UNIVERSIDADE DO ALGARVE

Aluminum bioaccumulation and tolerance in *Plantago algarbiensis* Samp. and *Plantago almogravensis* Franco, assessed by using *in vitro* cultures and micropropagated plants

Neusa Elisabete José do Nascimento Martins

Tese para obtenção do grau de Doutor em Ciências Biotecnológicas
(Especialidade em Biotecnologia Vegetal)

Trabalho efetuado sob a orientação de Professora Doutora Anabela Romano
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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

The author acknowledges the Portuguese Foundation for Science and Technology (FCT) for funding the PhD fellowship (Grant SFRH/BD/48379/2008).

This work was partially supported by the FCT project "Is *Plantago almogravensis* an aluminum hyperaccumulator plant? Elucidation of the tolerance mechanism using micropropagated plants" (PTDC/AGR-AAM/102664/2008).

ACKNOWLEDGMENTS

I would like to thank my supervisor, Professor Dr. Anabela Romano for accepting me under her supervision at the Plant Biotechnology Laboratory, for giving me the opportunity to develop this work and for her scientific guidance and advices throughout this work. I also thank for her support and encouragement over the last years.

I would like to express my heartfelt gratitude to my co-supervisor, Dr. Sandra Gonçalves for all the guidance throughout this work and for her incessant help. I also thank for always being there to give me the advice, support and encouragement needed to continue in my research and be able to overcome several difficulties.

I am grateful to Professors Drs. Leonor Osório and Júlio Osório for performing the chlorophyll fluorescence analysis and for their help in the interpretation of results.

I want to express my gratitude to Professors Drs. Paula Andrade and Patrícia Valentão for welcoming me at the Laboratory of Pharmacognosy (University of Porto), for putting the HPLC equipment at my disposal and for the help given with the identification and quantification of organic acids.

I would like to thank Professor Dr. Maria Clara Costa for providing access to the Hach LANGE DR 2800 spectrophotometer.

I would like to thank Eng. Castelão Rodrigues and Dr. João Leonel from Direcção Regional de Agricultura e Pescas do Algarve for the collaboration in the nutrients quantification at the laboratory 'Área de Apoio à Produção Agrícola'.

I would like to acknowledge Tânia Palma for their help with the maintenance of *in vitro* cultures and in several biochemical experiments. Also, I would like to thank my lab colleagues for their help and friendship.

A special thanks to all my family and friends for their support and encouragement.

ABSTRACT

This thesis aims to understand the aluminum (Al) bioaccumulation capacity, toxicity effects and tolerance mechanisms in two rare species endemic from Portugal, *Plantago algarbiensis* Samp. and *Plantago almogravensis* Franco. Firstly, two protocols were developed to propagate *in vitro* these species (Chapter 2). Afterwards it was investigated the influence of low pH on *in vitro* growth (Chapter 3). The seed germination requirements and the effects of Al on the germination and early development of seedlings were then evaluated in Chapters 4 and 5, respectively. The impact of low pH and Al on physiological responses, oxidative stress status and capacity for Al accumulation in micropropagated shoots and plantlets was investigated in the subsequent chapters (Chapters 6, 7 and 8). Finally, in Chapter 9 the possible implications of organic acids in Al detoxification were evaluated.

Both species were successfully micropropagated showing high shoot multiplication rates and rooting frequencies and the micropropagated shoots of both species were able to tolerate and grow in low pH conditions. The seeds of both species germinate readily at 15 ºC and Al showed no impact on the germination percentage in either species but oxidative stress during early seedling development was detected. Although, both species accumulated considerable Al amounts and showed to be moderately tolerant to low pH and Al, *P. almogravensis* appeared to be more able to manage the oxidative stress and, therefore, adapted to maintain cellular physiology and growth under these stress conditions. Results suggested that Al detoxification in *P. almogravensis* implies both secretion of organic acids from roots and Al intracellular chelation by organic acids, while in *P. algarbiensis* only the internal mechanism seems to be involved. This research contributes to the understanding of the mechanisms underlying *P. algarbiensis* and *P. almogravensis* adaptation to acidic Al-rich conditions.

Keywords: antioxidant enzymes; endemic species; *in vitro* propagation; pH; organic acids; Al tolerance.

RESUMO

O alumínio (Al) é o metal mais abundante na crosta terrestre, onde permanece maioritariamente insolúvel. Quando os solos se tornam ácidos, como é o caso de cerca de 40% do solo arável, o Al, dependendo do pH, pode hidrolisar-se em vários complexos que podem provocar diferentes efeitos biológicos. O complexo $Al(H_2O)_6^{3+}$ (ou simplesmente Al^{3+}) é o mais fitotóxico, afetando o crescimento e o desenvolvimento das plantas. A sua toxicidade é explicada pela elevada afinidade do Al^{3+} para as paredes celulares, membranas plasmáticas e metabolitos, interferindo em importantes processos morfológicos, fisiológicos e moleculares. Apesar disso, algumas plantas desenvolveram mecanismos para combater o stress causado pelo Al sendo capazes de crescer em áreas com elevado teor deste metal. As espécies selvagens podem apresentar fenótipos mais tolerantes que as plantas cultivadas, e ser úteis para compreender os mecanismos de tolerância ao Al, fornecendo informação fundamental para aumentar a tolerância das espécies cultivadas.

Plantago almogravensis Franco e *Plantago algarbiensis* Samp. são duas espécies endémicas do sudoeste costeiro Português e do Algarve centro-ocidental, respetivamente, que se encontram em risco de extinção global. *P. almogravensis* cresce em solos podzólicos com elevado teor em ferro e Al, e *P. algarbiensis* ocorre em solos argilosos, preferindo zonas a jusante de pequenas nascentes de água ou clareiras de matos baixos acidófilos. De acordo com a "Nova Flora de Portugal" *P. algarbiensis* e *P. almogravensis* são morfologicamente duas espécies distintas. No entanto, atualmente a taxonomia destas espécies deixou de ser consensual, uma vez que, recentemente, na "Flora Ibérica" foram classificadas como sendo uma única espécie, *P. algarbiensis*. Tendo em conta que um melhor conhecimento destas espécies pode ajudar a elucidar a sua taxonomia, são de grande importância os estudos que comparem ambas as espécies de *Plantago*. Num estudo conduzido com plantas a crescer no habitat natural, concluiu-se que a espécie *P. almogravensis* é hiperacumuladora de Al, sendo no entanto, necessários estudos mais detalhados que avaliem os efeitos da toxicidade do Al e os mecanismos de tolerância nesta espécie. Neste trabalho pretendeu-se avaliar a capacidade de *P. algarbiensis* e *P. almogravensis* como bioacumuladoras de Al, perceber como se manifesta a toxicidade deste metal e contribuir para uma melhor compreensão dos mecanismos de tolerância envolvidos.

Ambas as espécies em estudo neste trabalho estão em risco de extinção e encontram-se legalmente protegidas sob a Diretiva Europeia de Habitats 92/43/CEE e pela lei Portuguesa (referência 140/99, de 24 abril). Assim, as populações naturais destas espécies não suportam a colheita massiva de material vegetal para a realização de estudos de investigação sendo imperativo o desenvolvimento de processos alternativos de obtenção de material vegetal. A micropropagação é considerada uma ferramenta adequada para a propagação de espécies ameaçadas, dado que permite a produção em larga-escala de material vegetal a partir de um explantado inicial, tornando possível a realização dos mais diversos estudos. Simultaneamente, a cultura *in vitro* serve também de base à implementação de diversas estratégias de conservação. Assim, o primeiro objetivo deste trabalho foi o desenvolvimento de protocolos de propagação *in vitro* para ambas as espécies de *Plantago* (Capítulo 2).

As culturas foram iniciadas a partir de rebentos provenientes da germinação *in vitro* de sementes. Para ambas as espécies testou-se o meio de cultura Murashige and Skoog (MS) suplementado com as citocininas 6-benziladenina (BA), cinetina (Kin) e zeatina (Zea) nas concentrações 0,2 e 0,5 mg l^{-1}. A citocinina BA revelou-se a mais adequada para multiplicar *in vitro* culturas de ambas as espécies (8,5 ± 1,1 e 9,2 ± 1,2 rebentos por explantado para *P. algarbiensis* e *P. almogravensis*, respetivamente). Para o enraizamento dos rebentos foi testado o meio MS total e reduzindo para metade os macronutrientes (½MS), suplementados com as auxinas ácido indol 3-acético (IAA) ou ácido indol 3-butírico (IBA), nas concentrações 0,2 e 0,5 mg l^{-1}. Os rebentos apresentaram uma elevada resposta rizogénica (100 e 80% de frequência de enraizamento para *P. algarbiensis* e *P. almogravensis*, respetivamente) que não foi influenciada pela composição do meio de cultura base nem pela auxina utilizada. A aclimatização de plantas de ambas as espécies foi bem-sucedida, com percentagens de sobrevivência de 95 e 80% para *P. algarbiensis* e *P. almogravensis*, respetivamente. Os rebentos e plântulas produzidos de acordo com estes protocolos de micropropagação foram utilizados nos estudos subsequentes de avaliação de toxicidade e de tolerância ao Al.

A toxicidade do Al tem sido objeto de inúmeros estudos nas últimas décadas. Os efeitos do Al ocorrem apenas a pH baixo e em condições em que a toxicidade por protões (H^+) também se manifesta. Elevadas concentrações de H^+ no solo podem causar a inibição do crescimento das raízes e uma reduzida absorção e translocação de nutrientes. Como a

maioria dos fatores que afetam o crescimento *in vitro* são semelhantes aos que afetam o crescimento *in vivo*, a avaliação do efeito do baixo pH do meio no crescimento *in vitro* das espécies *P. algarbiensis* e *P. almogravensis* é útil para entender a tolerância a elevadas concentrações de H^+, sem a interferência de outros fatores indiretos como o Al. No Capítulo 3 deste trabalho investigou-se a capacidade dos rebentos micropropagados de ambas as espécies crescerem *in vitro* em condições de pH abaixo dos valores usualmente utilizados. Para tal os rebentos foram cultivados em meio de multiplicação e de enraizamento (MS suplementado com 0,2 mg l^{-1} de BA ou 0,5 mg l^{-1} de IAA, respetivamente) com diferentes valores de pH (4,50; 5,00 e 5,75). Após 6 semanas verificou-se que as culturas apresentaram um crescimento normal sem danos visíveis em todos os valores de pH testados e, em geral, o pH do meio não influenciou os parâmetros de multiplicação e enraizamento *in vitro*. Estes resultados demonstram que ambas as espécies de *Plantago* são capazes de crescer *in vitro* em meios de cultura com valores de pH consideravelmente mais baixos do que os normalmente utilizados na cultura de tecidos (pH 5,70 - 5,80).

As plantas tolerantes ao Al devem expressar os mecanismos de tolerância em todas as fases do desenvolvimento, particularmente na fase mais vulnerável de estabelecimento das plantas. Assim, é essencial avaliar os efeitos do Al durante a fase de germinação das sementes de *P. algarbiensis* e *P. almogravensis*. Inicialmente foram estudados os requisitos de germinação das sementes tendo em vista a otimização das percentagens de germinação (Capítulo 4). Testaram-se as temperaturas constantes de 15 ou 25 ºC e um regime de temperatura alternada de 25/15 ºC, combinadas com um fotoperíodo de 16/8 h luz/escuro ou escuro constante. Verificou-se que as sementes de ambas as espécies germinam rapidamente a 15 ºC na presença ou ausência de luz, alcançando 100% de germinação, não sendo por isso consideradas dormentes. Posteriormente, no Capítulo 5, avaliaram-se os efeitos do Al na germinação de sementes de ambas as espécies de *Plantago*, no crescimento dos germinantes e em vários parâmetros fisiológicos. Para tal, as sementes foram germinadas numa solução de $CaCl_2$ contendo diferentes concentrações de Al (0, 100, 200 ou 400 μM) durante 30 dias a 15 ºC. Verificou-se que a presença de Al não influenciou a percentagem de germinação nem a morfologia dos germinantes. No entanto, em ambas as espécies observou-se a acumulação deste metal nos ápices das raízes e a inibição do crescimento da raiz de uma forma dependente da concentração de Al. Na presença de Al verificou-se também um

incremento da atividade da superóxido dismutase (SOD) e manutenção da atividade da catalase (CAT) que explicam a observada acumulação de H_2O_2. Os resultados sugerem uma correlação entre a absorção de Al, a produção de H_2O_2 e a inibição do crescimento da raiz durante a fase inicial de desenvolvimento dos germinantes de ambas as espécies, embora a espécie *P. almogravensis* tolere concentrações mais elevadas do metal.

Conhecidos os efeitos do Al na germinação e crescimento dos germinantes, tornava-se essencial avaliar a toxicidade e tolerância deste metal em fases posteriores de desenvolvimento. As culturas *in vitro* têm demonstrado um enorme potencial em estudos que visam selecionar espécies tolerantes ao Al e esclarecer os seus mecanismos de tolerância. Utilizando culturas *in vitro* elimina-se a influência de fatores ambientais, os ensaios decorrem em condições controladas e o acesso ao sistema radicular está facilitado. Como uma primeira abordagem iniciaram-se os estudos com rebentos, devido à facilidade de obtenção e manuseamento deste tipo de material vegetal. Neste contexto, no Capítulo 6 investigou-se a capacidade bioacumuladora de Al em rebentos de *P. algarbiensis* e *P. almogravensis*, o efeito do baixo pH e de diferentes concentrações de Al no nível de peroxidação lipídica, acumulação de prolina e hidratos de carbono, e a atividade de vários enzimas antioxidantes. Os rebentos foram cultivados em meio de cultura ¼MS líquido a pH 5,75 (controlo) ou a pH 4,0 suplementado, com 0, 100, 200 ou 400 μM de Al. O método colorimétrico aluminon permitiu quantificar elevados teores de Al (1000 - 4500 μg g^{-1} DW) nos rebentos de ambas as espécies. Verificaram-se algumas diferenças entre as duas espécies em termos de sensibilidade ao baixo pH e ao Al. A acumulação de prolina e o incremento da atividade de enzimas antioxidantes induzidos pelos stresses não foram suficientes para suprimir os danos na membrana plasmática em *P. algarbiensis*, enquanto que em *P. almogravensis* nenhum dano foi detetado. Apesar do método aluminon ser usado em estudos de quantificação de Al em material vegetal, verificaram-se algumas inconsistências na reprodutibilidade dos resultados levando a colocar em causa o rigor do método. Assim, em estudos posteriores foi utilizada a espetrofotometria de absorção atómica, técnica analítica mais precisa.

Diversos estudos têm demonstrado que o Al inibe o crescimento do sistema radicular das plantas, alterando a absorção de água e nutrientes, e por consequência reduzindo o desenvolvimento das plantas. Assim, no Capítulo 7 analisaram-se os efeitos da exposição ao baixo pH e ao Al no crescimento, no conteúdo de nutrientes (cálcio,

fósforo, potássio e magnésio), na acumulação de prolina e hidratos de carbono e na capacidade fotossintética de rebentos e plântulas. Quantificou-se ainda o Al acumulado por espetrofotometria de absorção atómica. Os rebentos e plântulas foram cultivados durante 7 dias em meio de cultura ¼MS líquido a pH 5,75 (controlo) ou pH 4,0 suplementado com 0 ou 400 μM de Al. Em ambas as espécies, os rebentos e as folhas das plântulas demostraram acumular menores quantidades de Al (120 - 220 $\mu g\ g^{-1}$ DW) comparativamente com as elevadas concentrações detetadas nas raízes (900 - 1200 $\mu g\ g^{-1}$ DW). Neste capítulo, os teores de Al acumulado nos rebentos utilizando a espetrofotometria de absorção atómica foram inferiores aos obtidos com o método do aluminon, no capítulo anterior, confirmando a falta de rigor deste método para a análise de amostras vegetais. A espetrofotometria de absorção atómica é uma técnica bem estabelecida na análise elementar ao nível vestigial de diferentes matrizes, sendo vulgarmente usada na determinação de metais. Este método tem sido extensamente utilizado para quantificar Al em amostras vegetais, tendo como principais vantagens a elevada sensibilidade e seletividade, e a possibilidade de quantificar um elevado número de elementos com considerável rapidez. Verificou-se que o Al inibiu o alongamento da raiz apenas em *P. algarbiensis*. A espécie *P. almogravensis* mostrou-se mais tolerante ao Al, visto possuir alguns ajustamentos internos como a redução da pressão de extinção ($1 - q_p$), o aumento do coeficiente de extinção não fotoquímica (NPQ), a acumulação de hidratos de carbono e de nutrientes, que parecem ter sido suficientes para manter as funções fisiológicas perante a toxicidade causada pelo baixo pH e o Al.

A exposição das plantas à toxicidade do Al pode levar ao incremento dos níveis de espécies reativas de oxigénio (ROS) nas células por meio de processos como a fotossíntese, gerando stress oxidativo. Apesar das ROS serem importantes moléculas sinalizadoras podem causar danos oxidativos em biomoléculas, levando à peroxidação da membrana plasmática, perda de iões, oxidação proteica e até à quebra da cadeia de DNA. No entanto, a existência de um sistema antioxidante eficiente capaz de as eliminar tem sido considerado um mecanismo importante em plantas tolerantes ao Al. Assim, no Capítulo 8 analisaram-se os efeitos da exposição ao baixo pH e ao Al na partição de energia no fotossistema II, conteúdo de H_2O_2, biomarcadores de stress oxidativo e na atividade de vários enzimas antioxidantes. Estes estudos realizaram-se em plântulas de ambas as espécies cultivadas durante 7 dias em meio ¼MS líquido a pH

5,75 (controlo) ou pH 4,0, suplementado com 0 ou 400 μM de Al. Nem o Al nem o baixo pH influenciaram a função do fotossistema II, tendo o metabolismo oxidativo sido mais afetado pelo Al do que pelo baixo pH. A presença de Al induziu um aumento do conteúdo de H_2O_2 nas raízes de *P. algarbiensis*, enquanto nas folhas e raízes de *P. almogravensis* induziu uma diminuição. Além disso, o Al levou a um incremento da oxidação de proteínas e atividade dos enzimas SOD e CAT nas raízes de *P. algarbiensis*. Contrariamente, este metal diminuiu o nível de peroxidação lipídica nas raízes e a atividade da SOD nas folhas e raízes de *P. almogravensis*. Os resultados demonstraram que as plântulas de *P. almogravensis* foram capazes de minimizar a acumulação de H_2O_2, prevenindo assim danos celulares em resposta ao baixo pH e ao Al. Por outro lado, nas raízes de *P. algarbiensis* o sistema antioxidante não foi capaz de suprimir totalmente a toxicidade imposta pelo Al, levando à acumulação de H_2O_2 e à oxidação de proteínas.

As plantas nativas de solos ácidos com elevado teor em Al desenvolveram adaptações de forma a evitar a interação dos iões Al^{3+} com estruturas vitais e processos metabólicos. Dois mecanismos têm sido sugeridos, os que impedem a entrada do Al pela raiz (mecanismo de destoxificação externa ou de exclusão), e os que permitem a acumulação de Al no interior da planta (mecanismo de destoxificação interna ou de tolerância interna). A capacidade de ácidos orgânicos quelatarem o Al e o seu envolvimento na destoxificação do Al tanto internamente, no simplasto, como externamente, na rizosfera, foi comprovada em diversas espécies. Com o objetivo de avaliar a possível implicação dos ácidos orgânicos na destoxificação do Al em *P. algarbiensis* e *P. almogravensis*, no Capítulo 9 procedeu-se à identificação e quantificação por HPLC de ácidos orgânicos secretados e acumulados por ambas as espécies em resposta ao Al. Para tal os rebentos e plântulas foram cultivados durante 7 dias em meio de cultura ¼MS líquido (pH 4,0) suplementado com 0 ou 400 μM de Al. Adicionalmente, e com o objetivo de relacionar a acumulação de ácidos orgânicos com o incremento da atividade dos enzimas envolvidos no seu metabolismo (citrato sintase, malato desidrogenase, isocitrato desidrogenase, fumarase e fosfoenolpiruvato carboxilase), determinou-se a sua atividade. Verificou-se um aumento da secreção dos ácidos cítrico, sucínico e málico pelas raízes de *P. almogravensis* em resposta ao Al, enquanto em *P. algarbiensis* apenas a secreção de malónico foi incrementada. Foram também observados incrementos nos teores de ácido cítrico, oxálico, malónico e

fumárico nas folhas e raízes de ambas as espécies. De uma maneira geral, os enzimas do metabolismo dos ácidos orgânicos parecem não estar diretamente envolvidos na secreção e acumulação de ácidos orgânicos em resposta ao Al. Os resultados sugerem que na espécie *P. almogravensis* a secreção de ácidos orgânicos pelas raízes e a quelatação intracelular do Al pelos ácidos orgânicos, estão envolvidos na destoxificação do Al, enquanto na espécie *P. algarbiensis* apenas se verifica a quelatação interna.

A propagação *in vitro*, mostrou-se uma ferramenta útil e adequada para estudar a capacidade bioacumuladora de Al e os seus mecanismos de toxicidade e tolerância nas espécies *P. algarbiensis* e *P. almogravensis*. As culturas *in vitro* de ambas as espécies acumularam quantidades consideráveis de Al, embora em quantidades inferiores às observadas em plantas de campo. Este resultado não é, no entanto, surpreendente dadas as diferenças entre as condições naturais e as condições *in vitro,* as quais são assépticas e controladas, possuindo uma concentração de Al no meio e um período de exposição específicos. Desta forma, será de todo o interesse realizar estudos de resposta ao Al em plantas micropropagadas já a crescer no ambiente *ex vitro* em solos ácidos com teores de Al semelhantes aos observados nos solos onde estas plantas crescem, de forma a mimetizar as condições de campo. Além disso, considerando que durante o crescimento as raízes encontram diferentes concentrações de Al no solo seria interessante no futuro estudar os mecanismos de recuperação da toxicidade causada pelo Al em ambas as espécies.

De uma maneira geral, os resultados demonstraram que ambas as espécies são tolerantes ao Al e ao baixo pH, apesar de todos os estudos indicarem que a espécie *P. almogravensis* está melhor adaptada para manter o crescimento e as funções fisiológicas nestas condições de stress. Além disso, verificou-se que *P. almogravensis* é capaz de destoxificar o Al através de quelatação interna e externa com ácidos orgânicos, enquanto na espécie *P. algarbiensis* apenas a quelatação interna parece estar envolvida. Para melhor entender estes mecanismos de tolerância envolvendo ácidos orgânicos é essencial identificar em estudos futuros o ácido orgânico ligado ao Al e se ocorrem trocas deste ligando durante a absorção, translocação e acumulação do Al pela planta, bem como localizar o órgão específico onde o complexo ácido orgânico-Al é armazenado.

Como conclusão geral, pensa-se que este trabalho é um contributo importante para a compreensão dos mecanismos envolvidos na adaptação de espécies que colonizam solos ácidos com elevado teor em Al.

Palavras-chave: ácidos orgânicos; enzimas antioxidantes; espécies endémicas; pH; propagação *in vitro*; tolerância ao Al.

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Al - aluminum

ALMT - aluminum-activated malate transporter

ANOVA - analysis of variance

APX - ascorbate peroxidase

BA - 6-benzyladenine

BSA - bovine serum albumin

Ca - calcium

CAT - catalase

Chl - chlorophyll

CS - citrate synthase

DNA - deoxyribonucleic acid

DNPH - 2,4-dinitrophenyldrazine

DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT - dithiothreitol

DTZ - distal part of the transition zone

DW - dry weight

EDTA - ethylenediaminetetraacetic acid

EL - electrolyte leakage

F_v/F_m - maximum photochemical efficiency of PSII

FW - fresh weight

GPX - guaiacol peroxidase

$HO_2^{\bullet}$ - perhydroxy radical

HPLC - high performance liquid chromatography

H_2O_2 - hydrogen peroxide

IAA - indole-3-acetic acid

IBA - indole-3-butyric acid

K - potassium

Kin - kinetin

MATE - multidrug and toxic compound extrusion

MDA - malondialdehyde

MDH - malate dehydrogenase

Mg - magnesium

MGT - mean germination time

MS - Murashige and Skoog medium

NADP-IDH - NADP-isocitrate dehydrogenase

NBT - nitrotetrazolium blue chloride

NPQ - non-photochemical quenching

OAs - organic acids

$^{\bullet}OH$ - hydroxyl radical

$O_2^{\bullet-}$ - superoxide radical

$^1O_2$ - singlet oxygen

P - phosphorus

PEP - phosphoenolpyruvate

PEPC - phosphoenolpyruvate carboxylase

PSII - photosystem II

PVPP - polyvinylpolypyrrolidone

q_p - photochemical quenching

$RO^{\bullet}$ - alkoxy radical

ROS - reactive oxygen species

RWC - relative water content

SE - standard error

SOD - superoxide dismutase

SPSS - statistical package for the social sciences

TaALMT1 - *Triticum aestivum* aluminum-activated malate transporter

TBA - thiobarbituric acid

TCA - trichloroacetic acid

TW - turgid weight

Tween-20 - polyoxyethylenesorbitan monolaurate

Zea - zeatin

$1 - q_p$ - PSII excitation pressure

ϕ_{NO} - quantum yield of non-regulated energy dissipation of PSII

ϕ_{NPQ} - quantum yield of regulated energy dissipation of PSII

ϕ_{PSII} - actual quantum efficiency of PSII

CHAPTER 1

GENERAL INTRODUCTION

1.1. Acidic soils and aluminum

Acid soils (soils with pH < 5.5) comprise about half of the world's arable land and up to 60% of which occur in developing countries (Kochian et al. 2005). In Portugal, approximately 40% of the soils are acidic, being located mostly in the Northern regions (Almeida 1955). Soil acidity represents a major plant growth limiting factor, threatening agricultural processes and natural ecosystems. In addition, soil acidification is gradually increasing due to acid rain, removal of natural plant coverage and the inappropriate use of ammonium-based fertilizers (Kochian et al. 2004; Zheng and Yang 2005). These factors outstrip the buffering capacity of soils, leading to a combination of toxicities (H^+, Al^{3+} and Mn^{2+}) and deficiencies (NH_4^+-N, PO_4^{3-}, Ca^{2+}, Mg^{2+} and MoO_4^{2-}) (Foy 1984; Kidd and Proctor 2001). Among these stresses, low-pH (H^+ toxicity) and Al^{3+} are the main causes of reduced plant growth (Rangel et al. 2005).

Aluminum (Al) is the most abundant metal in the earth's crust and the third chemical element behind oxygen and silicon. Soils contain an average of 7% total Al, mostly present as a harmless oxide and aluminosilicate. However, in acid soils (especially pH < 4.5) Al is solubilized into soil solution and becomes toxic to sensitive plants (Kochian et al. 2005). Al toxicity not only depends on the concentration, but also on the Al chemical forms, as Al hydrolysis in a pH-dependent manner to form various complexes with hydroxyl groups that show different biological impacts (Kochian 1995). The most phytotoxic form of Al is $[Al(H_2O)_6]^{3+}$, commonly known as Al^{3+}, which predominates in soil solutions below pH 4.5. When the pH of a solution is raised, Al forms the mononuclear species $Al(OH)^{2+}$, $Al(OH)_2^+$, $Al(OH)_3$ and $Al(OH)_4^-$, as well as a very toxic polynuclear Al species, $AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$ (referred as Al_{13}), but its natural occurrence is unknown (Delhaize and Ryan 1995).

In addition to Al toxicity, plants growing on acidic soils are subject to proton (H^+) toxicity. However, the understanding of H^+ toxicity and the mechanisms of low pH tolerance are limited when compared to those of Al. It is known that high H^+ concentrations cause root growth inhibition (e.g. Kidd and Proctor 2001; Rangel et al. 2005; Sawaki et al. 2009) and reduces nutrient uptake and translocation, as it competes with cations for absorption sites in the root apoplast (Poschenrieder et al. 1995; Kidd and Proctor 2001). Moreover, it was observed that proton toxicity induced H^+ influx into the root tissues, depolarization of plasma membrane and cytoplasmic acidification in *Arabidopsis thaliana* (Bose et al. 2010).

The exposure to low pH damages the plasma membrane of *A. thaliana* root tip cells within a short time (Koyama et al. 2001), whereas Al causes swelling of cells without plasma membrane damage (Kobayashi et al. 2005). Furthermore, an *Arabidopsis* quantitative trait locus analysis showed that Al and H^+ tolerance are controlled by different genetic factors (Ikka et al. 2007). On the other hand, the proton-hypersensitive *Arabidopsis stop1* mutant is also hypersensitive to Al (Sawaki et al. 2009) and it was detected an interference of H^+ toxicity during the selection and evaluation of Al resistant cultivars in spinach (Yang et al. 2005) and common bean (Rangel et al. 2005). Hence, it is difficult to conclude that resistance to H^+ toxicity is independent from Al resistance (Ikka et al. 2007) and, therefore, it is important to evaluate the low pH effects, *per se,* for a greater understanding and correct interpretation of Al toxicity (Samac and Tesfaye 2003).

As resistance to both proton and Al toxicity are necessary for a plant to survive in acidic Al-rich soils, finding ways to alleviate the adverse effect of both stresses on plant growth has become an important purpose. Application of lime in acidic soils can increase the soil pH and hence reduce the Al solubility. However, this practice is costly,

ineffective in the subsoil and may have a deleterious effect on soil structure or cause deficiencies in certain nutrients (Wang et al. 2006). A complementary strategy is the genetic improvement of plants in order to increase their Al tolerance (Inostroza-Blancheteau et al. 2010).

1.2. Aluminum toxicity in plants

1.2.1. Plant growth inhibition

Inhibition of root growth has been reported as the earliest and the most dramatic symptom of Al toxicity in plants, arising after a few minutes of exposure to micromolar concentrations of the metal (Delhaize and Ryan 1995). Therefore, root growth has been widely used as a suitable criterion for assessing plant species and/or genotypic differences in Al resistance (e.g. Tahara et al. 2008; Li et al. 2009). Additionally, roots have been the focus of study to decode the Al toxicity and tolerance mechanisms.

The root apex (including root cap, meristematic and elongation zones) accumulates more Al than the mature tissues and is, therefore, the primary site of Al-induced injury (Delhaize and Ryan 1995). Ryan et al. (1993) found the root apex as the most Al-sensitive zone and Sivaguru and Horst (1998) identified the distal part of the transition zone (DTZ, 1-2 mm), where cells are switching from cell division to cell elongation, as the specific Al-sensitive apical root zone. Al interaction with the apical root zone leads to the inhibition of both root cell division in the meristem and cell elongation within minutes or a few hours (e.g. Sivaguru and Horst 1998; Silva et al. 2000; Doncheva et al. 2005). How Al interacts with the DTZ is still unknown, but several mechanisms have been predicted such as the decrease of cell wall extensibility, the inhibition of phospholipase C activity, the reorganization of cell cytoskeleton, the inhibition of auxin

transport, the disruption of calcium homeostasis, and the callose formation (Samac and Tesfaye 2003; Poschenrieder et al. 2008)

Long exposures to Al toxicity can lead to severe modifications in root morphology. Roots become stunted and brittle, lateral roots may turn thick and brown, fine branching and root hairs development are reduced, the root apices become swollen and damaged, and cracks can be detected in the epidermis (Vitorello et al. 2005; Chaffai and Marzouk 2009). This extensive root damage leads to inefficient water and mineral nutrient absorption and eventually to limited growth of the whole plant (Barceló and Poschenrieder 2002). Thus, symptoms of Al injury are also manifested in the shoots, but after longer exposures to Al and usually regarded as a result of root damage and consequent mineral nutrition deficiencies (Vitorello et al. 2005). Al toxicity in the shoots may lead to chlorosis and foliar necrosis, cellular modifications in leaves, reduced stomatal opening, chloroplast malformations and decreased photosynthetic activity (Vitorello et al. 2005).

1.2.2. Changes on nutrient uptake

Al has been found to disturb the uptake, accumulation and translocation of essential nutrients (particularly calcium - Ca, magnesium - Mg, potassium - K or phosphorus - P) in several plant species, such as pigeonpea (Choudhary and Singh 2011), barley (Ali et al. 2011), wheat (Silva et al. 2010) and maize (Giannakoula et al. 2008). The ability to maintain a less inhibited uptake and accumulation of nutrients in the presence of Al has been widely associated with Al resistant genotypes (e.g. Shamsi et al. 2007; Ali et al. 2011; Choudhary and Singh 2011).

The Al interference on Ca uptake has received considerable attention because the Al toxicity symptoms resemble Ca deficiency and the exogenous Ca application mitigates the Al toxicity effects (Rengel and Zhang 2003). It is well known that the exposure of plant roots to Al leads to Ca uptake reduction (e.g. Giannakoula et al. 2008; Ali et al. 2011). Kawano et al. (2004) observed that Al acts as a specific inhibitor of TPC1 (two-pore channel 1) Ca channels in transgenic tobacco BY-2 cells. Moreover, Rengel (1992) observed that Al acts as a Ca-channel blocker in *Amaranthus tricolor* by binding to the verapamil-specific channel-receptor site and by interfering with the action of GTP-binding proteins involved in the regulation of transmembrane Ca fluxes.

Inhibition of Mg uptake and accumulation was also widely reported in distinct plant species in response to Al (Giannakoula et al. 2008; Olivares et al. 2009; Silva et al. 2010; Ali et al. 2011). Al can lead to reduced Mg uptake and translocation through the Al binding to the cell wall and accumulation in the apoplast, high sensitivity of Mg membrane transporters to Al and inhibition of the basipetal transport of auxin to the root meristematic zone (Bose et al. 2011).

