as well as for characterizing, subcellular distribution, compartmentalization and speciation of metals in plants (Vázquez *et al.*, 1999; Kopittke, *et al*., 2009; Zhenga *et al*., 2012.). Data on Al induced structural and ultrastructural alterations in Brachiaria species are scarce. Here we used SEM-EDX and TEM techniques for better characterizing the time dependent responses to Al in *B. decumbens* and *B. brizantha.*

3.2. Materials and methods

3.2.1. *Transmission electron microscopy (TEM)*

Root tips of approximately 3mm length were collected for transmission electron microscopy (TEM). The samples were fixed in 2.5% glutaraldehyde in 2% paraformaldehyde overnight 0.1 M potassium phosphate buffer (pH 7.1) for 8h, and post fixed with OsO_4 with 0.8% potassium hexacyano-ferrate (III) in phosphate buffer. The samples were dehydrated in an ethanol series using 30%, 50%, 60%, 70% and 90% ethanol and 100% acetone, and embedded in Epoxy (Epon, Spurr) resin. Ultrathin sections were obtained using an ultramicrotome and stained with uranyl acetate and basic lead citrate for observation under JEOL TEM. According method described by Rodríguez-Cariño *et al.*, (2011)

3.2.2. *Scanning electron microscopy coupled with spectrometer of energy dispersive X-ray microanalysis (SEM-EDX)*

Root tips from control or 200 µM Al-treated plants of *B. decumbens* and *B. brizantha* were used for SEM studies. The root tips were fixed in 2%

paraformaldehyde, 2.5 % (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2h at 4 ºC, washed 4 times for 10 min each time in 0.1 M phosphate buffer, postfixed in 1% (wt/vol) osmium tetraoxide, washed in distilled water, dehydrated in an ascending ethanol series (50, 70, 80, 90 and 95 % for 10 min each and twice with 100 % ethanol), and dried by critical-point drying with CO_2. All samples were mounted on adhesive carbon films and then coated with gold 5 nm. According method described by Julián *et al.*, (2010). Samples were observed with a JMS – 6300 scanning electron microscope (Jeol LTD. Tokio, Japan) coupled with spectrometer of energy dispersive X-ray microanalysis EDX Link ISIS–200 (Oxford Instruments, Bucks, England) an accelerating voltage of 20 kV.

3.3. Results

3.3.1. *Effect of Al on root of Brachiria species structural changes*

The SEM micrographs of root samples of *B. brizantha* showed an increase of border cell (Fig. 9 d arrow) comparing to the control (without Al^{3+}) (Fig.) by 24h of aluminium treatment. Root apex surfaces of *B. brizantha* did not show morphological changes under aluminium treatment. Contrastingly *B. decumbens* showed significant structural changes in epidermal cell surfaces when exposed to Al for 24h (Fig. 9 b) compared to the control (without Al^{3+}). Additionally these Al exposed plants produced less border cells (Fig. 9 b arrow) than both the control plants (Fig. 9 a, arrow) and the plant exposed to Al for 96h (Fig. 11 b). The root cap was covered by abundant border cells attached to the root surface (Fig. 13 C, and Fig. 9 a) After 96h Al treatment the morphology of the root tips of *B. decumbens* had completely recovered and root surface looked like those of controls (Fig. 11 b)

Scanning electron microscopy images of root tip surfaces of *B. decumbens* revealed the presence of root hair initials at a distance of 1.7 mm or higher from the apex in control plants (Fig. 13 A arrow). Contrastingly, in plants exposed for 24h and 96h to Al, on the surface abundant root hair initials were found in the region between 100 and 500 µm of the root apex (Fig. 13 B and Fig. 11) some of the hairs were cone-shaped (Fig. 13 B).

X-ray microanalysis scanning of the whole root tip surface did not reveal significant Al signal in any of both species (Fig. 10 a, b). In both species, X-ray microanalysis of small white deposits that occasionally were found on the root tip

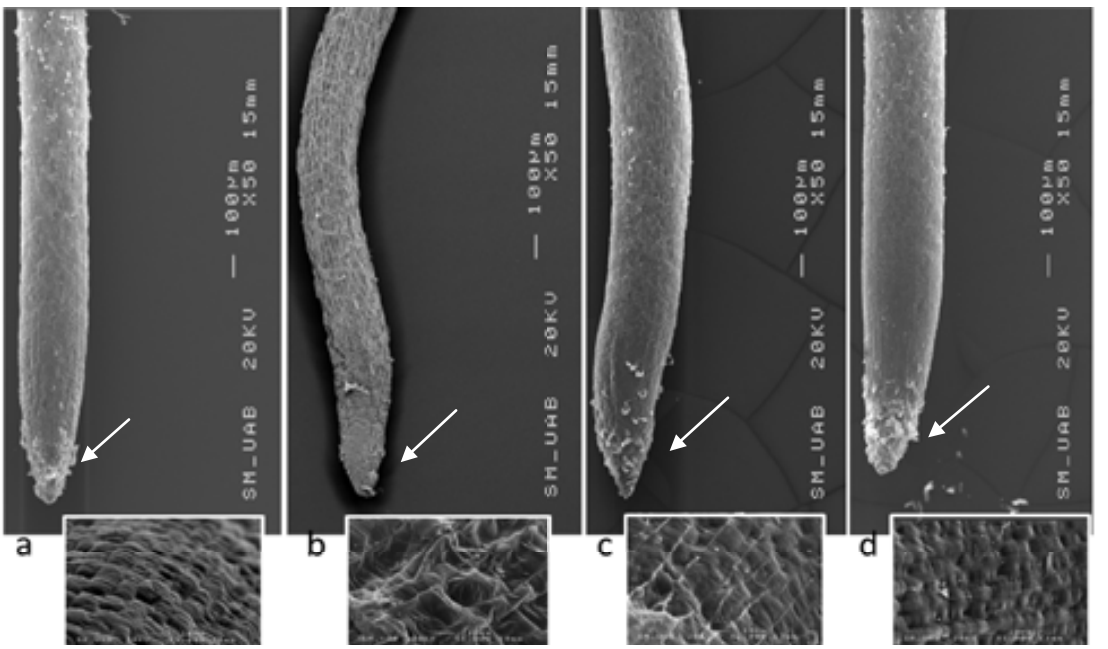


Fig.9 . SEM micrographs showing the surface of the root apex of control (a. and c.), Al (b. and d.) root apex treated of Brachiaria species. b) The root apex of Al-treated *B. decumbens* show breakdown of epidermal, change morphological compared to the control root (a) after 24h of $AlCl_3$ to 200 µM treatment. d) The changes observed of *B. brizantha* Al-treated roots were an increase of border cells (arrow) of root tip surface compared to control (c) root apex after 24h of $AlCl_3$ treatment (d). Note the micrographs below of the whole root apex (a, b, c, d) were observed of root at 0.865 mm from apex. (n=3) 1000X

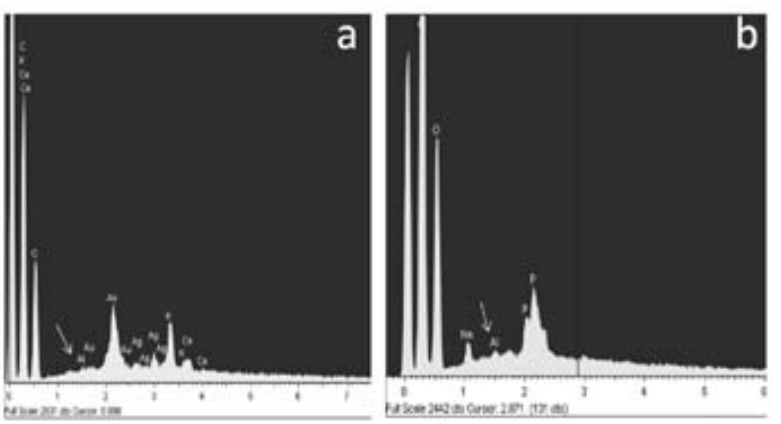


Fig.10 . SEM micrographs showing the surface of the root apex of control (e. and g.). Aluminium (f. and h.) root apex treated of Brachiaria species. e) Root apex control of *B. decumbens* and f) root apex after 96 h of $AlCl_3$ treatment. h) The root apex of Al-treated *B. brizantha* after 96h Al-treatment to 200 µM treatment, it show breakdown of epidermal. For *B. brizantha* change morphological compared to the control root g). Note the micrographs below of the whole root apex (e, f, and g, h) were observed of root at 0.865 mm from apex. (n=3) 1000X h)

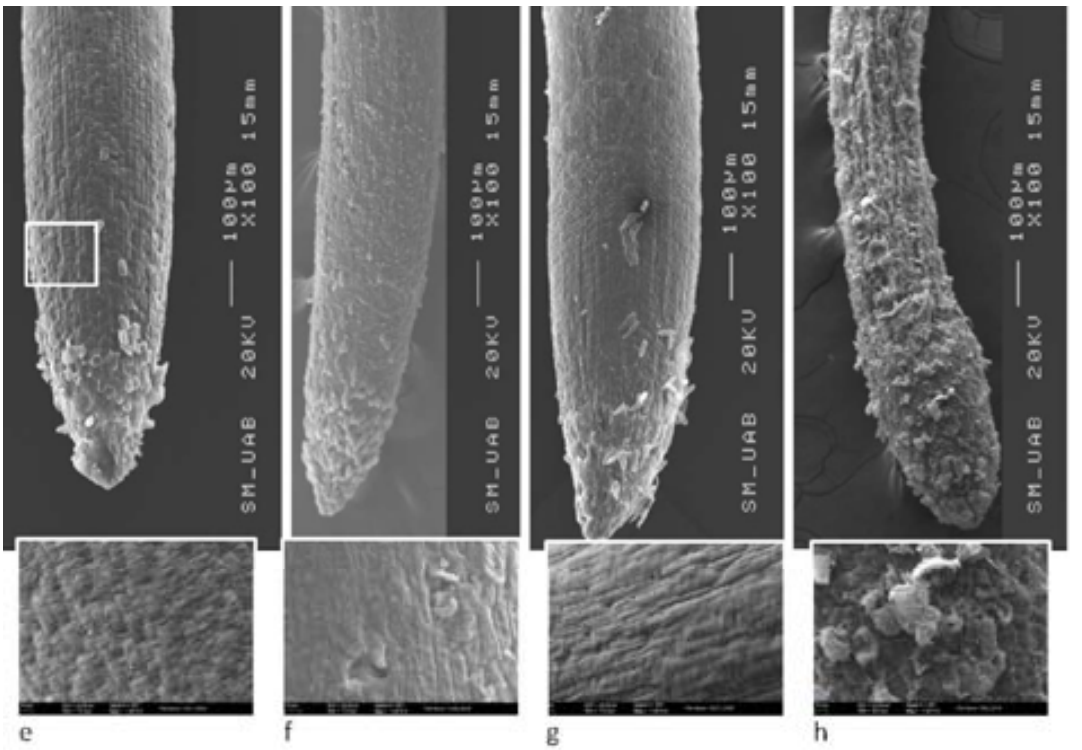


Fig.11. Aluminium was detected on the root surface with scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM - EDX). X-ray spectrum of whole root after 24h exposure to Al. a) *B. decumbens*. b) *B. brizantha*. Analysis of aluminium plots of the relative elemental concentration (arrow)

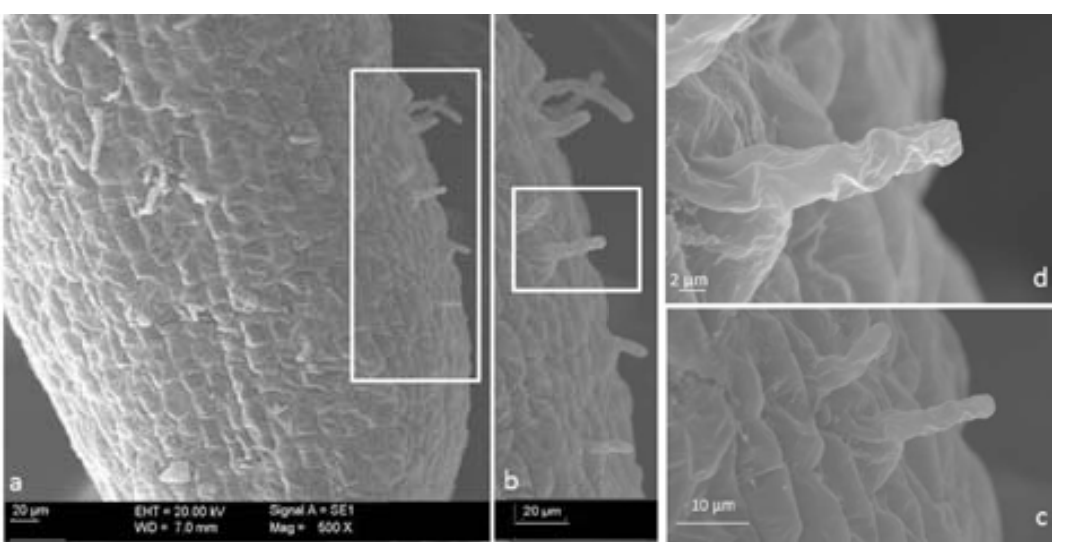


Fig.12.SEM micrographs show the surface of the root apex of *B. decumbens*. The root apex of Al-treated *B. decumbens* after 96h Al-treatment to 200 µM treatment, it shows root hairs are extensions of epidermis. (n=6)

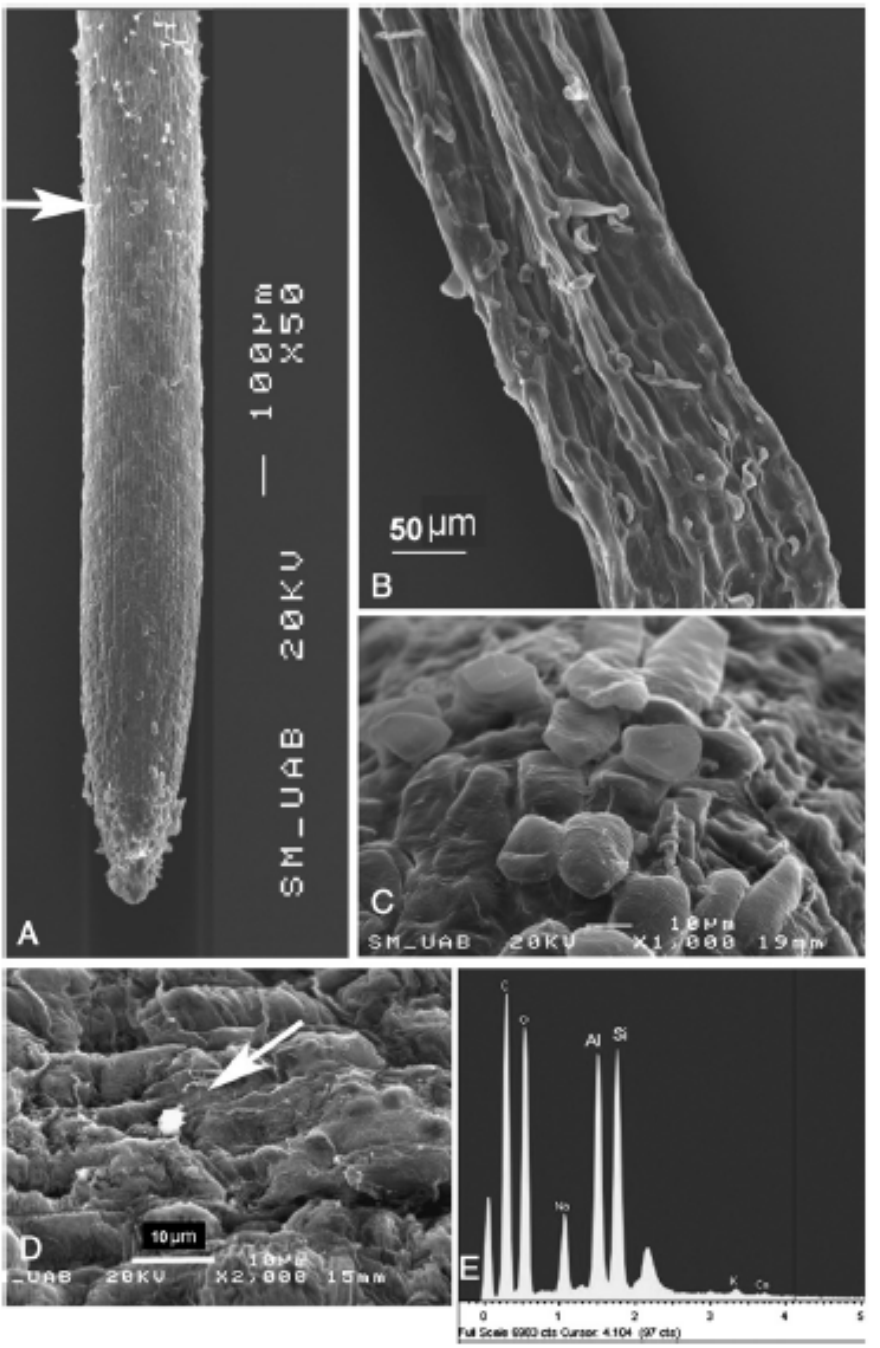


Fig.13. Scanning electron micrographs of root surface of *B. decumbens* after 24h treatment with $AlCl_3$ 200 µM. A) Control showing smooth surface and root hair initials at 1.7 mm from apex (arrow). B) Root at 100 to 500 µm from apex after 24h exposure to Al. Note rough surface, abundant root initials and cone-shaped hair. C) Root apex of Al-treated plant; note abundant border cells adhering to the root tip surface. D) Small Al-silicate particle on rough surface of Al-treated root. E) X-ray spectrum of this particle.

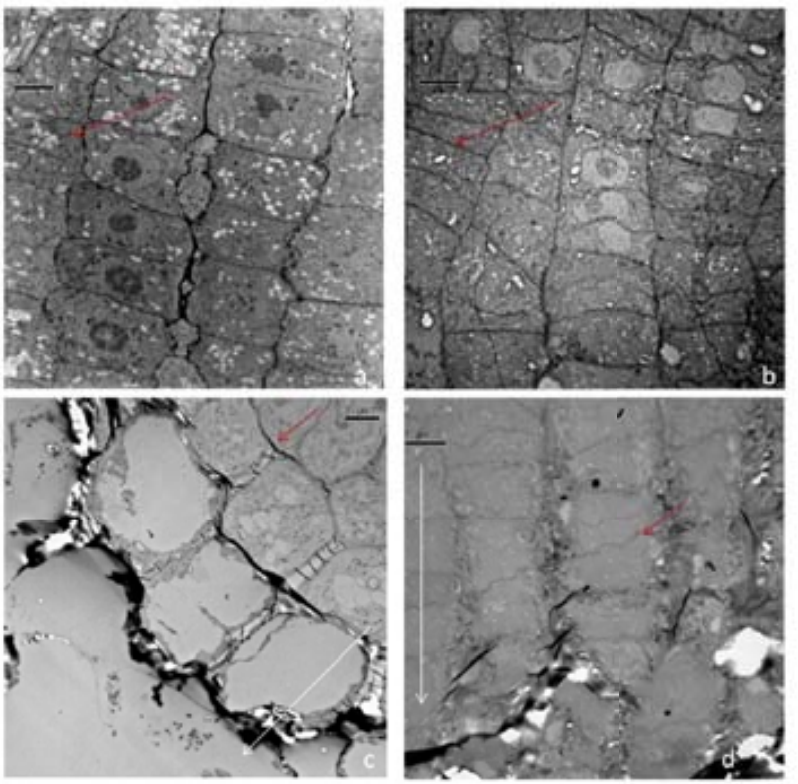


Fig.14. TEM images from longitudinal sections of root tips. Effect of Al on ultrastructure of root cells of *B. decumbens*. (a and c) control cells. (b and d) white arrow show the direction where to star of root apex. Ultrastructural alterations of root apex cells. Plants exposed to 200 μM Al at 24h. (a and b) showed epidermal (red arrowhead) root and the transitions zone. The root cells in Al-treatment change of morphology like a cylinder (b) treated plants compared to control (a). (c and d) showed the apex, meristematic zone 0-1 mm (red arrowhead).

surface, showed high Al and Si signals (Fig. 13 D, E). These aluminium silicate deposits of about 2 to 5 μm were much smaller than the surface structures of around 30–50 μm in roots of *B. decumbens* that stained with haematoxylin (see chapter 2).

TEM observations of fixed root sections showed changes in the morphology of cells from the root apex (Fig. 14 b and d). These results confirm that aluminium affected the morphology of *B. decumbens* after 24h under Al. Most of the root tip cortex cells were barrel shaped due to inhibition of cell elongation. Besides being smaller than control cells, the root apex cells from plants exposed to Al for 24h were irregular in outline (Fig. 14).

3.4. Discussion

Morphological changes induced by Al in roots of sensitive plants have frequently been described. Cracks in the epidermal cell layer have been attributed to Al-induced cell wall stiffening leading to inhibition of root elongation. In *B. decumbens* Al induced a rough surface but no cracks: this alteration of root surface morphology was transient and restoration of a smooth surface was coincident with the recovering of the root elongation rate.

Previous studies following root elongation recovering in Al treated maize seedlings by transfer from Al to control nutrient solution showed that in the sensitive variety two components of the Al-induced root growth inhibition can be distinguished: a reversible and an irreversible component (Amenós *et al.,* 2009). There was a coincidence in time between the reversible component of root cell elongation and the transient inhibition of vesicle recycling of boron linked RGII pectins. However, a causal relationship between both processes remains to be established.

Results provided here give further support to the view that there is a reversible component in Al–induced inhibition of root elongation. However, in the case of the Al hyperresistant *B. decumbens* it is not necessary that plants are transferred to Al free medium to achieve recover. This means that after a lag time of more than 24h (48 to 72h) an Al-induced tolerance mechanism gets effective that allows re-growth and restoration of a normal surface morphology. The relatively large lag time is not in line with an Al-induced activation of a previously formed enzyme or membrane channel (e.g. malate efflux channel). Probably gene activation and the formation of new proteins are required for this recovering mechanism.

This root elongation pattern under Al stress is in line with the behaviour of plants with a model Pattern II-type exudation of Al-binding organic acids by root tips (Delhaize *et al*., 2007). Nonetheless, previous investigations failed to relate Al tolerance in *B. decumbens* with Al-induced enhancement of root exudation of organic acids (Wenzl *et al*., 2003). Our observation that surface structure recovered under Al-treatment after 96h and the induction of abundant root hairs (Fig. 12) suggests that *B. decumbens* present a particular mechanism of epidermal cell patterning that responds under aluminium treatment. Furthermore, less Al tolerant *B. brizantha,* showed a considerably more disturbed epidermal cell pattern after 96h under aluminium (Fig. 11 d).

A surface X-ray scanning of the entire root tips of Al exposed plants did not give a significant Al signal (Fig. 10) and we can exclude massive Al precipitation on the root surface. Only a few small aluminium silicate deposits were detected on the root surface by SEM followed by X-ray microanalysis (Fig. 13 D). These deposits (2–5 µm) were much smaller and less abundant than the hot spots of Al accumulation with a size of (30–50 µm) observed by morin (Fig. 7 a Chapter 2) or haematoxylin (Fig. 5 a) staining. These bigger Al-rich structures correspond to root hair initials that developed close to the apex (Fig. 6B, C; Fig. 7G).

Root hairs are extensions of epidermis cells of roots that are considered to be an important structure for nutrient uptake (Gowda, 2011). Abundant root hairs and development closer to the tip has been observed in *B. decumbens* exposed to potentially toxic Pb or Cd concentrations (Kopittke *et al*., 2007; Kopittke *et al*., 2010). Lead accumulation in the root hairs of *B. decumbens* was observed and Pb translocation to the shoot was higher in the more Pb tolerant *B. decumbens* than in the sensitive *Rhodes grass* (Kopittke *et al*., 2007). Here we also observed a preferential accumulation of Al in the root hairs for *B. decumbens* (Fig. 7 a Chapter 2) and it seems that root hair development close to the root tip may be a more general response to ion toxicity in *B. decumbens* not only limited to Al^{3+}.