Al induced decreases in P content has been described in the roots of several plant species (Silva et al. 2010; Ali et al. 2011; Choudhary and Singh 2011). Other studies reported that Al exposure leaded to increases in the P content in roots and decreases in shoots (Liang et al. 2001; Quartin et al. 2001) which was thought to be related with the formation of P-Al complexes in roots that inhibit P transport from root to shoot (Liang et al. 2001). No consistent relationship between Al toxicity and K content is evident in plants since Al induced either inhibition (Giannakoula et al. 2008; Silva et al. 2010; Choudhary and Singh 2011) or increase in K content (Olivares et al. 2009).

1.2.3. Photosynthesis inhibition

The decline in growth of plant species subjected to stressful environment is often associated with a reduction in photosynthetic capacity. In fact, Al induced inhibition of CO_2 assimilation in many plant species resulting from stomatal and/or non-stomatal factors (Simon et al. 1994; Peixoto et al. 2002; Chen et al. 2005a; Silva et al. 2012). In some plant species it was observed a decrease in the intercellular CO_2 concentration in response to Al, showing that stomatal closure was partially responsible for the Al induced reduction in CO_2 assimilation (Simon et al. 1994; Ali et al. 2008; He et al. 2011). However, most studies reported that the inhibition of the photosynthetic process by Al is primarily caused by non-stomatal factors. The Al induced reduction in CO_2 assimilation was associated with structural damage to the thylakoids in citrus (Pereira et al. 2000) and changes in chloroplast ultrastructure in wheatgrass (Moustakas et al. 1997). Jiang et al. (2009) proposed that the photosynthesis impairment observed in pummelo exposed to Al was mainly caused by impaired photosynthetic electron transport chain in photosystem II (PSII). Photosynthesis inhibition by Al, as a result of both closure of PSII reaction centers and reduced electron transport rate through PSII, was reported in tangerine (Chen et al. 2005b), while a combination of factors such as impaired PSII photochemistry, reduced chlorophyll (Chl) content and distribution of enzymatic machinery was suggested in sorghum (Peixoto et al. 2002). Therefore, it seems that the PSII is the most vulnerable component of the photosynthetic apparatus to Al stress.

Al toxicity can also lead to decreases in the Chl content (e.g. Ali et al. 2008; Mihailovic et al. 2008; Reyes-Diaz et al. 2009), but not always associated with decreased CO_2 assimilation (Simon et al. 1994; Chen et al. 2005a). The Al-induced Chl content decreases may be due to reduced Chl synthesis (Mihailovic et al. 2008) or inhibited

absorption of Mg that is a constituent of Chl molecule (Vitorello and Haug 1996). Pereira et al. (2006) demonstrated that Al inhibits the activity of δ-aminolevulinic acid dehydratase responsible for the formation of Chl molecule and the cytochromes resulting in decreased Chl synthesis.

The impact of Al on the photosynthetic apparatus seems to depend on the plant species and/or genotype. Shamsi et al. (2007) reported that the Chl content, the photosynthesis rate and the stomatal conductance were more inhibited in the soybean Al-sensitive cultivar Zhechun 2 than in the Al-tolerant Liao 1. The PSII activity and the Chl content were also more affected in Al-sensitive maize line (Mihailovic et al. 2008). In addition, Reyes-Diaz et al. (2009) found that the photochemical efficiency of PSII was less affected in the Al-tolerant highbush blueberry cultivar.

1.2.4. Oxidative stress

Plants, like other aerobic organisms, constantly produce reactive oxygen species (ROS) as natural byproducts of various physiological pathways, such as photosynthesis and photorespiration (Gill and Tuteja 2010). The ROS comprises both free radical (superoxide radical - $O_2^{\bullet-}$, alkoxy radical - $RO^{\bullet}$, perhydroxy radical - $HO_2^{\bullet}$ and hydroxyl radical - $^{\bullet}OH$) and non-radical forms (singlet oxygen - $^1O_2$ and hydrogen peroxide - H_2O_2) (Gill and Tuteja 2010). At low concentrations, ROS can play a significant role in plant growth, development, abiotic stress responses, pathogen defense and systemic signaling (Gill and Tuteja 2010), whereas their high accumulation may affect many cellular functions by causing lipid peroxidation, protein and DNA oxidation and ultimately lead to cell death (Sharma et al. 2012). Thus, whether ROS will act as damaging or signaling factors depends on the equilibrium between ROS production and metabolism (Sharma et al. 2012).

Plants subjected to environmental stresses may fail to successfully scavenge ROS, disrupting redox homeostasis of cell and thus producing oxidative burst. Despite being a non-transition metal, Al is known to have pro-oxidant activity in biological systems facilitating superoxide- and iron-driven oxidation (Exley 2004). Studies tried to explain the pro-oxidant activity of this metal via the formation of an Al superoxide semireduced radical cation, $AlO_2^{\bullet 2+}$ (Exley 2004). In fact, increasing evidence has showed that Al induces ROS accumulation and concomitant oxidative stress in various plant species, being most evident in Al-sensitive plant species and/or genotypes (e.g. Tahara et al. 2008; Giannakoula et al. 2010; Xu et al. 2012).

Although, 30 - 90% of the Al absorbed in root tissues is localized in the apoplasm, it has been well established that Al rapidly accumulates in the plasma membrane and in the symplasm of Al-sensitive species (Kochian 1995; Samac and Tesfaye 2003). It was recently identified in rice an Nrat1 transporter (Nramp aluminum transporter 1) localized at the plasma membrane that enables the Al uptake into root cells symplasm (Xia et al. 2010). Al showed a strong affinity for the negative charges of carboxyl and phosphate groups of the plasma membrane (560-fold greater affinity than other cations such as Ca^{2+}) to which binds irreversibly (Zheng and Yang 2005). The binding of Al to the plasma membrane can account for changes on fluidity and charges, causing altered surface negativity and membrane potential (Sivaguru et al. 2003; Krtková et al. 2012), inhibition of H^+-ATPase activity (Ahn et al. 2002) and disturbing uptake of ions such as Ca^{2+}, K^+, Mg^{2+} and NH_4^+ (Piñeros and Tester 1997). Al-induced impairment of membrane functions can also be associated to membrane lipid peroxidation caused by Al-enhanced ROS accumulation (Jones et al. 2006). Lipid peroxidation in response to Al stress is the most prominent symptom of oxidative damage and has been extensively reported in various plant species (e.g. Achary et al. 2012; Xu et al. 2012).

Al-induced ROS accumulation can also cause damage to DNA and proteins. Al has been shown to induce chromosome stickiness and breaks in rice root cells (Mohanty et al. 2004), DNA damage and variations on nucleoli in onion (Achary et al. 2008) and increases in the frequencies of micronuclei and chromosome aberrations in broad bean (Yi et al. 2010). Al-induced protein oxidation was observed in the root cells of maize (Boscolo et al. 2003) and onion (Achary et al. 2008), and in barley seedlings (Achary et al. 2012).

Plants have developed a complex defense antioxidant system, including antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) as well as low-molecular mass antioxidants, such as ascorbic acid, reduced glutathione, flavonoids and carotenoids, that work in concert to scavenge ROS (Sharma et al. 2012). Antioxidant enzymes have been shown to be the most important components in the ROS scavenging system to combat metal induced oxidative injury. SOD performs the first step in the detoxifying process, converting superoxide radicals to H_2O_2, which is then reduced by CAT, GPX or APX to water. As opposed to other antioxidant enzymes, CAT does not require cellular reducing equivalent to transform H_2O_2 to H_2O and O_2, and has a high reaction rate but a low affinity for H_2O_2, thereby only removing the bulk of H_2O_2 (Achary et al. 2008). In contrast, APX has a high affinity for H_2O_2, allowing the detoxification of low H_2O_2 concentrations in more specific locations.

The plant ability to reduce the oxidative stress under Al through the regulation of the antioxidant enzymes is an important component of Al tolerance in distinct plant species (e.g. Jones et al. 2006; Giannakoula et al. 2010; Xu et al. 2012). Furthermore, it has been well documented that genes encoding antioxidant enzymes are activated by Al (e.g. Simonovicova et al. 2004; Ezaki et al. 2005) and that transgenic plants of

Arabidopsis and *Brassica napus* overexpressing such genes showed improved Al resistance (Ezaki et al. 2000; Basu et al. 2001).

The accumulation of compatible solutes, such as proline, glycine betaine and carbohydrates is regarded as a nearly universal strategy for the protection and survival of plants under several abiotic stresses (Chen et al. 2007). These solutes are able to stabilize proteins and cellular structures and/or to maintain cell turgor through osmotic adjustment. Most compatible solutes also seem to play an important role in ROS scavenging defending plants against oxidative injury (Chen et al. 2007). Therefore, the enhanced content of these solutes may probably ameliorate the tolerance through improving oxidative status. Proline and carbohydrates accumulation, as a response to Al, has been described in several plant species, including wheat (Tabuchi et al. 2004), mung bean (Ali et al. 2008) and maize (Giannakoula et al. 2008), and correlated with Al tolerance. The alleviation of water deficit and reduction of ROS levels mediated by compatible solutes, may also contribute to the adaptation of plants to Al stress conditions.

1.3. Aluminum hyperaccumulation

Metal hyperaccumulating plant species are able to tolerate and accumulate high amounts of metals in their aboveground tissues, far in excess of the levels found in the majority of species, without suffering damage and are usually endemic to metalliferous soils (Rascio and Navarri-Izzo 2011). These species belong to distantly related families, showing that the hyperaccumulation trait has developed independently under the spur of selective ecological factors. The evolutionary reasons that gave rise to hyperaccumulating plants are unknown, but metal tolerance, drought resistance,

allelopathy and protection against herbivores or pathogens have been suggested to describe the role of high metal contents in shoots (Rascio and Navarri-Izzo 2011).

Generally, plants are classified as Al hyperaccumulators if they accumulate at least 1,000 μg Al g^{-1} dry shoot (Foy 1984; Baker and Brooks 1989). Under these criteria most plants are Al non-accumulators, with levels below 300 μg g^{-1} dry weight (DW) (Jansen et al. 2004). More than 100 plant species, usually from tropical humid areas, have been identified as Al accumulators (Barceló and Poschenrieder 2002; Jansen et al. 2004). These hyperaccumulator plants can be found within certain families of plant orders, such as Ericales, Euphorbiales and Myrtales, where the shrub-type of woody plants are predominant, as represented by *Melastoma malabathricum* and *Camellia sinensis* plants (Jansen et al. 2004). *M. malabathricum* grows in tropical acidic soils and accumulates more than 10,000 μg Al g^{-1} DW in leaves and roots (Watanabe et al. 1998). The tea plant, *C. sinensis*, takes up Al throughout its life span, and mature leaves contain up to 30,000 μg Al g^{-1} DW without experiencing Al toxicity (Matsumoto et al. 1976).

The studies on potentially Al-hyperaccumulator plants are of outmost importance in order to highlight physiological and molecular mechanisms by which this metal is taken up, transported, sequestered and tolerated, to discover the adaptive functions performed by hyperaccumulation and to find new Al hyperaccumulating taxa (Rascio and Navarri-Izzo 2011). Furthermore, without the courses of breeding for agronomic features wild plants well adapted to Al toxicity can conserve high Al resistance genetic information, and therefore will be good resources to understand the multi-Al resistance mechanisms and to discover and isolate tolerant genes that can be used to increase Al tolerance in other species, particularly crops (Ezaki et al. 2008).

Metal hyperaccumulator plants can also be used for the practical implementation of phytoremediation techniques such as phytoextraction, phytostabilization or

rhizofiltration in contaminated or natural metal-rich soils (Shah and Nongkynrih 2007). However, the potential use of metal hyperaccumulator plants as means of soil decontamination is clearly limited by plant productivity since these plants are generally of small biomass (Rascio and Navarri-Izzo 2011). A promising biotechnological approach for enhancing the potential for metal phytoremediation may be to improve the hyperaccumulator growth through selective breeding, or by the transfer of metal hyperaccumulation genes to high biomass species (Rascio and Navarri-Izzo 2011). Even so, metal hyperaccumulator plants are quite rare, with small populations, often growing in geographically remote areas and having a restricted distribution in threatened areas (Shah and Nongkynrih 2007). Thus, it is important to implement measures for the conservation of these species and protocols aiming the mass production of plants to be used in future investigations and applications.

1.4. Aluminum detoxification mechanisms

Plant species that grow in acid soils with high levels of Al have developed mechanisms for its detoxification, which can be divided into mechanisms of external detoxification, exclusion or resistance, and mechanisms of internal detoxification or tolerance (Ryan and Delhaize 2010). Resistance mechanisms avoid Al from entering the symplast and reaching sensitive intracellular sites. Tolerance mechanisms can safely accommodate Al, once it is taken up by the plant, by chelating it in the cytoplasm with Al-chelating ligands and then compartmentalizing it in organelles.

1.4.1. Mechanisms of aluminum resistance

The mechanisms of resistance comprise the immobilization of Al at the cell wall, increased selective permeability of the plasma membrane, mucilage secretion,

rhizosphere pH barrier formation, active efflux of Al ions or the secretion of Al-chelating compounds such as organic acids (OAs), phenolic compounds and phosphate into the rhizosphere (Taylor 1991; Kochian 1995).

Among all of the proposed mechanisms, the secretion of Al-chelating ligands, particularly low molecular weight OAs, from the root apex is the most well-characterized and effective way to reduce the impact of Al on cellular components (Ma et al. 2001; Poschenrieder et al. 2008). Tolerant genotypes of several plant species have been shown to secrete larger amounts of OAs anions than sensitive ones (Zhao et al. 2003; Li et al. 2009), supporting the secretion of these anions as a mechanism of resistance to Al (Ryan and Delhaize 2010). The OAs anions chelate toxic Al in the rhizosphere, forming stable nonphytotoxic complexes with the metal (Delhaize et al. 1993; Ma et al. 2001). Continual secretion of OAs is confined to the root apex (Delhaize et al. 1993; Zheng et al. 1998; Li et al. 2009), providing therefore sufficient protection to the most Al sensitive root zone (Taylor 1991). Hue et al. (1986) showed that the most effective OAs to detoxify Al were citric and oxalic acid, followed by tartaric and malic acid. However, the kinds of OAs secreted by Al-exposed roots and the secretion pattern (rates and amounts) vary among species, cultivars and genotypes (Zhao et al. 2003; Tahara et al. 2008; Li et al. 2009). The OAs commonly secreted from roots are malic in wheat (Delhaize et al. 1993) and cowpea (Yu et al. 2012), citric in maize (Chaffai and Marzouk 2009), barley (Zhao et al. 2003) and stylo (Li et al. 2009), oxalic in buckwheat (Zheng et al. 1998), and citric and malic in rye (Li et al. 2000).

Two patterns of Al-induced secretion of OAs have been suggested in terms of time required for the secretion (Ma et al. 2001). In Pattern I, there was no marked delay between the Al exposure and the OAs release, such as in wheat (Delhaize et al. 1993) and buckwheat (Zheng et al. 1998), where the OAs secretion is rapidly activated

(15 - 30 min) after Al exposure and the rate of release remains constant with time. By contrast, in Pattern II, OA secretion is delayed for several hours after Al exposure such as in rye (Li et al. 2000), stylo (Li et al. 2009) and cowpea (Yu et al. 2012). In Pattern I, Al may induce an anion channel located in the plasma membrane to initiate OA secretion, while activation of genes involved in OAs metabolism or in the transport of these anions seems to be required to the secretion begin in Pattern II (Ma et al. 2001).

An important part of the Al resistance mechanism, is the activation of a specific organic permeable plasma membrane channel (Kochian et al. 2005). The OAs in the cytosol exist primarily as anions and due to the large negative-inside transmembrane electrical potential in plant cells, there is a very strong gradient directed out of the cell for anions (Kochian et al. 2005). Thus, an anion channel that opens upon exposure to Al would be sufficient to mediate this transport. The genes encoding anion channel proteins that are specifically activated by Al have been identified and characterized in several plant species. The first gene controlling Al resistance in plants was isolated in 2004 from wheat (Sasaki et al. 2004). The *TaALMT1* (*Triticum aestivum* aluminum-activated malate transporter) gene isolated from wheat encodes a member of the ALMT family that consists of membrane-bound proteins (Sasaki et al. 2004). More recently, Pereira et al. (2010) transformed an Al-sensitive wheat cultivar with *TaALMT1* gene and found that the resulting T_2 lines showed increased Al resistance. Several other members of the ALMT family also contribute to the Al resistance in other species including *Arabidopsis* (Hoekenga et al. 2006) and *Hordeum vulgare* (Furukawa et al. 2007). Some genes of the multidrug and toxic compound extrusion (MATE) family also seems to contribute to Al resistance, being responsible for citrate efflux in several Al resistant plant species (e.g. Furukawa et al. 2007; Liu et al. 2009; Ryan et al. 2009). In some species, genes from both families contribute to Al resistance (Liu et al. 2009; Ryan et al. 2009).

1.4.2. Mechanisms of aluminum tolerance

The mechanisms of tolerance may involve the chelation of Al in the cytosol, sequestration within the vacuole, Al-binding proteins, increased enzyme activity or Al-tolerant enzyme isoforms (Kochian 1995; Ma et al. 1997). These tolerance mechanisms allow the species endemic to acidic Al-rich soils to grow properly without showing symptoms of Al toxicity despite the high accumulation of this metal in their tissues (Kochian 1995). Although the free Al^{3+} concentration at the near neutral pH of the symplasm is less than 10^{-10} M owing to the pH-dependent formation of the insoluble $Al(OH)_3$, very low Al^{3+} concentrations can be phytotoxic due to the strong affinity for oxygen ligands such as inorganic phosphate, ATP, DNA, proteins, carboxylic acids and phospholipids (Martin 1988).

Internal tolerance mechanisms can detoxify Al^{3+} ions in the symplasm by chelating it with OAs anions, phenolic compounds or silicon (Barceló and Poschenrieder 2002). However, OAs anions seem to be the most common and stable Al ligands in many Al hyperaccumulator plant species such as *Fagopyrum esculentum*, *Hydrangea macrophylla*, *M. malabathricum* and *C. sinensis*. In *F. esculentum*, the Al captured by the root cells is internally chelated in both roots and leaves by oxalate, forming a nonphytotoxic 1:3 Al-oxalate complex (Ma et al. 1998). This complex is converted to Al-citrate (1:1) in the xylem sap and then transported towards the leaf cells, where it is converted back to Al-oxalate and stored in the vacuole (Shen et al. 2002). In *M. malabathricum* (Watanabe et al. 1998) and *C. sinensis* (Morita et al. 2004) Al accumulates in the form of Al-oxalate (1:1, 1:2 and 1:3) and Al-citrate (1:1) complexes, respectively. High citrate concentrations have been reported in *H. macrophylla* leaves whose sepals turn from pink to blue due to Al accumulation when the soil is acidified (Ma et al. 1997).

Some Al accumulators such as *F. esculentum* (Zheng et al. 1998), *C. sinensis* (Morita et al. 2004) and *M. malabathricum* (Watanabe et al. 2001) not only complex Al in the symplast but also release OA anions from the root tips to chelate Al. Considering that the Al accumulation occurs simultaneously with mechanisms of Al resistance that avoid Al from entering the symplast can be questionable for common opinion. However, Klug and Horst (2010a,b) investigated this apparent contradiction in *F. esculentum* and observed that Al exclusion and Al accumulation mechanisms are spatially co-localized and that oxalate secretion from root tips confers protection from Al toxicity and allow Al accumulation in the symplast. Although the interrelationship between Al exclusion and Al accumulation is not well understood, these authors hypothesized that the binding of Al^{3+} in the apoplast triggers the release of oxalate via a still unidentified anion permease. The oxalate secreted complexes Al to form the 1:1 complex $Al(Ox)^{+}$ which reduces Al binding to Al-sensitive apoplastic binding sites, but this complex is not stable enough to fully protect the root tip from Al injury and is readily transported and accumulated in the symplast via an unknown cation transporter.

1.4.3. Organic acids metabolism

The enhanced activity of the enzymes involved in OAs synthesis, such as citrate synthase (CS), phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), fumarase and NADP-isocitrate dehydrogenase (NADP-ICDH) has been suggested to be an important component of the Al resistance (Tesfaye et al. 2001). For instance, the Al-induced citrate secretion from *Citrus junos* (Deng et al. 2009) and *Secale cereale* (Li et al. 2000) roots was accompanied by increased citrate content and CS activity. Additionally, the overexpression of genes encoding enzymes involved in OA metabolism in transgenic plants has been shown to result in enhanced Al resistance.

Transgenic *Arabidopsis thaliana* and *Nicotiana benthamiana* overexpressing the mitochondrial *CS* gene showed increased citrate secretion (Koyama et al. 2000; Deng et al. 2009). Moreover, the overexpression of MDH in alfafa (Tesfaye et al. 2001) and tobacco (Wang et al. 2010), and PEPC in transgenic rice (Begum et al. 2009) resulted in enhanced OAs synthesis, secretion and Al resistance. Therefore, transgenic plants with an increased capacity to produce and secrete OAs that chelate and detoxify Al in the rhizosphere are a promising strategy to produce Al-resistant plants. However, the role of the OAs metabolism in Al resistance is still a matter of discussion, since some studies demonstrated no clear relationship between the root content and secretion of OA anions. In an Al-resistant soybean, which secreted citrate in response to Al, internal citrate content was not affected by Al while CS activity was increased (Yang et al. 2001). Hayes and Ma (2003) found that internal citrate is increased by Al in an Al-resistant triticale, but the activity of CS is not affected.

1.5. The genus *Plantago*

Plantago is the principal genus of the Plantaginaceae family and comprises about 275 species distributed worldwide. These species are small perennial or annual herbs, with alternate leaves forming a basal rosette and inflorescence as a bracteate spike (Castroviejo 1986). Most species are widely distributed and grow as weeds, while others are restricted to a specific area such as the Portuguese endemics *P. almogravensis* and *P. algarbiensis* (Hoggard et al. 2003; ICN 2007). Some *Plantago* spp. have medicinal properties and have long been used to treat diversified diseases related to the skin, digestive tract, reproductive system, blood circulation disorders and even cancer (Chiang et al. 2003). The use of some species as natural bioindicators of environmental pollution, particularly to monitor the amount of heavy metals in soil, is also described

(e.g. Kurteva 2009; Malizia et al. 2012). For instance, Remon et al. (2007) observed multi-tolerance to heavy metals in a population of *P. arenaria* growing in a metallurgical landfill.

1.5.1. The species *Plantago almogravensis* and *Plantago algarbiensis*

Plantago almogravensis Franco, or the common "Diabelha do Almograve", is a small woody hemicryptophyte forming a cushion less than 15 cm wide and 7 cm high, characterized by an inconspicuous woody stem, with short branches that end in rosettes and are covered by decomposing leaf sheaths (Figure 1.1 A, B) (Franco 1984). When old plants are densely branched and usually found buried in the soil with the terminal leaf rosettes standing out. The leaves look like small stars, 15 - 50 mm long by 0.5 - 0.7 mm wide (Figure 1.1 C), and the inflorescences are spikes with 20 - 40 mm long by 4 - 7 mm wide (Figure 1.1 D). The main root is woody and long and several fine absorbing roots are formed at the surface.

It is only known one small population of this species with 3,000 to 4,000 individuals restricted to an area of 7 ha in the vicinity of Vila Nova de Milfontes in the southwest coast of Portugal (Figure 1.1 E, Figure 1.2) (ICN 2007). It is a very rare species with a very restricted distribution and thus the risk of extinction due to catastrophes of human or natural origin is high. Additionally, several threats lead to a continuing decline in the habitat extent and quality, such as pollution with agricultural chemicals, intrusion by humans and their vehicles, trampling, and fires (ICN 2007).

P. almogravensis occupies undisturbed sandy podzolic soils preferentially in geochemical islands of superficial hardpans enriched in Al and iron (Fe), lacking other kinds of vegetation. The surrounding areas of these geochemical islands are colonized by coastal brushes mostly constituted by *Ericaceae* and *Cistaceae* species. According to

Serrano et al. (2011) *P. almogravensis* is an obligate metallophyte because it is predominantly found in the geochemical islands rich in Al and outside these areas it is not capable of existing with the surrounding vegetation. This species tolerates high Al soil concentrations by displaying an Al-hyperaccumulative character with more than 3,000 μg g^{-1} DW of Al in the aboveground parts and was reported as the only representative of the Plantaginaceae family with this trait (Branquinho et al. 2007; Serrano et al. 2011). It was also described as an unusual Fe accumulator since it accumulates high Fe amounts (Serrano et al. 2011). Contrasting with most of the Al hyperaccumulator plants that are found in tropical areas generally with high rainfall, *P. almogravensis* is one of the few Al hyperaccumulator plants to endure a Mediterranean climate subject to drought conditions (Serrano et al. 2011).

Plantago algarbiensis Samp., commonly known as "Diabelha do Algarve", is a rosulate hemicryptophyte that grows up to 7 - 30 cm long and has woody stems, undivided or rarely bifid (Figure 1.1 F, G) (Franco 1984). The leaves are linear, sharp and rigid with 30 - 90 mm long by 0.5 - 0.8 mm wide (Figure 1.1 H) and the inflorescences are spikes with 15 - 33 mm long by 3 - 5 mm wide (Figure 1.1 I). The species occurs on clay-rich soils that are temporarily flooded in winter and spring and it prefers areas that are located downstream from small springs or clearings containing acidophilic brushes dominated by *Stauracanthus boivinii* and *Calluna* spp. (Figure 1.1 J) (ICN 2007).

Figure 1.1. Different aspects of *Plantago almogravensis* (A, B, C, D and E) and *P. algarbiensis* (F, G, H, I and J) plants: mature plant, flowering plant, leaves detail, inflorescence detail and general aspect of the sampled population, respectively.

This species is endemic from the West-Central Algarve region, where three populations widespread through a 50 ha area (Figure 1.2). One of these populations is located within the Site of Community Importance of Barrocal and has a very restricted effective population that is estimated to be below 10,000 individuals and occupies about 0.1 ha. The risk of extinction due to catastrophes of human or natural origin, or caused by unpredictable biological and demographic phenomenoms, are major threats to its conservation. Mining of clay soils for the production of construction materials, urbanization, trampling and grazing by livestock are also considered important threats (ICN 2007).

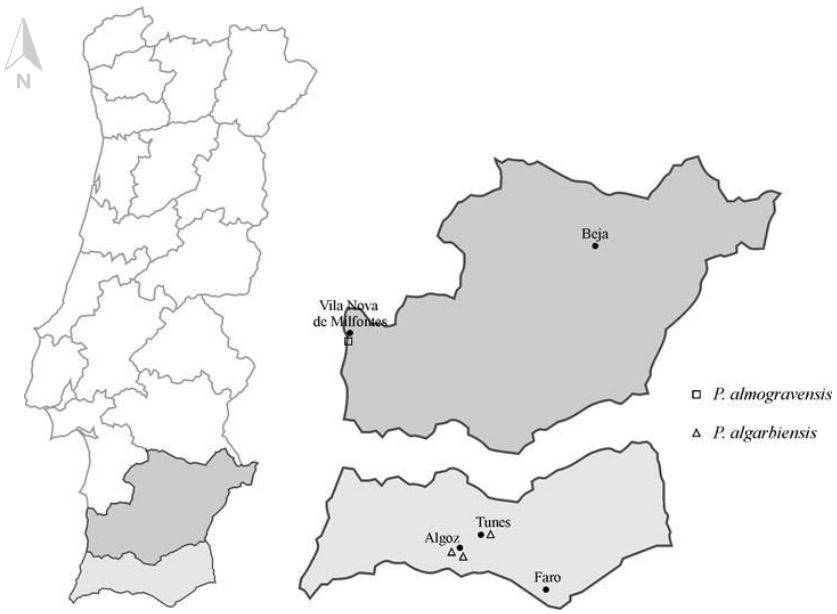

Figure 1.2. Distribution of *Plantago almogravensis* and *P. algarbiensis* in Portugal.

According to Rabinowitz (1981), the strategy for classifying species rarity is based on 3 attributes: geographic range, local abundance and habitat specificity. Both *P. algarbiensis* and *P. almogravensis* are considered geographically rare, since their distribution is restricted to a very small area (*P. almogravensis* ~7 ha and *P. algarbiensis* ~50 ha). They are also demographically rare as in each scattered

population a low number of individuals occur. Moreover, they are ecologically rare since their distribution is consistent with a specific habitat at a regional comparison scale level. *P. almogravensis* is assessed as Critically Endangered and *P. algarbiensis* as Endangered (IUCN 2012), and both species are protected under the European Habitats Directive 92/43/CEE and by Portuguese law (reference 140/99 of April 24).

Although *P. almogravensis* and *P. algarbiensis* are considered two independent species in "Nova Flora de Portugal", recently doubts have emerged about the taxonomic status of these species after their classification as a single species in "Flora Iberica". However, according to "Flora Iberica" taxonomists the plants from Vila Nova de Milfontes differ from the Algarve by having shorter bracts, as well as being cushioned, which gives them a very characteristic appearance and therefore they may deserve recognition at infraspecific level. Since these classifications are based only on morphological characters, more detailed studies are needed in order to clarify the taxonomy of *P. almogravensis* and *P. algarbiensis*.

1.6. Micropropagation

Micropropagation is the propagation of plants in a defined growth medium under aseptic and controlled environment conditions. It is based on the concept of totipotency, which is the ability of a single cell to divide and differentiate to originate a whole plant. Single cells, protoplasts, tissues, embryos and organs can be used as explant to generate a new plant. There are two principal methods of *in vitro* propagation: propagation from axillary or terminal buds and propagation via the formation of adventitious shoots or somatic embryos (George and Debergh 2008). The first method requires pre-existing meristems in the explants, whereas the second involves adventitious shoots or embryos formation either directly, from explant tissues without previously-formed callus (direct

organogenesis or direct embryogenesis), or indirectly, when shoots or embryos regenerate on previously-formed callus or in cell culture (indirect organogenesis or indirect embryogenesis).

Micropropagation offers many advantages over traditional plant propagation techniques, namely: i) the rapid production of a large number of plants starting from a single explant; ii) the production may be carried out throughout the year and independently of seasonal constraints; iii) the possibility to propagate species impossible to clone *in vivo*; iv) the production of disease-free plants; and v) the virus eradication and maintenance of plants in a virus-free-state (Hartmann et al. 1997; George and Debergh 2008). The mother plant is not destroyed in the process, a factor of considerable importance in the case of threatened plants. However, micropropagation is not always the most adequate method to propagate plants, especially for recalcitrant plants that cannot regenerate *in vitro* even under appropriate conditions. Moreover, this method presents other limitations such as the requirement of specialized and expensive production facilities, high mortality rates during the acclimatization to *ex vitro* conditions, production of off-type individuals and high losses due to contaminations (Hartmann et al. 1997; George and Debergh 2008).

A micropropagation protocol can be divided into five main stages: collection and preparative stage; culture initiation; shoots multiplication; shoots elongation and rooting and acclimatization (Debergh and Maene 1981). Each stage is influenced by several factors, namely the composition of the culture medium and the environmental conditions.

The first stage involves the selection and preparation of the mother plant in order to obtain high quality explants for a successful establishment of aseptic cultures. To reduce contaminations in the next stage mother plants or its parts should be maintained under

more hygienic conditions before explants excision. Changing the physiological status of the mother plant through the manipulation of light, temperature, mineral nutrition and growth regulators can also make the explant more suitable or reliable as starting material (Debergh and Read 1991).

The following step in the micropropagation process is the successful establishment of an explant into aseptic conditions by avoiding contamination and then providing an *in vitro* environment that promotes stable shoot production (Hartmann et al. 1997). The developmental stage, physiological age and size of the initial explant as well as the season of explant collection are important in this stage and can determine the success of *in vitro* culture (Debergh and Read 1991). Exogenous and endogenous contaminations are important obstacles for *in vitro* culture of plants, particularly when dealing with threatened species since the source of material is often limited and located in the wild (Sarasan et al. 2006). Seeds can be used as explants to initiate *in vitro* cultures. Indeed, the seeds present several advantages compared with the vegetative material as they provide a wider genetic diversity, crucial for successful conservation of threatened species, and this material is usually free of most of the pests and diseases which may have affected their parents (Fay 1992; George and Debergh 2008). Stress situations such as the explant excision from the mother plant can induce the production of phenolic compounds, which lead to the blackening and growth inhibition of the explants and ultimately to their dead (Debergh and Read 1991; George and Debergh 2008). It is possible to overcome this problem through frequent transfer of explants to fresh medium, pre-treatment of the explant with sterile water, adding activated charcoal or polyvinylpyrrolidone to the medium, modifying the redox potential and reducing phenolase activity of the tissue (Debergh and Read 1991).

The purpose of the next stage is the multiplication of shoots to a number required for subsequent rooting. During this stage several environmental factors should be considered including temperature, light intensity, photoperiod and CO_2 concentration. Growth regulators are added to the medium to support a basic level of growth and to direct the developmental response of the explant (Debergh and Read 1991; Hartmann et al. 1997). Multiplication of shoots is achieved by subculturing them in medium containing a specific cytokinin concentration at regular intervals that may vary from two to eight weeks depending on the shoot development (Hartmann et al. 1997). The plant material may be subcultured several times to new medium however variant plants may arise from cultures maintained *in vitro* for long periods.

The shoots produced in the multiplication stage are usually small, and not capable of self-supporting growth in soil or compost (George and Debergh 2008). Therefore, the shoots are subsequently elongated, rooted and prepared for *ex vitro* transplantation (Hartmann et al. 1997). The auxins play an essential role in this stage and the choice of the auxin, the concentration supplied to the medium, and the period of exposition influence the success of the rooting process. Rooting may occur in the *in vitro* or *ex vitro* environment. For *in vitro* rooting, shoots are subcultured in a root-inducing medium with reduced or omitted cytokinin and increased auxin concentration, in a root-inducing medium for a few days and then transfer to an auxin-free medium or, alternatively, after basal immersion in an auxin-rich solution for a few minutes shoots are subcultured in auxin-free medium. In the e*x vitro* procedure the rooting occur directly in the acclimatization substrate after basal immersion in auxin-rich solution. This procedure provides an excellent transition from the culture environment to the open air since the root system produced *in vitro* may not be functional and saves labor requirements for handling plants (Debergh and Read 1991).

The last stage involves the acclimatization of *in vitro* produced plants to the external environment (Hartmann et al. 1997). Plants propagated *in vitro* are exposed to controlled growth conditions including high concentrations of organic and inorganic nutrients, plant growth regulators, a defined carbon source, high humidity, low light and poor gaseous exchange, which induce structural and physiological changes (e.g. non-functional roots, less leaf epicuticular wax or wax with an altered composition, atypical leaf stomata and poor photosynthetic efficiency) that render the plants unfit to survive when transferred directly to the field (Hazarika 2006). Thus, it is necessary to acclimatize plantlets to the natural environment in order to ensure high survival rates, subsequent growth and allow the micropropagated plants to become established in their new environment (Hazarika 2006). For many species the plant acclimatization to *ex vitro* conditions can be successfully achieved by gradually reducing the relative humidity and increasing the light intensity (Hartmann et al. 1997).

Micropropagation is particularly important to propagate endangered plant species. Micropropagated plants can be used in re-establishment programs, to establish botanic garden living collections and for research and other activities that could compromise or deplete wild populations (Pence 2011). Indeed, *in vitro* propagation techniques have proved to be a powerful tool for germplasm conservation and mass-multiplication of many threatened plant species (e.g. Coelho et al. 2012; Coste et al. 2012). Additionally, *in vitro* culture has shown an enormous potential to study plant responses to metal ions, offering several advantages such as easy access to the root system, rapid means of producing thousands of plants from a single explant, controlled conditions (strict control over nutrient availability and pH), possibility to eliminate the influence of environmental factors, non-destructive measurements and large number of experiments in a small area and over short periods (Santos-Díaz et al. 2007; Smykalova et al. 2010).

In fact, *in vitro* cultures have been previously used for the selection of Al-tolerant genotypes, the study of Al tolerance mechanisms and development of Al-tolerant variants (Wen et al. 2009; Tabaldi et al. 2011). However, these studies require the use of culture medium with low ionic strength to more closely match Al availability and toxicity in acid soils and to minimize the problems related with Al speciation, precipitation and polymerization. Thus, several modifications to the standard culture medium should be considered such as the decrease of pH to 4.0 and reduce phosphate and calcium concentrations (Conner and Meredith 1985).

1.7. Objectives

P. almogravensis and *P. algarbiensis* are two Portuguese endemic species that are in risk of extinction. Probably due to their rarity, no studies regarding *P. algarbiensis* are known and only two recent works reporting *P. almogravensis* as an Al hyperaccumulator are known (Branquinho et al. 2007; Serrano et al. 2011). Due to their morphological similarities *P. almogravensis* and *P. algarbiensis* are currently classified as a single species in "Flora Iberica" contradicting the classification of "Nova Flora de Portugal". Since both *Plantago* species colonize acid soils and are morphologically similar, it is interesting to compare their response to Al. Therefore, the main objectives of this study are to increase the knowledge on *P. algarbiensis* and *P. almogravensis* species regarding Al bioaccumulation and the mechanisms of toxicity and tolerance. The specific objectives of this research work are:

i) to develop micropropagation protocols using seeds as explants in order to contribute to their conservation and to produce enough number of shoots and plantlets for the Al toxicity and tolerance studies;

ii) to investigate the ability of *in vitro* cultures to tolerate and grow in low pH conditions;

iii) to evaluate the Al bioaccumulated in shoots and plantlets;

iv) to study the impact of low pH and Al stress on the growth, metabolism and physiological traits;

v) to elucidate Al detoxification mechanisms.

It is also expected that this work contributes for the clarification of the controversial taxonomy of *P. algarbiensis* and *P. almogravensis* and to the implementation of conservation strategies.

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CHAPTER 2

Micropropagation and conservation of endangered species

Plantago algarbiensis and *P. almogravensis*

Gonçalves S, Martins N, Romano A (2009) Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. Biologia Plantarum 53:774-778

2.1. Abstract

Plantago algarbiensis and *P. almogravensis* are endemic species from the West-Central Algarve region (South of Portugal) and Portuguese southwest coast, respectively, which are in risk of global extinction. The aim of this work was to establish an efficient protocol to *in vitro* propagate these species using shoots obtained from *in vitro* germinated seeds. The best results in terms of multiplication response were afforded in Murashige and Skoog medium (MS) supplemented with 6-benzyladenine (8.5 and 9.2 shoots per explant in *P. algarbiensis* and *P. almogravensis*, respectively). Shoots of both species showed a great rooting capacity (100 and 80% for *P. algarbiensis* and *P. almogravensis*, respectively) that was not significantly influenced by the concentration of MS macronutrients or auxins. Plants were acclimatized to *ex vitro* conditions, exhibited normal development (survival rate of 95 and 80% in *P. algarbiensis* and *P. almogravensis*, respectively), and were successfully reintroduced in their natural habitat.

2.2. Introduction

Plantago algarbiensis Samp. and *Plantago almogravensis* Franco are endemic species from the West-Central Algarve region (South of Portugal) and Portuguese southwest coast, respectively. Both species are in risk of global extinction and were included in the Red List of Threatened Species (Walter and Gillet 1998). These species are legally protected under the European Habitats Directive 92/43/CEE and by the Portuguese law. The distribution of these species is restricted to a very small area and the number of individuals in each scattered population is very low.

Al-tolerant wild plants could be interesting sources of genes conferring tolerance (Ezaki et al. 2008). Recently, *P. almogravensis* was described as an Al hyperaccumulator (Branquinho et al. 2007). Tissue culture techniques have previously been found useful in the selection of metal tolerant plants (Rout et al. 1999; Taddei et al. 2007; Gatti et al. 2008; Xu et al. 2008) and for their propagation in large-scale (Bidwell et al. 2001; Bhatia et al. 2002; Gatti et al. 2008; Xu et al. 2008). Moreover, micropropagation is a suitable tool for the propagation of threatened species providing complementary conservation options (Filho et al. 2005; Kaliamoorthy et al. 2008; Dutra et al. 2008).

Micropropagation of other *Plantago* species, especially those with medicinal properties, was described in the literature (Mederos et al. 1997; Makowczyńska and Andrzejewska-Golec 2003; 2006, Budzianowska et al. 2004; Khawar et al. 2005; Makowczyńska et al. 2008), however, to the best of our knowledge, the *in vitro* propagation of *P. almogravensis* and *P. algarbiensis* has not been reported. Thus, the aim of this study was to establish an effective micropropagation protocol for these two species in order to produce large number of plants to reintroduce them in their natural habitat. Moreover, *in vitro* produced plants could be used in future to investigate in detail the Al accumulation capacity of these species.

2.3. Materials and methods

2.3.1. Establishment of *in vitro* seedlings and culture conditions

Seeds of *P. algarbiensis* and *P. almogravensis* were collected from wild populations growing in Algoz and Odemira (Portugal), respectively. Collection was made from several plants to ensure the capture of a broad range of genetic variation. Seeds were surface sterilized with 15% (v/v) commercial bleach (5% sodium hypochlorite) and a few drops of Tween-20 for 15 min and then washed three times in sterile water. Thereafter, seeds were germinated in test tubes containing 10 ml of ¼MS medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose and solidified with 1% (w/v) agar. pH was adjusted to 5.8 before autoclaving at 121 ºC and 1.1 kg cm^{-2} for 20 min. Cultures were incubated under a 16 h photoperiod at a photon flux density of 55 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps and a temperature of 25 ± 2 ºC.

2.3.2. Shoot multiplication

After 2 months of germination, roots were discarded, and shoots were used in several multiplication assays. The effect of three cytokinins (6-benzyladenine - BA, kinetin - Kin and zeatin - Zea) at 0.2 or 0.5 mg l^{-1} was evaluated. MS medium without growth regulators was used as control. Shoots were cultured in 250 ml Erlenmeyer flasks with 50 ml of culture medium and capped with aluminum foil. After 6 weeks, shoot multiplication was assessed. In each assay 4 repetitions with 10 shoots each were tested.

2.3.3. Rooting

For root induction, individual shoots with identical size, harvested at the end of the

multiplication stage in MS medium with 0.2 mg l^{-1} BA, were used. During this phase the effect of MS and ½MS medium supplemented with 0.2 or 0.5 mg l^{-1} indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was evaluated. Shoots were grown in 500 ml Erlenmeyer flasks, containing 80 ml of medium, capped with aluminum foil and rooting was evaluated after 6 weeks. To test each medium 3 repetitions with 10 shoots each were used.

2.3.4. Acclimatization

At the end of rooting period, plantlets of both species were removed from the culture flasks and the roots were cleared of agar to prevent pathogenic contamination. Plantlets were transplanted to 350 ml plastic pots containing a mixture of peat and vermiculite (3:1, v/v). The acclimatization took place in a plant growth chamber at 25 ± 2 ºC and a 16 h photoperiod (100 µmol m^{-2} s^{-1}, Grow-Lux F18W/GRO lamps). The relative humidity, initially at 98%, was decreased to 70% during the acclimatization period. After 4 months of acclimatization micropropagated plants were planted in natural conditions.

2.3.5. Statistical analysis

The data were subjected to analysis of variance (ANOVA) with the general linear model procedure (SPSS statistical package for Windows, release 15.0; SPSS Inc., Chicago, IL, USA) to assess treatment differences and interactions. Significant differences between means were determined using Duncan's New Multiple Range Test. Before analysis of percentage data, arcsin square root transformation was used.

2.4. Results and discussion

After 2 months of germination 80 and 58% seedlings were obtained for *P. algarbiensis* and *P. almogravensis*, respectively. Embryos grew into normal seedlings without morphological abnormalities and the entire shoots were used in the multiplication assays. Cultures of both species had a great multiplication capacity in all tested media even without cytokinins (Table 2.1; Figure 2.1 A, B). Regardless the cytokinin type and concentration, the multiplication percentage ($P < 0.001$) and the mean number of shoots ($P < 0.01$) were significantly higher in *P. almogravensis*.

In *P. algarbiensis* both multiplication percentage and mean number of shoots were significantly higher in media with BA, regardless the concentration (Table 2.1). As compared with the control, shoots of *P. almogravensis* also showed a multiplication percentage significantly higher in media with BA, however, no significant differences were observed in the mean number of shoots (Table 2.1).

Regardless the multiplication medium, shoots of *P. algarbiensis* had longer leaves, while *P. almogravensis* had higher number of leaves. In *P. algarbiensis* no significant differences were observed between all the media tested and the control, regarding the mean number of leaves per shoot. Moreover, in this species only in medium containing 0.5 mg l^{-1} Zea the length of the longest leaf was significantly higher than the control. The mean number of leaves of *P. almogravensis* shoots cultured in medium with 0.2 mg l^{-1} Zea was significantly higher than the value obtained in the control, although no differences were observed between these results and those observed in media with BA. In relation to the leaf length, in this species differences were not observed between the control and the media containing 0.2 mg l^{-1} Kin, Zea and BA.

Table 2.1. Effect of cytokinin type and concentration on multiplication rate, number of shoots and leaves and longest leaf length of *Plantago algarbiensis* and *P. almogravensis* shoots. Control - medium without growth regulators.

Plant species	Cytokinin	(mg l^{-1})	Multiplication (%)	Shoot number	Leaf number	Length (cm)
P. algarbiensis	Control	-	55.00 ± 2.89 b	4.45 ± 0.69 b	27.91 ± 2.70 abc	5.33 ± 0.42 bc
	Kin	0.2	55.00 ± 6.45 b	4.00 ± 0.53 b	27.33 ± 3.46 abc	5.40 ± 0.53 abc
		0.5	57.50 ± 4.79 b	3.74 ± 0.44 b	26.73 ± 3.52 abc	4.62 ± 0.35 c
	Zea	0.2	65.00 ± 2.89 b	4.04 ± 0.55 b	30.25 ± 3.75 ab	6.10 ± 0.43 ab
		0.5	67.50 ± 2.50 b	4.67 ± 0.60 b	33.57 ± 4.46 a	6.57 ± 0.38 a
	BA	0.2	80.00 ± 4.08 a	8.50 ± 1.09 a	19.00 ± 1.24 c	4.89 ± 0.38 bc
		0.5	82.50 ± 4.79 a	7.21 ± 0.96 a	22.79 ± 1.48 bc	5.25 ± 0.34 bc
P. almogravensis	Control	-	65.00 ± 6.45 b	6.38 ± 1.28 a	36.58 ± 2.96 bc	4.13 ± 0.30 a
	Kin	0.2	77.50 ± 4.79 ab	5.74 ± 0.63 a	31.10 ± 1.86 c	3.81 ± 0.18 ab
		0.5	72.50 ± 8.54 ab	5.17 ± 0.66 a	35.86 ± 3.49 bc	3.07 ± 0.23 c
	Zea	0.2	87.50 ± 4.79 ab	7.31 ± 1.10 a	45.86 ± 2.12 a	3.87 ± 0.21 ab
		0.5	67.50 ± 7.50 b	7.00 ± 0.76 a	35.85 ± 2.83 bc	3.36 ± 0.22 bc
	BA	0.2	90.00 ± 7.07 a	9.22 ± 1.15 a	44.22 ± 2.54 ab	3.44 ± 0.19 abc
		0.5	90.00 ± 5.77 a	7.11 ± 0.88 a	38.08 ± 3.39 abc	3.18 ± 0.28 bc
Plant species (A)			***	**	***	***
Cytokinin type (B)			***	***	**	***
Cytokinin concentration (C)			ns	ns	ns	ns
A × B × C			ns	ns	ns	ns

Values represent means ± SE of 4 replications with 10 shoots. *, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively. Values followed by the same letter are not significantly different at $P \geq 0.05$ according to Duncan's Multiple Range Test.

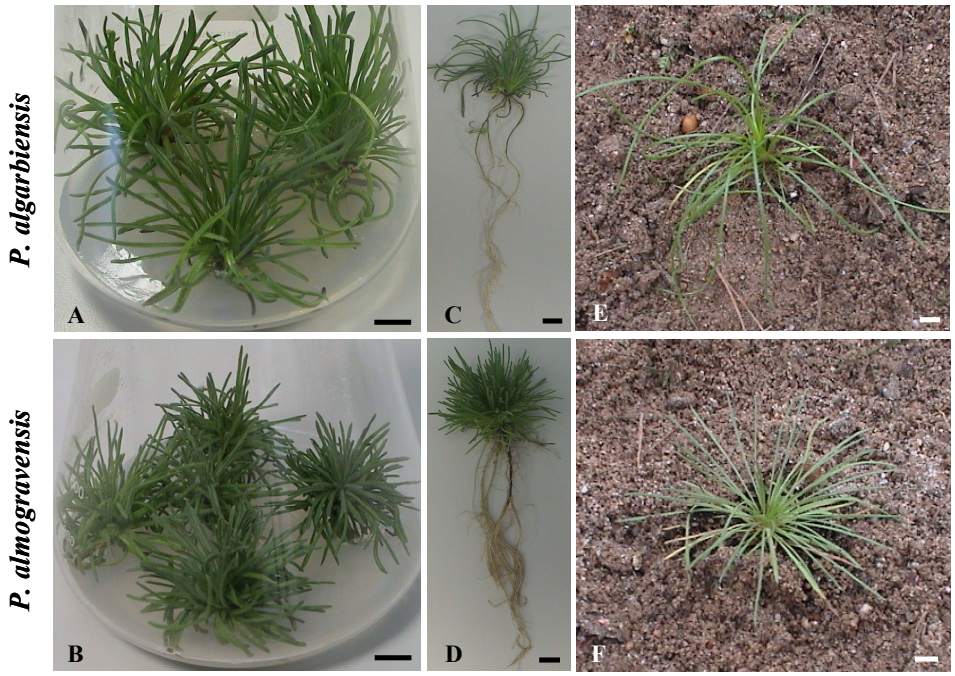

Figure 2.1. Micropropagation of *Plantago algarbiensis* and *P. almogravensis*. Shoots at the end of the multiplication phase (A, B) (bar = 1 cm); rooted shoots after 6 weeks in ½MS medium (C, D) (bar = 1 cm); plants reintroduced in the nature (E, F) (bar = 2 cm).

Spontaneous rooting was observed during the multiplication phase in both species. In *P. algarbiensis* rooting percentage was not influenced by the presence of cytokinins, while in *P. almogravensis* rooting was significantly higher in medium with 0.5 mg l^{-1} Kin or 0.2 mg l^{-1} BA. The good rooting results observed during multiplication are probably due to the high contents of endogenous plant growth regulators (Centeno et al. 1996). The produced plantlets could be directly acclimatized without passing through a rooting phase. Likewise, shoot production and rooting in one-phase was also observed for *Thapsia garganica* (Makunga et al. 2003), *Pinguicula lusitanica* (Gonçalves et al. 2008) and *Crithmum maritimum* (Grigoriadou and Maloupa 2008).

In order to improve the rooting frequencies fully developed shoots, with similar size, were selected and used in a range of rooting assays. As expected rooting occurred in the absence of auxins (control) and, surprisingly, auxins did not improve rooting significantly (Table 2.2). These results are not in agreement with those observed by

Mederos et al. (1997) in *Plantago major* where no rooting was obtained in medium without auxins. However, similar results were reported by Grigoriadou and Maloupa (2008) in *Crithmum maritimum* where IBA at several concentrations did not improve rooting.

Table 2.2. Effect of MS macronutrients concentration (MS total and ½MS) and auxins (IAA and IBA) on *in vitro* rooting, root number and longest root length of *Plantago algarbiensis* and *P. almogravensis* micropropagated shoots. Controls - media without growth regulators.

Plant species	Medium	Auxin	(mg l^{-1})	Rooting (%)	Root number	Length (cm)
P. algarbiensis	MS	Control	-	96.67 ± 3.33 a	4.17 ± 0.36 c	23.85 ± 1.00 a
		IAA	0.2	93.33 ± 3.33 a	4.68 ± 0.53 c	13.11 ± 0.90 b
			0.5	96.67 ± 3.33 a	7.45 ± 0.74 b	12.29 ± 1.01 b
		IBA	0.2	96.67 ± 3.33 a	10.55 ± 1.25 a	13.61 ± 0.80 b
			0.5	96.67 ± 3.33 a	8.66 ± 0.73 ab	12.97 ± 0.77 b
	½MS	Control	-	93.33 ± 6.67 a	6.46 ± 1.15 b	11.81 ± 0.82 a
		IAA	0.2	93.33 ± 3.33 a	8.39 ± 0.80 b	9.75 ± 0.86 a
			0.5	96.67 ± 3.33 a	7.93 ± 0.94 b	9.57 ± 0.63 a
		IBA	0.2	96.67 ± 3.33 a	8.45 ± 0.79 b	11.52 ± 0.75 a
			0.5	100.00 ± 0.00 a	14.77 ± 1.89 a	9.41 ± 0.62 a
P. almogravensis	MS	Control	-	46.67 ± 14.53 a	8.29 ± 1.86 a	5.95 ± 1.16 a
		IAA	0.2	43.33 ± 14.53 a	8.00 ± 1.74 a	7.77 ± 2.00 a
			0.5	66.67 ± 8.82 a	11.25 ± 1.86 a	6.26 ± 1.06 a
		IBA	0.2	63.33 ± 12.02 a	13.58 ± 1.92 a	6.07 ± 0.78 a
			0.5	63.33 ± 17.64 a	11.79 ± 2.07 a	4.77 ± 0.92 a
	½MS	Control	-	50.00 ± 10.00 a	8.27 ± 1.88 a	10.86 ± 1.59 a
		IAA	0.2	73.33 ± 8.82 a	12.91 ± 1.37 a	10.51 ± 1.08 a
			0.5	80.00 ± 5.77 a	14.79 ± 1.43 a	9.20 ± 1.07 a
		IBA	0.2	70.00 ± 0.00 a	16.57 ± 2.34 a	9.24 ± 1.00 a
			0.5	80.00 ± 11.55 a	12.58 ± 1.90 a	5.31 ± 0.88 b

Values represent means ± SE of 3 replications with 10 shoots. Means followed by the same letter are not significantly different at $P \geq 0.05$ according to Duncan's Multiple Range Test.

Contrarily to previous results in several species (Gonçalves and Romano 2005; Gonçalves et al. 2008; Zhang et al. 2008), in this study the concentration of MS macronutrients did not affect rooting frequency significantly (Table 2.2). As observed during shoot proliferation, *P. algarbiensis* showed higher rooting frequencies (93 - 100%) when compared with *P. almogravenis* (47 - 80%). The mean number of roots developed by *P. algarbiensis* shoots was significantly affected by MS strength and auxin type, with ½MS and IBA being the most effective. The highest number of roots was afforded in ½MS supplemented with 0.5 mg l^{-1} IBA (Table 2.2). On the contrary, the longest roots were obtained in MS medium without auxins (Table 2.2). In *P. almogravensis* the mean number of roots was not affected by any of the factors tested whereas root length was significantly higher in ½MS media, except when supplemented with 0.5 mg l^{-1} IBA. In both species, the number and length of the roots were higher in shoots rooted during the rooting phase than in shoots spontaneously rooted during the multiplication phase (data not shown). Additionally, greater rooting frequencies were attained during the rooting assays. Therefore, we can conclude that an independent rooting phase must be included in the micropropagation protocols of both *Plantago* species.

In vitro produced plantlets (Figure 2.1 C, D) were transplanted to substrate in pots and higher survival rates were achieved after 6 weeks of transplantation (95 and 80% for *P. algarbiensis* and *P. almogravensis*, respectively). The micropropagated plants produced in this work were successfully transferred into the field conditions exhibiting good performance (Figure 2.1 E, F). Plans are underway to reintroduce a high number of *in vitro* produced plants in selected locations in their natural habitat.

This work describes for the first time two effective protocols for the mass propagation of *P. algarbiensis* and *P. almogravensis* providing a valuable contribution to the

preservation of these species.

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CHAPTER 3

The influence of low pH on *in vitro* growth and biochemical parameters of *Plantago almogravensis* and *P. algarbiensis*

Martins N, Gonçalves S, Palma T, Romano A (2011) The influence of low pH on *in vitro* growth and biochemical parameters of *Plantago almogravensis* and *P. algarbiensis.* Plant Cell, Tissue and Organ Culture 107:113-121

3.1. Abstract

The effects of low medium pH (4.50, 5.00 and 5.75) on *in vitro* growth and on several biochemical parameters (lipid peroxidation, proline and carbohydrate content, antioxidant enzymes activities and total soluble protein) of *Plantago almogravensis* and *P. algarbiensis* micropropagated shoots were investigated. Overall, it was observed that medium pH did not affect *in vitro* proliferation and rooting. Interestingly, cultures of both species modify the initial pH value to the same final value. Results have shown that the lowest pH tested induced an increase in the level of lipid peroxidation in roots of both species and in shoots of *P. algarbiensis*, indicating plasma membrane damage. An accumulation of carbohydrates was observed in roots of *P. almogravensis* cultured in pH 4.50 and 5.00. It was observed a slight response of the enzymatic system to medium pH, particularly in *P. almogravensis*. Based on the results obtained we can conclude that *Plantago* species are apt to grow *in vitro* in medium with pH values much lower than the usually used in tissue culture, which is in agreement with the fact that both species colonize acid soils.

3.2. Introduction

Low pH (high H^+ activity) may directly inhibit plant growth and development (Kidd and Proctor 2001; Pavlovkin et al. 2009), probably by adverse effects at the root plasmalemma level. Additionally, low pH can affect plant growth indirectly by elevated levels of aluminum (Al) and manganese, as well as by limited mineral nutrient uptake and translocation. Among these complex factors, the combined Al^{3+} and H^+ stresses are the major causes of poor plant growth. However, there is growing evidence suggesting that these two stresses differ in their inhibition of plant growth (Bose et al. 2010) and that resistance to Al and to protons is controlled by separate mechanisms (Lazof and Holland 1999; Kidd and Proctor 2001). Hence, it is necessary to evaluate the effects of low pH separately from the combination of low pH and Al to better understand H^+ toxicity.

Acid soils are found throughout the world and the atmospheric inputs of natural nitric and sulphuric acids, anthropogenic pollutants, and certain fertilization practices are increasing the area affected by acidity (Marschner 1995). Species differ in their physiological tolerance to acidity and examples of tolerance to low pH in crops are numerous (Llugany et al. 1995; Osaki et al. 1997; Fageria et al. 2009). However, reduced information is known regarding H^+ tolerance in wild species. *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. are endemic species from the Portuguese southwest coast and the West-Central Algarve region (Portugal), respectively, that are in risk of global extinction. *P. almogravensis* colonizes a sandy eroded podzol-like soil, enriched in iron and Al (Buurman and Jongmans 2002; Branquinho et al. 2007), and *P. algarbiensis* occurs in clay-rich soils and prefers areas that are located downstream from small spring or clearings of low acidophilic brushes (ICN 2007). Branquinho et al. (2007) showed with field plants that *P. almogravensis* is

an Al hyperaccumulator species, however its tolerance to Al and H^+ stress remains unclear.

The micropropagation protocol for both *Plantago* species utilizing the medium pH usually used in *in vitro* culture (pH = 5.75) was recently published by our group (Gonçalves et al. 2009). Most factors affecting *in vitro* growth are similar to those limiting the growth *in vivo* (Hew and Yong 1997), thus the effect of low medium pH on *in vitro* growth of both *Plantago* species will be useful to understand their tolerance to low pH, without the interference of indirect factors. Cell and/or tissue culture have been extensively used to evaluate the abiotic stress tolerance of many species, since responses are relatively fast, the generation times are short, and the environment is controlled (Cui et al. 2010; Lokhande et al. 2010; Xu et al. 2011).

Besides affecting growth, environmental adverse conditions can induce physiological and biochemical effects on plants (Çiçek and Çakirlar 2008). Therefore, the aim of this work was to investigate the ability of *P. algarbiensis* and *P. almogravensis* micropropagated shoots to grow *in vitro* in low pH conditions, as well as to elucidate the impact of this condition on the degree of membrane damage through lipid peroxidation level, proline and carbohydrates content and antioxidant enzyme activities.

3.3. Materials and methods

3.3.1. Plant material

The cultures of *P. algarbiensis* and *P. almogravensis* were initiated *in vitro* as described earlier (Gonçalves et al. 2009), and were proliferated and maintained on MS medium (Murashige and Skoog 1962) with 0.2 mg l^{-1} 6-benzyladenine (BA) (pH 5.75), at an interval of 6 weeks, under a 16 h photoperiod (cool white fluorescent lamps at 69 μmol m^{-2} s^{-1}) and a temperature of 25 ± 2 ºC.

3.3.2. Influence of medium pH on shoot proliferation and rooting

To investigate the effect of medium pH on proliferation, *P. algarbiensis* and *P. almogravensis* shoots (about 5 and 3 cm of size, respectively) were inoculated in MS medium supplemented with 0.2 mg l^{-1} BA. Five shoots were cultured per 250 ml Erlenmeyer flask with 50 ml of culture medium, capped with aluminum foil.

For root induction, shoots of *P. algarbiensis* and *P. almogravensis* with identical size (about 7 and 6 cm of size, respectively) were inoculated in ½MS medium (half-strength macronutrients) supplemented with 0.5 mg l^{-1} indole-3-acetic acid (IAA). Five shoots were cultured per 500 ml Erlenmeyer flask with 80 ml of culture medium, capped with aluminum foil.

All the media were supplemented with 2% (w/v) sucrose and solidified with 0.5% (w/v) gelrite. The pH was adjusted to 4.50, 5.00 or 5.75 before autoclaving, at 121 ºC and 1.1 kg cm^{-2} for 20 min. The post-autoclave pH values were determined after medium cooling until 40 ºC.

The cultures were grown in the conditions described above. After 6 weeks, the shoot proliferation was assessed by the proliferation frequency, the number of shoots and the length of the longest shoot, and the rooting was evaluated in terms of rooting frequency, root number and the longest root length. The pH of the culture media was measured at the end of the proliferation or rooting phases after harvesting the cultures. Media were liquefied and cooled until 40 ºC before pH measurements. As control, the pH values in the culture media maintained during 6 weeks in the same incubation conditions but without cultures were also measured.

3.3.3. Effect of medium pH on biochemical parameters

The level of lipid peroxidation, proline and carbohydrates content, and antioxidant enzyme activities were analyzed in samples of shoots and roots randomly collected from different Erlenmeyers within each medium.

3.3.3.1. Determination of lipid peroxidation

The lipid peroxidation was estimated by determining the malondialdehyde (MDA) content according to the thiobarbituric acid-reactive-substances method (Hodges et al. 1999). Fresh tissue was ground in 0.1% (w/v) trichloroacetic acid (TCA) using mortar and pestle and then centrifuged at 10,000 *g* for 5 min. After centrifugation, the supernatant was mixed to either 20% (w/v) TCA (- TBA solution) or 0.5% (w/v) thiobarbituric acid (TBA) prepared in 20% (w/v) TCA (+ TBA solution). The mixture was heated at 95 ºC for 30 min and then quickly cooled on ice. After centrifugation at 3,000 *g* for 10 min, the concentration of MDA was calculated from the absorbance at 532, 600 and 440 nm using the extinction coefficient of 157 $mM^{-1}cm^{-1}$ and expressed as nmol MDA g^{-1} fresh weight (FW).

3.3.3.2. Proline and carbohydrate estimations

The shoots and roots were repeatedly extracted with 80% (v/v) ethanol, and heated at 80 ºC for 30 min in each extraction for free proline and carbohydrates determination.

The free proline accumulation was determined using the acid-ninhydrin reagent method (Troll and Lindsley 1955) modified by Magné and Larher (1992). The extract was reacted with 1% (w/v) ninhydrin reagent, in 60% (v/v) acetic acid, for 1 h at 100 ºC and the reaction was terminated in an ice bath. The mixture was extracted with toluene and

the chromophore optical density was measured at 520 nm. The content of proline was determined from a standard curve in the range of 0 - 750 µM.

The carbohydrates content was determined based on the anthrone method (Dreywood 1946; Yemm and Willis 1954). The extract was reacted with 75% (v/v) sulphuric acid and 0.01 M anthrone reagent for 15 min at 100 ºC. After cooled at room temperature the absorbance was read at 578 nm. Glucose solutions at different concentration (0 - 500 µM) were used as standards.

3.3.3.3. Enzyme assays and soluble protein

The activities of the superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX; EC 1.11.1.7) were evaluated in extracts from shoots and roots after 6 weeks of culture. Fresh tissue (100 mg) was ground in a prechilled mortar using a homogenization medium consisting of 50 mM sodium phosphate (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpolypyrrolidone and 2.5 mM dithiothreitol. For APX the homogenizing solution contained 5 mM ascorbate. The homogenate was centrifuged at 20,000 *g* for 10 min at 4 ºC. The supernatant was used for enzyme activities and protein content assays. The SOD activity was assayed using the method of Beauchamp and Fridovich (1971) by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 10 mM methionine, 0.075 mM NBT, 0.2 mM riboflavin and 20 µl enzyme extract. One unit of SOD was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm in the presence of riboflavin in the light during 6 min. The CAT activity was determined by the method described by Aebi (1983). The

reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 40 mM H_2O_2 and 20 µl enzyme extract. The CAT activity was measured by following the decrease in absorbance at 240 nm (ε = 39.4 mM^{-1} cm^{-1}) due H_2O_2 decomposition. One unit of CAT was defined as the amount of enzyme which breaks down 1 µmol H_2O_2 per min. The activity of APX was determined as described previously (Nakano and Asada 1981). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.5 mM H_2O_2 and 20 µl enzyme extract. The oxidation of ascorbic acid in the reaction mixture was measured using the rate of decrease in absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). One unit of APX was defined as the amount of enzyme which breaks down 1 µmol of ascorbate per min. The GPX activity was defined as outlined by Egley et al. (1983) through monitoring the increase in absorbance at 470 nm due to tetraguaiacol formation (ε = 26.6 mM^{-1} cm^{-1}). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 8 mM guaiacol, 8 mM H_2O_2 and 20 µl enzyme extract. One unit of GPX was defined as the amount of enzyme to produce 1 µmol tetraguaiacol per min. These assays were conducted in a total volume of 1 ml at 25 ± 2 ºC. The specific enzyme activity for all enzymes was expressed as unit mg^{-1} protein. Detection of total soluble protein was determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard.

3.3.4. Statistical analysis

To evaluate the influence of medium pH on *in vitro* proliferation and rooting 3 repetitions with 10 shoots each (two flasks) were performed. To investigate the effect of medium pH on the biochemical parameters plantlets (shoots and roots separately) were analysed in all the experiments and the values obtained were expressed as the mean ± standard error of five replicates. The results were subjected to one-way analysis

of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 15.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test.

3.4. Results and discussion

The pH of the culture medium is an important factor for the *in vitro* shoot and root formation and healthy culture growth. Some plants can tolerate a broader pH range, while in others pH tolerance is limited. Proliferation frequencies of both *Plantago* species were very high, near 100%, in all the medium pH tested (Figure 3.1 A), and no significant differences ($P \geq 0.05$) were observed in the mean number of shoots (values from 7.43 ± 0.58 to 8.90 ± 0.77 in *P. algarbiensis* and 6.30 ± 0.53 to 7.48 ± 0.51 in *P. almogravensis*) and shoot length (6.44 ± 0.31 to 7.54 ± 0.35 cm in *P. algarbiensis* and 5.74 ± 0.26 to 6.49 ± 0.29 cm in *P. almogravensis*) (Figure 3.1 C, E). Plantlets developed normally in all the medium pH tested, without visually noticed damages caused by low pH (Figure 3.2). Xu et al. (2008) in *Malus zumi* also observed that shoot growth was not significantly affected within a broad range (5.0 - 7.0) of initial medium pH. In contrast, Naik et al. (2010) observed that shoot regeneration of *Bacopa monnieri* was significantly affected by medium pH, obtaining the best results under pH 4.5.