3.5. References chapter 3

Amenós M., Corrales, I., Poschenrieder, C., Illes, P., Baluska, F., Barceló J. 2009. Different effects of aluminum on the actin cytoskeleton and brefeldin A-sensitive vesicle recycling in root apex cells of two maize varieties differing in root elongation rate and aluminum tolerance. Plant Cell Physiol 50: 528-540

Delhaize, E., Gruber, B., Ryan PR. 2007. The roles of organic anion permeases in aluminium resistance and mineral nutrition. FEBS Lett 581: 2255–2262

Doncheva, S., Amenós, M., Poschenrieder, C., Barceló, J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. J Exp Bot 56: 414 1213–1220

Gowdaa, R., Henrya, A., Yamauchic, A., Shashidharb, H., Serra, R. Root biology and genetic improvement of drought avoidance in rice. Field Crop Res 122: 1–13

Julián, E., Roldán, M., Sánchez-Chardi, A., Astola, O., Agustí, G., Luquin, M. 2010. Microscopic Cords, a Virulence-Related Characteristic of *Mycobacterium tuberculosis*, Are Also Present in Nonpathogenic Mycobacteria. J Bacteriol 192: 1751–1760

Kidd, P., Llugany, M., Poschenrieder, Ch., Gunsé, B., Barceló, J. 2001. The Role Exudates in Aluminum resistance and Silicon – induced amelioration of Aluminum toxicity in three varieties of Maize (Zea mays L.). J Exp Bot 52: 1339 – 1352

Kochian, L. 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol. Plant Mol Biol 46: 237-260

Kopittke, P., Asher, C., Blamey, F., Menzies, N. 2009. Toxic effects of Cu^{2+} on growth, nutrition, root morphology, and distribution of Cu in roots of Sabi grass. Sci Total Environ 407: 4616–4621

Kopittke, M., Asher, C., Kopittke, A., Menzies, N. 2007. Toxic Effects of Pb^{2+} on Growth of Cowpea (*Vigna unguiculata*). Environ Pollut 150: 280-287

Kopittke, P., Blamey, E., Menzies, N. 2010. Toxicity of Cd to signal grass (*Brachiaria decumbens Stapf*.) and Rhodes grass (*Chloris gayana Kunth*.). Plant Soil 330: 515–523

Huang, C., Yamaji, N., Nishimura, M., Tajima, S., Ma, J. 2009. A Rice Mutant Sensitive to Al Toxicity is Defective in the Specifi cation of Root Outer Cell Layers. Plant Cell Physiol 50: 976–985

Rodríguez-Cariño, Duffy, C., Sánchez-Chardi, A., McNeilly, F., Allan, G., Segalés, J. 2011. Porcine circovirus type 2 morphogenesis in clone derived from the L35 lymphoblastoid cell line. J Xomp Path 144: 91-102

Zhenga, L., Peera, T., Seyboldb, V., Lütz-Meindlc, U. 2012. Pb-induced ultrastructural alterations and subcellular localization of Pb in two species of Lespedeza by TEM-coupled electron energy loss spectroscopy. Environ Exp Bot 77: 196– 206

Chapter 4

A role for Phenolic Substances in Al Tolerance of *Brachiaria*

Chapter 4

4. Introduction

4.1. *Phenolic substances in roots*

Aluminium tolerance implies the ability of the plant to bind the trivalent Al^{3+} in a non toxic form, either or both in the rhizosphere and inside the plant tissues (Barceló and Poschenrieder, 2002). As a hard Lewis acid, Al^{3+} forms stable complexes with oxygen donor ligands. Organic acids such as citrate, malate and oxalate are strong chelators for Al. In fact, differential Al resistance in varieties of several crop species like wheat bean or barley have been related to the ability of plant root tips to respond to Al with the activation of efflux of organic acids from root tips into the rhizosphere (Ryan *et al*., 2001; Delhaize *et al*., 2007).

Besides organic acids, other organic and inorganic substances can bind Al and are potential candidates for detoxifying Al in the rhizosphere and inside plants e.g., phosphate, silicon, fluoride, hydroxamates and phenolic substances (Barceló and Poschenrieder, 2000; Ma *et al*., 2001; Jung *et al*., 2003; Poschenrieder *et al*., 2005; Tolrà *et al.,* 2009).

Cyclic hydroxamates have been related to both iron acquisition and defence against metal toxicity in plants. Higher concentrations of the cyclic hydroxamate 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) have been detected in root tips of some, but not all, Al resistant maize varieties (Poschenrieder *et al*., 2005). A more intense, Al-induced enhancement of root tip exudation of flavonoid-type phenolics has been observed in Al resistant, acid soil adapted maize varieties than sensitive ones (Kidd *et al.,* 2001).

Many species able to withstand high tissue concentrations of Al, such as tea, Melastomataceae species, or *Hydrangea sp*. have constitutively high phenolics concentrations in their tissues. The phenolic structure is characterized by at least one aromatic ring bearing one or more hydroxyl groups (Fig. 15). These hydroxyl groups form strong complexes with Al^{3+}, especially at the neutral pH inside plants. (Barceló and Poschenrieder, 2002¸ Kochian *et al*., 2004; Tolrá *et al.,* 2009). Phenolics may provide protection against the Al fraction that is able to surpass the exclusion mechanisms operating in Al resistant varieties. Phenolics have been proposed to be involved in the detoxification of Al in leaves of *Rumex acetosa* and there is some evidence that phenolics may be active in both Al binding in non-

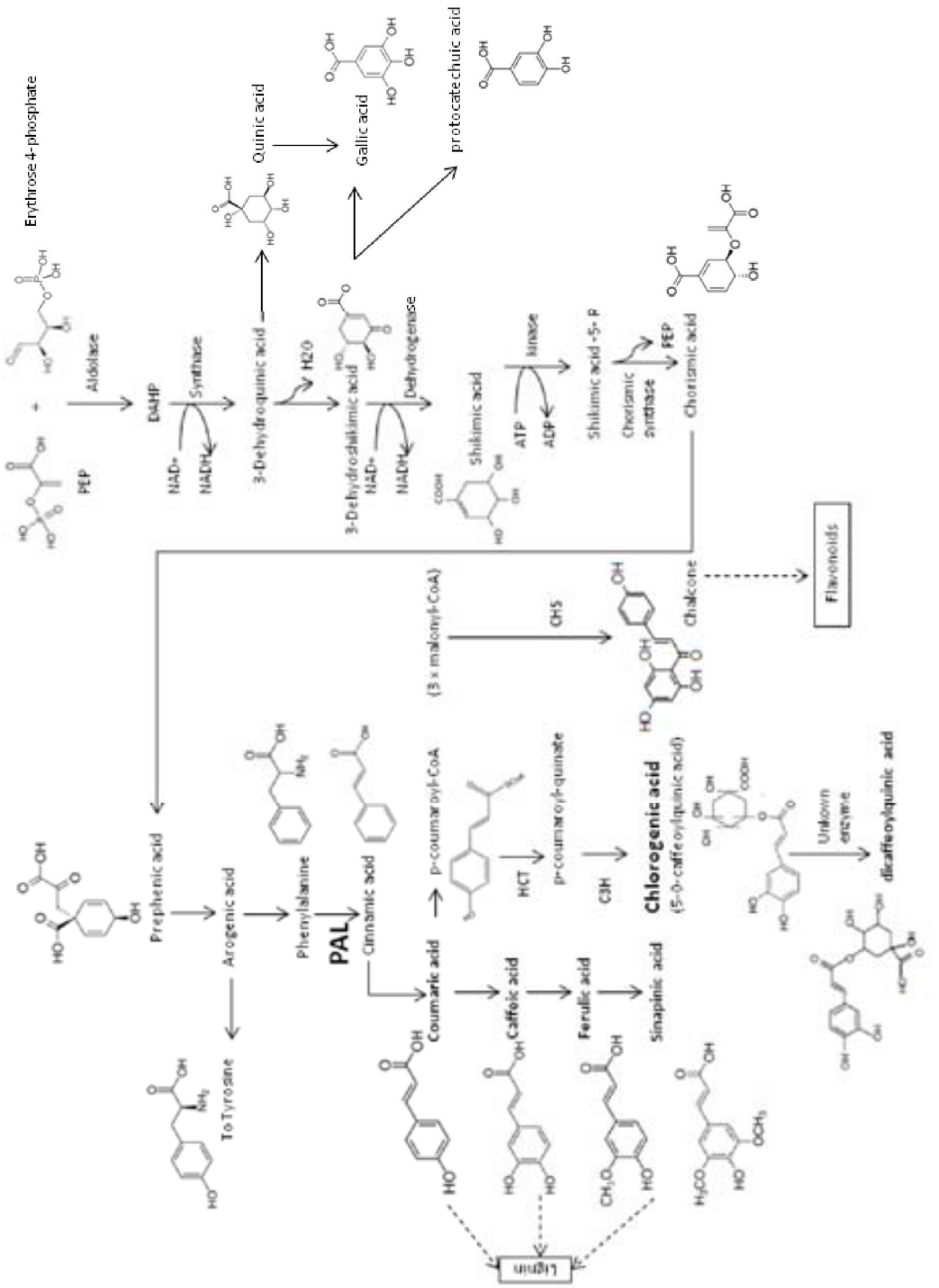


Fig.15. Biosynthetic phenolics pathway

toxic form and as scavengers of reactive oxygen species caused by Al stress in the plant tissues (Tolrà *et al*., 2005, 2011). Besides these direct roles in Al stress response, the importance of phenolics in cell wall stability has to be taken into account, moreover, in the view of the cell wall as a primary target of Al toxicity.

The aim of the present study was to explore the possible implication of phenolic substances in the differential Al tolerance of *B. decumbens* and *B. brizantha* in order to contribute to a better understanding of this hyperresistance to Al in *B. decumbens*.

4.2. Materials and methods

4.2.1. *Determination of capacity for Fe III complexation in root tips (cyclic hydroxamates content in Brachiaria species)*

Roots tips were analysed according to Poschenrieder *et al.,* (2005) after 24h exposure to nutrient solutions that contained 200 µM $AlCl_3$.The presence of cyclic hydroxamates in the seedlings of *Brachiaria* was visualized by squashing roots tips on filter paper impregnated with ferric chloride 0.1 M. The appearance of a dark blue-purple (Fig.15.) colour on the filter paper shows the tip release of cyclic hydroxamates able to complex Fe III. The squashed root tips of control and Al-treated plants were observed under a lens Nikon H55OS.

4.2.2. *Analysis of phenolics substances in roots*

4.2.2.1. Phenolics *soluble*

Extraction of soluble phenolics from roots of *B. decumbens* and *B. brizantha* was achieved by homogenizing roots (0.5 g fresh weight) with 1.5 mL MeOH 70 % for three times in a test tube. Analysis, were performed according to Solecka *et al*., (1999) with modifications according to Tolrá *et al.,* (2009). The root concentrations of soluble phenolic substances of plants exposed to control or 200 µM Al for 24h were analysed by liquid chromatography equipped with an electrospray ionization source and a time-of-flight mass spectrometer (LC-ESI-QTOF) (LC 1200RR, Agilent; Micro-TOF-Q, Bruker ,Daltonics and an ESI Apollo 1/2 Bruker, Daltonics)

4.2.2.2. *Phenolics from cell wall in Brachiaria decumbens*

Cell wall bound phenolics in roots of *B. decumbens* were extracted by homogenizing roots (0.5 g fresh weight) with 1.5 mL MeOH 70 % for three times in a test tube (Solecka *et al*., 1999). After extraction, samples were air dried and re-extracted three times with ethylether to eliminate the remaining ether soluble lipids. The water phase containing total cell wall phenolics was treated with 2 M sodium hydroxide for basic hydrolysis of soluble conjugated phenolics. After extraction with ethyl acetate and drying, the residue was re-dissolved in 70% methanol. Concentrations of phenolics from cell wall were determined by HPLC on a Shimadzu system (System controller SCL-10ADvp equipped with a diode array detector (250–400 nm, Shimadzu SPD-M 10 Avp) an autoinjector (SIL 10 ADvp), and a Novapack C-18 column (60 Å., 4 µm; 3.9 x 150 mm, Waters Corporation). All extracts were analysed by HPLC-MS (Agilent 1100, Agilent Technologies, Inc. Palo Alto, USA) equipped with a mass spectrometer (MS Esquire 3000, Bruker Optik, Ettlingen Germany) and an electro spray ionization source. Given values are means ± SE of five replicated samples per specie, control, and Al-treatment.

4.2.3. *Autofluorescence*

Root apexes (10 mm) were cut with a razor blade from roots rinsed with distilled water. Single apexes were placed into a mould prepared using plastic foil. The mould's dimensions were: height about 20 mm, width about 5 mm diameter.

The root tip samples were embedded in a 2.5 % solution of agarose at a temperature near the point of solidification (approx. 45 °C). After pouring the agarose solution into the mould, root tips were carefully positioned to be parallel to the longitudinal axis of the mould. The mould was placed on ice for the agarose to solidify. After this step, the agarose embedded root tip samples were cut by hand (transversal section) using a razor blade. Samples could be cut immediately. Roots were cut together with agarose, the embedding medium, providing mechanical support. The cross-sections were placed into an Eppendorf with 70 % ethanol and were stored for maximum 1 month at 4 °C.

4.3. Results

4.3.1. *Iron complexation capacity*

Cyclic hydroxamates from Poaceae species have been described as ligands for iron and Al forming Fe III-complexes and Al III-complexes (Dunn *et al*., 1981; Poschenrieder *et al.,* 2005).