Although proton toxicity usually causes severe inhibition of root growth in various plant species (Kidd and Proctor 2001; Koyama et al. 2001; Sawaki et al. 2009), in this work high rooting frequencies were attained for both species regardless the pH of the rooting medium ($P \geq 0.05$) (Figure 3.1 B). Also the root number, in both species (Figure 3.1 D) and root length, in *P. almogravensis* (Figure 3.1 F), were not affected by pH levels ($P \geq 0.05$). In concordance with our results, Geneve and Heuser (1982) reported that pH ranging from 3.0 to 7.0 did not affect rooting of *Vigna radiana*. Furthermore Bennet et

al. (2003) observed that the pH of the medium did not affect the number of roots produced by *Eucalyptus globulus* shoots. Curiously, in *P. algarbiensis* roots obtained at pH 4.50 were longer than at pH 5.75 ($P < 0.05$) (Figure 3.1 F). Nutrient bioavailability often decreases at low pH, thus the increase in root surface area could be considered a strategy for enhancing nutrient acquisition (Marschner 1995).

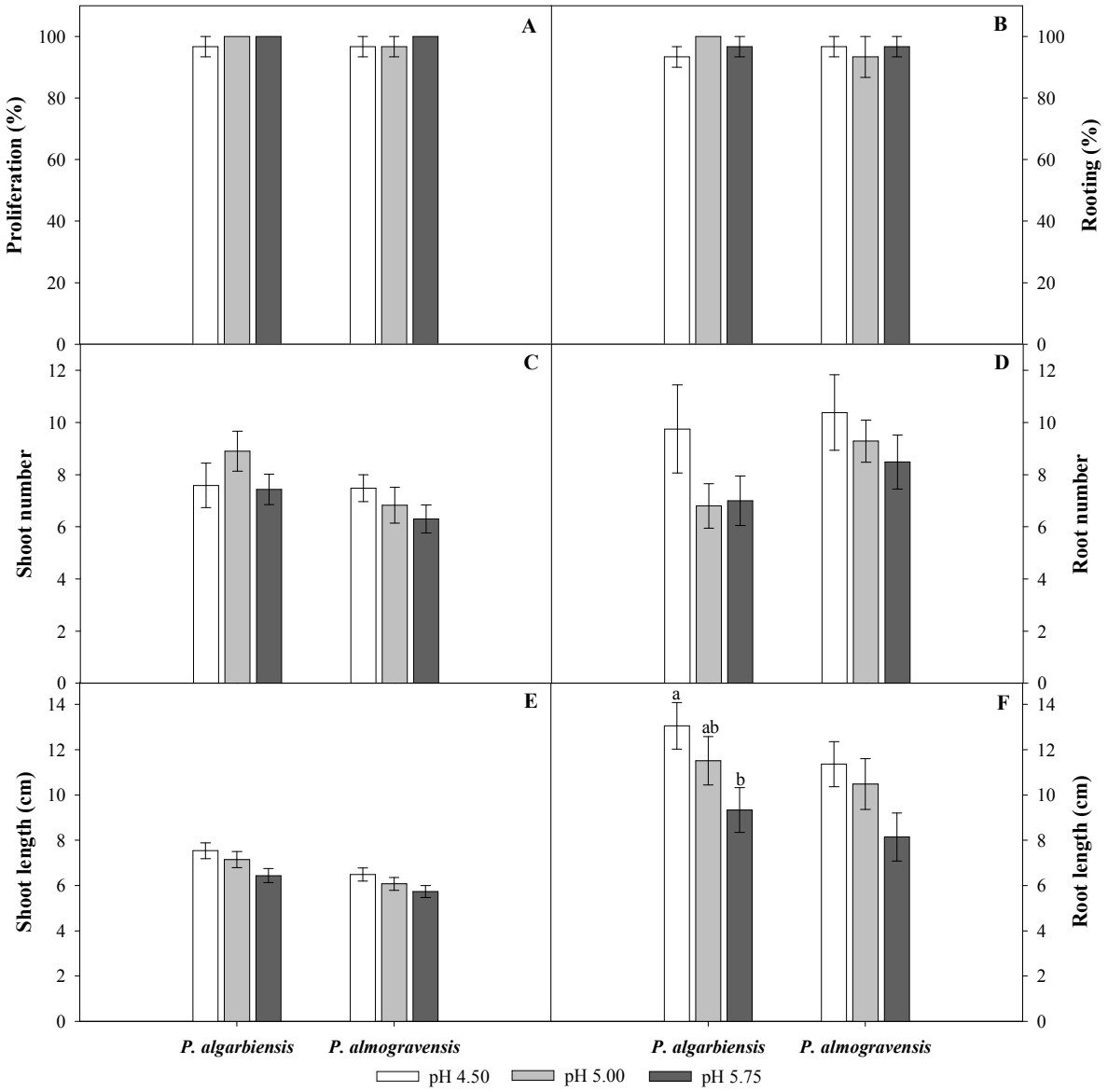

Figure 3.1. Effect of medium pH (4.50, 5.00 and 5.75) on proliferation and rooting of *Plantago algarbiensis* and *P. almogravensis* shoots. Values are expressed as the mean ± SE (n = 3). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed.

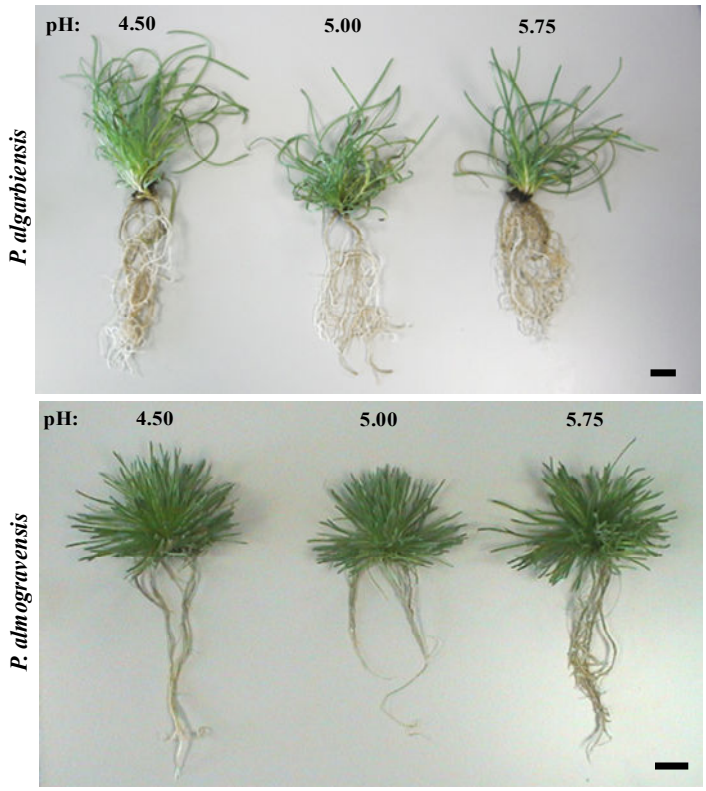

Figure 3.2. *Plantago algarbiensis* and *P. almogravensis* plantlets (bar = 2 cm) at the end of the rooting phase in medium with different pH values (4.50, 5.00 and 5.75).

Changes in medium pH can occur during culture, depending on the initial pH, medium composition and plant species (Schuch et al. 2010). Thus, in this work the pH was monitored after autoclaving and at the end of the subculture period. As expected post-autoclave pH values were lower (0.20 to 0.75 units) than the pre-autoclave in all the media tested (Table 3.1). Furthermore, in most media without plantlets the pH decreased to a more acid condition over the 6 week period (Table 3.1). This acidification was previously observed and can be caused by dehydration of media and/or by the precipitation of medium components such as mineral nutrients (Skirvin et al. 1986; Leifert et al. 1992; Shibli et al. 1999).

Independently of the initial pH, after 6 weeks of proliferation or rooting, there were not significant differences ($P \geq 0.05$) regarding the final pH value, for both species (Table

3.1). The shift of a wide range of initial pH to the same final pH value has been reported for other plant species (Skirvin et al. 1986; Minocha 1987). It is tempting to suggest that plant material has an active role in establishing an optimum pH environment. The direction and extent of the pH change might also have been influenced by the plant's *in vivo* optimum pH.

Table 3.1. Changes in the pH of proliferation and rooting media after autoclaving and after 6 weeks, with or without *Plantago algarbiensis* and *P. almogravensis* plantlets.

Medium	Original	Post-autoclave	After 6 weeks		
			Without plantlets	*P. algarbiensis*	*P. almogravensis*
Proliferation	4.50	4.20 ± 0.01 b	4.11 ± 0.02 b	3.65 ± 0.10* c	4.60 ± 0.07* a
	5.00	4.52 ± 0.01 ab	4.39 ± 0.04 b	3.72 ± 0.13* c	4.68 ± 0.07* a
	5.75	4.97 ± 0.02 a	4.74 ±0.01 a	3.50 ± 0.03* b	4.72 ± 0.18* a
Rooting	4.50	4.26 ± 0.02 a	4.08 ± 0.01 b	3.15 ± 0.05 d	3.46 ± 0.09 c
	5.00	4.74 ± 0.02 a	4.35 ± 0.06 b	2.92 ± 0.08 d	3.62 ± 0.09 c
	5.75	5.09 ± 0.01 a	4.85 ± 0.02 b	3.15 ± 0.10 d	3.72 ± 0.10 c

Values are expressed as the mean ± SE (n = 3). In each row, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test. * indicates a significant difference between proliferation and rooting media for each original pH ($P < 0.05$).

When cultures were inoculated the pH of the media significantly decreased ($P < 0.05$) (Table 3.1) after 6 weeks, with the exception of *P. almogravensis* during proliferation. Moreover, the lowest pH values were observed in the rooting media for both species ($P < 0.05$). These results can be due to differences in the composition of the proliferation and rooting media but also to the root biomass in the medium. In fact, soil acidification by roots has been demonstrated for many plant species (Marschner et al. 1986; Hauter and Mengel 1988). Under *in vivo* conditions, plant roots not only respond

to pH changes in the soil, but also contribute to the modulation of ambient soil by a variety of mechanisms including: differential uptake of NO_3^- and NH_4^+, release of H^+ and/or OH^-, changes in the uptake and release of CO_2, and release of various organic acids and organic compounds (Minocha 1987; Yan et al. 1992).

It has been suggested that the excess of H^+ competes with other cations for root absorption sites, interfering with ion transport and uptake, and causes root membranes to become leaky in sensitive plants (Foy 1992). According to Koyama et al. (2001) the exposure to low pH damages the plasma membrane of root tip cells irreversibly within a short time. Furthermore, changes in biochemical parameters would occur before any visible symptom of toxicity appears (Lin et al. 2007), and conclusions based on these parameters may be more reliable than morphological observations in revealing H^+ toxicity. Thus, in this work, although *in vitro* proliferation and rooting were not affected by medium pH, its effect on lipid peroxidation was investigated. Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids, has been utilized as a biomarker for lipid peroxidation (Sivanesan et al. 2011). The MDA content was significantly higher ($P < 0.05$) in shoots of *P. algarbiensis* and in roots of both species cultured at pH 4.50 in comparison with shoots cultured at the remaining pH (Table 3.2). This indicates membrane damage stress and was analytically detectable before growth differences appeared.

The accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress conditions (Chen et al. 2007). In addition to the role of compatible solutes in cell osmotic adjustment they can also function as reactive oxygen species (ROS) scavengers (Hong et al. 2000). Therefore, the enhanced content of these solutes may probably ameliorate the tolerance through improving oxidative status. Although the accumulation of proline and carbohydrates in

response to abiotic stress has been well documented (Ghnaya et al. 2010; Lokhande et al. 2010; Somboonwatthanaku et al. 2010), few studies related to H^+ stress conditions were reported.

A significant increase ($P < 0.05$) in proline content was observed in *P. almogravensis* roots cultured at pH 5.75, while the roots grown at pH 4.50 and 5.00 showed a significant ($P < 0.05$) increase in carbohydrates content (Table 3.2). The proline and carbohydrate contents in *P. algarbiensis* shoots and roots remained unchanged after grown under different pH values (Table 3.2).

Table 3.2. Contents of MDA, proline and carbohydrate in *Plantago algarbiensis* and *P. almogravensis* plantlets (shoot and root) cultured in media with different pH.

pH	MDA (nmol g^{-1} FW)		Proline (μmol g^{-1} FW)		Carbohydrates (μmol g^{-1} FW)	
	Shoot	Root	Shoot	Root	Shoot	Root
P. algarbiensis						
4.50	70.52 ± 8.11 a	40.60 ± 3.50 a	0.50 ± 0.09 a	0.20 ± 0.03 a	91.85 ± 9.73 a	285.90 ± 11.46 a
5.00	26.97 ± 4.01 b	29.12 ± 4.12 b	0.57 ± 0.07 a	0.16 ± 0.04 a	100.83 ± 5.54 a	269.08 ± 35.61 a
5.75	26.05 ± 5.68 b	30.90 ± 2.08 b	0.54 ± 0.10 a	0.21 ± 0.04 a	87.90 ± 14.39 a	240.90 ± 62.45 a
P. almogravensis						
4.50	59.51 ± 8.96 a	47.10 ± 4.11 a	0.20 ± 0.01 a	0.16 ± 0.03 b	33.25 ± 3.93 a	168.22 ± 27.15 a
5.00	48.32 ± 9.06 a	31.21 ± 2.99 b	0.33 ± 0.08 a	0.26 ± 0.04 ab	57.09 ± 12.20 a	190.57 ± 14.72 a
5.75	42.12 ± 4.22 a	29.76 ± 3.02 b	0.35 ± 0.06 a	0.39 ± 0.10 a	42.19 ± 5.54 a	86.30 ± 22.73 b

Values are expressed as the mean ± SE (n = 5). For each species and in each column, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

In *P. almogravensis* no significant differences ($P \geq 0.05$) were observed in protein content between all the pH values assayed (Figure 3.3 B), while in *P. algarbiensis* that content was significantly higher ($P < 0.05$) in roots developed at pH 4.50 and 5.00 (Figure 3.3 A). Soluble protein content is an important indicator of changes in metabolism, and it is known to respond to a wide variety of stresses (Singh and Tewari 2003). A higher content of soluble protein has been reported in several plant species under adverse growth conditions (Ashraf and Harris 2004; He and Huang 2007).

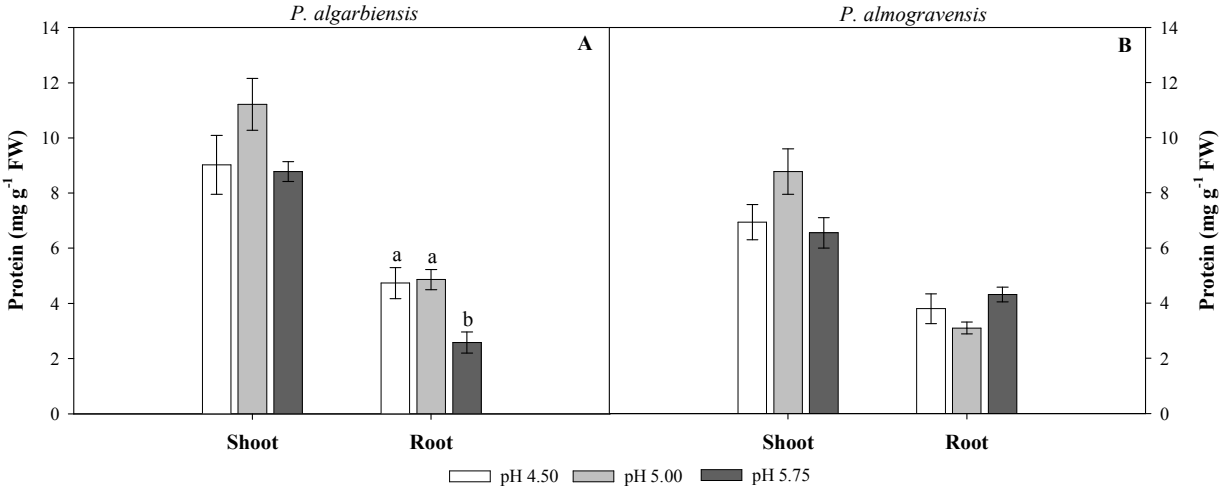

Figure 3.3. Effect of medium pH (4.50, 5.00 and 5.75) on protein content in shoots and roots of *P. algarbiensis* (A) and *P. almogravensis* (B). Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed.

One of the plant responses to environmental stresses is the rapid and increased generation of ROS (Yang et al. 2010). These free radicals have an important role in the metabolism and development of aerobic organisms; however, their uncontrolled production leads to oxidative stress (Abbasi et al. 2011). Different plants develop

different protection mechanisms to eliminate ROS and prevent oxidative damage (Yang et al. 2010). Antioxidant enzymes like SOD, CAT, APX and other enzymes are efficiently involved in ROS scavenging and can act as one of the main tolerance mechanisms against oxidative stress in plants (Lokhande et al. 2010). It is known that pH affects nutrient uptake as well as enzymatic and hormonal activities in plants (Bhatia and Ashwath 2005). However, reports about the response of antioxidant system for plants subjected to H^+ stress are scarce.

To investigate the antioxidant response of *P. algarbiensis* and *P. almogravensis* shoots and roots to medium pH the activities of SOD, CAT, APX and GPX were measured (Figure 3.4). No significant changes ($P \geq 0.05$) in SOD activity were observed in shoots and roots of both *Plantago* species cultured in medium with different pH values (Figure 3.4 A, B). The CAT activity was not affected ($P \geq 0.05$) by the pH of the medium in *P. algarbiensis* (Figure 3.4 C), while a significant activation ($P < 0.05$) of this enzyme was observed in roots of *P. almogravensis* developed under pH 5.00 as compared with pH 5.75 (Figure 3.4 D). In *P. algarbiensis* there was a significant increase ($P < 0.05$) in APX and GPX activity in roots and shoots, respectively, grown at pH 4.50 (Figure 3.4 E, G). The APX and GPX activity of *P. almogravensis* shoots and roots was not affected by medium pH (Figure 3.4 F, H).

The level of antioxidative response depends on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Reddy et al. 2004). Our results demonstrate that after 6 weeks of culture SOD activity was unaffected by the pH of the culture medium in both *Plantago* species but minor changes were detected in the activity of the other enzymes (Figure 3.4). This is quite acceptable because superoxide anions could also be mitigated through non-enzymatic pathways (Costa et al. 2010).

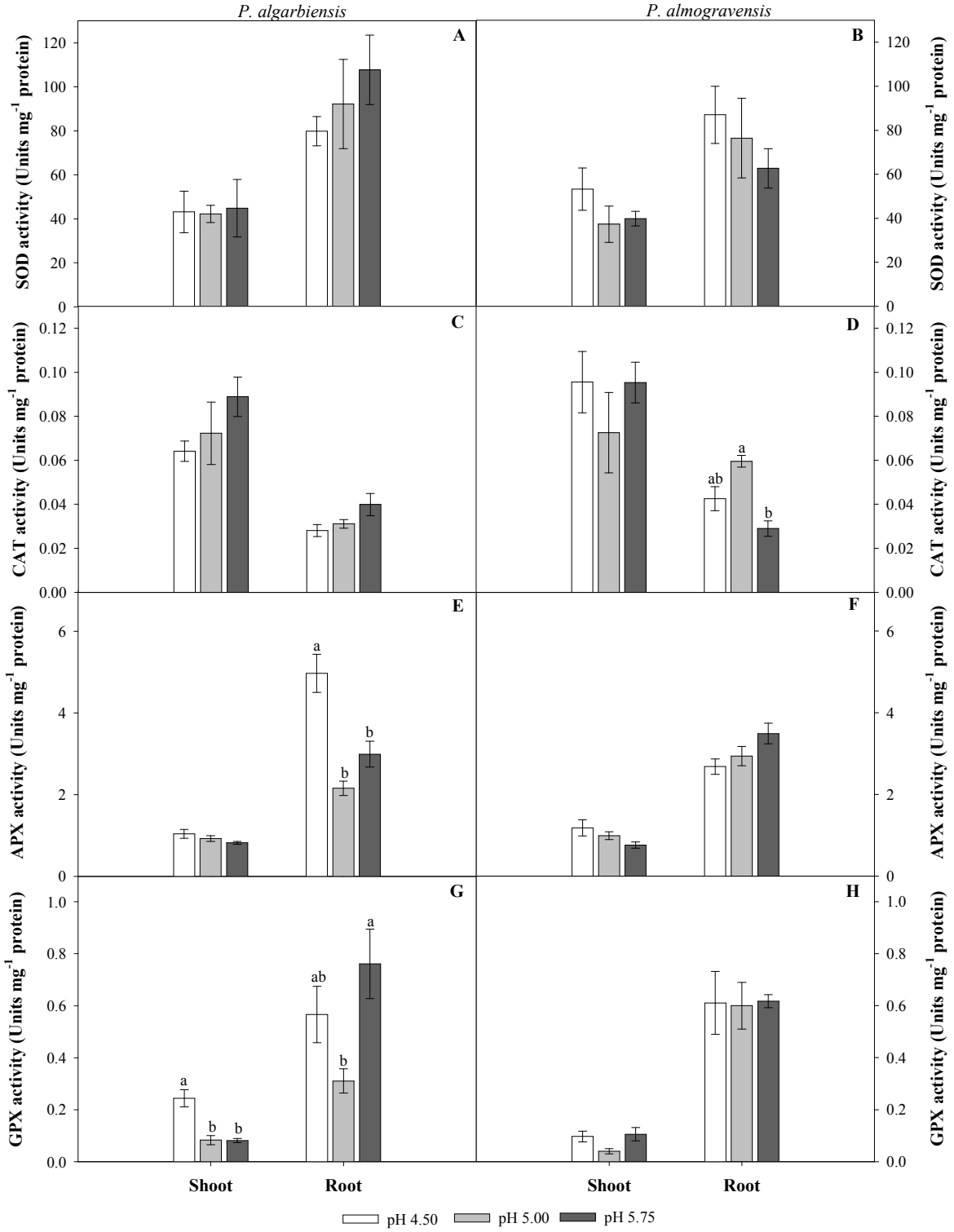

Figure 3.4. Effect of medium pH (4.50, 5.00 and 5.75) on SOD (A, B), CAT (C, D), APX (E, F) and GPX (G, H) activities in shoots and roots of *Plantago algarbiensis* and *P. almogravensis*. Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed.

The general conclusion of this investigation is that medium pH did not affect *in vitro* proliferation and rooting of micropropagated shoots of *P. almogravensis* and *P. algarbiensis*. Nevertheless, plasma membrane damage was observed in both species at the lowest pH (pH 4.50), which is controversial because at the end of the rooting period pH values were similar in all the media, regardless the initial value. Although this remains unexplained and should be clarified in future works, from these results it seems evident that both *Plantago* species studied are apt to grow *in vitro* in medium with pH values much lower than the usually used in tissue culture (pH 5.70 - 5.80). This is further reinforced by the fact that medium pH even decreases to a more acid condition during rooting and is in agreement with the fact that both species colonize acid soils.

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CHAPTER 4

Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae)

Martins N, Gonçalves S, Palma T, Romano A (2012) Seed germination of two critically endangered plantain species, *Plantago algarbiensis* and *P. almogravensis* (Plantaginaceae). Seed Science and Technology 40:144-149

4.1. Abstract

The aims of this study were to determine the seed size and the effects of temperature and light on germination of *Plantago algarbiensis* and *P. almogravensis* seeds, two endemic species from Portugal that are at risk of extinction. The temperatures tested were a constant 15 or 25 ºC and an alternating temperature regime of 25/15 ºC, combined with 16/8 h light/dark photoperiod (light) or constant darkness. The results showed that *P. algarbiensis* and *P. almogravensis* have non-dormant seeds. Although *P. algarbiensis* seeds are larger and heavier, seeds of both species have similar germination requirements. The best germination results were obtained at 15 ºC under either light or darkness, along with the shortest mean germination time. The results are crucial for developing conservation strategies for these *Plantago* species.

4.2. Introduction

Plantago algarbiensis Samp. and *Plantago almogravensis* Franco are endemic species from West-Central Algarve, South Portugal, and the Portuguese southwest coast, respectively. Both species are restricted to a very small area (*P. algarbiensis* ~50 ha and *P. almogravensis* ~7 ha) and are protected by both the European Habitats Directive 92/43/CEE and Portuguese law (reference 140/99 of April 24). Practical measures to ensure the conservation of the maximum genetic diversity of these species, are urgently required. Seed cryopreservation is an important tool to store germplasm for long periods and has been successfully applied to the preservation of seeds of several wild and endangered species (Gonçalves et al. 2009a; Hirano et al. 2011). However, before cryopreservation can be implemented the seed germination requirements must be known.

Field studies by Branquinho et al. (2007) demonstrated that *P. almogravensis* is an aluminum (Al) hyperaccumulator species because it accumulates more than 1,000 $\mu g\ g^{-1}$ of this mineral in the aboveground parts. Furthermore, we have established a micropropagation protocol for both *Plantago* species (Gonçalves et al. 2009b) and recently demonstrated that *in vitro* shoots are able to grow in a medium with a low pH (Martins et al. 2011), which agrees with the fact that both species colonize acid soils. Since germination may be the first decisive step for survival of plants under Al stress at low pH (Kikui et al. 2005), it is important to determine the effect of Al during this stage to establish the Al tolerance. For that, the prior study of the germination requirements is necessary. No information is available on the germination of *P. algarbiensis* or *P. almogravensis* seeds. Thus, having in mind the application of conservation strategies and the evaluation of Al tolerance, the aims of this work were to study the effects of

temperature and light during germination of *P. algarbiensis* and *P. almogravensis* seeds and to study their size.

4.3. Materials and methods

4.3.1. Seed collection

Mature seeds of *P. algarbiensis* were collected in July 2009 and June 2010 from wild plants growing in a natural population in Algoz (Algarve region, Portugal). *P. almogravensis* seeds were collected from wild plants growing in Vila Nova de Milfontes (Portugal). Since just one population of this species is known and the over-harvesting of seeds may represent a threat, seeds were only collected in June 2010. Seeds were collected from 20 individuals of each species randomly selected within the population area. After their collection the seeds were stored in hermetic sealed glass jars inside paper bags and kept in the dark under laboratory conditions at 25 ± 2 ºC for two months.

4.3.2. Morphological features

The seed water content was determined in three replicates of 25 randomly selected seeds using the low temperature oven method (103 ºC for 17 h; ISTA 2003). For each species, 75 randomly selected seeds were observed using a stereomicroscope, the image was captured with a digital Motic camera and seed length and width measured using the software Motic Images 2000. The length-to-width ratio was also calculated. Three replicates of 100 seeds were used to quantify seed weight.

4.3.3. Germination trials

The seeds were sown in 9 cm diameter glass Petri dishes on 0.5% (w/v) bactoagar without nutrients. Germination tests were done at constant 15 or 25 °C and alternating 25/15 ºC under 16 h light / 8 h dark photoperiod (light) or continuous darkness. The light was provided by cool-white fluorescent lamps at a photon flux density of 69 μmol m^{-2} s^{-1}. Germination was recorded every 2 days over a total 40 days incubation period and the criterion for germination was visible radical protrusion. Due to the endangered nature of these species the availability of seeds is limited, thus for each treatment only three replicates of 10 seeds were used.

4.3.4. Statistical analysis

At the end of the germination period, results were expressed as final germination percentage (mean value ± standard error) and mean germination time (MGT, mean value in days ± standard error). The MGT was determined according to the formula described by Ellis and Roberts (1981). The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 19.0, SPSS Inc., California). For additional pairwise comparisons, Duncan´s New Multiple Range Test was used. Differences were considered significant at $P < 0.05$. Final germination percentages were arcsine transformed prior to statistical analysis.

4.4. Results and discussion

As expected, seed size differed between the two *Plantago* species (Table 4.1). *P. algarbiensis* seeds showed higher ($P < 0.05$) seed length, width, length-to-width ratio and weight. Seed water content was similar ($P \geq 0.05$) in both *Plantago* species with

values below 10% (Table 4.1). Moreover, a significant variation ($P < 0.05$) in the seed width, length-to-width ratio and weight was also observed among *P. algarbiensis* seeds collected in different years (Table 4.1), indicating that the size of these seeds vary each year within a single population.

P. algarbiensis and *P. almogravensis* seeds showed high final germination percentages without any pre-treatment (Table 4.2). Germination percentage was affected by temperature ($P < 0.001$), light ($P < 0.05$) and by their interaction ($P < 0.05$). Irrespective of the temperature and light conditions tested, seeds of both species have similar germination percentage ($P \geq 0.05$). Moreover, no significant differences ($P \geq 0.05$) were observed on the germination traits of *P. algarbiensis* with regard to the collection year.

Temperature affects the timing of germination by inhibiting it under unsuitable environmental conditions and subsequently promoting germination under suitable conditions (Marone et al. 2000; Carasso et al. 2011). Independently of light conditions, *P. algarbiensis* and *P. almogravensis* seeds reached the maximum germination percentage (100%) at 15 ºC (Table 4.2). This behavior is a typical strategy of Mediterranean species with optimal temperatures ranging between 15 and 20 ºC (Pérez-García et al. 1995; Zaidi et al. 2010). Moreover, the ideal seed germination temperature for most species of *Plantago* ranges from 15 to 25 ºC (Arnold 1973; Fons et al. 2008). According to Baskin and Baskin (2004) seeds that have the capacity to germinate when the appropriate set of environmental conditions (temperature, light/dark) are present can be considered non-dormant. Since *P. algarbiensis* and *P. almogravensis* seeds reached 100% germination at 15 ºC without any pre-treatment, they can be considered non-dormant.

Table 4.1. Length, width, weight and water content of *Plantago algarbiensis* and *P. almogravensis* seeds.

Species	Collection year	Seed length (μm)	Seed width (μm)	Length-to-width ratio	Weight of 100 seeds (mg)	Seed water content (% fwb)
P. algarbiensis	2009	1796.4 ± 19.16	995.8 ± 10.51	1.8 ± 0.02 †	73.4 ± 1.16	9.7 ± 0.91
	2010	1768.0 ± 13.11*	1067.6 ± 9.26 *, †	1.7 ± 0.01*	79.3 ± 0.22 *, †	8.8 ± 0.75
P. almogravensis	2010	1538.8 ±12.82	963.4 ± 7.46	1.6 ± 0.02	66.8 ± 0.29	9.1 ± 1.08

Values are expressed as the mean ± SE. *: indicates significant differences ($P < 0.05$) between results of seeds of different species collected in the same year (2010). †: indicates significant differences ($P < 0.05$) between results of *P. algarbiensis* seeds collected in different years. The absence of symbols indicates that no significant differences were observed.

Table 4.2. Effects of temperature on the final germination percentages and mean germination time (MGT) of *Plantago algarbiensis* and *P. almogravensis* seeds. Results after 40 days of incubation under 16 h light photoperiod (light) or constant darkness.

Species	Collection year	Temperature (ºC)	Germination (%)			MGT (days)		
			Light	Dark	S[(1)]	Light	Dark	S
P. algarbiensis	2009	15	100.0 ± 0.00 a	100.0 ± 0.00 a	ns	3.3 ± 0.24 a	3.3 ± 0.18 b	ns
		25	66.7 ± 3.33 c	80.0 ± 11.55 a	ns	10.5 ± 4.47 a	22.1 ± 2.33 a	ns
		25/15	83.3 ± 3.33 b	40.0 ± 10.00 b	*	12.6 ± 0.83 a	26.4 ± 0.44 a	***
	2010	15	100.0 ± 0.00 a	100.0 ± 0.00 a	ns	3.8 ± 0.23 b	3.1 ± 0.44 b	ns
		25	83.3 ± 6.67 b	90.0 ± 5.77 a	ns	15.8 ± 0.80 a	16.2 ± 0.85 a	ns
		25/15	66.7 ± 6.67 b	33.3 ± 8.82 b	*	18.2 ± 2.43 a	21.6 ± 3.22 a	ns
P. almogravensis	2010	15	100.0 ± 0.00 a	100.0 ± 0.00 a	ns	3.9 ± 0.07 b	2.7 ± 0.07 b	***
		25	90.0 ± 5.77 a	66.7 ± 17.64 ab	ns	13.0 ± 1.37 a	19.7 ± 1.39 a	*
		25/15	90.0 ± 0.00 a	53.3 ± 3.33 b	***	12.4 ± 0.97 a	20.7 ± 1.99 a	*

Values are expressed as the mean ± SE. For each species and the same year of collection, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test. For each temperature, the significance level (S) between results from light and darkness is showed. [(1)]S: ***Significantly different at $P < 0.001$; $^{*}P < 0.05$; ns, not significant.

No significant differences ($P \geq 0.05$) in MGT values were observed between species. Overall, the seeds from both species germinated significantly ($P < 0.05$) faster (i.e. lower MGT values) at 15 ºC. In accordance with our results, Pérez-García et al. (1995) showed that seed germination of several Iberian species was more rapid at low temperatures (around 15 ºC) and slower or ceased at temperatures higher than 20 ºC. The alternating temperature regime 25/15 ºC did not improve the final germination percentage compared with the constant temperatures (Table 4.2), consistent with the observation that germination of *P. maritime* seeds are not stimulated by alternating temperatures (Arnold 1973). However, MGT values were higher in the alternating temperature regime.

There were no significant differences ($P \geq 0.05$) in the final germination percentages between light and dark regimes at the constant temperatures of 15 and 25 ºC, for either species (Table 4.2). This indicates that *P. algarbiensis* and *P. almogravensis* seeds can germinate under these temperatures without light, showing no photoblastic response. Similarly, *P. lanceolata* have the ability to germinate under light or darkness (Blom 1992), while other *Plantago* species are strongly light requiring (Arnold 1973; Gutterman and Shem-Tov 1996). Conversely, light had a positive effect on the final germination percentage under the alternating temperature regime of 25/15 ºC in both species (Table 4.2). The alternating regime did not favor germination in either species, but changed their light sensitivity. Moreover, light had no effect ($P \geq 0.05$) on MGT values in *P. algarbiensis* seeds collected in 2010, while, an enhancement ($P < 0.001$) was observed in the seeds collected in 2009 at the alternate temperature of 25/15 ºC. In *P. almogravensis*, the light regime influenced the values of MGT under all the temperatures tested (Table 4.2).

Based on our results we conclude that *P. algarbiensis* and *P. almogravensis* seeds germinate readily at 15 ºC under either light or darkness, reaching the 100% germination, thus showing no intrinsic dormancy. These results must however be interpreted with caution given the low number of seeds tested. Significant differences were detected in the seed size between the two species with *P. algarbiensis* seeds showing the higher size and weight. However, no differences were observed between both species in terms of germination capability. In *P. algarbiensis* the seed size differed between the two collection years, although the germination was not affected. The insight of the optimal germination conditions of *P. algarbiensis* and *P. almogravensis* will be useful to further studies of seed cryopreservation and Al tolerance evaluation.

4.5. References

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CHAPTER 5

Aluminum inhibits root growth and induces hydrogen peroxide accumulation in *Plantago algarbiensis* and *P. almogravensis* seedlings

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Martins N, Gonçalves S, Romano A (2013) Aluminum inhibits root growth and induces hydrogen peroxide accumulation in *Plantago algarbiensis* and *P. almogravensis* seedlings. Protoplasma (in press). doi: 10.1007/s00709-013-0511-1

5.1. Abstract

We have evaluated the impact of aluminum (Al) on germination, relative root growth, Al accumulation in roots tips, H_2O_2 levels, plasma membrane integrity, pigment levels, protein content and the activities of superoxide dismutase (SOD) and catalase (CAT) in seedlings of the endemic Portuguese species *Plantago algarbiensis* and *P. almogravensis*. We found that up to 400 μM Al had no impact on the germination percentage in either species but inhibited root growth in a concentration-dependent manner (more severely in *P. algarbiensis*). Al accumulation in the root tips of both species was concentration dependent up to 200 μM but declined thereafter despite the absence of membrane damage. We observed a concentration-dependent induction of SOD activity but no change in CAT activity resulting in the accumulation of H_2O_2 (a known growth inhibitor) although its impact in *P. almogravensis* may be partially ameliorated by the accumulation of carotenoid pigments. Our data suggest a correlation between Al uptake, H_2O_2 production and the inhibition of root growth during early seedling development in *P. algarbiensis* and *P. almogravensis*, although the latter is more tolerant towards higher concentrations of the metal.

5.2. Introduction

Germination is a complex process involving the coordination of physiological, biochemical and molecular mechanisms that are particularly susceptible to adverse environmental factors (Bewley 1997). Although the impact of metal toxicity on the growth of vegetative tissues such as shoots and roots has been well documented, there have been few studies focusing on metal toxicity and germination (Kranner and Colville 2011). Physiological metal tolerance strategies in plants depend on age and the nature of the metal ions, but metal-tolerant species are thought to deploy their adaptive mechanisms throughout development, particularly during germination when the young plant is most vulnerable (Lefèvre et al. 2009).

Aluminum (Al) is a major metal component in soils and is solubilized as phytotoxic ions (predominantly Al^{3+}) which inhibit plant growth under low pH conditions (Tahara et al. 2008). Both germination and seedling growth can be inhibited if the medium surrounding the seed is contaminated with Al (Marciano et al. 2010; Gui et al. 2011). Germination and root growth tests have been used to evaluate the effect of Al in several plant species, particularly crops (Marciano et al. 2010; Zhang et al. 2010). Furthermore, Ezaki et al. (2007, 2008) used germination and relative root growth tests to measure Al tolerance in 49 wild species and in panels of *Arabidopsis thaliana* mutants.

Plantago almogravensis Franco is an endemic species that grows along the southwest coast of Portugal in acidic, podzolic soils naturally enriched in bioavailable Al and iron (Pimentel et al. 1996; Buurman and Jongmans 2005; Serrano et al. 2011). This species is described as an Al hyperaccumulator based on the results of field testing (Branquinho et al. 2007). *Plantago algarbiensis* Samp. is an endemic species from the West-Central Algarve region, and its sensitivity to Al is unknown. Both species are currently endangered, so a micropropagation protocol was established (Gonçalves et al. 2009) and

it was also demonstrated that shoots from each species can grow in low pH medium (Martins et al. 2011).

Although the requirements for germination in both species have been studied (Martins et al. 2012) the behavior of germinating seeds in the presence of Al has not been investigated. We therefore set out to evaluate the effect of Al on germination and seedling development in both species to gain insight into their Al tolerance strategies.

5.3. Materials and methods

5.3.1. Plant material

P. algarbiensis and *P. almogravensis* seeds were collected in June 2010 from wild plants growing among natural populations in Algoz (Algarve Region, Portugal) and Vila Nova de Milfontes (Portugal), respectively. Several plants from both species were selected to ensure the seeds were genetically diverse. The seeds were stored in hermetic glass jars inside paper bags and were kept in the dark under laboratory conditions at 25 ± 2 ºC for 2 months.

5.3.2. Seed germination and seedling growth

Seeds were sown in 9 cm glass Petri dishes on 1 mM $CaCl_2$ solution (pH 4.5) solidified with 0.5% (w/v) bactoagar (Difco, USA) supplemented with $AlCl_3$ (0, 100, 200 or 400 μM). Seeds were incubated under optimal germination conditions (15 ± 2 ºC, 16 h photoperiod, 69 μmol m^{-2} s^{-1}) as previously described (Martins et al. 2012). The seeds were monitored every 2 days over 30 days and germination was scored on the basis of visible radical protrusion.

For each treatment, we recorded the final germination percentage (%) and the mean germination time (MGT), which was determined using the formula MGT = ΣDN / ΣN

(Ellis and Roberts 1981), where D is the number of days from sowing and N is the number of seeds that have germinated by day D. Root length was measured for each treatment after 30 days of germination and relative root growth was estimated by calculating the root lengths of Al treated seedlings as a percentage of the root length of untreated seedlings (Ezaki et al. 2007).

5.3.3. Aluminum uptake

The content was determined by hematoxylin staining (Polle et al. 1978). Roots were washed in distilled water for 30 min at room temperature, stained in 0.2% (w/v) hematoxylin solution containing 0.02% (w/v) KIO_3 for 30 min and washed again as above. Stained roots were photographed using a Motic camera (Moticam 350, China) under a stereomicroscope (Olympus SZ40, Japan). For quantitation, 5 mm root tips were excised and soaked in 1 M HCl for 1 h to release the hematoxylin, and the concentration was determined by measuring absorbance at 490 nm.

5.3.4. H_2O_2 content

The H_2O_2 content of the seedlings was determined as described by Loreto and Velikova (2001). Fresh plant material (100 mg) was homogenized in 1 ml 0.1% (w/v) trichloroacetic acid at 4 ºC and centrifuged at 12,000 *g* for 15 min. We then mixed 0.2 ml of the supernatant with 0.2 ml 10 mM sodium phosphate buffer (pH 7.0) and 0.4 ml 1 M KI. The reaction was developed for 30 min in darkness and the H_2O_2 content was determined by measuring absorbance at 390 nm against a set of H_2O_2 standards, and subtracting a blank sample lacking the plant extract. The results were expressed as $\mu mol\ g^{-1}$ fresh weight (FW).

5.3.5. Plasma membrane integrity

Plasma membrane integrity was evaluated using Evans blue as described by Baker and Mock (1994). Intact roots were stained with 0.25% (w/v) Evans blue solution for 10 min at room temperature, washed three times with distilled water for 10 min and photographed under a stereomicroscope as above. For quantitation, 5-mm root tips were excised and soaked in N,N-dimethylformamide for 1 h at room temperature to release the stain, and the concentration was determined by measuring absorbance at 600 nm.

5.3.6. Photosynthetic pigments

Photosynthetic pigments were extracted with 100% acetone from fresh plant material (25 mg) and quantified by measuring the absorbance at 661.6, 644.8 and 470 nm (Lichtenthaler 1987).

5.3.7. SOD and CAT activities and soluble protein levels

Superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) activities were evaluated in extracts from seedlings after 30 days of germination. Fresh tissue (100 mg) was ground in a pre-chilled mortar containing 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid, 1% (w/v) polyvinylpolypyrrolidone and 2.5 mM dithiothreitol. The homogenate was centrifuged at 20,000 *g* for 10 min at 4 ºC and the supernatant was used for subsequent enzyme assays. The specific activity for both enzymes was expressed as enzyme units per milligram of protein, based on the measurement of total soluble protein levels according to the method of Bradford (1976) using bovine serum albumin as standard.

SOD activity was measured by the reduction of nitroblue tetrazolium chloride (NBT) as described by Beauchamp and Fridovich (1971). The reaction mixture comprised 50 mM

sodium phosphate buffer (pH 7.8), 10 mM methionine, 0.075 mM NBT, 0.2 mM riboflavin and 20 µl of the enzyme extract. The reaction was monitored by measuring absorbance at 560 nm for 6 min. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%.

CAT activity was measured by the degradation of H_2O_2 as described by Aebi (1983). The reaction mixture comprised of 50 mM sodium phosphate buffer (pH 7.0), 40 mM H_2O_2 and 20 µl of the enzyme extract. The reaction was monitored by measuring absorbance at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). One unit of CAT was defined as the amount of enzyme required to degrade 1 µmol H_2O_2 per min.

5.3.8. Statistical analysis

Final germination percentages, MGT and relative root growth values were based on data from 10 replicates of 10 seeds or seedlings, whereas other values were determined from five replicates of plant material randomly collected from different Petri dishes. The values obtained were expressed as means ± standard errors. The impact of different treatments were tested by one-way analyses of variance (ANOVA) using the SPSS statistical package for Windows (release 19.0, SPSS Inc., California). Significant differences between means were identified using Duncan's New Multiple Range Test or Dunnett's test. Final germination percentages were arcsine transformed prior to statistical analysis.

5.4. Results and discussion

Germination assays are often used to evaluate the toxicity of exogenous compounds including metals such as Al (Labra et al. 2006; Ezaki et al. 2008). We exposed *P. algarbiensis* and *P. almogravensis* seeds to different concentrations of Al during

germination, but found no statistically significant differences in either species ($P \geq 0.05$) when we compared the final germination percentages and MGT values between the Al treated seeds and controls (Table 5.1). This reflects the behavior of other Al-tolerant plants, which have also been shown to germinate successfully despite high concentrations of Al ions in the environment (Ezaki et al. 2008).

Table 5.1. Effect of Al on the final germination percentage and mean germination time (MGT) of *Plantago algarbiensis* and *P. almogravensis* seeds.

Al (μM)	Germination (%)	MGT (days)
P. algarbiensis		
0	98.00 ± 1.33	5.35 ± 0.20
100	100.00 ± 0.00	5.26 ± 0.24
200	96.00 ± 2.21	5.53 ± 0.20
400	99.00 ± 1.00	5.50 ± 0.50
P. almogravensis		
0	97.00 ± 2.13	4.49 ± 0.31
100	98.00 ± 1.33	4.35 ± 0.32
200	98.00 ± 1.33	4.28 ± 0.27
400	96.00 ± 2.67	4.72 ± 0.37

Values are expressed as the mean ± SE (n = 10). For each species, the absence of letters indicates that no significant differences were observed between treatments according to Duncan's test ($P \geq 0.05$).

Seedling growth is more sensitive to metal toxicity than germination (Kranner and Colville 2011) and seedling root growth is the most relevant indicator because roots are generally the first point of contact between plants and toxic metals in the environment (Lamhamdi et al. 2011). We exposed seedlings of both species to different

concentrations of Al, but even at the highest concentration we tested (400 μM) there were no overt signs of toxicity, which usually manifests as chlorotic shoots and stubby, brittle and brown roots (Figure 5.1 A). We investigated the quantitative impact of Al loading by measuring root growth in Al treated seedlings relative to root growth in untreated controls. In *P. almogravensis* seedlings, there was no difference in relative root growth at 100 μM Al, a negligible reduction at 200 μM Al and even at 400 μM Al the relative root growth was still ~60% (Figure 5.2). In *P. algarbiensis* seedlings, there was a slight reduction in relative root growth at 100 μM Al, and the values fell to approximately 63% and 41% at 200 and 400 μM Al, respectively. These data indicated that *P. almogravensis* is more tolerant towards Al than *P. algarbiensis*, consistent with the observation that *P. almogravensis* colonize soils naturally enriched in bioavailable Al.

To monitor the accumulation and distribution of Al ions in the two species, we incubated seedling roots with the Al-specific dye hematoxylin, which has been widely used to study Al tolerance (Polle et al. 1978; Marciano et al. 2010). The intensity of hematoxylin staining in the root tips of both species increased in concert with Al loading (Figure 5.1 B) and the corresponding quantitative data showed that Al accumulates in the roots in a concentration-dependent manner in both species when exposed to media containing up to 200 μM Al (Table 5.2). This suggests that both *Plantago* species lack effective aluminum exclusion mechanisms. However, in both species there was a significant reduction in hematoxylin staining ($P < 0.05$) between 200 to 400 μM Al (Table 5.2), which could reflect either the induction of Al exclusion mechanisms under high loading conditions or the consequences of root apex damage caused by metal toxicity.

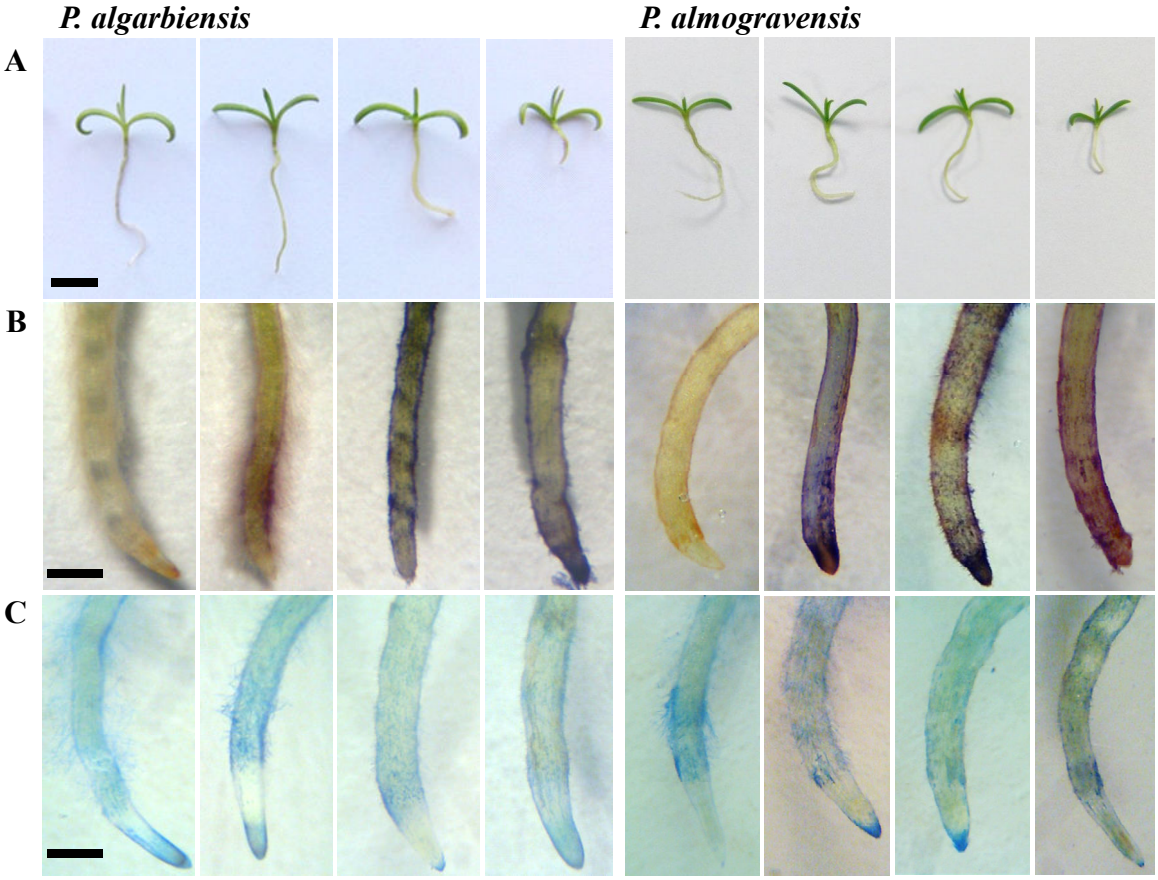

Figure 5.1. Effect of Al on *Plantago algarbiensis* and *P. almogravensis* seedling growth (A) (bar = 1 cm). Al accumulation in roots tips revealed by hematoxylin staining (B) (bar = 1 mm). Evaluation of plasma membrane integrity in the roots tips using Evans blue (C) (bar = 1 mm). Panels from left to right show seedlings grown in medium containing 0, 100, 200 and 400 μM Al respectively, for 30 days. Images represent at least three seedlings or roots.

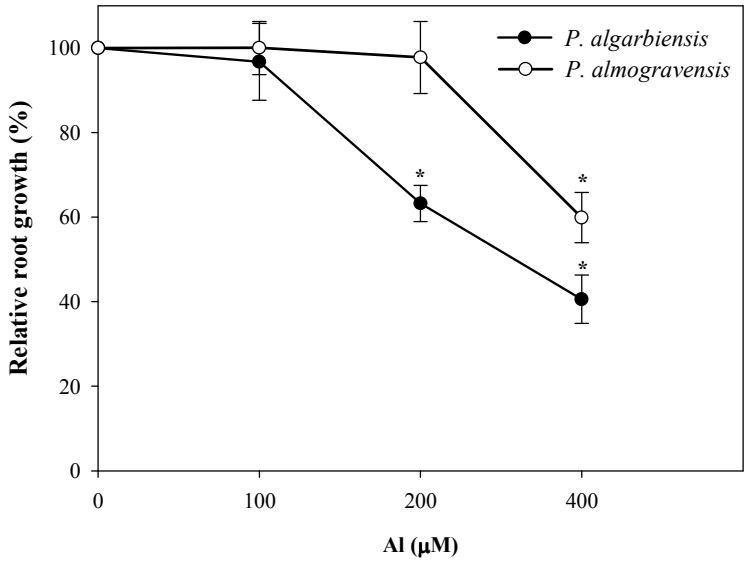

Figure 5.2. Effect of Al on relative root growth in *Plantago algarbiensis* and *P. almogravensis* seedlings. Values are expressed as the mean ± SE (n = 10). *Represents significant difference from control (Dunnett's test, $P < 0.05$).

Table 5.2. Quantitative determination of hematoxylin staining and the uptake of Evans blue in *Plantago algarbiensis* and *P. almogravensis* root tips in the presence of Al.

Al (µM)	Hematoxylin (*A* 490 nm)	Evans blue (*A* 600 nm)
P. algarbiensis		
0	0.013 ± 0.001 c	0.060 ± 0.006 a
100	0.021 ± 0.001 bc	0.061 ± 0.005 a
200	0.045 ± 0.005 a	0.059 ± 0.011 a
400	0.026 ± 0.003 b	0.064 ± 0.008 a
P. almogravensis		
0	0.008 ± 0.001 c	0.061 ± 0.003 a
100	0.040 ± 0.004 a	0.057 ± 0.003 a
200	0.036 ± 0.005 a	0.056 ± 0.007 a
400	0.021 ± 0.004 b	0.062 ± 0.005 a

Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

Excess Al is thought to cause oxidative damage to cells by triggering the production of reactive oxygen species (ROS) that interact with nucleic acids in the embryo as well as stored proteins and lipids in the seed (Pereira et al. 2010). These processes can interfere with germination and seedling development, and also generate potentially harmful mutations (Kranner and Colville 2011). Oxidative damage triggered by Al is primarily thought to result from the production of H_2O_2 (Yamamoto et al. 2003). We therefore investigated the H_2O_2 content of Al treated seedlings compared to untreated controls, and observed a significant increase ($P < 0.05$) in both species at the highest Al concentration and also at 200 µM in *P. algarbiensis* (Figure 5.3 A). H_2O_2 accumulation in response to Al loading has been observed in other plants (Pereira et al. 2010; Xu et al. 2011) and the resulting oxidative stress may be responsible for the inhibition of root growth (Zhang et al. 2010; Xu et al. 2011) as we observed in our experiments (Figure 5.2).

Membrane lipids and proteins are especially vulnerable to ROS, therefore damaged membranes are considered to be reliable indicators of cell death caused by oxidative stress in plants (Halliwell and Gutteridge 1993). We investigated the potential link between Al loading, the inhibition of root growth, ROS production and cell death by measuring plasma membrane integrity. The azo dye Evans blue is not taken up by living cells because it cannot cross functional lipid membranes, but it can leak through ruptured membranes and is therefore a sensitive indicator of membrane integrity and cell death (Xu et al. 2011; Zelinová et al. 2011). We found no differences in dye uptake between Al treated roots and controls ($P \geq 0.05$) suggesting that the plasma membrane was not damaged to a significant extent by the increased production of ROS in either species (Figure 5.1 C; Table 5.2). These data suggest that ROS production does not

inhibit root growth by damaging membranes, although it is possible that other targets including nucleic acids and cytosolic proteins may be involved (Gill and Tuteja 2010).

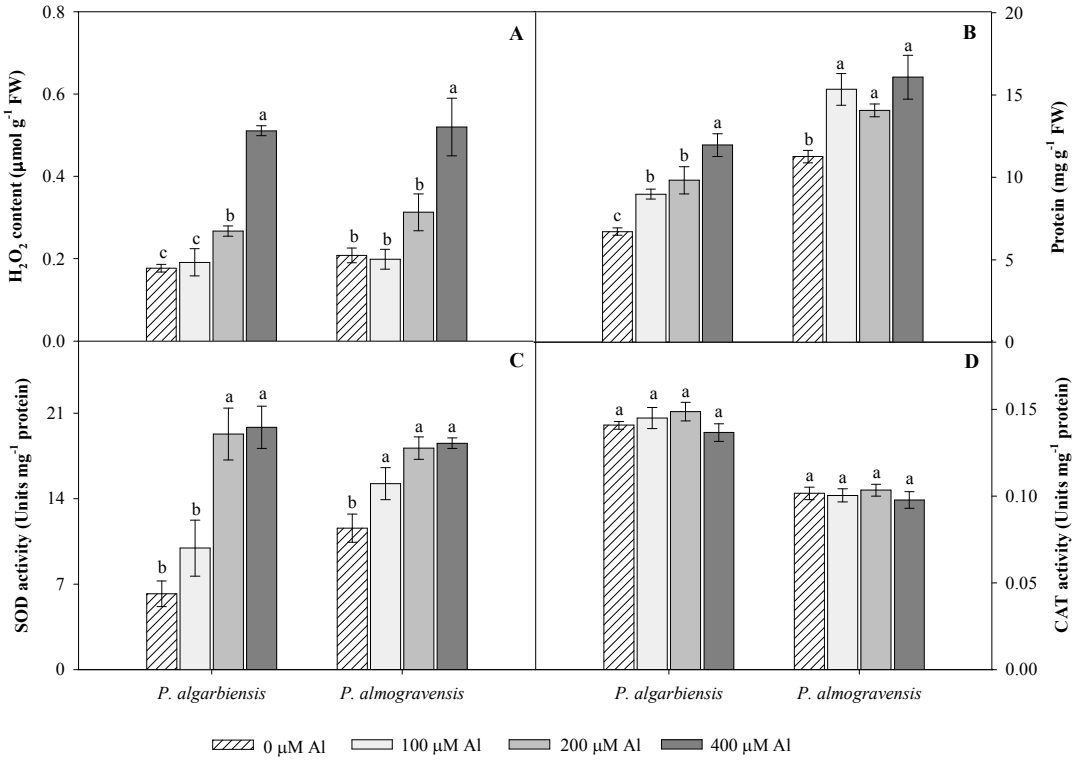

Figure 5.3. Effect of Al on H_2O_2 content (A), protein content (B), SOD activity (C), and CAT activity (D) in *Plantago algarbiensis* and *P. almogravensis* seedlings. Values are expressed as the mean ± SE (n = 5). For each species, mean values are significantly different at $P < 0.05$, according to Duncan's test.

Several studies have shown that photosynthetic pigment levels decline in plants exposed to Al (Yadav and Mohanpuria 2009; Pereira et al. 2010). We observed no significant differences ($P \geq 0.05$) in the total chlorophyll content when we compared Al treated plants and controls (Table 5.3) suggesting that Al does not interfere with early photosynthetic activity in these species, but we observed a significant ($P < 0.05$) increase in the carotenoid content of *P. almogravensis* seedlings (Table 5.3) suggesting

that the production of antioxidant pigments may be induced to prevent oxidative damage (Larson 1988).

Table 5.3. Effect of Al on the pigment content of *Plantago algarbiensis* and *P. almogravensis* seedlings.

Al (μM)	Total chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
P. algarbiensis		
0	0.51 ± 0.04	0.18 ± 0.01
100	0.50 ± 0.02	0.15 ± 0.01
200	0.54 ± 0.04	0.18 ± 0.01
400	0.52 ± 0.03	0.17 ± 0.00
P. almogravensis		
0	0.58 ± 0.02	0.17 ± 0.00 b
100	0.57 ± 0.03	0.20 ± 0.00 a
200	0.56 ± 0.03	0.21 ± 0.01 a
400	0.58 ± 0.01	0.21 ± 0.01 a

Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test and the absence of letters indicates that no significant differences were observed.

The soluble protein content of plants is an important indicator of metabolic changes which responds to a wide variety of stresses (Singh and Tewari 2003) and predicts the physiological status of seedlings (Nataraj and Parmar 2008). We observed a significant increase in protein content ($P < 0.05$) in the seedlings of both species in response to Al loading (Figure 5.3 B). This is likely to reflect the *de novo* synthesis of stress-induced proteins that mediate the adaptive physiological mechanisms of metal tolerance (Verma and Dubey 2003). Al may also be detoxified through the formation of complexes with cytosolic Al-binding proteins (Basu et al. 1999).

The oxidative damage caused by ROS can be prevented by the induction of both enzymatic and non-enzymatic antioxidant systems in plants (Apel and Hirt 2004). These systems are thought to play a substantial role during the germination and development of seedlings exposed to metals (Kranner and Colville 2011), although the precise roles of antioxidant enzymes in Al tolerance have not been investigated in detail. We therefore studied the activities of two pivotal antioxidant enzymes, namely SOD and CAT. SOD represents the first step in the detoxifying process, catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2, whereas CAT converts H_2O_2 into harmless oxygen and water. The complementary activities of SOD and CAT are therefore necessary to mitigate oxidative stress caused by superoxides (Benavides et al. 2005).

We measured the SOD (Figure 5.3 C) and CAT (Figure 5.3 D) activities in Al treated *P. algarbiensis* and *P. almogravensis* seedlings and compared the values to control seedlings growing without Al. In *P. almogravensis* seedlings, there was a significant increase in SOD activity ($P < 0.05$) at all three Al concentrations, whereas in *P. algarbiensis* the significant increase was observed at the two highest concentrations (Figure 5.3 C). Conversely, Al had no impact ($P \geq 0.05$) on CAT activity in either species (Figure 5.3 D). This suggests that the increase in H_2O_2 generation observed in *P. algarbiensis* and *P. almogravensis* seedlings exposed to Al reflects a parallel increase in SOD activity but the maintenance of CAT activity at normal levels.

In conclusion, our study describes for the first time the effect of Al on the germination and early development of *P. algarbiensis* and *P. almogravensis* seedlings. Al at concentrations up to 400 μM had no effect on the germination percentage or the overall morphology of the seedlings, but there was a quantitative impact on relative root growth that was more severe in *P. algarbiensis* than *P. almogravensis*. We found that the concentration-dependent inhibition of root growth was associated with a quantitative

increase in H_2O_2 levels resulting from the induction of SOD with no compensatory increase in the level of CAT activity. However, the higher levels of H_2O_2 were not sufficient to induce membrane damage indicating that the inhibition of root growth reflects the impact of oxidative stress on intracellular targets.

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CHAPTER 6

Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress

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Martins N, Gonçalves S, Romano A (2013) Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress. Biologia Plantarum 57:325-331

6.1. Abstract

We investigated the impact of low pH and aluminum (Al) on the metabolism and capacity for Al accumulation in shoots of the plantain species *Plantago algarbiensis* and *P. almogravensis*. We found that increasing the concentration of Al in the medium increased accumulation of it in the shoots of both plants (although more in *P. almogravensis* than in *P. algarbiensis*). The presence of Al in the medium induced proline and carbohydrate synthesis in *P. almogravensis* without affecting lipid peroxidation, but increased proline synthesis and lipid peroxidation in *P. algarbiensis* without affecting the carbohydrate content. Lipid peroxidation in *P. algarbiensis* was also enhanced at pH 4.0. The activity of antioxidant enzymes was increased as a response to low pH and Al in both species. Our data indicate that both species can accumulate high levels of Al but they have different sensitivities to low pH and/or the presence of Al in the growth medium.

6.2. Introduction

Aluminum (Al) is one of the most abundant elements in the earth's crust and at pH values below 5.5 becomes soluble in a phytotoxic form (predominantly Al^{3+}) that inhibits plant growth (Kochian et al. 2004; Xu et al. 2010). Anthropogenic activity is leading to the progressive acidification of environments, and acidic soils that currently account for ~30% of the global ice-free land area are gradually expanding (von Uexküll and Mutert 1995). Low pH stress (proton toxicity) and Al toxicity are therefore already becoming important constraints to crop productivity in several countries.

Some plant species have evolved strategies to reduce the impact of Al toxicity and can therefore grow well in acidic, Al-rich soils and accumulate high content of Al in their shoots (Kochian et al. 2004). Plants may be classified as Al hyperaccumulators if at least 1 mg Al g^{-1} dry weight can be stored in the shoots (Foy 1984; Baker and Brooks 1989). Wild plants are often more tolerant than crops towards environmental stresses (Mittova et al. 2004), and this includes low pH stress and excess Al (Ezaki et al. 2008; Olivares et al. 2009). Therefore, wild plants may be useful sources of Al/low pH tolerance genes (Ezaki et al. 2008).

In this context, we investigated the ability of two *Plantago* species (plantains) to grow under low pH and presence of Al as a first step towards determining the mechanisms of stress tolerance. *Plantago almogravensis* is an endemic plantain species that grows along the southwest coast of Portugal. It grows in podzolic soils with high content of iron and Al, and is considered as Al hyperaccumulator (Buurman and Jongmans 2002, Branquinho et al. 2007). *Plantago algarbiensis* is an endemic plantain species from the West-Central Algarve region. It grows in clay-rich soils preferably downstream from small springs or clearings containing acidophilic brushes, and its response to Al is unknown. Since both species belongs to endangered ones, we established *in vitro*

cultures (Gonçalves et al. 2009) and have recently demonstrated that they can grow in medium with low pH values (Martins et al. 2011). *In vitro* cultures have previously been used to select Al-tolerant species and to understand the tolerance mechanisms (Barnabas et al. 2000; Wen et al. 2009; Tabaldi et al. 2011). We therefore set out to evaluate the capacity of each species to accumulate Al, and the effects of low pH and Al^{3+} on *in vitro* shoots in terms of: 1) the degree of membrane damage through lipid peroxidation, 2) proline and carbohydrate accumulation and 3) the activity of antioxidant enzymes.

6.3. Materials and methods

6.3.1. Plant material and growth conditions

Plantago algarbiensis Samp. shoots (~6 cm in length) and *Plantago almogravensis* Franco shoots (~3 cm in length) were separated from *in vitro* cultures proliferating in MS medium (Murashige and Skoog 1962) containing 0.2 mg l^{-1} 6-benzyladenine as described by Gonçalves et al. (2009). The cultures were maintained at 25 ± 2 ºC and a 16 h photoperiod (cool white fluorescent lamps, irradiance of 69 µmol m^{-2} s^{-1}).

6.3.2. Stress treatments

Low pH stress was applied by transferring shoots to ¼MS liquid medium with the pH adjusted to 4.00 (stress) or 5.75 (control). Al stress was applied by transferring shoots to ¼MS liquid medium containing 100, 200 or 400 µM $AlCl_3$, which dissociates to form Al^{3+} ions when the pH is adjusted to 4.00. Shoots were inoculated individually in test tubes (32 × 200 mm) containing 20 ml of liquid medium on filter paper bridges, and incubated under the conditions described above.

6.3.3. Determination of Al content

Shoots were cultured for 7 days under stress or control conditions and then oven dried at 65 ºC until they reached a constant dry weight (DW). The dried material was ground, burned to ash in a muffle furnace at 500 ºC and acid digested. The Al content was then determined according to the aluminon method described by Kerven et al. (1989) using a commercial kit and a DR 2800 spectrophotometer (both provided by Hach LANGE, Düsseldorf, Germany). The accuracy of the results was verified against reference beech leaves BCR-100.

6.3.4. Measurement of lipid peroxidation

The lipid peroxidation was measured by determining the amount of malondialdehyde (MDA) available to react with 2-thiobarbituric acid (TBA) (Hodges et al. 1999). Fresh plant material was homogenized with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 5 min. The supernatant was added to either 20% (w/v) TCA (-TBA solution) or 0.5% (w/v) TBA in 20% (w/v) TCA (+TBA solution) and incubated at 95 ºC for 30 min. The reaction was quenched on ice and the samples were centrifuged at 3,000 *g* for 10 min. The absorbance of the supernatant was measured at 532, 600 and 440 nm and MDA equivalents were calculated as described by Hodges et al. (1999).