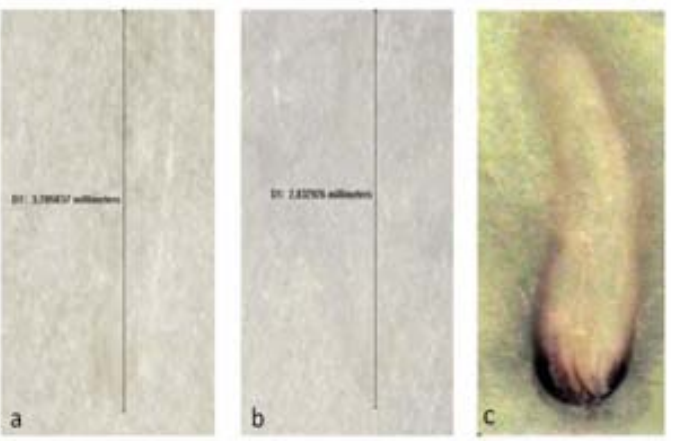


Fig.16. Iron complexation capacity of a. *B. decumbens* and b. *B. brizantha* root tips exposed for 24h to 200 µM $AlCl_3$. Tips were squashed on filter paper impregnated with $FeCl_3$. c. image for comparison showing iron complexation capacity of maize (Sikuani) (image taken from Poschenrieder *et al.,* 2005) root tips exposed for 24h 50 µM Al containing nutrient solution. The appearance of a dark blue-purple colour shows the capacity of the

The root tip squash test (Fe III-complex) for detection of cyclic hydroxamates in the tips, was used in the seedlings of Brachiaria sp. In any of the species cyclic hydroxamates were detectable (Fig. 16 a. and b). For comparison figure 16 c shows the result for a maize seedling that produced cyclic hydroxamates.

4.3.2. *Phenolics substances*

4.3.2.1. *Phenolics soluble in B. decumbens and B. brizantha*

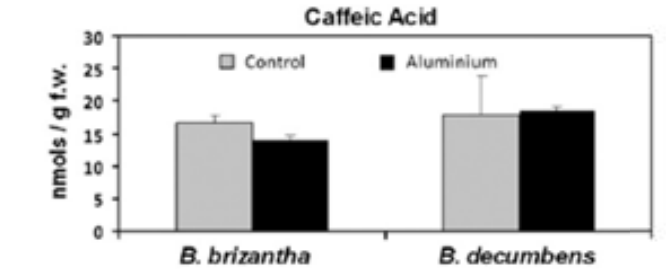
Excepting caffeic acid (Fig. 17), species and treatment differences in root concentrations of soluble phenolics were observed after 24h of exposure. Concentrations of sinapic acid were enhanced by Al in *B. brizantha* by about 50% reaching values similar to those observed in *B. decumbens* under both control and Al supply conditions (Fig. 17).

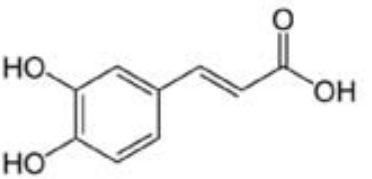
The low coumaric acid concentrations found in *B. brizantha* were not affected by Al. In *B. decumbens* free coumaric acid was not detected (Fig. 17). Aluminium exposure decreased ferulic acid concentrations in roots of *B. brizantha* by 25%

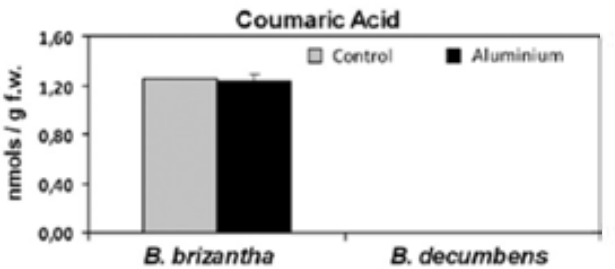
while the opposite effect was observed in *B. decumbens* (Fig. 16). In both species 1,3-di-O-trans-feruloylquinic acid (DFQA) was identified by its mass spectrum (Wenzl *et al*., 2000). As no appropriate standard was available only peak areas are compared (Fig. 17). In Al treated plants of both species peak areas for DFQA were lower than in controls. The most conspicuous differences were observed in the chlorogenic acid levels. No chlorogenic acid signal was obtained in the MS-spectra of *B. brizantha* roots (Fig. 17). In *B. decumbens* chlorogenic acid was found under control conditions and 24h Al supply caused a two fold increase of the root chlorogenic acid concentration (Fig. 17).

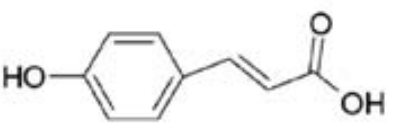
4.3.2.2. *Phenolics from cell wall in B. decumbens*

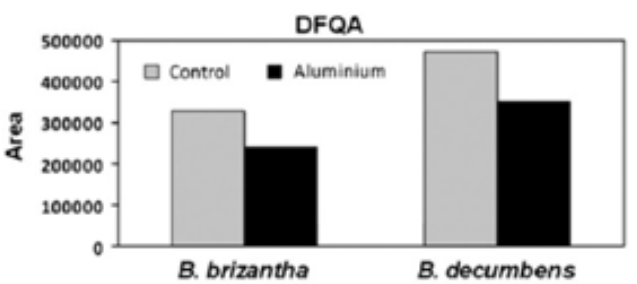
In contrast to the patterns of soluble phenolics observed in roots of *B. decumbens* (Fig. 18) no chlorogenic acid, sinapic acid and DFQA was detected in the cell wall bound fraction of phenolics. Only coumaric and ferulic acids bound to cell walls were detectable. Cell wall bound concentrations of both coumaric and ferulic acid decreased after 24h Al exposure but increased substantially 96h after start of Al supply.

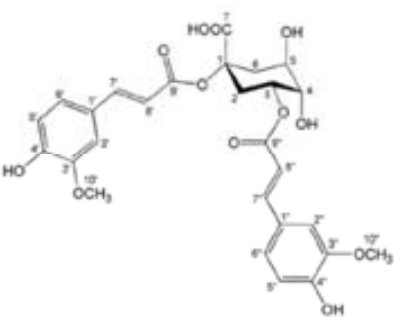

Caffeic Acid
Control
Aluminium
nmols / g f.w.
30
25
20
15
10
5
0
B. brizantha
B. decumbens

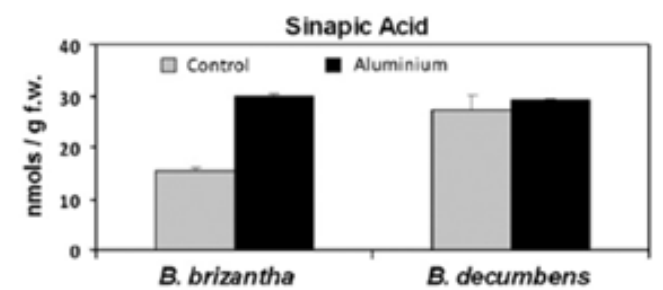

HO
HO
O
OH

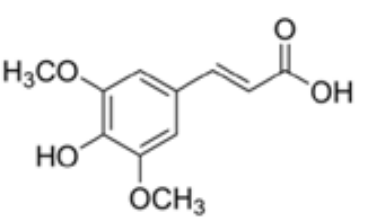

Coumaric Acid
Control
Aluminium
nmols / g f.w.
1,60
1,20
0,80
0,40
0,00
B. brizantha
B. decumbens

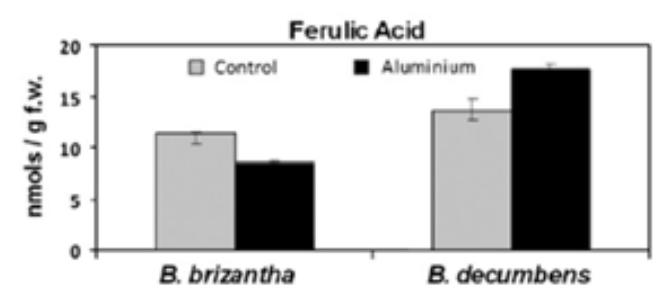

HO
O
OH

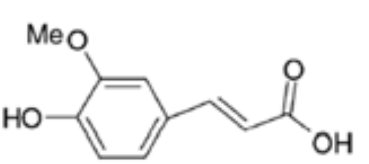

DFQA
Control
Aluminium
Area
500000
400000
300000
200000
100000
0
B. brizantha
B. decumbens


HOOC
OH
O
OH
HO
OCH3
OCH3
OH


Sinapic Acid
Control
Aluminium
nmols / g f.w.
40
30
20
10
0
B. brizantha
B. decumbens


H3CO
HO
OCH3
O
OH


Ferulic Acid
Control
Aluminium
nmols / g f.w.
20
15
10
5
0
B. brizantha
B. decumbens


Me
O
HO
O
OH

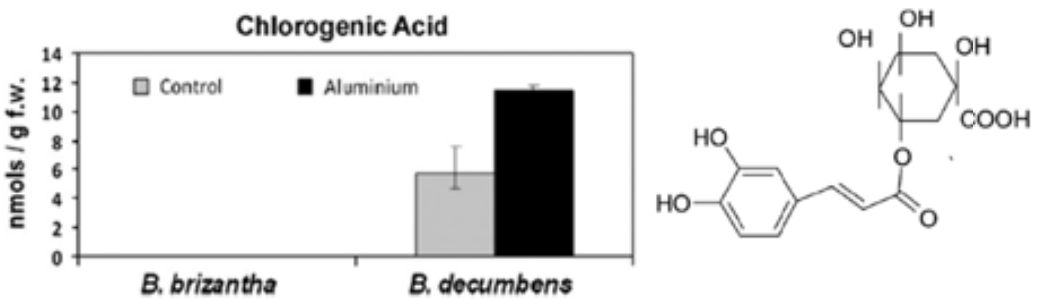


Fig.17. Concentrations of soluble phenolic in roots of *B. decumbens* and *B. brizantha*. Plants under control or after 24h Al-treatment. Structure image taken from Tolrà *et al*., 2005; DFQA structure from Wenzl *et al.,* 2000 (means±SE; n=3).

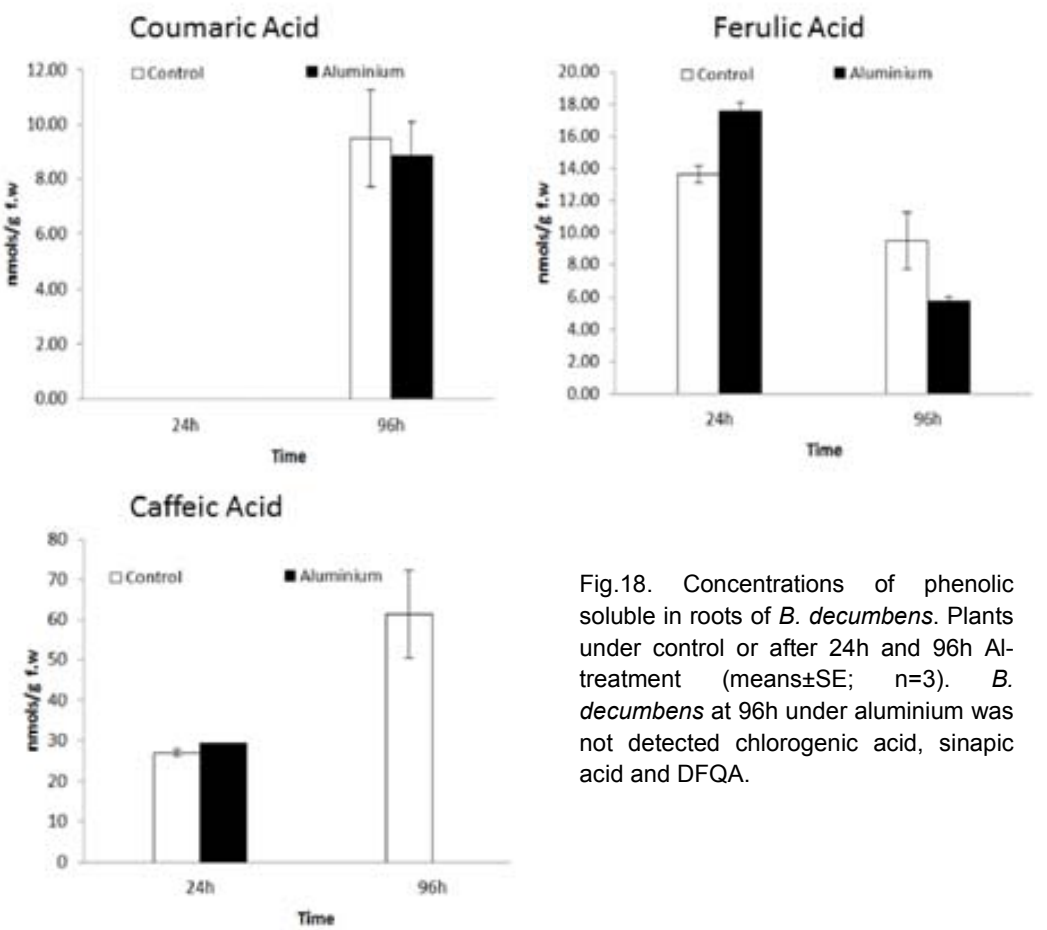


Fig.18. Concentrations of phenolic soluble in roots of *B. decumbens*. Plants under control or after 24h and 96h Al-treatment (means±SE; n=3). *B. decumbens* at 96h under aluminium was not detected chlorogenic acid, sinapic acid and DFQA.

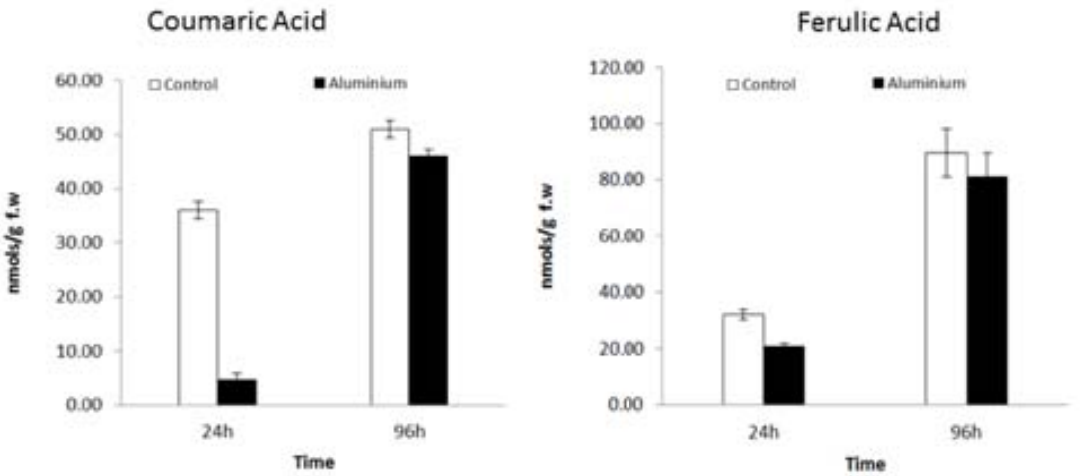


Fig.19. Concentrations of phenolic from cell wall of roots in *B. decumbens*. Plants under control or after 24 h and 96 h Al-treatment. (means±SE; n=3). *B. decumbens* at 96h under aluminium was not detect chlorogenic acid, sinapic acid, caffeic acid and DFQA.

4.4. Discussion

Previous investigations found high Al-induced organic acid production in root tips of *B. decumbens* (Wenzl *et al*., 2001); however no relation between the productions of Al-binding root exudates (e.g. containing organic acids or phenolics) and the hyperresistance to Al in *B. decumbens* could be established (Xia *et al*., 2010) Root tip production of cyclic hydroxamates, a characteristic of some Al-resistant maize varieties (Poschenrieder *et al.*, 2005) was also not observed neither in *B. decumbens* nor in *B. brizantha*. The mechanisms by which *B. decumbens* achieves both a preferential accumulation of morin or haematoxylin-stainable Al in the root hairs and an efficient exclusion from the Al-sensitive meristem and transition zones remains to be established. To what extent the development of a multiseriate exodermis (Fig. 21) can contribute to restriction of apoplastic Al accumulation requires further attention. In cotton seedlings to prevent the loss of water and/or solutes from roots by salinity, was reported that cotton can induce the formation of an exodermis (Reinhardt and Rost, 1995).

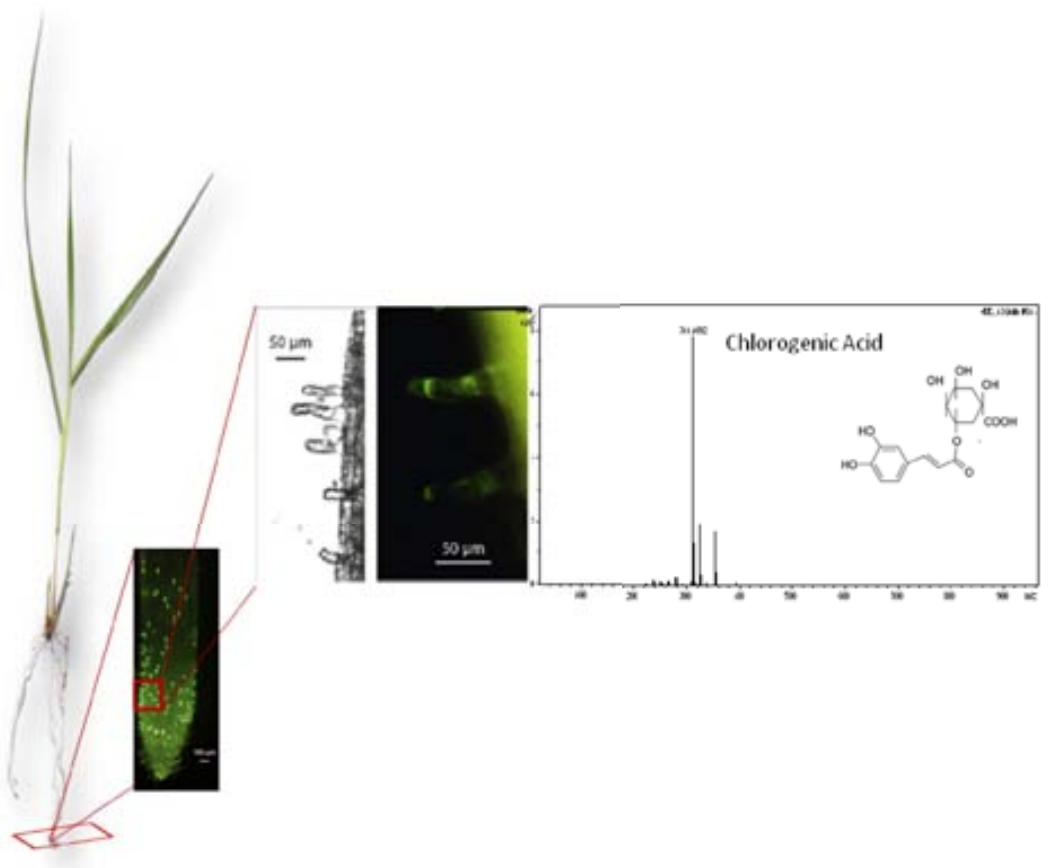


Fig. 20. Seedling of *B. decumbens* after 24 h treatment with $AlCl_3$ 200 µM. The phenylpropanoids such as chlorogenic acid stimulates root hair formation.

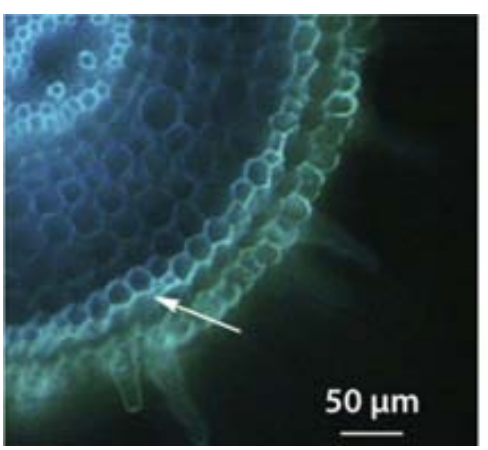


Fig.21. Autofluorescence of transversal section of Al-treated exposed for 24 h to 200 µM Al (32 µM Al^{3+} activity) in low ionic strength nutrient solution. Root from *B. decumbens* showing hypodermis with suberin layer (arrow).

The extent and rate at which apoplastic exodermal barriers (Casparian bands and suberin lamellae) are laid down in radial transverse and tangential walls depends on the response to conditions in a given habitat such as salinity, heavy metal or nutrient stresses (Hose *et al*., 2001). Huang *et al.*, (2009) found that the outer cell layers function as a physiological barrier, which prevents metals from entering into the inner cortical cells in rice.

Chlorogenic acid (caffeoylquinic-acid) esters have been found to accumulate under nitrogen, phosphorus, sulphur and potassium deprivation and may play an important role in preventing premature tissue senescence (Wenzl *et al.,* 2000). Recent investigations on root hair development in lettuce identified endogenous chlorogenic acid as a key factor in the induction of root hair formation (Narukawa *et al*., 2009). Here chlorogenic acid concentrations were found to increase two fold in response to Al supply in roots of the Al hyperresistant *B. decumbens* (Fig. 17). Contrastingly, chlorogenic acid was not detected in the roots of the less tolerant *B. brizantha* (Fig. 17). Chlorogenic acid is an inhibitor of auxin oxidation (Pilet, 1964) and thus may contribute to the Al-induced alteration of epidermal cell patterning. It is tentative to speculate that Al-induced enhancement of chlorogenic acid stimulates root hair formation in *B. decumbens* (Fig. 20). This may contribute to both more efficient uptake of essential nutrients such as P and a controlled intake of Al via the symplastic pathway in the root zone where the development of a suberized hypodermis may restrict the apoplastic Al transport. Moreover, a continuous turnover of the root hairs can favour the elimination of Al from sensitive zone of the root.

1,3-di-O-trans-feruloylquinic acid (DFQA) presents a similar structure to that of 1,3-di-O-trans-caffeoylquinic acid (Wenzl *et al*., 2000). DFQA was first time isolated from root cultivated in low-ionic-strength nutrient solutions of Brachiaria species *B. decumbens* and *B. ruziziensis*. This novel di-hydroxycinnamoyl ester of quinic acid was related to increasing root lifespan under nutrient-limited growth conditions. The phenylpropanoids, such as chlorogenic acid, may play an important role in preventing premature tissue senescence (Wenzl *et al*, 2000). These authors also found that the DFQA accumulates in older parts of the root system, but not in root apices or shoots. Synthesis of DFQA was stimulated in roots under nitrogen and phosphorus deficiency, but not by aluminium toxicity or deprivation of other nutrients.

As far as we know this is the first work reporting DFQA in *B. brizantha.* In both species (*B. decumbens* and *B. brizantha,* Fig. 17) concentrations of DFQA decreased when the roots were exposed to aluminium. This phenol was not detected in *B. decumbens* after 96h Al-treatment (Fig. 18). Our results also do not support a direct role for DFQA in Al hypertolerance of *B. decumbens*.

In grasses, ferulates play a main role in the cross-linking process that control wall organisation and structural integrity. Ferulates can cross-link wall polysaccharides and other components (Hatfield *et al*., 1999; Spollen *et al.*, 2008). Hossain *et al.,* (2006) found a higher content of ferulic acid in the cell walls of an Al-sensitive cultivar of wheat. Under Al stress ferulates may form extensive cross-linking with arabinoxylans and inhibit root growth. However in this study we found a reduction of the concentration of ferulic acid under Al-treatment (Fig. 19) and the transient inhibition of root elongation induced by Al in *B. decumbens* was not due to this mechanism.

However, after 96h exposure to Al (Fig. 19) cell wall bound ferulic acid and coumaric acid concentrations recovered, to control levels. This may be related to the recovering of the structure of the epidermis after 96h Al-treatment (Fig. 11 chapter 3).

In conclusion, the full expression of hyperresistance to Al in *B. decumbens* has an Al-inducible component related to a change in root epidermal cell patterning. Confinement of Al accumulation to root hairs and border cells can contribute to low Al accumulation in sensitive root zones. Al-induced enhancement of chlorogenic acid in roots may contribute to changes in epidermal cell patterning.