6.3.5. Measurement of proline and carbohydrate levels

Harvested shoots were exhaustively extracted with 80% (v/v) ethanol. The content of free proline was determined using the ninhydrin method (Troll and Lindsley 1955) as modified by Magné and Larher (1992). The extract was added to 1% ninhydrin (w/v, in 60% acetic acid) and the mixture was boiled for 1 h. The resulting chromogen was

extracted with toluene and the free proline was quantified at 520 nm against a set of proline standards (0 - 750 μM). The carbohydrate content was determined using the anthrone method (Yemm and Willis 1954). The sample was mixed with 75% (v/v) sulphuric acid containing 0.01 M anthrone reagent and was boiled until the reaction was complete. The resulting 5-(hydroxymethyl)furfural/anthrone complex was quantified at 578 nm against a set of glucose standards (0 - 500 μM) and the results were expressed as glucose equivalents.

6.3.6. Enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (GPX; EC 1.11.1.7) and ascorbate peroxidase (APX; EC 1.11.1.11) activities were evaluated after shoots were cultured for 1, 2 and 7 days. Fresh shoot tissue (100 mg) was homogenized with a pre-chilled mortar and pestle in 50 mM sodium phosphate buffer (pH 7) supplemented with 0.1 mM ethylenediaminetetraacetic acid, 1% (w/v) polyvinylpolypyrrolidone and 2.5 mM dithiothreitol (and 5 mM ascorbate in the case of APX). The homogenate was centrifuged at 20,000 *g* for 10 min at 4 ºC and the supernatant was used for subsequent enzymes assays. The specific enzyme activity for all enzymes was expressed as enzyme units per mg of protein, based on the measurement of total soluble protein content according to the method of Bradford (1976) using bovine serum albumin as a standard.

SOD activity was determined by the reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The photo-reduction of NBT was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. CAT activity was determined by the degradation of H_2O_2 using the method of Aebi (1983). The H_2O_2 decomposition was monitored at

240 nm (ε = 39.4 mM^{-1} cm^{-1}). One unit of CAT was defined as the amount of enzyme required to degrade 1 µmol H_2O_2 per min. GPX activity was measured by the modification of guaiacol as described by Egley et al. (1983). The tetraguaiacol formation was measured at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). One unit of GPX was defined as the amount of enzyme required to produce 1 µmol tetraguaiacol per min. APX activity was determined by the oxidation of ascorbate as described by Nakano and Asada (1981). The H_2O_2-dependent oxidation of ascorbate was monitored at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). One unit of APX was defined as the amount of enzyme required to degrade 1 µmol of ascorbate per min.

6.3.7. Statistical analysis

Five shoots were used in all the experiments and the values obtained were expressed as means ± standard errors. We carried out a one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 19.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test.

6.4. Results

Neither species accumulated detectable levels of Al in the shoots when cultivated on control MS medium without Al. However, both species accumulated large amounts of Al (1.0 - 4.5 mg g^{-1} DW) when cultivated in MS medium supplemented with Al and there was a positive correlation between the accumulation of Al *in planta* and the concentration of Al^{3+} in the medium (Figure 6.1). *P. almogravensis* shoots showed a tendency to accumulate more Al than *P. algarbiensis* shoots, although this only became

statistically significant ($P < 0.05$) in the medium containing 200 μM Al. The biomass increment after 7 days of culture in all the tested media was negligible in both *Plantago* species and, thus, it was not possible to evaluate the effect of low pH and Al on shoot growth.

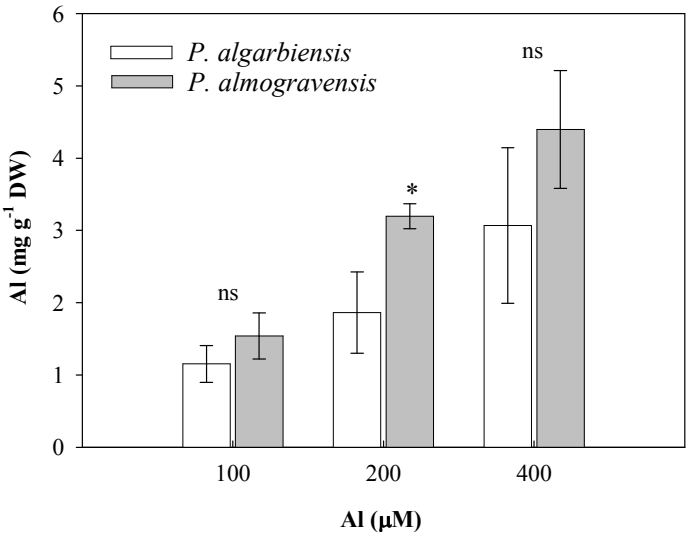

Figure 6.1. Al accumulation in *Plantago algarbiensis* and *P. almogravensis* shoots after 7 days of culture in medium containing different concentrations of $AlCl_3$ (100, 200 or 400 μM). Values are expressed as the mean ± SE (n = 5). * indicates a significant difference between species ($P < 0.05$), whereas ns indicates that the difference between species is not significant.

The lipid peroxidation (expressed as the content of MDA) in *P. algarbiensis* shoots was higher ($P < 0.05$) in the low pH medium with or without Al than in the control medium at pH 5.75 (Table 6.1). The MDA content was also affected by Al. Shoots cultivated in medium containing 100 μM Al showed lower lipid peroxidation than those cultivated in the absence of Al, but more severe lipid peroxidation occurred when the amount of Al in the medium further increased. In contrast, neither low pH stress nor the presence of Al had a significant ($P \geq 0.05$) impact on lipid peroxidation in *P. almogravensis* (Table 6.1).

In comparison to shoots cultured in the low pH medium lacking Al, there was a significant increase ($P < 0.05$) in the proline content of *P. algarbiensis* shoots cultured in medium containing 100 and 400 μM Al but no significant change ($P \geq 0.05$) in carbohydrate content regardless the Al concentration (Table 6.1). In contrast, *P. almogravensis* shoots cultured in medium containing 200 and 400 μM Al contained higher content ($P < 0.05$) of both proline and carbohydrates compared to shoots cultivated in the absence of Al (Table 6.1).

Table 6.1. Effect of low pH and Al on the contents of MDA, proline and carbohydrates in *Plantago algarbiensis* and *P. almogravensis* shoots after 7 days in culture.

pH	Al (μM)	MDA (nmol g^{-1} FW)	Proline (μmol g^{-1} FW)	Carbohydrates (μmol g^{-1} FW)
P. algarbiensis				
5.75	0.0	22.06 ± 2.72 c	0.43 ± 0.03 ab	107.18 ± 20.83 a
4.00	0.0	76.48 ± 4.36 a	0.31 ± 0.04 b	91.29 ± 9.76 a
4.00	100	55.13 ± 7.27 b	0.58 ± 0.07 a	117.13 ± 14.60 a
4.00	200	73.06 ± 7.25 ab	0.46 ± 0.03 ab	114.75 ± 7.99 a
4.00	400	82.55 ± 6.22 a	0.48 ± 0.06 a	113.33 ± 10.02 a
P. almogravensis				
5.75	0.0	48.50 ± 3.25 a	0.38 ± 0.09 b	39.33 ± 6.36 b
4.00	0.0	42.65 ± 7.05 a	0.51 ± 0.06 b	52.68 ± 1.98 b
4.00	100	42.60 ± 6.55 a	0.44 ± 0.06 b	48.75 ± 4.12 b
4.00	200	54.76 ± 2.76 a	0.80 ± 0.08 a	80.47 ± 6.53 a
4.00	400	53.13 ± 3.63 a	0. 87 ± 0.10 a	79.51 ± 1.78 a

Values are expressed as the mean ± SE (n = 5). Mean values with common letters are not significantly different at $P \geq 0.05$, according to Duncan's test. Carbohydrate content is expressed as μmol of glucose equivalents.

We observed a significant reduction ($P < 0.05$) in the protein content of *P. algarbiensis* shoots grown for 1 and 7 days in low pH medium either without Al or containing the highest test concentration, 400 µM Al (Figure 6.2 A). The same pattern was observed after 7 days in *P. almogravensis* shoots (Figure 6.2 B).

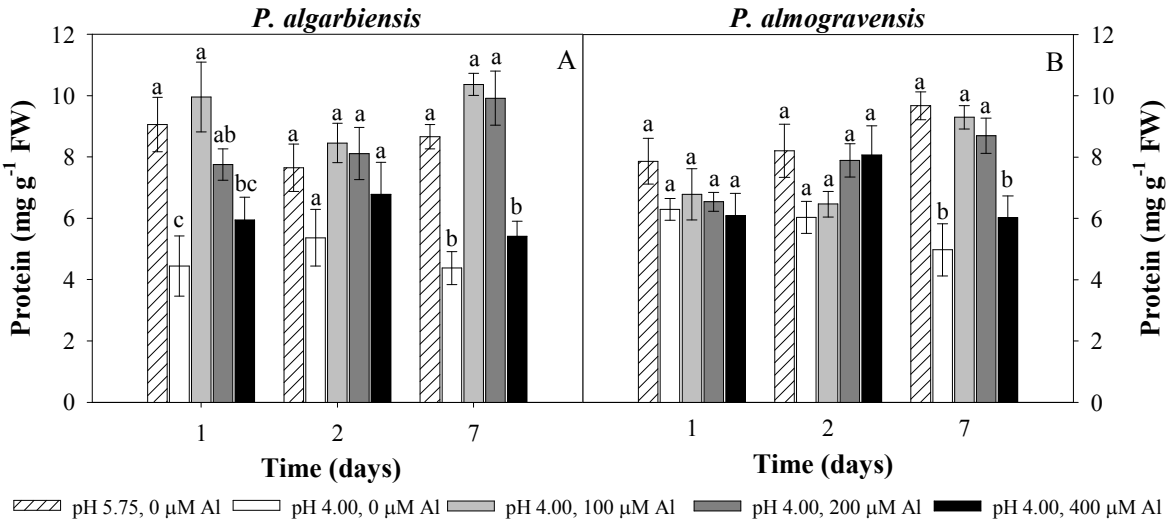

Figure 6.2. Effect of low pH and Al on the protein content of *Plantago algarbiensis* (A) and *P. almogravensis* (B) shoots after 1, 2 and 7 days of culture. Values are expressed as the mean ± SE (n = 5). Mean values followed by the same letter are not significantly different at $P \geq 0.05$.

We also observed significant changes in the activities of antioxidant enzymes in both species in response to low pH and Al stress (Figure 6.3). After cultivation for 1 or 2 days in the low pH medium with or without Al, *P. algarbiensis* shoots generally showed a three-fold increase in SOD activity compared to shoots growing at pH 5.75 (Figure 6.3 A), although after 2 days in medium containing 400 µM Al the SOD activity was no higher ($P \geq 0.05$) than in the control. There was also a significant increase ($P < 0.05$) in SOD activity in *P. almogravensis* shoots after cultivation for 1 day in low pH medium without Al or containing 200 µM Al (Figure 6.3 B). SOD activity also increased ($P < 0.05$) after cultivation for 7 days in low pH medium lacking Al.

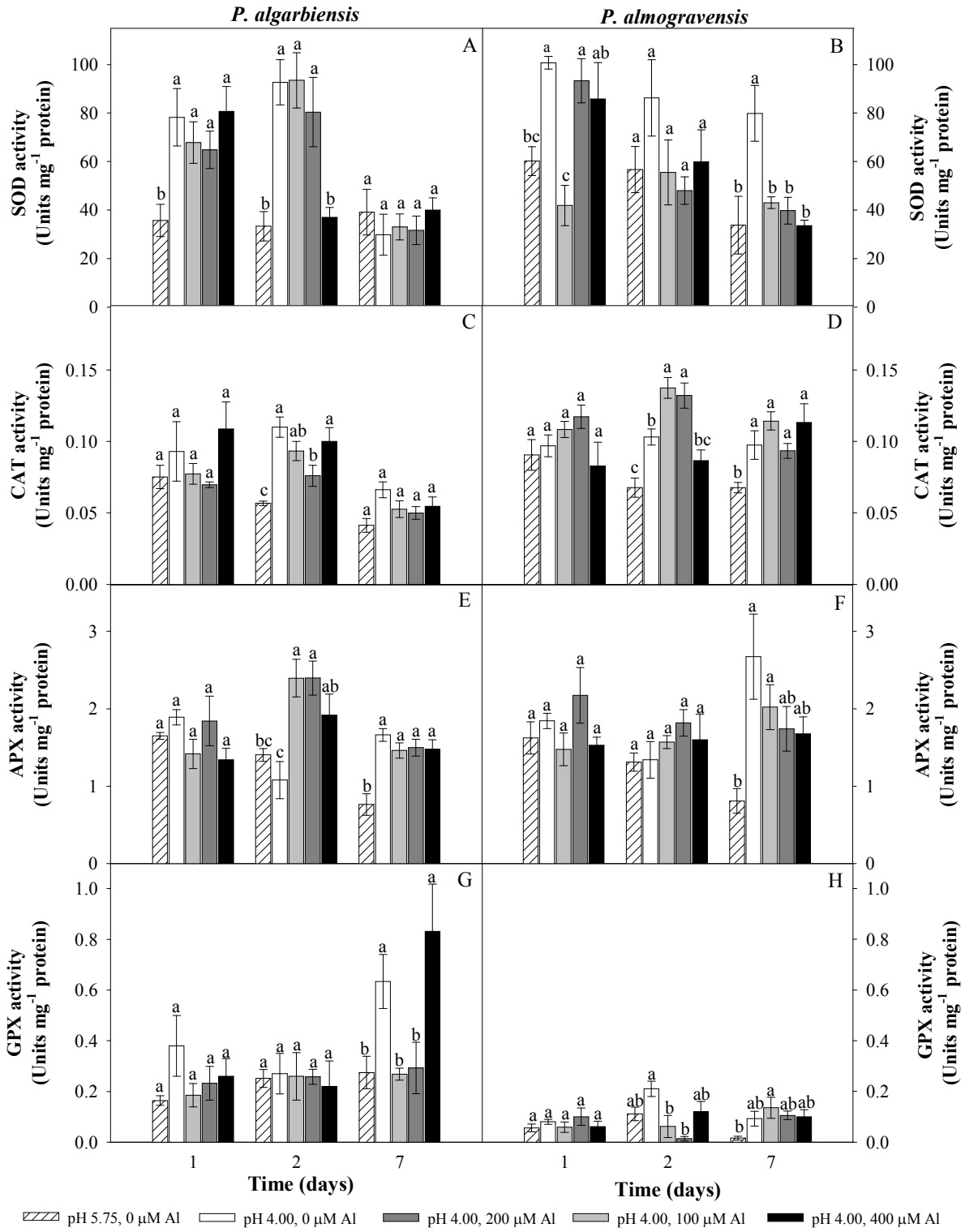

Figure 6.3. Effect of low pH and Al on the SOD (A, B), CAT (C, D), APX (E, F) and GPX (G, H) activities of *Plantago algarbiensis* and *P. almogravensis* shoots, after 1, 2 and 7 days of culture. Values are expressed as the mean ± SE (n = 5). Mean values followed by the same letter are not significantly different at $P \geq 0.05$.

We observed a two-fold increase in CAT activity when *P. algarbiensis* shoots were cultivated for 2 days in the low pH medium with or without Al (Figure 6.3 C). Similar results were observed for *P. almogravensis* shoots, although in this species the CAT activity did not increase in shoots cultivated in the low pH medium supplemented with 400 μM Al until 7 days of cultivation had passed (Figure 6.3 D).

We also observed a two-fold increase in APX activity in *P. algarbiensis* shoots cultivated for 2 days in low pH media containing 100 and 200 μM Al, and after 7 days in all the low pH media regardless of Al content (Figure 6.3 E). In *P. almogravensis*, a significant ($P < 0.05$) increase in APX activity occurred only after 7 days and only in the low pH media with no Al and 100 μM Al (Figure 6.3 F).

GPX activity in *P. algarbiensis* shoots remained low for the first few days of cultivation but showed a significant ($P < 0.05$) increase after 7 days in the low pH medium without Al or in the presence of the highest Al concentration (Figure 6.3 G). In *P. almogravensis* shoots, there was a significant increase ($P < 0.05$) in GPX activity after 7 days of cultivation in medium containing the lowest Al concentration (Figure 6.3 H).

6.5. Discussion

We studied the low pH and Al stress responses in two protected wild plantain species, *P. algarbiensis* and *P. almogravensis*, using *in vitro* shoot cultures. We found that both species were able to accumulate large amounts of Al without visible toxicity symptoms, in agreement with previous observations of Al accumulation in field-grown *P. almogravensis* plants (Branquinho et al. 2007).

One of the main symptoms of Al toxicity in plants is lipid peroxidation. The damaged membranes release MDA, which is therefore considered a good stress indicator (Liu et

al. 2008; Xu et al. 2012). We observed a striking difference in the content of MDA induced by low pH and Al stress when the two *Plantago* species were compared (Table 6.1). There was no significant change in MDA content when *P. almogravensis* shoots were exposed to low pH with or without Al, suggesting this species was protected from oxidative stress and the resulting membrane damage. In contrast, there was a significant increase in MDA content when *P. algarbiensis* shoots were exposed to low pH with or without Al, suggesting this species is susceptible to this stress. In a previous work we also observed an increase in the content of lipid peroxidation in shoots of *P. algarbiensis* grown for 6 weeks in medium with low pH (Martins et al. 2011). There was less membrane damage in *P. algarbiensis* at the lowest Al concentration that has also been reported in other species (Andersson and Brunet 1993; Kinraide 1993). The protective mechanism is unclear, but the trivalent Al^{3+} ion may stabilize the membrane by reducing the surface negative charge, reducing the interaction with protons and therefore reducing exosmosis (Kinraide 1993; Liu et al. 2003).

Carbohydrates and proline are scavengers of reactive oxygen species, helping to prevent the oxidative damage that results from many forms of abiotic stress (Smirnoff 1998). In accordance with our previous results (Martins et al. 2011) we observed that the proline and carbohydrate content was not affected by the reduction of medium pH in both *Plantago* species. Nevertheless, the proline content of *P. algarbiensis* shoots increased in the low pH medium containing 100 and 400 μM Al compared to the same without Al, whereas the proline content of *P. almogravensis* shoots increased when the shoots were cultured in media with the highest Al concentrations. These results corroborated the role of proline on the protection of plants against Al stress (Khan et al. 2000; Yadav and Mohanpuria 2009; Giannakoula et al. 2010). The carbohydrate content of *P. almogravensis* shoots exposed to the two highest Al concentrations was higher than

that of shoots cultivated in the low pH medium without Al (Table 6.1). The modulation of carbohydrate metabolism in response to Al stress has been reported previously (Khan et al. 2000; Giannakoula et al. 2010).

Al toxicity can also induce changes in protein synthesis (Delhaize and Ryan 1995) and protein degradation by proteolytic enzymes (Basu et al. 1994). We observed a significant reduction in total protein content in the shoots of both species in consequence of low pH with or without Al (Figure 6.2).

The oxidative stress caused by Al ions induces a well characterized response pathway that includes the production of antioxidant enzymes (Shamsi et al. 2008; Xu et al. 2012). However, little is known about the induction of antioxidants in response to low pH. Our data suggest that SOD, CAT, APX and GPX are induced by both Al and low pH. The most significant induction of all four enzymes occurs when shoots growing in the low pH medium are compared to those growing at pH 5.75. Again, it appears that the presence of both Al^{3+} and H^{+} reduces the overall stress, suggesting that each ion alleviates the toxicity of the other to a certain extent.

We conclude that both *Plantago* species can accumulate high content of Al, but that there are differences in terms of sensitivity to low pH and/or Al and in the specific responses. In *P. algarbiensis*, the high content of proline and antioxidant enzyme activity induced by stress were not sufficient to suppress oxidative membrane damage whereas no damage was detected in *P. almogravensis* suggesting this is the more stress-tolerant of the two species.

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CHAPTER 7

Physiological responses of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets to low pH and aluminum stress

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Martins N, Osório ML, Gonçalves S, Osório J, Palma T, Romano A (2013) Physiological responses of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets to low pH and aluminum stress. Acta Physiologiae Plantarum 35:615-625

7.1. Abstract

We investigated the impact of low pH and aluminum (Al) stress on the growth, nutrients concentration, chlorophyll *a* fluorescence, photosynthetic pigment contents, proline and carbohydrate accumulation in shoots and plantlets (leaves and roots) of *Plantago almogravensis* and *P. algarbiensis*. Both species accumulated considerable and similar amounts of Al in their tissues, mainly in the roots. The presence of Al caused a significant reduction on root elongation in *P. algarbiensis*. Low pH and Al induced significant changes on nutrient accumulation, but no significant alterations on the maximum efficiency of PSII (F_v/F_m), quantum yield of PSII photochemistry (ϕ_{PSII}), quantum yield of regulated energy dissipation (ϕ_{NPQ}) and quantum yield of non-regulated energy dissipation (ϕ_{NO}) were detected in both species in response to these stresses. However, Al increased significantly the non-photochemical quenching (NPQ) and the chlorophyll *b* content and decreased the PSII excitation pressure ($1 - q_p$) in *P. almogravensis* leaves. Both stress treatments induced carbohydrate accumulation in the shoots and roots of this species, but not in leaves. In *P. algarbiensis*, low pH and Al decreased the photosynthetic pigment contents in the shoots, whereas Al stimulated the carbohydrate accumulation in the leaves. Although our data showed that both species are tolerant to Al^{3+} and H^+, *P. almogravensis* appeared to be more adapted to maintain cellular physiology and growth under those conditions.

7.2. Introduction

Soil acidity is a major growth-limiting factor for plants in many parts of the world (Foy 1984). It comprises 30 - 40% of the world's arable lands and is an increasing problem due to several anthropogenic factors (von Uexküll and Mutert 1995). Multiple abiotic stress factors contribute to phytotoxicity in these soils, but aluminum (Al) is the main cause of toxicity because at pH below 5.5 it is solubilized into the toxic species Al^{3+} (Matsumoto 2000). Much attention has been given to Al^{3+} toxicity, but not much is known about how plants cope with high H^+ ions concentration. However, the evaluation of low pH effects is also important for a greater understanding and correct interpretation of Al toxicity (Samac and Tesfaye 2003).

Al and low pH stresses are highly correlated with poor plant growth and biomass reduction (Moustakas et al. 1995; Chen et al. 2005a, 2010), revealing possibly alterations in photosynthesis. Inhibition of CO_2 assimilation rate due to Al toxicity was reported to distinct plant species (Pereira et al. 2000; Chen et al. 2005b; Zhang et al. 2007; Jiang et al. 2008; Jin et al. 2011; Silva et al. 2012). However, the nature of limitation of photosynthesis (stomatal or non-stomatal) by Al is still debated. It is generally assumed that the decrease in photosynthesis in response to Al is due to direct effects on the capacity of photosynthetic apparatus rather than stomatal closure. Several reports have shown that photosystem II (PSII) is the most vulnerable component of the photosynthetic apparatus to this stress. An exposure to Al can induce damage to PSII (Zhang et al. 2007; Reyes-Dias et al. 2010), decrease in electron transport rate through PSII and increase in closure of PSII reaction centers (Moustakas et al. 1995; Chen et al. 2005b). As chlorophyll *a* fluorescence emitted from chloroplast thylakoid membranes reflects the primary process of photosynthesis, its changes can be utilized as an early and non-destructive sign of stress injury. Since stressors modify the partitioning of

absorbed light energy on leaves, assessment of the contribution of different pathways for energy utilisation/dissipation in PSII complexes is of huge importance for analysis of the photosynthetic apparatus (Kornyeyev and Hendrickson 2007). A few years ago, Kramer et al. (2004) developed new fluorescence parameters to examine the partitioning of absorbed light energy into three fractions which add up to unity: (i) utilised by PSII photochemistry (ϕ_{PSII}), (ii) thermally dissipated via ΔpH and xanthophyll-dependent energy quenching (ϕ_{NPQ}) and (iii) absorbed light going neither to ϕ_{PSII} nor ϕ_{NPQ} i.e. non-regulated energy dissipation (ϕ_{NO}). The values of these quantum yields allow deep insights into the plant's capacity to cope with excess excitation energy. The ϕ_{PSII} and ϕ_{NPQ} pathways work as protective mechanisms whereas ϕ_{NO} can be regarded as excess energy. Therefore, the use of PSII fluorescence emission has been considered a sensitive and reliable method for detection and quantification of Al induced damages to the PSII (Pereira et al. 2000; Jiang et al. 2008). In addition, Al can also indirectly affect photosynthetic activity, by decreasing the content of photosynthetic pigments (Jiang et al. 2008), reducing the absorption and accumulation of nutrients essential for chlorophyll synthesis and chloroplast assembly and maintenance (Mihailovic et al. 2008) and increasing the carbohydrates accumulation (Liu et al. 2006).

A thorough understanding of Al toxicity and associated tolerance mechanisms is crucial for developing appropriate tests for selecting tolerant germplasm and for developing plants with enhanced performance in acid soils (Samac and Tesfaye 2003). Wild plants growing vigorously on acid soils with high amounts of Al evolved important physiological mechanisms to reduce the impact of proton and Al toxicity and therefore provide good materials to better understand the Al tolerance (Ezaki et al. 2008).

Plantago almogravensis Franco is an endemic plantain species that grows along the southwest coast of Portugal and is considered an Al hyperaccumulator (Branquinho et

al. 2007). Serrano et al. (2011) had reported that *P. almogravensis* is the only known representative of the Plantaginaceae with this trait, but recent results of our group showed that also *Plantago algarbiensis* Samp., another endemic plantain species from Portugal (West-Central Algarve region), accumulates high amounts of Al (unpublished data). However their tolerance to Al and H^+ stress remains unclear. Since both species are protected under the European Habitats Directive and by Portuguese law, we established micropropagation protocols for these species (Gonçalves et al. 2009) to have enough number of plants. We have showed that they can grow in medium with pH values much lower than those typically used in tissue culture (Martins et al. 2011). Moreover we have also proved the capacity of *in vitro* produced shoots and plantlets of *P. algarbiensis* and *P. almogravensis* to accumulate Al.

This work aims to evaluate the impact of both low pH and Al stress on photosynthetic performance, growth, nutrients concentration, photosynthetic pigment contents, proline and carbohydrate accumulation in shoots and plantlets of these two plantain species. Specifically, we have addressed the following key research questions: (i) Is *P. algarbiensis* and *P. almogravensis* growth affected by low pH and Al? (ii) Is there any impact on photosynthetic apparatus? (ii) Are both species resistant to these stresses and if so, which are the protection mechanisms?

7.3. Materials and methods

7.3.1. Plant material and growth conditions

P. algarbiensis shoots (~6 cm in length) and *P. almogravensis* shoots (~3 cm in length) were separated from *in vitro* cultures proliferating for 6 weeks in MS medium (Murashige and Skoog 1962) containing 0.2 mg l^{-1} 6-benzyladenine as described by Gonçalves et al. (2009). Plantlets were obtained by cultivating the shoots for 3 weeks in

rooting medium (½MS containing 0.5 mg l^{-1} indole-3-acetic acid). The shoots and plantlets were maintained at 25 ± 2 ºC with a 16 h photoperiod (cool white fluorescent lamps, 69 µmol m^{-2} s^{-1}).

7.3.2. Stress treatments

Low pH stress was applied by transferring shoots and plantlets to ¼MS liquid medium with the pH adjusted to 4.00 (stress) or 5.75 (control). Al stress was applied by transferring shoots and plantlets to ¼MS liquid medium with the pH adjusted to 4.00 containing 400 µM $AlCl_3$, that correspond to 140 µM Al^{3+} activity, as estimated by Geochem-EZ (Shaff et al. 2010). Shoots and plantlets were inoculated individually in 32 × 200 mm test tubes containing 20 ml liquid medium on filter paper bridges, and incubated for 7 days under the conditions described above.

7.3.3. Plant growth and relative water content

The length of shoots and plantlets (leaves and roots) were measured before and after treatments. Shoots and plantlets were then collected and dried at 63 ºC until they reached a constant weight and the dry weight (DW) determined. Plant water status was assessed by measuring relative water content (RWC) in five fresh leaves and calculated as 100 × (FW - DW)/(TW - DW), where FW is the fresh weight, TW is turgid weight (determined after submersing the petioles for 24 h in distilled water) and DW is the dry weight (determined after drying at 63 ºC until constant weight).

7.3.4. Determination of Al and nutrients contents

The Al, calcium (Ca), phosphorus (P), potassium (K) and magnesium (Mg) contents in plant tissues were determined by atomic absorption spectrophotometry (GBC, Avanta-

Sigma, USA) following standard methods (A.O.A.C. 1990). Samples were ground, dry-ashed at 500 ºC and digested in an acidic solution.

7.3.5. Chlorophyll fluorescence and photosynthetic pigments analysis

Chlorophyll (Chl) fluorescence was determined using a mini blue version of *Imaging*-PAM Chl fluorometer (IMAG-MIN/B, Walz, Effeltrich, Germany) in shoots and leaves after 20 min of darkness. To evaluate spatial and temporal heterogeneity four areas of interest (AOIs) were selected. Images of F_0 were obtained by applying measuring light pulses modulated at 1 Hz, while images of the maximal fluorescence yield (F_m) were obtained with the help of a saturating blue pulse (10,000 µmol m^{-2} s^{-1} PPFD) at 10 Hz and F_v/F_m were derived from that. Afterwards, actinic illumination [103 µmol (photon) m^{-2} s^{-1}] was switched on and saturating pulses were applied at 20 s intervals for 5 min in order to determine the maximum fluorescence yield (F_m') and the Chl fluorescence yield (F_s) during illumination. The actual PSII quantum efficiency in light-adapted leaves (ϕ_{PSII}) was calculated using the $(F_m' - F_s)/F_m' = (\Delta F/F_m')$ ratio (Genty et al. 1989). Since measurements were done under constant PPFD, ϕ_{PSII} and the electron transport rate (ETR) are equivalent. The photochemical quenching (q_p), was calculated as described by Bilger and Schreiber (1986) and PSII excitation pressure that reflects the proportion of the primary electron acceptor Q_A in the reduced state, is calculated as $1 - q_p$ (Demmig-Adams et al. 1990). The Stern-Volmer equation (Cornic 1994) was used to calculate non-photochemical quenching (NPQ), which is an estimate of the thermal energy dissipation at PSII. The quantum yields of regulated energy dissipation (ϕ_{NPQ}) and of non-regulated energy dissipation (ϕ_{NO}) in PSII were calculated by the equations $\phi_{NPQ} = 1 - \phi_{PSII} - 1/[NPQ + 1 + q_L (F_m/F_0 - 1)]$ and $\phi_{NO} = 1/[(NPQ + 1 + q_L) (F_m/F_0 - 1)]$, respectively (Kramer et al. 2004).

Photosynthetic pigments were extracted with 100% acetone from fresh plant material (25 mg) and the absorbance of the extract solutions was measured in a spectrophotometer at 661.6, 644.8 and 470 nm. Pigment contents were estimated as described by Lichtenthaler (1987).

7.3.6. Proline and carbohydrate content determinations

Shoots and plantlets (leaves and roots) were repeatedly extracted with 80% (v/v) ethanol. The concentration of free proline was determined using the ninhydrin method (Troll and Lindsley 1955) omitting phosphoric acid to avoid interference with concentrated sugars (Magné and Larher 1992). The reaction mixture containing the plant extract and 1% ninhydrin (w/v, in 60% acetic acid) was boiled for 1 h. After cooling, the resulting chromogen was extracted with toluene and the free proline in the organic phase was quantified by spectrophotometry at 520 nm against a set of proline standards (0 - 750 μM). The carbohydrate content was determined using the anthrone method (Dreywood 1946; Yemm and Willis 1954). The plant extract was added to 75% (v/v) sulphuric acid containing 0.01 M anthrone and the mixture was boiled for 15 min. The resulting 5-(hydroxymethyl)furfural/anthrone complex was quantified by spectrophotometry at 578 nm against a set of glucose standards (0 - 500 μM) and the results were expressed as glucose equivalents.

7.3.7. Statistical analysis

The plant growth parameters and the RWC were recorded using 15 replicates, the nutrients and Al contents were performed in 3 replicates, whereas each of the other parameters were analysed in 5 replicates. The values obtained were expressed as mean ± standard errors. We carried out a one-way analysis of variance (ANOVA) on

the results to assess treatment differences using the SPSS statistical package for Windows (release 19.0; SPSS Inc., Chicago, IL, USA). Where appropriate, significant differences between means were determined using Duncan's New Multiple Range Test. Differences were considered significant at $P < 0.05$.

7.4. Results and discussion

The MS medium does not contain any Al source, thus neither species accumulated detectable levels of this metal in the shoots and plantlets when cultivated in Al-unsupplemented media. However, shoots and plantlets of both *P. algarbiensis* and *P. almogravensis* accumulated considerable amounts of Al (120 - 1,200 μg g^{-1} DW) when cultivated in medium supplemented with Al (Figure 7.1). No significant differences ($P \geq 0.05$) between species were found for Al content in each of the three types of plant material analyzed (shoots, leaves and roots). However, in both species, shoots and plantlets leaves showed the lowest Al concentrations (120 - 220 μg g^{-1} DW), whereas the roots displayed higher ($P < 0.05$) concentrations (> 900 μg g^{-1} DW), suggesting the existence of a mechanism that immobilizes and sequesters Al preventing its translocation to the aboveground plant parts. The Al contents detected in plantlets produced *in vitro* were lower than the previously observed in field-grown plants of *P. almogravensis* (Branquinho et al. 2007) and *P. algarbiensis* (Martins et al. unpublished data). This is not surprising considering the differences between the controlled axenic *in vitro* conditions and the field conditions.