4.5. References chapter 4

Dunn, G., Long, B., Routlet, D. 1981. Inheritance of cyclic hydroxamates in *Zea mays* L. Can. J Plant Sci 61: 583-593

Hatfield, R., Ralph, J.,Grabber, J. 1999. Review Cell wall cross-linking by ferulates and diferulates in grasses. J Sci Food Agric 79: 403–407

Hose, E., Clarkson, D., Steudle, E., Schreiber, L., Hartung., W. 2001. The exodermis: a variable apoplastic barrier. J Exp Bot 52: 2245-2264

Hossain, Z., Koyama, H., Hara, T. 2006. Growth and cell wall properties of two wheat cultivars differing in their sensitivity to aluminum stress. J Plant Physiol 163: 1 39-47

Huang, C., Yamaji, N., Nishimura, M., Tajima, S., Ma, J. 2009. A Rice Mutant Sensitive to Al Toxicity is Defective in the Specifi cation of Root Outer Cell Layers. Plant Cell Physiol 50: 976–985

Kochian, L., Hoekenga, O., Piñeros, M. 2004. How do Crop Plants Tolerate Acid Soils? Mechanisms of Aluminum Tolerance and Phosphorous Efficiency. Ann Rev Plant Biol 55: 459-493

Jung, C., Maeder, V., Funk, F., Frey, B., Sticher, H., Frossard, E. 2003. Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. Plant Soil 252: 301–312

Ma, J., Ryan, P., Delhaize, E. 2001. Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci. 6: 273-278

Narukawa, M., Kanbara, K., Tominaga, Y., Aitani, Y., Fukuda, K., Kodama, T., Murayama, N., Nara, Y., Arai, T., Konno, M., Kamisuki, S., Sugawara, F., Iwai, M., Inoue, Y.2009. Chlorogenic acid facilitates root hair formation in lettuce seedlings. Plant Cell Physiol 50: 504–514

Pilet, P. 1964. Effect of chlorogenic acid on the auxin catabolism and the auxin content of root tissues. Phytochem 3: 617–681

Poschenrieder, C., Tolrà, R., Barceló, J. 2005. A role for cyclic hydroxamates in aluminium resistance in maize? J Inorg Biochem 99: 1830–1836

Reinhardt D., Rost T.1995. Salinity accelerates endodermal development and induces an exodermis in cotton seedling roots. Environ Exp Bot 35: 563–574

Solecka, D., Boudet, A., Kacperska, A. 1999. Phenylpropanoid and anthocyanin changes in low temperature treated winter oilseed rape leaves. Plant Physiol Biochem 37: 491–496

Spollen, W., Tao, W., Valliyodan, B., Chen, K., Hejlek, L., Kim, L., LeNoble, M., Zhu, J., Bohnert, B., Henderson, D., Schachtman, D., Davis, G., Springer, G., Sharp, R.,

Nguyen, T. 2008. Spatial distribution of transcript changes in the maize primary root elongation zone at low water potential. BMC Plant Biol 8: 32

Tolrà, R., Poschenrieder, C., Luppi, B., Barceló, J. 2005. Aluminium-induced changes in the profiles of both organic acids and phenolic substances underlie Al tolerance in *Rumex acetosa L*. Environ Exp Bot 54: 231–238

Tolrà, R., Vogel-Mikus, K., Hajiboland, R., Kump, P., Pongrac, P., Kaulich, B., Gianoncelli, A., Babin, V., Barcelo, J., Regvar, M., Poschenrieder, C. 2011. Localization of aluminium in tea (*Camellia sinensis*) leaves using low energy X-ray fluorescence spectro-microscopy. J Plant Res 124: 165–172

Tolrà, R., Poschenrieder, C., Barceló, J. 2009. Constitutive and aluminium-induced patterns of phenolic compounds in two maize varieties differing in aluminium tolerance. J Inorg Biochem 103: 1486–1490

Wenzl, P., Chávez, L., Mayer, J., Rao, I. and Nair, M. 2000. Roots of nutrient-deprived Brachiaria species accumulate 1,3-di-0-trans-feruloylquinic acid. Phytochemistry 55: 389-395

Xia, J., Yamaji, N., Kasai, T., Ma, J. 2010. Plasma membrane-localized transporter for aluminum in rice. PNAS 107: 18381–18385

Chapter 5

DIGE Analysis of Root Proteome in *Brachiaria decumbens*

Chapter 5

5. Introduction

5.1. Proteomic analysis of *Brachiaria decumbens* roots under aluminium stress

Aluminium-tolerant varieties detoxify Al through multiple mechanisms that are currently not well understood at genetic and molecular levels. Al stress induces multiple cellular physiological, and biochemical disorders in roots. Under aluminium stress roots are characterized by different cellular activities involved in stress/defence, signal transduction, transport, protein folding, gene regulation, and primary metabolisms, which are critical for plant survival under Al toxicity. These changes are controlled by alterations in gene expression at transcriptional, post-transcriptional, translational, and post-translational levels (Zhen et al., 2007; Zhou et al., 2009).

Knowledge of aluminium resistance mechanisms will contribute to the general understanding of the interactions between the molecular pathways responsible for signalling abiotic stress factors and the expression of resistance mechanisms controlling crop yield in acidic environments. In this manner it will improve agricultural production and contribute to better adaptation of crops to unpredictable conditions under climate change.

Proteome analysis has been employed to study alterations in protein expression in response to different stress (Konishi *et al.,* 2005). Two-dimensional (2D) gel electrophoresis is a powerful technique enabling simultaneous visualization of relatively large portions of the proteome (Marouga et al., 2005)

To enhance our understanding of the molecular mechanisms involved in Brachiaria Al resistance and toxicity, we conducted proteomic analysis of *B. decumbens* roots under Al stress after different exposure times (24h and 96h) using 2-D-DIGE, MALDI-TOF analysis.

5.2. Materials and methods

5.2.1. *Harvest*

Brachiaria decumbens plants were grown under the same conditions described in chapter 1. After harvest, 1 gram of fresh root tissue (pooled from several plants) from each treatment (control without aluminium, 24h and 96h under Al-

treatment) was frozen in liquid N_2 and stored at -85 °C. This was used for analysing protein profiles.

5.2.2. *Protein extraction*

For each treatment a 1 g of the frozen root sample was ground to powder in liquid N_2 using Mikro-Dismembrator S–Sartorius by 4.5 minutes. This process was segmented in three times of 1.5 minutes each; with the objective to put more liquid N_2, so to prevent thawing of the sample. Protein extraction of roots was done according to the method described by Chaves (2010). Protein concentrations of samples were determined using the Bradford method (Bradford, 1976)

5.2.3. *2D DIGE analysis*

2D DIGE analyses were carried out in the proteomics service facility of Autonomous University of Barcelona, a member of ProteoRed-ISCIII network. All the reagents used were from GE Healthcare–Barcelona.

5.2.3.1. *Sample preparation and CyDye labelling*

An internal standard for assay normalization was created by mixing equal amounts of protein from each sample. For each 9 sample, 50 µg of protein was mixed with 1.0 µL (400 pmols) of diluted CyDye (GE Healthcare) and kept in the dark on ice for 30 min. Proteins from the control and treated samples were labelled with Cy3 and Cy5, respectively, whereas the internal standard was labelled with Cy2. The labeling reaction was stopped by adding 1.0 µL of 10mM Lysine and incubation in the dark on ice for an additional 10min. The labelled samples from the control and treated groups and the internal standard were blended for a run of three samples per gel. Protein samples were dissolved to 140 µL of 2D cell lysis buffer (stock de TrisHCl 40mM pH 9.5 + 20 mM DTT + 0.5 % IPG). Samples were applied by Cup Loading (BioRad)

	Cy 3	Cy 5	Cy 2	
Gel 1	50 µg control 1	50 µg Al-treat -24h1	50 µg control 1 + 50 µg control 2 +50 µg control 3 + 25 µg Al-treat -24h 1 + 25 µg Al-treat-24h 2 + 25 µg Al-treat-24h 3 + 25 µg Al treat-96h 1 + 25 µg Al-treat-96h 2 + 25 µg Al-treat-96h 3 = 300 µg mixed	1/6
Gel 2	50 µg control 2	50 µg Al-treat -24h2		1/6
Gel 3	50 µg Al-treat -24h3	50 µg control 3		1/6
Gel 4	50 µg control 1	50 µg Al-treat -96h1		1/6
Gel 5	50 µg Al-treat -96h2	50 µg control 2		1/6
Gel 6	50 µg control 3	50 µg Al-treat 96h 3	**300 µg mixed + 6 µL CyDye 2**	1/6

Cy 2: Mixed 9 samples

50 µg control 1 + 50 µg control 2 +50 µg control 3 + 25 µg Al-treatment-24h 1 + 25 µg Al-treatment--24h 2 + 25 µg Al-treatment-24h 3 + 25 µg Al-treatment-96h 1 + 25 µg Al-treatment-96h 2 + 25 µg Al-treatment-96h 3 = 300 µg mixed

300 µg mixed + 6 µL CyDye 2

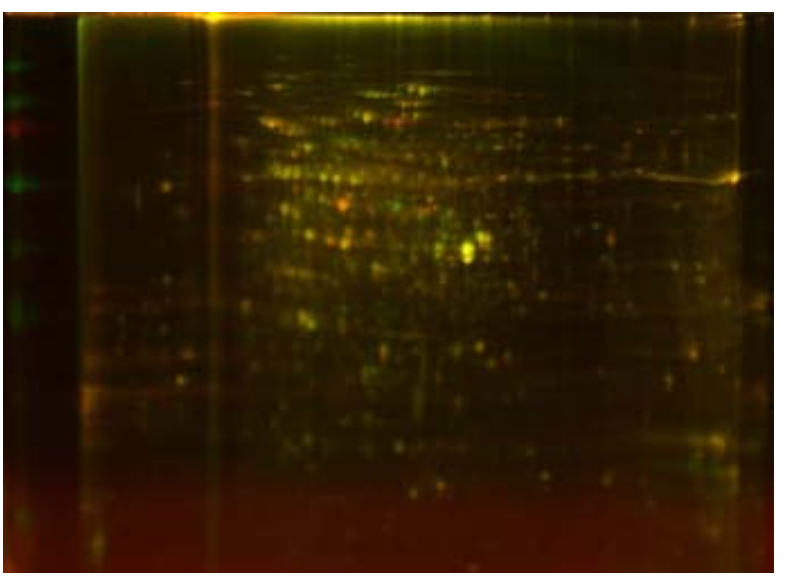


Fig. 22. 2D profile of aluminium regulated-proteins in *B. decumbens* 24h and 96 h Al-treatment. 2D-DIGE to label 3 samples with different dyes and electrophoresis all the samples on the same 2D gel, thus reducing spot pattern variability.

5.2.3.2. *Isoelectric focusing*

First-dimension was using Ettan DaltSix IpG Phor 3 system (GE Healthcare). Immobilized pH gradient IPG strips (GE Healthcare) of 24 cm in length with linear pH 3.0–10.0 gradients were used for this first-dimension separation. Strips were rehydrated for 24h at 20 ° C in 450 µL of rehydration buffer (lysis solution * + 0.5% IPG + 1.4% Destreak) containing 125 µg of root extract proteins and bromophenol blue 0.002% (w/v), and then transferred onto a strip tray Isoelectric focusing (IEF) was carried out at 20 ° C with voltage to 60.000 Vhr.

* *Lysis solution*: Urea 7M, thiourea 2M, CHAPS 2.5%, Tris HCl 30mM pH 8.5

After IEF, for reduction and alkylation of proteins, strips were incubated in fresh equilibrated for 25 min with gentle shaking, in equilibration solution I (equilibration solution** + DTT (100mg/10ml). The strips were hatched in equilibration solution II (equilibration solution** + IAM (250mg/10mL) for 25 min with gentle shaking.

***Equilibration solution*: 6 M urea, 2% SDS, Tris HCl 50mM pH 8.5, bromophenol Blue 0.002% (w/v)

Afterwards, the IPG strips were rinsed with the SDS-gel running buffer and transferred to SDS-polyacrylamide gels 12% (without stacking, GE Healthcare). Paper Whatman (size pads same of strips) was prepared with 15 µL of Benchmark. Labelled molecular weight 8 µL using EZ-Run pre-stained (Fisher Bioreagents). SDS-gels were run at 16 C until the dye front began running out of the gel. The gels were run on a Ettan DALT Six system (GE Healthcare) using the following protocol: 16 °C at 60 mA / 80V / 6W for 1h then at 240 mA / 500V / 78W for 5 h until the bromophenol blue front marker reached the bottom of the gel.

For expression analysis, proteins were labelled with Cy-dyes as previously described. Digital gel images were obtained using a Typhoon 9400 laser scanner (GE Healthcare, Barcelona Vall d'Hebron). The scanned images were then analysed by DataAnalysis program (version 4.0, Barcelona Institute of Biotechnology and Biomedicine UAB). The fold changes of protein expression levels were obtained from program Progenesis Same Spots version 4.1.3884 de Nonlinear Dynamics (Newcastle, UK). Protein spots that showed a change of at least 1.5-fold between control and treated samples with $p < 0.05$ were selected for digestion and identification by MALDI-TOF analysis. For protein identification, separate picking

gels were run. Spots of interest were excised from gel 2 after staining with instant blue (ThermoFisher). The protein spots were picked manually.

5.2.3.3. *In-gel digestion with trypsin*

Spots showing statistically significant changes ($p < 0.05$) were excised and then digested. Gel pieces were destained in 50% ACN (250 µl Acetonitrile + 250 µl doubly deionized H_2O) containing 50 mM ammonium bicarbonate by 20 min. After washing, for reduction and alkylation of cysteine were using to reduction DTT 20 mM /20 min /60ºC and to alkylation Iodoacetamide 25 mM / 15 min / 37ºC. The coomassie stained protein spots were excised from the acrylamide gel, destained and digested with 20 ng/sample trypsin sequencing grade (Promega) for 4 hours at 37ºC. Peptides were eluted by centrifugation with 40 µl of ACN:H_2O (1:1)* + 0.2%TFA.