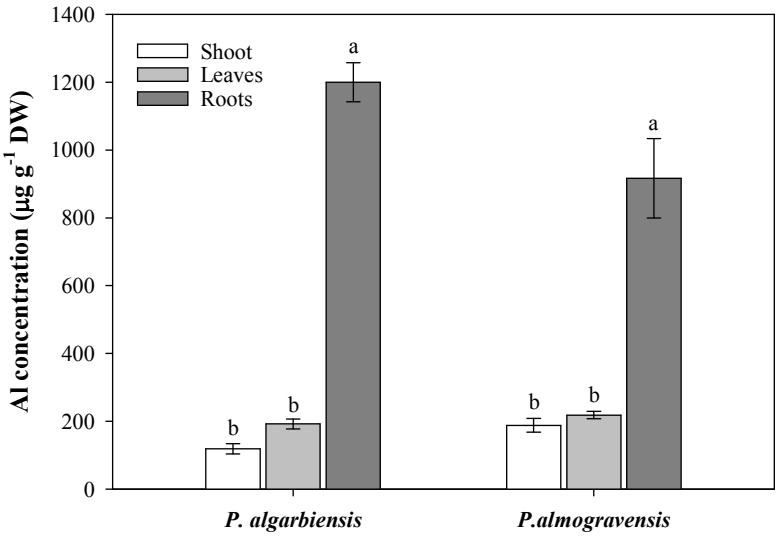

Figure 7.1. Aluminum accumulation in *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets (leaves and roots) after 7 days of culture in medium containing 400 µM Al. Values are expressed as the mean ± SE (n = 3). For each species, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test. No significant differences ($P \geq 0.05$) were observed between species in the same organ.

The effect of low pH and Al on *P. algarbiensis* and *P. almogravensis* growth was evaluated in terms of DW and elongation (Table 7.1). Neither of the stress treatments had a significant impact ($P \geq 0.05$) on the DW of both species, but a significant decrease ($P < 0.05$) of root elongation was observed in *P. algarbiensis* as a response to Al. Although the *P. algarbiensis* Al treated roots have their elongation inhibited, they were thicker and stubby enough compared with the roots cultured in medium without Al to result in similar DW. Root growth inhibition is the major effect of Al toxicity in plants and may lead to reduced absorption of water and mineral nutrients (Barceló and Poschenrieder 2002). However, the RWC was not affected ($P \geq 0.05$) by the stress treatments in both species (data not shown).

Table 7.1. Effect of low pH and Al on the elongation and DW of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets.

pH	Al (µM)	Elongation (mm)			DW (mg)		
		Shoot	Plantlet		Shoot	Plantlet	
			Leaves	Roots		Leaves	Roots
P. algarbiensis							
5.75	0	2.13 ± 0.38 a	11.33 ± 2.30 a	36.27 ± 2.75 a	60.81 ± 5.55 a	85.11 ± 8.88 a	11.51 ± 0.90 a
4.00	0	2.60 ± 0.65 a	8.13 ± 1.11 a	39.93 ± 1.91 a	65.17 ± 7.64 a	81.38 ± 9.50 a	13.28 ± 1.13 a
4.00	400	1.87 ± 0.29 a	9.27 ± 1.83 a	26.93 ± 1.60 b	51.24 ± 4.87 a	86.89 ± 5.97 a	10.94 ± 0.99 a
P. almogravensis							
5.75	0	2.07 ± 0.48 a	6.93 ± 0.77 a	24.67 ± 3.07 a	84.67 ± 7.35 a	131.37 ± 10.14 a	17.75 ± 2.08 a
4.00	0	2.67 ± 0.46 a	5.13 ± 0.72 a	26.60 ± 4.52 a	74.21 ± 8.65 a	118.59 ± 10.37 a	16.37 ± 1.08 a
4.00	400	1.73 ± 0.49 a	4.20 ± 0.88 a	21.27 ± 4.23 a	80.45 ± 5.94 a	125.44 ± 9.12 a	14.15 ± 1.38 a

Values are expressed as the mean ± SE (n = 15). For each species and in each column, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

We also investigated the effect of low pH and Al on the P, Mg, Ca and K contents in the shoots and plantlets of *P. algarbiensis* and *P. almogravensis* (Table 7.2). In shoots, low pH *per se* or in combination with Al induced a significant ($P < 0.05$) reduction in P in *P. algarbiensis* and in Ca and Mg in *P. almogravensis*. We observed that the *P. algarbiensis* plantlets were more affected by both stress treatments than the *P. almogravensis* plantlets indicating that the inhibition of root elongation found in *P. algarbiensis* may be associated with nutrient homeostasis break. In *P. algarbiensis*, low pH and Al increased ($P < 0.05$) Mg in both leaves and roots, and decreased P only in leaves. In this species, the Ca and K were enhanced ($P < 0.05$) in low pH treated leaves, whereas K was higher and Ca was lower in Al treated roots. In *P. almogravensis*, both treatments increased ($P < 0.05$) P in roots, the P and K were depressed ($P < 0.05$) as a response to low pH in leaves, while K enhanced ($P < 0.05$) in roots under Al.

Overall, nutrient accumulation in both *Plantago* species were markedly affected by low pH and Al, however, the response was quite variable. It is known that low pH and/or Al toxicity may lead to nutrient deficiencies in many plant species (Poschenrieder et al. 1995; Schroth et al. 2003) but, as in our results, a clear trend cannot be found (Silva et al. 2010; Ali et al. 2011). In addition, the nutrients studied have been reported as key nutrients involved in Al toxicity and the genotypes with the ability to maintain or increase the uptake of these nutrients had been considered tolerant (Chen et al. 2011; Choudhary and Singh 2011). Thus, the maintenance or increase of Ca, Mg, P and K contents observed in the *Plantago* species studied are in agreement with the fact that both species colonize acidic Al-rich soils.

Table 7.2. Effect of low pH and Al on the Ca, P, K and Mg contents (mg g^{-1} DW) of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets.

Nutrient	pH	Al (μM)	*P. algarbiensis*			*P. almogravensis*		
			Shoot	Plantlet		Shoot	Plantlet	
				Leaves	Roots		Leaves	Roots
Ca	5.75	0	3.67 ± 0.12 a	3.63 ± 0.03 b	2.80 ± 0.06 a	3.93 ± 0.03 a	3.87 ± 0.03 a	2.53 ± 0.03 a
	4.00	0	3.57 ± 0.03 a	3.87 ± 0.09 a	2.70 ± 0.06 a	3.57 ± 0.03 b	4.10 ± 0.12 a	2.77 ± 0.03 a
	4.00	400	3.33 ± 0.07 a	3.60 ± 0.06 b	2.07 ± 0.09 b	3.53 ± 0.03 b	4.03 ± 0.03 a	2.60 ± 0.10 a
P	5.75	0	5.00 ± 0.06 a	5.97 ± 0.03 a	8.10 ± 0.38 ab	3.00 ± 0.06 a	4.30 ± 0.06 a	6.10 ± 0.06 c
	4.00	0	4.03 ± 0.03 b	5.70 ± 0.12 b	7.53 ± 0.09 b	3.13 ± 0.03 a	3.77 ± 0.03 b	8.33 ± 0.20 a
	4.00	400	3.87 ± 0.07 b	5.50 ± 0.06 b	8.70 ± 0.10 a	3.03 ± 0.03 a	4.20 ± 0.00 a	7.37 ± 0.07 b
K	5.75	0	27.33 ± 0.52 a	24.60 ± 0.10 b	28.93 ± 0.29 b	27.33 ± 0.64 a	29.57 ± 0.41 a	32.30 ± 0.49 b
	4.00	0	27.70 ± 0.55 a	26.10 ± 0.10 a	27.23 ± 0.98 b	28.27 ± 0.43 a	27.83 ± 0.13 b	31.67 ± 0.58 b
	4.00	400	25.83 ± 0.82 a	24.60 ± 0.21 b	34.60 ± 1.21 a	28.73 ± 0.43 a	29.73 ± 0.34 a	37.60 ± 1.77 a
Mg	5.75	0	1.50 ± 0.00 a	1.40 ± 0.00 c	1.07 ± 0.03 b	1.67 ± 0.03 a	1.53 ± 0.03 a	1.17 ± 0.03 a
	4.00	0	1.53 ± 0.03 a	1.60 ± 0.00 a	1.20 ± 0.00 a	1.53 ± 0.03 b	1.47 ± 0.03 a	1.17 ± 0.03 a
	4.00	400	1.43 ± 0.03 a	1.50 ± 0.00 b	1.20 ± 0.00 a	1.40 ± 0.00 c	1.50 ± 0.00 a	1.20 ± 0.00 a

Values are expressed as the mean ± SE (n = 3). For each nutrient and in each column, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

Photosynthesis is a highly regulated and integrated process that aims to maximize the use of the light, optimize the use of carbon resources and minimize the damaging effects of excess energy (He et al. 2011). This important process is greatly sensitive and responsive to environmental stresses, including Al (Ali et al. 2008; Jiang et al. 2008). Thus, the effect of low pH and Al on the PSII photochemistry was evaluated through Chl fluorescence parameters in the shoots and plantlets leaves of both *Plantago* species (Figure 7.2). The maximum photochemical efficiency of PSII (F_v/F_m) is known to be a sensitive indicator of plant photosynthetic performance, with optimal values around 0.84 for most species (Björkman and Demmig 1987). Plants exposed to stress present values lower than this, which is closely related to the degree of photoinhibition (Papageorgiou and Govindjee 2004). Decreases in F_v/F_m are well described for Al stressed plants (Reyes-Diaz et al. 2010; Jin et al. 2011). In both *Plantago* species, neither of the stress treatments studied had a significant impact ($P \geq 0.05$) on the F_v/F_m values (Figure 7.2 A, B), which points to an efficient conversion of light energy at PSII level. Moreover, the mean values of F_v/F_m in both species were close to maximum (0.77 - 0.84) in all treatments, indicating healthy and non-stressed leaves. Therefore, non-reversible photoinhibitory damage to PSII reaction centers did not occur in consequence of low pH (4.00) or Al stress (400 μM Al^{3+}). Our results are in agreement with several authors (Lidon et al. 1999; Silva et al. 2012), which found that F_v/F_m was little affected by Al in other plant species. In contrast, other authors observed that Al induced reductions of F_v/F_m (Peixoto et al. 2002; Reyes-Diaz et al. 2010). The discrepancy between these results may be related to species differences or different experimental conditions, such as Al concentration in the medium or Al exposure time.

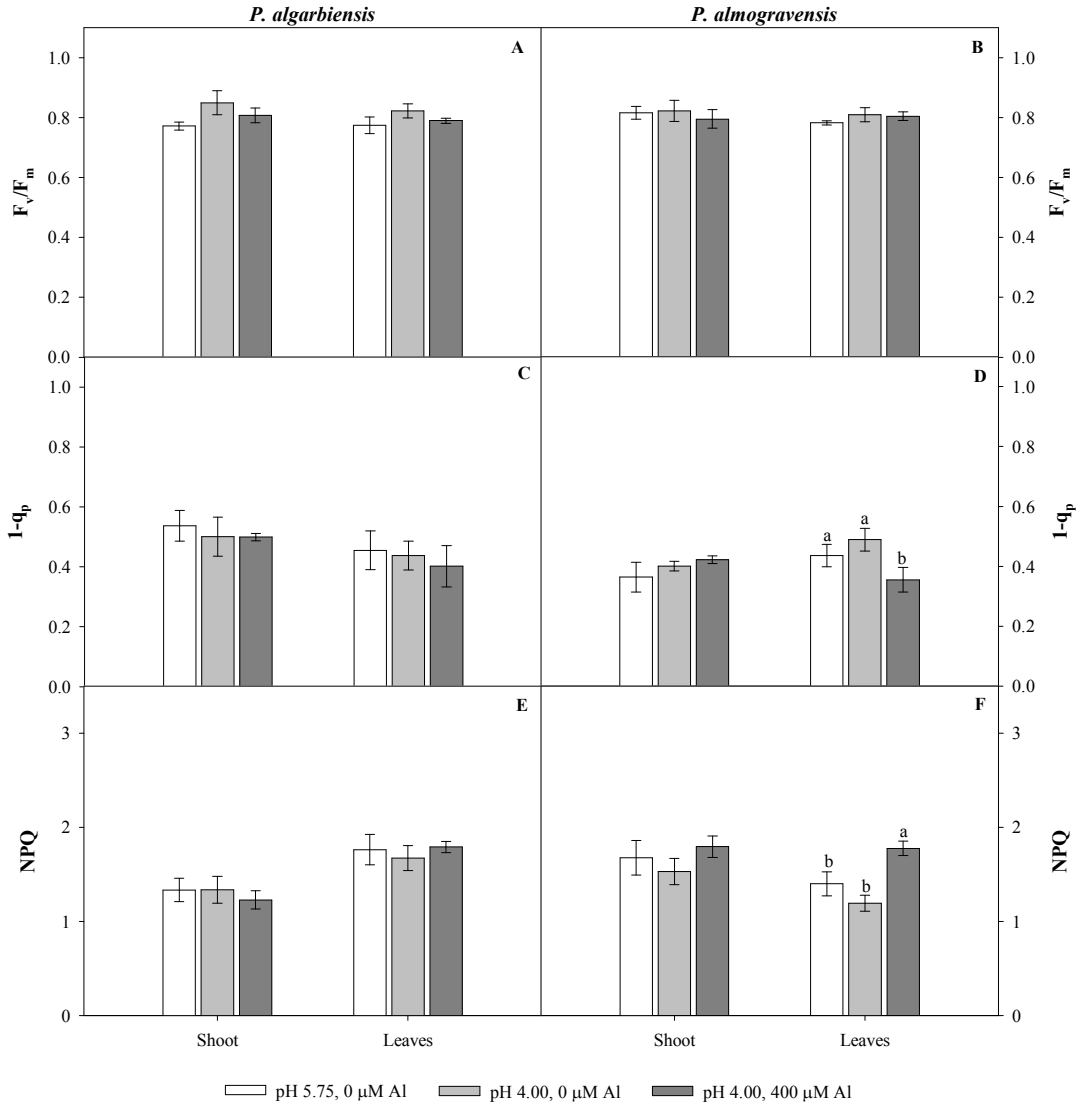

Figure 7.2. Effect of low pH and Al on the maximum photochemical efficiency of PSII (F_v/F_m) (A, B), PSII excitation pressure ($1 - q_p$) (C, D) and non-photochemical quenching (NPQ) (E, F) of *Plantago. algarbiensis* and *P. almogravensis* shoots and leaves. Values are expressed as the mean ± SE (n = 5). Mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test. The absence of letters indicates that the difference between treatments is not significant.

In the same way, the actual PSII quantum efficiency (ϕ_{PSII}) that can indirectly reflect linear electron transport was not significantly ($P \geq 0.05$) influenced by low pH and Al (Figure 7.3 A, B), which confirms that photoinhibition was not induced by these conditions. It is noteworthy that ϕ_{PSII} was positively affected by Al, although not significantly, in leaves of *P. almogravensis* plantlets.

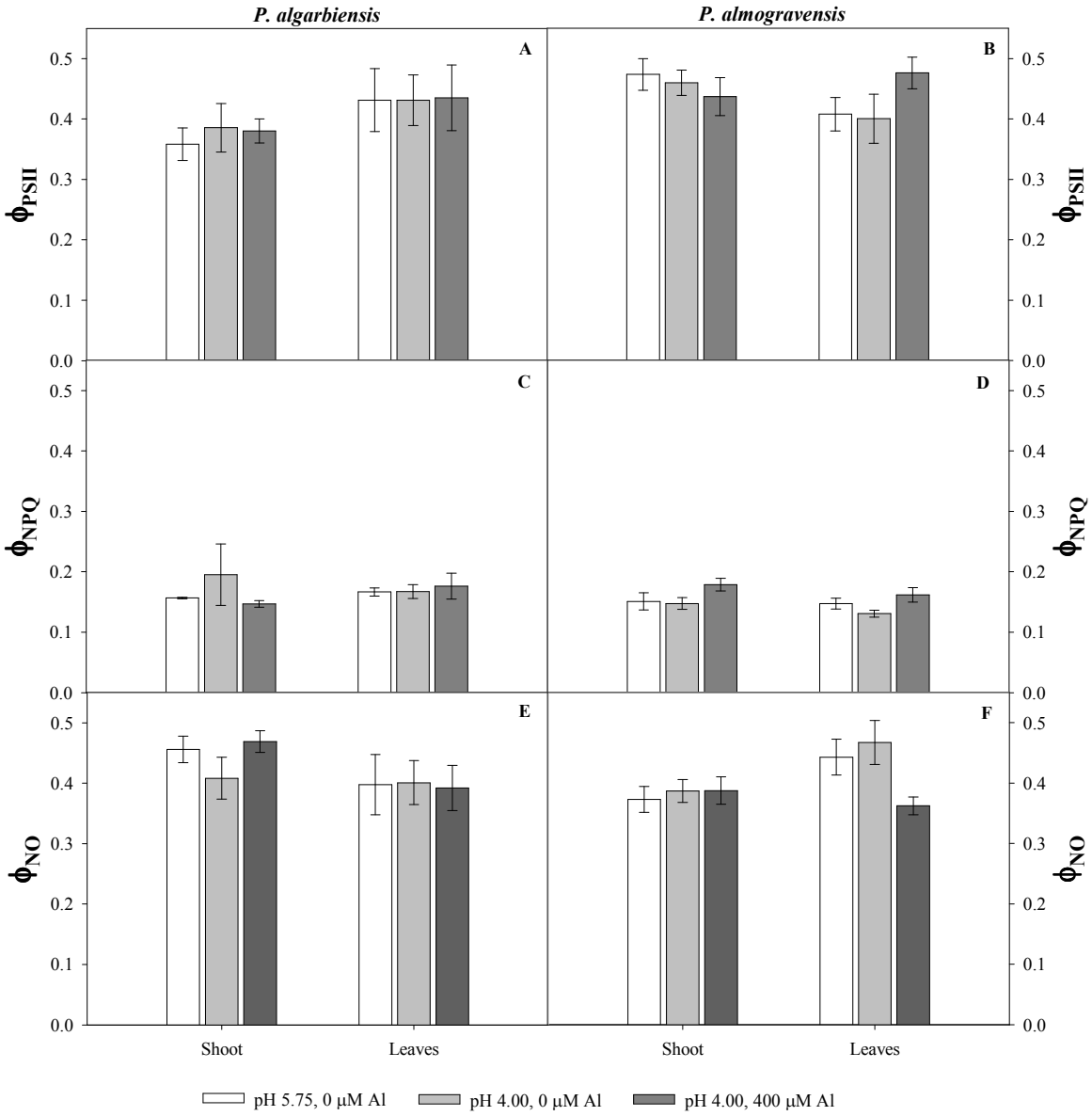

Figure 7.3. Effect of low pH and Al on PSII energy partitioning of *Plantago algarbiensis* and *P. almogravensis* shoots and leaves: actual quantum yield (ϕ_{PSII}) (A, B), quantum yield of regulated energy dissipation (ϕ_{NPQ}) (C, D) and quantum yield of non-regulated energy dissipation (ϕ_{NO}) (E, F). Values are expressed as the mean ± SE (n = 5). The absence of letters indicates that the difference between treatments is not significant at $P \geq 0.05$, according to Duncan's test.

The non-photochemical quenching (NPQ) is a process that regulates energy conversion in photosystem II to protect plants from photoinhibition, thus serving as an indicator of the plant ability to dissipate light energy in excess of that required for CO_2 assimilation (Müller et al. 2001). In our study, Al induced a significant ($P < 0.05$) increase of ~27% in the NPQ value in *P. almogravensis* plantlets leaves (Figure 7.2 F), indicating an enhancement of thermal energy dissipation by antenna pigments which prevent the photosynthetic structure from being affected under Al stress. Increases of NPQ in response to Al were also detected in diverse plant species (Moustakas et al. 1996; Jin et al. 2011), while in other NPQ was slightly decreased by Al stress (Chen et al. 2005a, b). As early postulated (Maxwell et al. 1995), modulation of excitation pressure ($1 - q_p$) is a central component of a general signaling mechanism to initiate appropriate alterations to the photosynthetic apparatus at the physiological, biochemical, and molecular levels in response to environmental change. Previous studies (Kato et al. 2003) proved that the rate of PSII photoinactivation was determined by excess energy, identified as excitation pressure ($1 - q_p$) in a broad sense of the term. Our results show that $1 - q_p$ drop (~19%) in Al treated leaves of *P. almogravensis* (Figure 7.2 D). This may indicate that energy reaching closed PSII reaction centers diminished, which is important to photoprotection. In fact, plants can modulate the rate of PSII photoinactivation by changing the partitioning of the light energy absorbed by PSII antennae (Kornyeyev et al. 2010). In our study, the pattern of energy partitioning did not significantly change either at low pH alone or combined with Al (Figure 7.3). However, in *P. almogravensis* leaves, Al had a positive effect, although not significant, in linear electron transport (increase of ϕ_{PSII}) and in the level of regulated thermal energy (increase of ϕ_{NPQ}), which minimized the proportion of light that was excessive (decrease of ϕ_{NO}) (Figure 7.3 B, D, F). Hence, in leaves of Al treated *P. almogravensis*,

the absorbed energy that was converted into non-regulated energy is reduced, which hinders the production of reactive oxygen species. Besides, more energy was distributed to both PSII photochemistry (CO_2 fixation, photorespiration, water-water cycle) and heat dissipation via the xanthophylls cycle, contributing to the protection of PSII against photoinactivation. Overall, our findings indicate that at PSII photochemistry level, both species have a relatively adequate capacity to cope with low pH and Al stresses, and hence a good physiological state of its photosynthetic apparatus. Nevertheless, the plantlets of *P. almogravensis* have a higher capacity for energy dissipation by regulatory mechanisms than *P. algarbiensis*.

Photosynthetic pigments are important components of the photosynthetic light-harvesting complex responsible for absorbing and trapping light energy in the early steps of photosynthesis (Bowyer and Leegood 1997). Al induced decreases in the photosynthetic pigment contents are reported for several plant species (Jiang et al. 2008; Mihailovic et al. 2008). We observed a significant decrease ($P < 0.05$) in all photosynthetic pigment contents in *P. algarbiensis* shoots as a consequence of low pH and Al stresses (Table 7.3). This decrease indicates that *P. algarbiensis* need to reduce the efficiency to harvest light to protect leaves from photooxidative damage, which is a sign of high sensitivity to H^+ and Al^{3+} toxicity. Nevertheless, although light absorption had decreased due to lowering Chl concentration, the energy absorbed was enough to maintain the photosynthetic performance (Figure 7.2). On the contrary, in *P. almogravensis* the Chl *b* and Chl *a* + *b* contents were higher ($P < 0.05$) in leaves of Al treated plantlets (Table 7.3).

Table 7.3. Effect of low pH and Al on the photosynthetic pigment contents of *Plantago algarbiensis* and *P. almogravensis* shoots and leaves.

pH	Al (µM)	Chl *a* (mg g^{-1} FW)		Chl *b* (mg g^{-1} FW)		Chl *a* + *b* (mg g^{-1} FW)		carotenoids (mg g^{-1} FW)	
		Shoot	Leaves	Shoot	Leaves	Shoot	Leaves	Shoot	Leaves
P. algarbiensis									
5.75	0	0.66 ± 0.06 a	0.60 ± 0.04 a	0.22 ± 0.02 a	0.19 ± 0.02 a	0.89 ± 0.08 a	0.78 ± 0.06 a	0.22 ± 0.02 a	0.19 ± 0.01 a
4.00	0	0.51 ± 0.01 b	0.56 ± 0.05 a	0.16 ± 0.01 b	0.18 ± 0.02 a	0.67 ± 0.01 b	0.73 ± 0.06 a	0.16 ± 0.00 b	0.18 ± 0.02 a
4.00	400	0.43 ± 0.05 b	0.62 ± 0.05 a	0.13 ± 0.01 b	0.19 ± 0.02 a	0.56 ± 0.06 b	0.81 ± 0.06 a	0.13 ± 0.01 b	0.20 ± 0.02 a
P. almogravensis									
5.75	0	0.64 ± 0.04 a	0.58 ± 0.05 a	0.22 ± 0.01 a	0.19 ± 0.02 b	0.86 ± 0.05 a	0.77 ± 0.07 b	0.19 ± 0.01 a	0.17 ± 0.01 a
4.00	0	0.66 ± 0.06 a	0.56 ± 0.04 a	0.22 ± 0.02 a	0.19 ± 0.01 b	0.88 ± 0.08 a	0.75 ± 0.05 b	0.20 ± 0.02 a	0.17 ± 0.01 a
4.00	400	0.58 ± 0.05 a	0.72 ± 0.05 a	0.21 ± 0.01 a	0.25 ± 0.01 a	0.79 ± 0.06 a	0.97 ± 0.06 a	0.18 ± 0.01 a	0.22 ± 0.02 a

Values are expressed as the mean ± SE (n = 5). For each species and in each column, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

The increase of Chl would enhance the capacity of the leaves to absorb incident radiation and hence the risk of photoinhibition if other protective mechanisms are not acquired. However, *P. almogravensis* developed competent mechanisms for non-radiative energy dissipation, as is shown by the increase of the NPQ value in this species (Figure 7.2 F). In concordance with our results, Liu et al. (2006) reported that Al at middle or low concentrations increased the Chl content, whereas Al at high concentration caused remarkable decreases of the Chl contents in four herbaceous plant species. The authors propose that adjustments of Chl contents could be one of the important physiological basis of plant adaptation to Al stress. Thus, our results suggest that *P. almogravensis* is better adapted to high Al concentration than *P. algarbiensis*.

Stressful environment affect carbohydrates storage, translocation and metabolism (Mishra and Dubey 2008). As final products of photosynthesis, they are used as indicators of plant growth potential. Liu et al. (2006) observed that Al inhibited the use of carbohydrates for growth and their translocation from the leaves to other parts of the plant. In addition, carbohydrates accumulation in leaves can lead to decreased expression of photosynthetic genes, and hence to a depression in photosynthetic activity (Paul and Foyer 2001). Conversely, the accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress conditions (Chen et al. 2007). Both low pH and Al induced a significant increase ($P < 0.05$) in carbohydrate content in the shoots and roots of *P. almogravensis* (Table 7.4), while in *P. algarbiensis*, only Al increased ($P < 0.05$) the carbohydrate content in the leaves. Similarly to the described by other authors (Tabuchi et al. 2004; Giannakoula et al. 2008), Al-induced carbohydrates accumulation does not seems to be correlated with photosynthesis inhibition but instead with the Al tolerance, inasmuch as they can act as osmotic adjusters or oxidative stress regulators.

Table 7.4. Effect of low pH and Al on the proline and carbohydrate contents of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets.

pH	Al (µM)	Proline (µmol g^{-1} FW)			Carbohydrates (µmol g^{-1} FW)		
		Shoot	Plantlet		Shoot	Plantlet	
			Leaves	Roots		Leaves	Roots
P. algarbiensis							
5.75	0	0.43 ± 0.03 a	0.45 ± 0.06 a	0.63 ± 0.11 a	107.18 ± 20.83 a	74.00 ± 16.63 b	148.34 ± 26.25 a
4.00	0	0.31 ± 0.04 a	0.45 ± 0.05 a	0.40 ± 0.06 a	91.29 ± 9.76 a	99.80 ± 7.43 ab	114.55 ± 23.97 a
4.00	400	0.48 ± 0.06 a	0.54 ± 0.05 a	0.47 ± 0.05 a	113.33 ± 10.02 a	123.17 ± 11.16 a	184.48 ± 10.87 a
P. almogravensis							
5.75	0	0.38 ± 0.09 b	0.51 ± 0.07 a	0.77 ± 0.15 a	39.33 ± 6.36 c	112.10 ± 15.22 a	91.34 ± 13.52 b
4.00	0	0.51 ± 0.06 b	0.62 ± 0.10 a	0.82 ± 0.09 a	52.68 ± 1.98 b	101.49 ± 6.27 a	158.15 ± 7.51 a
4.00	400	0.87 ± 0.10 a	0.62 ± 0.12 a	0.93 ± 0.12 a	79.51 ± 1.78 a	90.18 ± 7.82 a	171.32 ± 19.94 a

Values are expressed as the mean ± SE (n = 5). For each species and in each column, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

Plants can also accumulate proline to decrease cell osmotic potential, keep pressure potential, retain and stabilize macromolecules, participate in chlorophyll synthesis and maintain normal functions of cellular membrane (Liu and Yang 2000). In our study, Al stress induced a significant two-fold increase ($P < 0.05$) in the proline content in *P. almogravensis* shoots, which agrees with previous reports showing that proline accumulation is correlated with Al tolerance (Ali et al. 2008; Giannakoula et al. 2008).

In conclusion, both *Plantago* species accumulate considerable and similar amounts of Al, however this metal induced inhibition of root elongation only in *P. algarbiensis*. *P. almogravensis* was proved to be more resistant than *P. algarbiensis* to Al since it has some internal adjustments, such as decreases in 1 - q_p, increases in NPQ, carbohydrate accumulation and nutrients concentration that at least partially enabled this species to cope with Al^{3+} and H^+ toxicity. In contrast, fewer effective protective mechanisms were detected in *P. algarbiensis*. Our results contributed to elucidate the physiological response of *P. algarbiensis* and *P. almogravensis* to low pH and Al stresses and to understand their tolerance to acidic Al-rich soils. However, to clarify differences between *P. algarbiensis* and *P. almogravensis,* and fully understand the ecophysiological mechanisms of Al-accumulation and tolerance in *Plantago* spp., field studies are necessary.

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CHAPTER 8

Impact of low pH and aluminum on the oxidative stress, energy partitioning and antioxidant responses in roots and leaves of *Plantago algarbiensis* and *P. almogravensis*

Martins N, Osório ML, Gonçalves S, Osório J, Romano A (2013) Differences in Al tolerance between *Plantago algarbiensis* and *P. almogravensis* reflect their ability to respond to oxidative stress. Biometals 26:427-437

8.1. Abstract

We evaluated the effects of low pH and aluminum (Al) on the energy partitioning on photosystem II, H_2O_2 content, lipid peroxidation, electrolyte leakage, protein oxidation, total soluble protein content and antioxidant enzyme activities in leaves and roots of *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. Overall, Al triggered more changes on the oxidative metabolism than low pH alone in both species, mainly in the roots. We found that Al supply increased the H_2O_2 content in *P. algarbiensis* roots, while in *P. almogravensis* decreased in leaves and roots. Chlorophyll fluorescence images did not show any spatial heterogeneity or quantitative changes on the maximum photochemical efficiency of PSII (F_v/F_m), actual quantum efficiency of PSII (ϕ_{PSII}) and quantum yields of regulated (ϕ_{NPQ}) and nonregulated (ϕ_{NO}) energy dissipation, in response to low pH and Al in either species. As well, no significant alterations on total soluble protein content and electrolyte leakage were detected. In *P. algarbiensis*, Al increased the carbonyl content and the activities of superoxide dismutase (SOD) and catalase (CAT) in the roots, and also the CAT, ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities in the leaves. In *P. almogravensis* Al decreased the MDA level in the roots and the SOD activity in leaves and roots. Our data showed that *P. almogravensis* plantlets were able to manage the oxidative stress caused by low pH and Al, whereas in *P. algarbiensis* the antioxidant system was unable to fully suppress the toxicity imposed by Al, leading to H_2O_2 accumulation and protein oxidation in roots.

8.2. Introduction

Acid soils cover 30 - 40% of the word's arable lands and are increasing on a global scale as a result of natural, agricultural and industrial processes (von Uexküll and Mutert 1995). Aluminum (Al) is the main cause of toxicity in these soils because at pH below 5 it is solubilized into the toxic cation Al^{3+} (von Uexküll and Mutert 1995). Al has harmful effects on plant growth and on a wide range of physiological processes, via Al interactions with cellular targets, disturbing the redox homeostasis and inducing the accumulation of reactive oxygen species (ROS) (Mittler 2002; Achary et al. 2012). Al stress in plants can affect the functionality of the photosynthetic apparatus, by reducing the photochemical efficiency of photosystem II (PSII) and restricting electron flow often due to structural damage of the thylakoids (Pereira et al. 2000; Inostroza-Blancheteau et al. 2011). This photooxidative damage can be prevented by dissipation of excess excitation energy as heat in the antenna pigment complexes through xanthophylls cycle (Demmig-Adams and Adams 1996) or via alternative routes as photorespiration, water-water cycle and cyclic electron transport (Murchie and Niyogi 2011). A few years ago, Kramer et al. (2004) developed a model in which energy absorbed was partitioned in three fractions: (1) utilized photochemically by PSII (ϕ_{PSII}); (2) dissipated thermally by regulated quenching mechanisms (ϕ_{NPQ}) and (3) dissipated by nonregulated quenching mechanisms (ϕ_{NO}), satisfying the condition $\phi_{PSII} + \phi_{NPQ} + \phi_{NO} = 1$. The ϕ_{PSII} and ϕ_{NPQ} pathways work as protective mechanisms, whereas ϕ_{NO} can be regarded as excess energy. Therefore, the use of this model may be considered a sensitive and reliable method for understanding and quantifying the Al induced damages to the PSII. If photochemical and nonphotochemical capacities are exceeded, the surplus of energy is transferred to O_2, and ROS are produced (Wilson et al. 2006). Although ROS are important signaling molecules, they can affect a variety of

physiological functions (Gill and Tuteja 2010). To mitigate oxidative injury, all plant cells have developed mechanisms to scavenge ROS via non-enzymatic and enzymatic antioxidant systems (Ma et al. 2012). Increasing evidence has shown that Al stress induced the up-regulation of the antioxidant enzymes, superoxide dismutase (SOD; E.C.1.15.1.1) that acts on superoxide anion producing hydrogen peroxide (H_2O_2) as well as catalase (CAT; E.C. 1.11.1.6), ascorbate peroxidase (APX; E.C. 1.11.1.11) and guaiacol peroxidase (GPX; E.C. 1.11.1.7) responsible for H_2O_2 elimination (Giannakoula et al. 2010; Inostroza-Blancheteau et al. 2011), constituting therefore an important protective mechanism to minimize ROS production. When the production of ROS exceeds the removing capacity of scavenging systems, the excessive ROS accumulation may lead to lipid peroxidation, protein oxidation and DNA mutation (Ma et al. 2012).