*(H_2O: ACN (1:1) 250 µl acetonitrile + 250 µl doubly deionized H_2O + 0.2% TFA)

5.2.3.4. *Protein identification by MALDI-TOF mass spectrometry*

All mass spectrometry samples were prepared mixing 0.5µL of sample with the same volume of a solution of alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix (10 mg/ml in 30% acetonitrile, 60% water + 0.1% trifluoroacetic acid) and were spotted onto a ground steel plate (Bruker,Daltonics Inc., Bremen) and allowed to air-dry at room temperature (Kussmann and Roepstorff, 2000). MALDI-mass spectra were recorded in the positive ion mode on an UltrafleXtreme time-of-flight instrument (Bruker). Ion acceleration was set to 25 kV. All mass spectra were externally calibrated using a standard peptide mixture (Bruker,)

5.2.3.5. *Database Search*

For peptide mass fingerprint analysis, Mascot search engine (Matrix Science; http://www.matrixscience.com) was used with the following parameters: Biotechnology Information non-redundant protein database (NCBInr) database, 3 maximum missed trypsin cleavages, and cysteine carbamidomethylation and methionine oxidation as variable modifications and 50 ppm tolerance. Positive identifications were accepted with P values higher than 0.05.

5.3. Results

5.3.1. *Protein affected by Al stress*

The experiments for proteomic analysis were designed taking into account our previous finding that the expression of Al resistance in *B. decumbens* requires a lag time of several hours exhibiting inhibition of root elongation after 24h, but recovering after 96h exposure to Al. To characterize proteomic changes that underlay this behaviour proteome profiles were analysed after two exposure two times, 24h and 96h after Al supply, as a well as under control conditions. The roots were sampled, and proteins were extracted and separated by 2-DE (Fig. 22).

#	Anova (p)	Fold	Average Normalised Volumes		
			control	24hr_treated	96hr_treated
913	3,819e-004	3,5	1,379	0,716	0,394
1657	4,600e-004	4,7	0,570	2,680	0,894
1198	6,368e-004	2,2	1,561	0,867	0,720
535	9,392e-004	3,6	0,578	1,100	2,061
1375	0,001	2,3	0,936	0,911	2,124
567	0,002	2,9	1,850	0,639	1,727
552	0,002	2,7	1,755	0,658	1,648
1638	0,002	2,0	0,723	1,460	1,013
1380	0,002	2,4	0,709	0,693	1,652
779	0,003	2,0	0,962	0,936	1,916
589	0,003	2,1	0,963	2,052	1,755

Table.1. Aluminium regulated-proteins detected in *B. decumbens* in two times (24h – 96h) Al stress experiment. Spots showing changes statistically significant p <0.05.

Resultantly, eleven proteins were differentially expressed under Al stress. A master image, which was produced as a composite of all analysed gel images, is shown in Fig. 22. Eleven protein spots showed significant different intensities (2.1–3.5-fold, ANOVA $p < 0.05$) of root proteomes from the Al-treatments (24h and 96 h under Al) and control without Al (Table 1). The Al-up-regulated proteins after 24h of Al exposure were spots number 1657, 535, 1638 and 589 and Al-down-

regulated proteins were spots number: 913, 535, 1198, 567 and 552 (Table 1). After 96 h of aluminium supply, the Al-down-regulated root proteins were (913 and 1198) while Al-up-regulated proteins were spot numbers: 1657, 535, 1375, 1638, 1380, 779, and 589 (Table 1). The use of proteomic analysis resulted, up to date, in the identification of one protein (Table 1). This protein corresponds to spot number 567. It was specifically down-regulated 2.9 fold after 24 h exposure to Al and recovered to near control values after 96 h Al exposure. In all three samples the protein nº 567 was identified as phenylalanine ammonia-lyase (PAL).

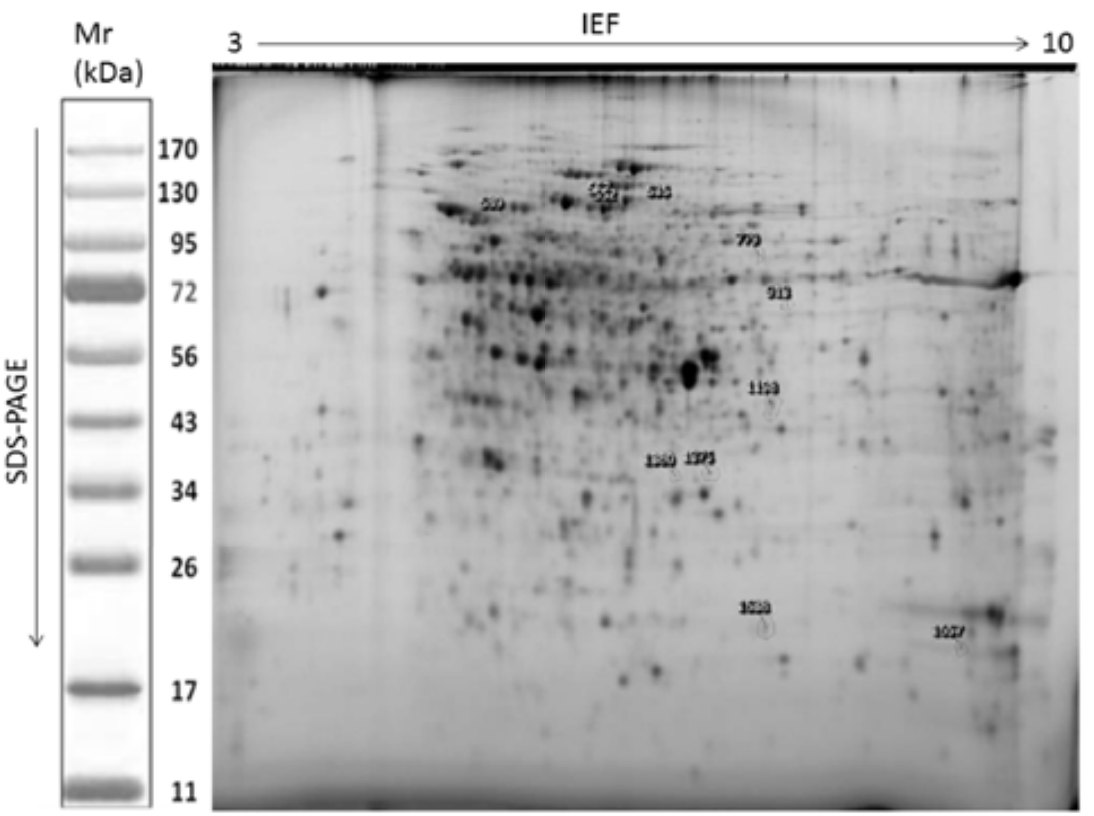


Fig.23. Image of gel showing all protein spots that had significant difference ($p < 0.05$) between Al-treatment and without aluminium in *Brachiaria decumbens* root samples. The image was generated in Progenesis Samespots.

Unfortunately, it was no possible yet to identify the other ten proteins, because the concentration in the gel was low.

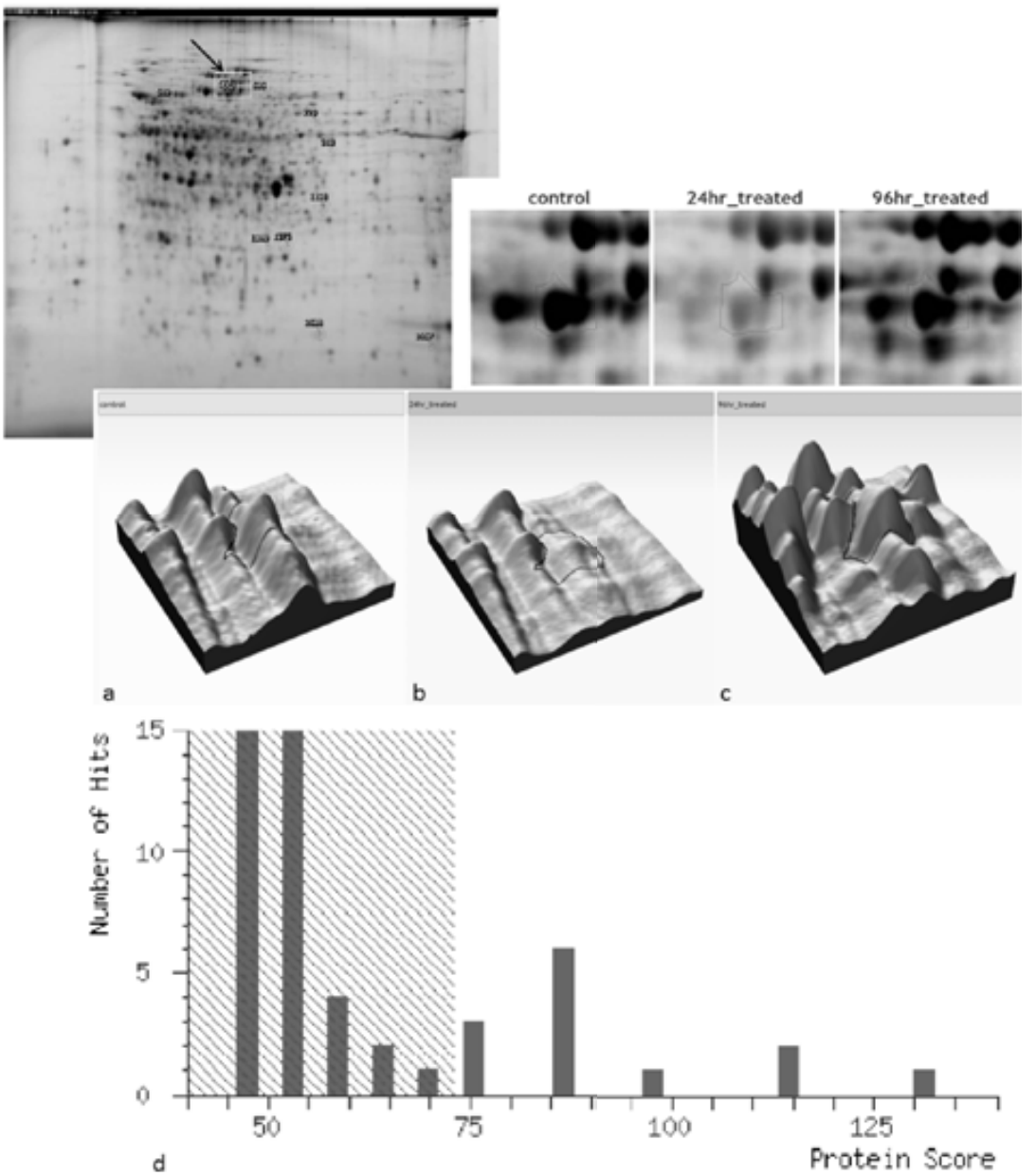


Fig.24. Position of spot (1421, 553), Anova ($p < 0.05$), 2.9 –fold, average normalised volumes control 1.850, Al treatment 24 h 0,639 and Al-treatment 96h 1.727. a. control b. 24h Al-treatment. c. 96h Al-treatment. a.b.c. Spot 567 3D. d. Mascot score histogram protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 73 are significant ($p<0.05$).

5.4. Discussion

In recent studies, several research groups have attempted to investigate the proteome of roots under Al stress in order to enhance the understanding of the molecular mechanisms involved in Al tolerance and toxicity. Duressa *et al.,* (2009) found that protein profile changes revealed aluminium-induced Al-tolerance-related proteins and enzymes in soybean. Specifically, Al up-regulated proteins were malate dehydrogenase, enolase, malate oxidoreductase, and pyruvate dehydrogenase; this was not observed in sensitive genotypes. In another study, also with soybeans, a total of 17 differentially expressed proteins associated with aluminium stress were identified (Zhen *et al.,* 2007). In rice 56 protein spots extracted from roots were found to change under Al stress conditions (Fukuda *et al.*, 2007). Change in cysteine synthase suggested that this enzyme plays a functional role in the mechanism of adaptation of rice to Al. This protein is a key enzyme in sulphur assimilation responsible for the production of antioxidants and metal chelators such as metallothionein (MT), phytochelatin (PC) (Yang *et al.,* 2007). Aluminium-induced changes in the proteome of tomato roots mainly were related to Al-induced oxidative stress because main changes in antioxidant enzymes such as dehydroascorbate reductase, glutathione reductase, and catalase were observed (Zhou *et al*., 2009).

The genome and proteome of Brachiaria species are poorly investigated. For *B. decumbens* only four proteins are reported in the NCBI database. (http://www.ncbi.nlm.nih.gov/protein/?term=Brachiaria%20decumbens).

In this study we identified phenylalanine ammonia-lyase (PAL) by homology with a high score (Fig. 24) as one of the proteins that changed substantially during the first hours of exposure to Al. As far as we know this is the first report on PAL in *Brachiaria decumbens*. This enzyme is very important in the secondary metabolism (Fig. 15 chapter 4); the chemical reactions and pathways involving aromatic derivatives of trans-cinnamic acid. The initial step of phenylpropanoid synthesis is mediated by phenylalanine ammonia-lyase. Despite the importance of PAL in the plant metabolism of antioxidant phenolic compounds and in lignin synthesis, information related to PAL activity under aluminium stress is scarce.

Aluminium supply to *B. decumbens* roots not only caused changes in the cell wall-bound fraction of phenolics, but also in the soluble fraction (Fig. 19 chapter 4).

Chlorogenic acid and DFQA concentrations were substantially enhanced by Al at the 24h, but not at the 96h time sample.

Abiotic stress factors like Al, Cd or Cu toxicity, but also N and P starvation have been found to up-regulate PAL and enhance the concentrations of soluble phenolics in plants (Yamamoto *et al*., 1998; Kováčik and Bačkor 2007a; Kováčik and Bačkor, 2007; Kováčik *et al.,* 2010).

In monocot sorghum Al caused a decrease of PAL activity in both in the root system of the Al-sensitive and Al-tolerant cultivars of sorghum. The authors (Pereira *et al*., 1999) suggested that the reduction of the PAL activity observed in the root system in the presence of Al in both cultivars maybe the result of a feedback inhibitory effect of accumulated phenolic compounds. Recently genetic evidence has been provided for a flavonol-dependent feedback inhibition of PAL (Yin *et al.*, 2012).

By proteomic analyses of *B. decumbens* roots we found that PAL was down-regulate after 24h exposure to Al, but recovered after 96h. Concentrations of cell wall bound ferulic acid and coumaric acid also were lower in roots exposed to Al for 24h than in controls. Coincident in time, a transient Al-induced decrease of root elongation was observed after 24h exposure.

The pathway of phenolic acid metabolism in plants requires the initial steps of general phenylpropanoid metabolism and provides the precursors for lignin biosynthesis (Tamagnone *et al*., 1998). Enhanced lignification is a general response of plants to abiotic and biotic stress and stress-induced inhibition of root elongation has been related to enhanced lignification. Aluminium-induced inhibition of root elongation in Al sensitive wheat has been found to be accompanied by enhanced lignifications of the root elongation zone (Sasaki *et al*., 1997). Contrastingly, here in the Al resistant *B. decumbens* inhibition of root elongation was accompanied by down regulation of PAL and a decrease of cell wall bound coumaric acid, a major component of grass lignin.

Taken together these results do not support a role for Al-induced lignification in the inhibition of root elongation after 24h exposure to Al in our plants. This was to be expected taking into account the transient character of this growth reduction.

Recent investigations in rapidly growing shoots of rice seedlings have established a close positive relationship between elongation growth, PAL, an increase of cell

wall bound ferulic acid. (Wakabayashi *et al.,* 2012). The progressive establishment of a ferulate network will finally lead to cessation of expansion when the cell walls get more mature.

The Al-induced strong, but transient, enhancement of root boron concentrations, coincident with a decrease in cell wall bound ferulate, suggests a fast re-structuration of the cross-links among cell wall polymers in the cell walls of *B. decumbens* exposed to Al. Ferulic acid esters are important components of glucoarabinxilans (GAX), the major hemicellulose in grasses. Ferulate esters may covalently cross-link GAX to lignin (Scheller and Ulvskov, 2010). Recent investigations in Arabidopsis have identified hemicelluloses as a major Al binding fraction in cell walls and Al-induced inhibition of root elongation was associated with considerable inhibition of xyloglucan endotransglucosylase (XET) activity (Yang *et al.,* 2011).

Extrapolation of results on cell walls obtained with dicots to monocots must be made with care because grass cell walls substantially differ from those in dicots. This is also reflected in the different mechanism of B-induced amelioration of Al toxicity in monocot and dicot species (Corrales *et al*., 2008). Boron-induced amelioration of Al toxicity in flax (dicot) was accompanied by a down regulation of PAL and lower concentrations of cell wall bound phenolics (Heidarabadi *et al.,* 2011). In cucumber supplemental B supply decreased Al-induced inhibition of root elongation, while in maize supplemental B did not enhance root elongation under Al stress, but improved membrane stability and enhanced antioxidant defences (Corrales *et al*., 2008).

At the present stage of our research we cannot establish whether the re-structuration of elements involved in the cross-linking of cell wall polymers as observed here in *B. decumbens* roots is cause or consequence of the Al-induced transient inhibition of root elongation. A further point for future research is to see to whether these Al-induced alterations of cell wall features are implied or not in the activation of the Al resistance mechanism i.e. if the inhibition of root elongation and the changes in wall structure are part of the stress perception mechanism leading to activation of the defence mechanism that allows to re-establish root elongation and wall structure 96h after the start of the Al supply.

Clearly further identification of the other 10 proteins that are specifically up or down regulated during the first 96h of exposure to Al will help us in the near future to clarify these complex interactions.

5.5. References chapter 5

Bradford, M. 1976. A rapid and sensitive method for rapid quantification of micrograquantities of protein utilizing the principle of protein-dye binding. Ann of Biochem 72: 248–254

Chaves, L. 2010. Estudio Fisiológico y evaluación del cambio proteómico en dos híbridos de maíz (*Zea mays, L*.) bajo crecimiento de déficit de nitrógeno. Tesis Doctoral. Universidad Autónoma de Barcelona.

Corrales, I., Poschenrieder, C., Barceló, J. 2008. Boron-induced amelioration of aluminium toxicity in a monocot and a dicot species. J Plant Physiol 165: 504-513

Duressa, D., Soliman, K., Taylor, R., Senwo, Z. Proteomic Analysis of Soybean Roots under Aluminum Stress. 2011. J Plant Genomics ID 282531 doi:10.1155/2011/282531

Fukuda, T., Saito, A., Wasaki, J., Shinano, T., Osaki, M. 2007. Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. Plant Sci 172: 1157–1165

Heiderabadi, MD., Ghanati, F., Fujiwara, T. 2011. Interaction between boron and aluminium and their effects on phenolic metabolism of Linum usitatissimum L. roots. Plant Physiol Biochem 49: 1377-1383

Konishi, H., Kitano, H., Komatsu, S. 2005. Identification of rice root proteins regulated by gibberellin using proteome analysis. Plant, Cell and Environ 28: 328–339

Kovaçik, J., Baçkor, M. 2007a. Phenylalanine ammonia-lyase and phenolic compounds in chamomile tolerance to cadmium and copper excess. Water Air Soil Pollut 185: 185-193

Kovaçik, J., Baçkor, M., 2007b. Changes of phenolic metabolism and oxidative status in nitrogen-deficient Matricaria chamomilla plants. Plant Soil 297: 255-265

Kovaçik, J., Klejdus, B., Hedbavny, J. 2010. Effectof aluminium uptake on physiology, phenols and aminoacids in Matricaria chamomilla plants. J Hazardous Mat 178: 949-955

Kussmann, M and Roepstorff, P. 2000. Sample Preparation Techniques for Peptides and Proteins Analyzed by MALDI-MS. Mass spectrometry of proteins and peptides. Method Mol Biol 2000. 146: 405-424. DOI: 10.1385/1-59259-045-4:405

Marouga, R., David, S., Hawkins, E. 2005. The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382: 669–678

Pereira P., Cambraia, J., Sant'Anna, R., Mosquim PR., Moreira, MA. 1999. Aluminum effects on lipid peroxidation and the activities of enzymes of oxidative metabolism in sorphum. Rev Bras Fisiol Veg 11: 137-143

Sasaki, M., Yamamoto, Y., Ma, JF., Matsumoto, H. 1997. Early events induced by aluminum in wheat roots. Soil Sci Plant Nutr 43: 1009-10014

Scheller, H., Ulsvskov, P. 2010. Hemicelluloses. Annu Rev Plant Biol 61: 263-289

Tamagnonea, L.,l Merida, A., Staceya, N., Plaskitta, K., Parr2, A., Chang, S., Lynn, D., Dowa, J., Robertsa, K., Martina, C. 1998. Inhibition of Phenolic Acid Metabolism Results in Precocious Cell Death and Altered Cell Morphology in Leaves of Transgenic Tobacco Plants. Plant Cell 10: 1801-1816

Wakabayashi, K., Soga, K., Hoson, T. 2012. Phenyalanine ammonia-lyase and cell wall pèroxidae are cooperatively involved in the extensive formation of ferulate network in walls of developing rice shoots. J Plant Phsyiol 169: 262-267

Yang, LL., Zhu, XF., Peng YX., Zheng C., Li GX., Liu, Y., Shi YZ., Zheng SJ. 2011. Cell wall hemicelluloses contributes significantly to aluminum adsorption and root growth in Arabidopsis. Plant Physiol 155: 1885-1892

Yin, R., Messner, B., Faus-Kessler, T., Hoffmann, T., Schwab, W., Hajirezaei, M-R., von Saint Paul, V., Heller, W., Schäffber, AR. 1012. Feedback inhibition of the general phenylpropanoid and flavonol biosynthetic pathway upon a compromised flavonol-3-O glycosilation. J Exp Bot doi:10.1093/jxb/err416 1-14

Zhen, Y., Qi , J., Wang, S., Su, J., Xu, G., Zhang, M., Miao, L., Peng, X., Tian, D., Yang, Y. 2007. Comparative proteome analysis of differentially expressed proteins induced by Al toxicity in soybean. Physiol Plant 131: 542–554

Zhou, S., Sauvé, R., Thannhauser, T. 2009. Proteome changes induced by aluminium stress in tomato roots. J Exp Bot 60: 1849–1857

GENERAL CONCLUSIONS

General conclusions

Results from the present work allow drawing the following conclusions:

1) All three Brachiaria species, B decumbens, B. brizantha and B. ruziziensis can grow at low pH and under conditions of low nutrient supply. However, *B. decumbens* and *B. brizantha* are better adapted to these conditions than *B. ruziziensis*

2) The three Brachiaria species clearly differ in their resistance to high AL^{3+} availability following the order *B. decumbens* > *B. brizantha* > *B. ruziziensis*.

3) The ability of *B. decumbens* to maintain 83% of its control root elongation when exposed to 200 µM of total Al (32µM Al^{3+} activity) deserves the classification of this species as hyperresistant to Al. *Brachiaria brizantha* exhibits moderate Al resistance, while *B ruziziensis* can be scored as species with low sensitivity to Al.

4) Qualitative and quantitative evaluation of Al impact on cell membrane stability using Evans blue confirm these species differences in Al resistance.

5) Aluminium resistance in Brachiaria species is due to Al exclusion. Both *B. decumbens* and *B. brizantha* are more efficient in avoiding Al accumulation than *B. ruziziensis*. This was evidenced by Al tissue analysis and different Al- specific staining techniques.

6) In roots of hyperresistant B decumbens Al was specifically localized in root hairs, while Al was apparently excluded from root tip cell walls

7) Expression of Al hyperresistance in *B. decumbens* is characterized by the requirement of a lag time of at least 24 h. This lag time is characterized not only by a strong but transient inhibition of root elongation but also by temporal structural and chemical changes in the Al treated roots that get restored to near control levels after 72 to 96 h of Al exposure.

8) The most conspicuous transient changes are
    a. an inhibition of longitudinal expansion of root tip cortex cells
    b. a 2-3 fold increase of root boron concentration
    c. ruffling of the root epidermal cells
    d. induction of numerous root hairs close to the tip
    e. Increased concentrations of chlorogenic acid in the fraction of soluble root phenolics.
    f. lowering of ferulic acid and coumarilic acid concentrations in root cell walls

9) Proteomic analysis during the induction phase of the Al resistance mechanism (Control 24 and 96 h) revealed significant changes in a relatively small number of only 11 proteins.
10) Among that phenylalanine ammonia lyase was unequivocally identified. This key enzyme in phenolic metabolism in plants was down regulated after 24 h Al but recovered to control levels after 96 h.
11) Take together the results suggest that full expression of hyperresistance to Al in *B. decumbens* has an Al-inducible component related to changes in both cross-linking of cell wall polymers and patterning of root epidermal cells.
12) Clearly further identification of the other 10 proteins that are specifically up or down regulated during the first 96h of exposure to Al will help us in the near future to clarify these complex interactions.