During evolution, wild plants have developed morphological, physiological and biochemical mechanisms that allow them to survive in acidic Al-rich soils (Kochian 1995). In fact, it has been observed that Al tolerant plants are better equipped to deal with Al induced ROS accumulation, due to a faster response to Al exposure and an enhanced baseline redox capacity (Ramírez-Benítez and Hernández-Sotomayor 2008). Therefore, it is important to study functional aspects related with the capacity of wild Al-tolerant species to cope with Al to clarify the mechanisms underlying Al resistance. This knowledge should contribute to improve plant breeding strategies for dealing with acid soils.

Plantago almogravensis Franco and *Plantago algarbiensis* Samp., are species of the Plantaginaceae family endemic from Portugal (southwest coast and West-Central Algarve region, respectively). As these species are protected under the European Habitats Directive and by Portuguese law we established micropropagation protocols

(Gonçalves et al. 2009) in order to have enough number of plants and we demonstrated that they can grow properly in medium with low pH (Martins et al. 2011). Our recent results revealed that micropropagated shoots and plantlets accumulated considerable amounts of Al (Martins et al. 2012a, b). In addition, we investigated the impact of low pH and Al stress on the growth, nutrients concentration, chlorophyll *a* fluorescence, photosynthetic pigment contents, proline and carbohydrate accumulation in shoots and plantlets of both species (Martins et al. 2012b). Although our data showed that both species are tolerant to Al and H^+, *P. almogravensis* appeared to be more adapted to maintain cellular physiology and growth under those conditions (Martins et al. 2012b).

Differences in Al and low pH tolerance can be explained through the presence of different mechanisms. Many studies indicated that an efficient ROS scavenging system might be an important trait linked with plant tolerance to Al stress (Giannakoula et al. 2010; Inostroza-Blancheteau et al. 2011). The aim of this work was to investigate the impact of low pH and Al stress on the energy partitioning at photosystem II, H_2O_2 content, oxidative biomarkers (lipid peroxidation, electrolyte leakage and protein oxidation) and the activities of antioxidant enzymes in leaves and roots of *P. algarbiensis* and *P. almogravensis*. We made attempts to answer the following questions: (i) Does low pH alone or combined with Al induce oxidative stress in *P. algarbiensis* and *P. almogravensis*? (ii) Are those effects related to H_2O_2 accumulation? (iii) Is the enzymatic antioxidant system enough to suppress the oxidative stress?

8.3. Materials and methods

8.3.1. Plant material and growth conditions

P. algarbiensis shoots (~6 cm in length) and *P. almogravensis* shoots (~3 cm in length) were separated from *in vitro* cultures proliferating for 6 weeks in MS medium (Murashige and Skoog 1962) containing 0.2 mg l^{-1} 6-benzyladenine as described by Gonçalves et al. (2009). Plantlets were obtained by cultivating the shoots for 3 weeks in rooting medium (½MS containing 0.5 mg l^{-1} indole-3-acetic acid, Gonçalves et al. 2009). The plantlets were maintained at 25 ± 2 ºC with a 16 h photoperiod (cool white fluorescent lamps, 69 µmol m^{-2} s^{-1}).

8.3.2. Stress treatments

Low pH stress was applied by transferring plantlets to ¼MS liquid medium with the pH adjusted to 4.00 (stress) or 5.75 (control). Al stress was applied by transferring plantlets to ¼MS liquid medium with the pH adjusted to 4.00 containing 400 µM $AlCl_3$, that corresponds to 140 µM Al^{3+} activity, as estimated by Geochem-EZ (Shaff et al. 2010). Plantlets were inoculated individually in 32 × 200 mm test tubes containing 20 ml liquid medium on filter paper bridges, and incubated for 7 days under the conditions described above.

8.3.3. Chlorophyll fluorescence

Chlorophyll (Chl) fluorescence imaging was determined using a mini blue version of Imaging-PAM Chl fluorometer (IMAG-MIN/B, Walz, Effeltrich, Germany). In order to evaluate spatial heterogeneity three areas of interest (AOIs) were selected in leaves. Pixel value images of the fluorescence parameters were displayed with help of a false colour code ranging from black (0,000) through red, yellow, green, blue to pink (ending

at 1,000). Leaves were darkened for 20 min prior the measurement. Images of the minimum fluorescence (F_0) were obtained by applying measuring light pulses modulated at 1 Hz, while images of the maximal fluorescence yield (F_m) were obtained with the help of a saturating blue pulse (10,000 µmol quanta m^{-2} s^{-1} PPFD) at 10 Hz. The images of F_0 and F_m were subtracted and divided by F_m to generate the image of the maximum quantum efficiency of PSII photochemistry $F_v/F_m = (F_m - F_0)/F_m$. Afterwards, actinic illumination (103 µmol m^{-2} s^{-1}) was switched on and saturating pulses were applied at 20 s intervals for 5 min in order to determine the maximum fluorescence yield (F_m') and the Chl fluorescence yield (F_s) during illumination. For each interval, saturation pulse, images and values of various Chl fluorescence parameters were captured namely the actual quantum efficiency of PSII (ϕ_{PSII}), the quantum yield of regulated energy dissipation (ϕ_{NPQ}) and the quantum yield of non-regulated energy dissipation (ϕ_{NO}) at PSII (Kramer et al. 2004).

8.3.4. Determination of hydrogen peroxide content

The H_2O_2 content was determined using the method of Loreto and Velikova et al. (2001). Fresh plant material (100 mg) was homogenized in 1 ml 0.1% (w/v) trichloroacetic acid (TCA) at 4 ºC. The homogenate was centrifuged at 12,000 *g* for 15 min and 0.2 ml of supernatant was added to 0.2 ml 10 mM potassium phosphate buffer (pH 7.0) and 0.4 ml 1 M KI. The reaction was developed for 30 min in darkness and the H_2O_2 content was quantified at 390 nm against a set of H_2O_2 standards, and subtracting a blank sample lacking the plant extract. The results were expressed as µmol g^{-1} fresh weight (FW).

8.3.5. Determination of lipid peroxidation and electrolyte leakage

Oxidative damage to lipids was evaluated as lipid peroxidation, which was determined by the amount of malondialdehyde (MDA) available to react with 2-thiobarbituric acid (TBA) (Hodges et al. 1999). Fresh plant material (100 mg) was crushed in 0.1% (w/v) TCA and centrifuged at 10,000 *g* for 5 min. The supernatant was added to either 20% (w/v) TCA (-TBA solution) or 0.5% (w/v) TBA in 20% (w/v) TCA (+TBA solution). The mixture was heated at 95 ºC for 30 min and then cooled in an ice bath. The samples were centrifuged at 3,000 *g* for 10 min. The absorbance of the supernatant was measured at 532, 600 and 440 nm and MDA equivalents were calculated as described by Hodges et al. (1999).

Electrolyte leakage (EL) was measured using an electrical conductivity meter as described by Lutts et al. (1996). Fresh plant material (100 mg) was excised and washed 3 times with distilled water to remove surface contamination. Leaves and roots were cut into 1 cm segments and incubated in distilled water overnight at 25 ºC in a rotator shaker. The electrical conductivity (EC_1) of the bathing solution was recorded. These samples were then boiled for 30 min to release all electrolytes, cooled to 25 ºC and the final electrical conductivity (EC_2) was measured. The EL was expressed following the formula $EL= (EC_1/EC_2) \times 100$.

8.3.6. Protein oxidation

Protein oxidation was measured by the reaction of carbonyls with 2,4-dinitrophenyldrazine (DNPH), using the method of Levine et al. (1994). Fresh plant material (100 mg) was homogenized in 20 mM phosphate buffer (pH 6.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 20,000 *g* for

1 min at 4 ºC. Two equal aliquots of the supernatant were precipitated with equal volume of 20% (w/v) TCA. The pellets were suspended with 2 N HCl with or without (blank) 10 mM DNPH and left for 1 h at room temperature. The samples were then precipitated with 20% TCA for 10 min and the supernatant discarded. After washing with ethanol:ethyl acetate (1:1, v/v), the pellets were dissolved in 20 mM sodium phosphate buffer, pH 6.8 containing 6 M guanidine hydrochloride and centrifuged at 6,000 *g* at 4 °C. The carbonyl content was calculated from the difference in absorbance at 380 nm (ε = 22 mM^{-1} cm^{-1}) for DNPH-treated and HCl-treated (blank) samples and expressed as nmol carbonyl mg^{-1} protein.

8.3.7. Enzyme assays and soluble protein

SOD, CAT, GPX and APX activities were evaluated after plantlets were cultured for 7 days. Fresh tissue (100 mg) was ground in a pre-chilled mortar using a homogenization medium consisting of 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1% (w/v) PVPP and 2.5 mM dithiothreitol (and 5 mM ascorbate in the case of APX). The homogenate was centrifuged at 20,000 *g* for 10 min at 4 ºC and the supernatant was used for subsequent enzymes assays.

SOD activity was determined by the reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The photo-reduction of NBT was measured at 560 nm during 6 min. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. CAT activity was determined by the degradation of H_2O_2 using the method of Aebi (1983). The H_2O_2 decomposition was monitored at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). One unit of CAT was defined as the amount of enzyme required to degrade 1 µmol H_2O_2 per min. GPX activity was measured by the modification of guaiacol as described by Egley et al. (1983). The tetraguaiacol

formation was monitored at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). One unit of GPX was defined as the amount of enzyme required to produce 1 μmol tetraguaiacol per min. APX activity was determined by the oxidation of ascorbate as described by Nakano and Asada (1981). The H_2O_2-dependent oxidation of ascorbate was monitored at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of APX was defined as the amount of enzyme required to degrade 1 μmol of ascorbate per min. The specific enzyme activity for all enzymes was expressed as enzyme units per milligram of protein, based on the measurement of total soluble protein levels according to the method of Bradford (1976) using bovine serum albumin as a standard.

8.3.8. Statistical analysis

The values obtained were expressed as mean ± standard errors of five replicates. We carried out a one-way analysis of variance (ANOVA) on the results to assess treatment differences using the SPSS statistical package for Windows (release 19.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test.

8.4. Results

The fluorescence imaging technique was used to assess the heterogeneity of photosynthetic performance and the extent to which it is influenced by regulated or non-regulated energy dissipation at PSII under low pH alone or combined with Al. As shown in Figure 8.1, the images reveal a homogeneous distribution of F_v/F_m, ϕ_{PSII}, ϕ_{NPQ} and ϕ_{NO} over the screened leaf area in all treatments. Moreover, neither low pH nor the presence of Al had a significant impact ($P \geq 0.05$) on the ϕ_{PSII}, ϕ_{NPQ} and ϕ_{NO} values in both *P. algarbiensis* and *P. almogravensis* (Figure 8.2).

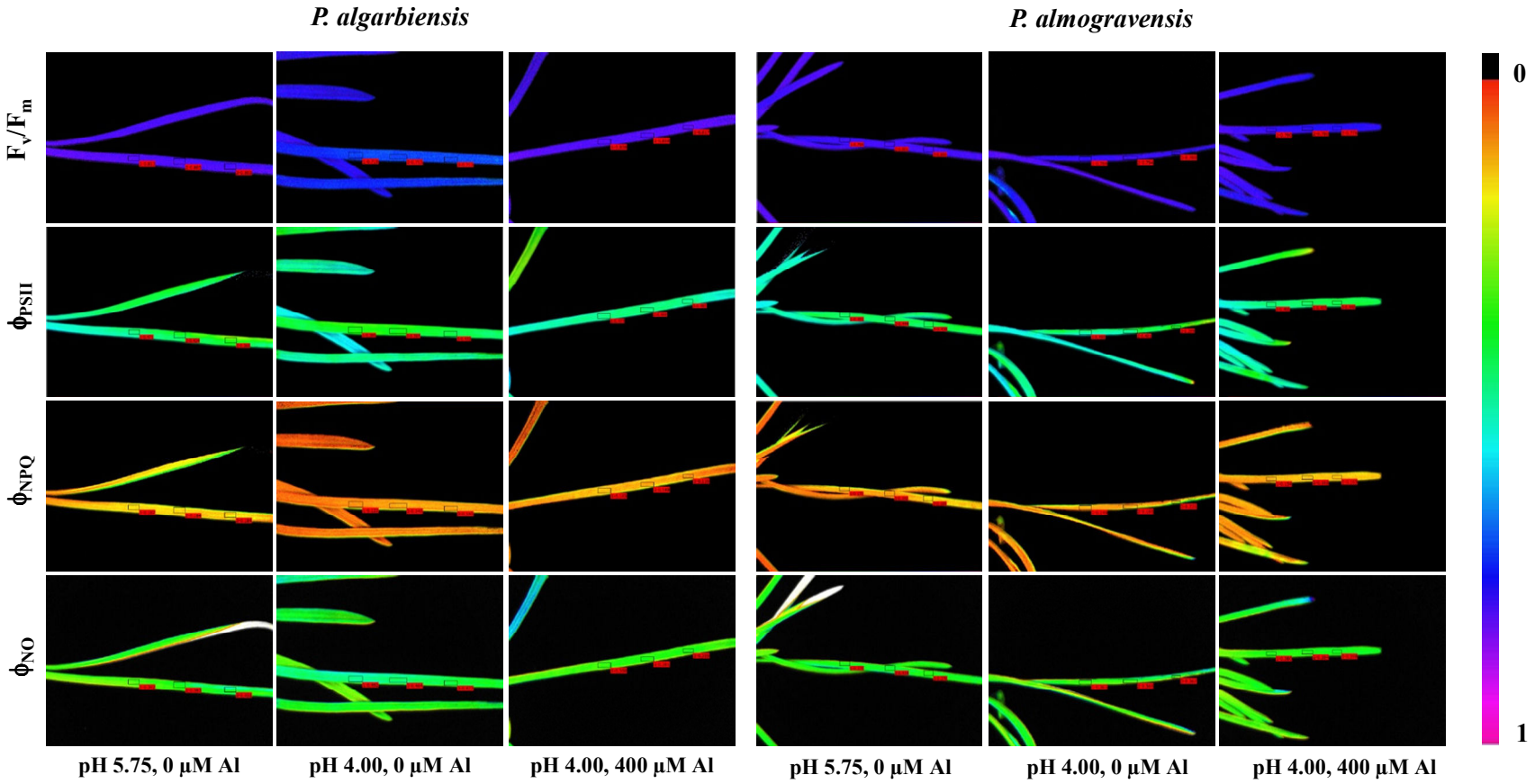

Figure 8.1. Chlorophyll fluorescence images of maximum PSII photochemical efficiency (F_v/F_m), actual PSII quantum yield (ϕ_{PSII}), quantum yield of regulated energy dissipation (ϕ_{NPQ}) and quantum yield of non-regulated energy dissipation (ϕ_{NO}) at steady-state captured on *Plantago algarbiensis* and *P. almogravensis* leaves. The false color code depicted at the right of images ranges from 0,000 (black) to 1,000 (pink). For each selected sample leaf, 3 areas of interest (AOIs) were defined and displayed as small rectangles in each image, accompanied by a little red box with the averaged values of fluorescence.

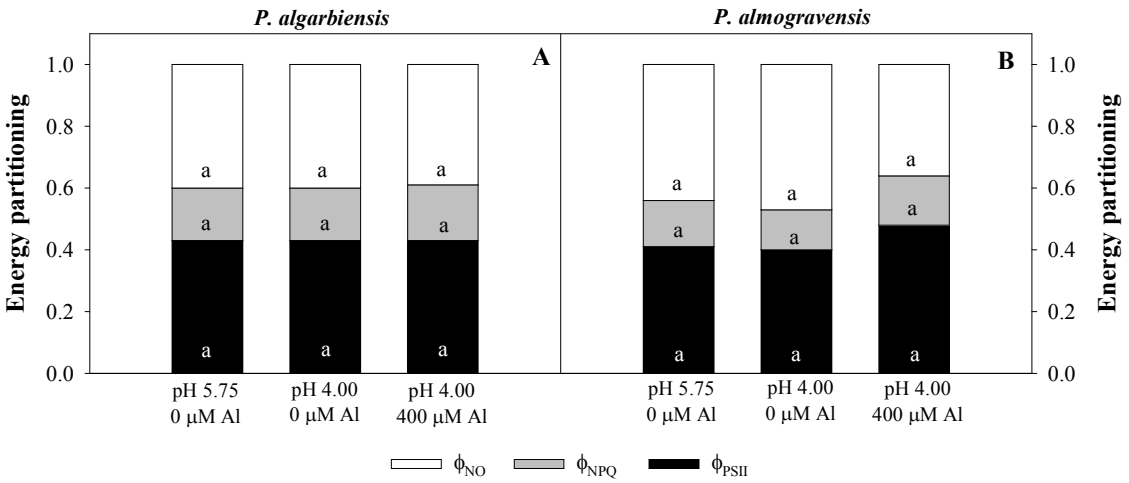

Figure 8.2. Complementary changes of ϕ_{PSII}, ϕ_{NPQ} and ϕ_{NO} with pH and Al in *Plantago algarbiensis* (A) and *P. almogravensis* (B). Stacked bars represent mean values (n = 5) for treatments. Mean values followed by different letters are significantly different at $P < 0.05$ according to Duncan's test.

The effect of low pH and Al on the H_2O_2 content was evaluated in leaves and roots of both *Plantago* species (Figure 8.3). Low pH combined with Al induced a significant ($P < 0.05$) 2-fold increase on the H_2O_2 content in *P. algarbiensis* roots whereas in leaves no significant ($P \geq 0.05$) effect was detected (Figure 8.3 A). In *P. almogravensis*, Al significantly ($P < 0.05$) decreased the H_2O_2 content, both in leaves and roots (Figure 8.3 B).

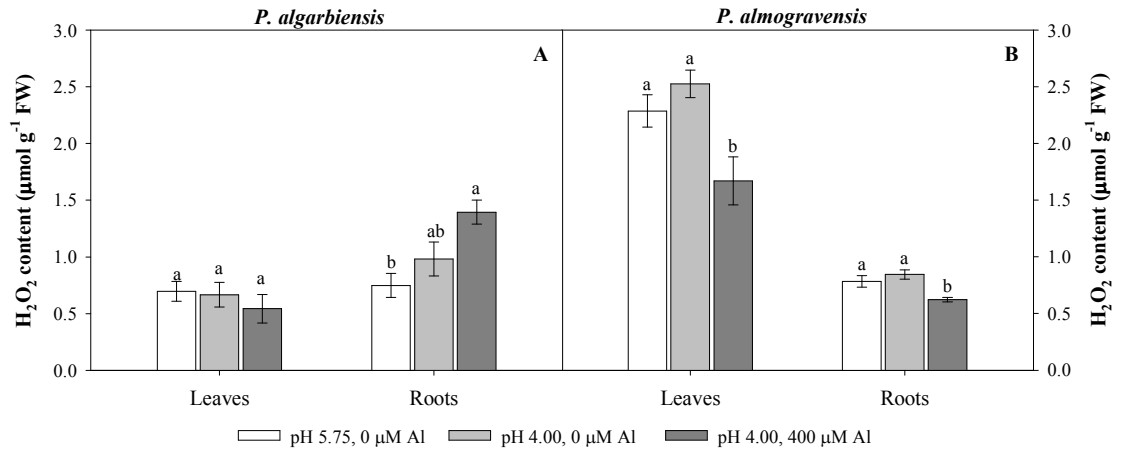

Figure 8.3. Effect of low pH and Al on the H_2O_2 content in leaves and roots of *Plantago algarbiensis* (A) and *P. almogravensis* (B). Values are expressed as the mean ± SE (n = 5). Mean values followed by different letters are significantly different at $P < 0.05$ according to Duncan's test.

To investigate the oxidative damage caused by low pH and Al stresses we measured the MDA level, EL, total protein content and protein oxidation in *P. algarbiensis* and *P. almogravensis* plantlets (Table 8.1). We observed a significant ($P < 0.05$, ~1.5-fold) decrease on the MDA level in *P. almogravensis* Al treated roots, whereas in *P. algarbiensis* the MDA level was not influenced ($P \geq 0.05$) by the stress treatments. Low pH combined with Al induced a significant ($P < 0.05$) increase on the protein oxidation, expressed by the carbonyl content, only in *P. algarbiensis* roots. In both *Plantago* species, neither of the stress treatments had a significant impact ($P \geq 0.05$) on EL and total protein content.

To evaluate if the antioxidant system of *P. algarbiensis* and *P. almogravensis* plantlets works efficiently to overcome the low pH and Al stresses, the activities of SOD, CAT, APX and GPX were measured (Figure 8.4). In *P. algarbiensis* the presence of Al significantly increased ($P < 0.05$, ~2-fold) the SOD activity in roots (Figure 8.4 A) and the CAT, APX and GPX activities in leaves (Figure 8.4 C, E, G). Moreover, both low pH and Al augmented ($P < 0.05$) CAT activity in roots of this species (Figure 8.4 C). In *P. almogravensis* Al induced a significant ($P < 0.05$) decrease in SOD activity (Figure 8.4 B) in both leaves and roots and neither of the stress treatments had a significant impact ($P \geq 0.05$) on the CAT, APX and GPX activities (Figure 8.4 D, F, H).

Table 8.1. Effect of low pH and Al on the MDA, electrolyte leakage, protein and carbonyl contents in leaves and roots of *Plantago algarbiensis* and *P. almogravensis*.

	pH	Al (μM)	*P. algarbiensis*		*P. almogravensis*	
			Leaves	Roots	Leaves	Roots
MDA	5.75	0	58.19 ± 1.74 a	83.30 ± 8.29 a	59.14 ± 5.35 a	55.18 ± 4.10 a
(nmol g^{-1} FW)	4.00	0	73.34 ± 7.93 a	105.85 ± 5.98 a	52.47 ± 3.37 a	57.12 ± 5.18 a
	4.00	400	71.70 ± 4.03 a	106.84 ± 8.16 a	52.87 ± 4.09 a	38.69 ± 5.03 b
Electrolyte leakage	5.75	0	11.42 ± 1.11 a	15.15 ± 0.67 a	9.01 ± 0.83 a	18.71 ± 1.57 a
(%)	4.00	0	12.41 ± 0.65 a	15.20 ± 1.00 a	8.51 ± 0.41 a	16.01 ± 1.12 a
	4.00	400	10.79 ± 0.49 a	17.62 ± 0.31 a	8.63 ± 0.70 a	22.87 ± 2.53 a
Protein	5.75	0	12.27 ± 0.34 a	6.32 ± 0.21 a	7.32 ± 0.81 a	6.41 ± 0.19 a
(mg g^{-1} FW)	4.00	0	10.90 ± 1.33 a	4.57 ± 0.56 a	7.43 ± 0.72 a	5.66 ± 0.43 a
	4.00	400	11.31 ± 0.69 a	6.00 ± 0.58 a	8.60 ± 1.01 a	6.35 ± 0.26 a
Carbonyl content	5.75	0	11.42 ± 2.19 a	15.44 ± 2.14 b	13.33 ± 2.23 a	28.09 ± 2.54 a
(nmol mg^{-1} protein)	4.00	0	10.56 ± 2.46 a	18.10 ± 1.24 ab	16.06 ± 2.13 a	21.77 ± 2.61 a
	4.00	400	12.63 ± 3.14 a	23.17 ± 1.64 a	16.65 ± 2.30 a	30.23 ± 1.29 a

Values are expressed as the mean ± SE (n = 5). Mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test.

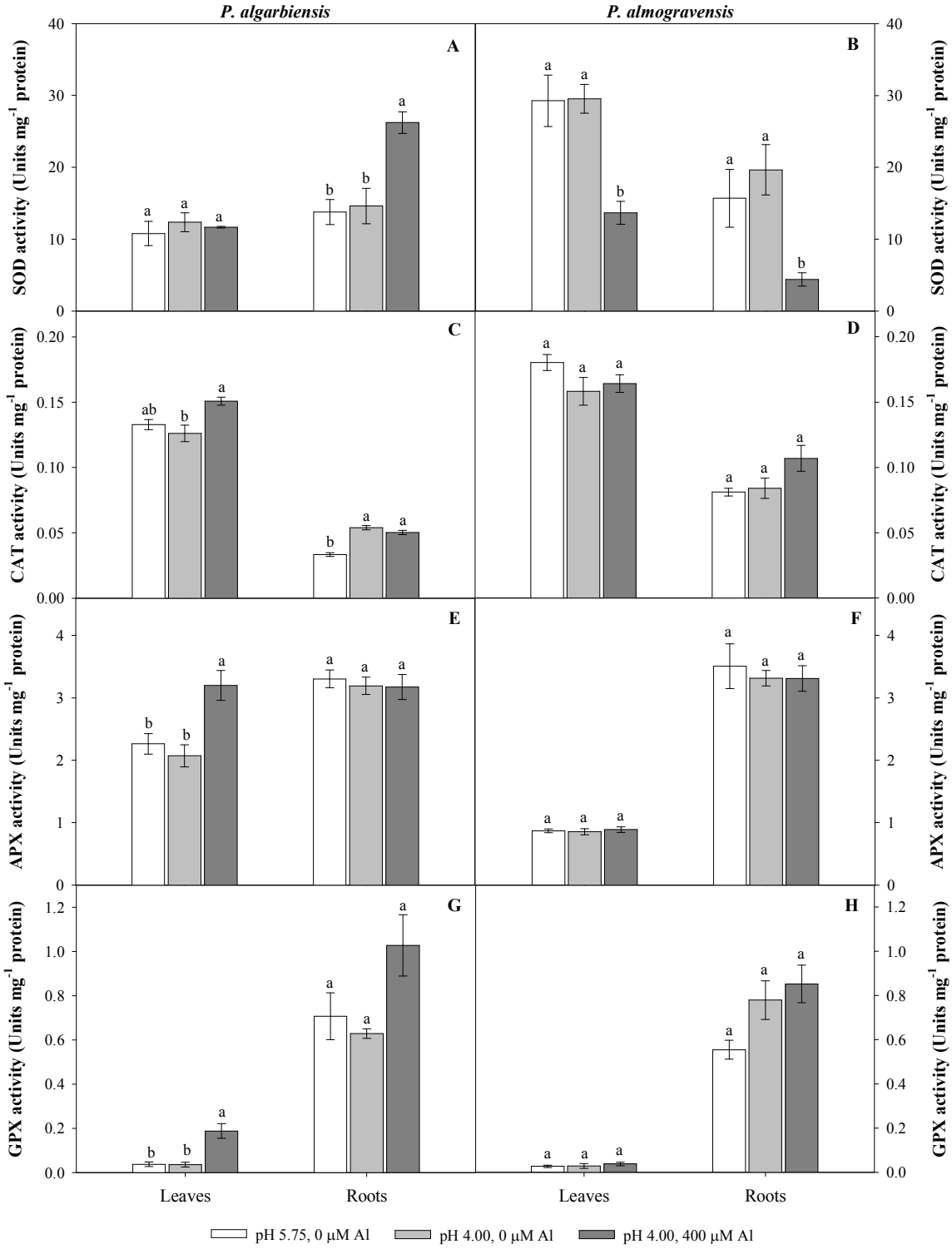

Figure 8.4. Effect of low pH and Al on the SOD (A, B), CAT (C, D), APX (E, F) and GPX (G, H) activities in leaves and roots of *Plantago algarbiensis* and *P. almogravensis*. Values are expressed as the mean ± SE (n = 5). Mean values followed by different letters are significantly different at $P < 0.05$ according to Duncan's test.

8.5. Discussion

It was shown that Al reduces the photochemical efficiency of PSII and electron transport in several plant species (Chen et al. 2005; Jiang et al. 2008; Inostroza-Blancheteau et al. 2011). Recently, our findings (Martins et al. 2012b) indicate that both *P. algarbiensis* and *P. almogravensis* have a relatively adequate capacity to cope with low pH and Al stresses, although *P. almogravensis* has a higher capacity for energy dissipation by regulatory mechanisms (increase in NPQ) than *P. algarbiensis*. Thus, in *P. algarbiensis* thermal dissipation may not be the main way to dissipate excess excitation energy in Al treated leaves, and water-water cycle and photorespiration may be up-regulated to cope with the increased excess due to high Al (Chen et al. 2005). Despite such processes may play a protective role by decreasing the degree of reduction of the PSII acceptor side and the risk of photoinhibition, it is well known that they are prone to lead to overproduction of ROS. Since limitations induced by stresses are not evenly distributed over the whole leaf area and direct measurement of ROS production is not currently feasible in plants, we used chlorophyll fluorescence imaging to monitor changes in absorbed energy distribution as a rapid and efficient tool to detect signs of oxidative stress. In fact, plants can modulate the rate of PSII photoinactivation by changing the partitioning of the light energy absorbed by PSII antennae (Kornyeyev et al. 2010; Osório et al. 2011). In this study, Chl fluorescence images revealed a quite homogeneous pattern of distribution of ϕ_{PSII}, ϕ_{NPQ} and ϕ_{NO} over the screened leaf area (Figure 8.1). Furthermore, neither of the stress treatments had a significant impact on the ϕ_{PSII}, ϕ_{NPQ} and ϕ_{NO} values in both *P. algarbiensis* and *P. almogravensis* (Figure 8.2), suggesting that low pH and Al stress did not affect the function of PSII and no energy was diverted to non-regulated energy dissipation processes. These findings suggest that both species have developed enzymatic and nonenzymatic scavenging systems to

quench active oxygen, and to eliminate the harmful effects of active oxygen at leaves. In fact, no accumulation in H_2O_2 content were observed in the leaves of both *Plantago* species (Figure 8.3) and the Al induced H_2O_2 accumulation in *P. algarbiensis* roots (Figure 8.3 A) did not seem to affect PSII behavior in the photosynthetic tissues.

The maintenance of cell membranes integrity and stability in plants under Al toxicity is an important component of tolerance (Tabaldi et al. 2007; Giannakoula et al. 2008). Since lipid peroxidation is one of the major outcomes of ROS actions on cell membrane (Blokhina et al. 2003) it major product, MDA, is used to evaluate the Al induced oxidative damage on lipids (Giannakoula et al. 2010; Pereira et al. 2010; Mukhopadyay et al. 2012). Furthermore, the degree of cell membrane injury brought by Al stress has been also evaluated through measurements of EL from the cells (Pereira et al. 2010; Yin et al. 2010; Mukhopadyay et al. 2012). Although severe lipid peroxidation was reported in many plant species under Al stress (Giannakoula et al. 2010; Achary et al. 2012), our results showed no significant changes on the MDA level in *P. algarbiensis* leaves and roots, and a decrease was even observed in *P. almogravensis* Al treated roots (Table 8.1), suggesting the existence of protective mechanism in this species. Similarly, Basu et al. (2001) found a correlation between decreased lipid peroxidation and increased resistance to Al in *Brassica napus*. In both species, no significant differences on the EL were detected in response to the stress treatments (Table 8.1), indicating no stress induced changes in the permeability of the cell membrane.

Abiotic stress may inhibit the synthesis of some proteins and promote others (Ericson and Alfinito 1984) with a general trend of decline in the overall content. The detrimental effect in total protein content could be the consequence of an increase in protein degradation or a decline on protein synthesis caused by ROS (Sgherri and Navari-Izzo 1995). In our study, the total protein content was not affected by the stress

treatments in both species (Table 8.1), despite a number of reports have showed the negative effects of Al on the total protein content of several plant species (Guo et al. 2007; Cruz et al. 2011). Additionally, ROS in excess can also lead to various structural modifications in proteins (Cargnelutti et al. 2006). These oxidative modifications are characterized by the formation of carbonyl derivates on side chains of histidine, arginine, lysine and proline residues, that are irreversible and increase the protein susceptibility to proteolytic degradation (Shacter et al. 1994; Rinalducci et al. 2008). The increased carbonyl content observed in *P. algarbiensis* Al treated roots (Table 8.1) are consistent with previous studies where the levels of carbonylated proteins increase in plants undergoing Al toxicity (Boscolo et al. 2003; Achary et al. 2012) and indicates that the quantity of ROS surpassed the capacity of the antioxidant system on the roots of this species.

In order to overcome the oxidative damage under Al stress, plants have developed an extensive network of antioxidant enzymes comprising SOD, CAT, APX and GPX that neutralize and scavenge the ROS (Sharma and Dubey 2007). In addition, many investigations indicate that the activity of these antioxidant enzymes is correlated with plant tolerance to Al stress (Giannakoula et al. 2010; Inostroza-Blancheteau et al. 2011). SOD represents the first step in the detoxifying process, catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. However, the product of SOD activity (H_2O_2) is still toxic and must be removed by conversion to H_2O in subsequent reactions involving CAT, and several peroxidases like APX and GPX (Wang et al. 2009). In our study, the increased H_2O_2 content observed in Al treated roots of *P. algarbiensis* (Figure 8.3 A) seems to be associated with enhanced SOD activity (Figure 8.4 A), since the increased CAT activity observed was not sufficient to decrease the production of this ROS. Whilst, in *P. almogravensis* plantlets treated with Al, the reduced SOD activity (Figure 8.4 B)

coupled with unchanged activities of CAT, APX and GPX (Figure 8.4 D, F, H) are in agreement with the low H_2O_2 content mentioned previously (Figure 8.3 B).

Overall, our findings indicate that *P. almogravensis* plantlets were able to minimize the H_2O_2 accumulation and therefore limit the oxidative damage on cellular targets under low pH and Al stress. On the other hand, in *P. algarbiensis* roots the balance between the ROS production and detoxification is lost, leading to H_2O_2 accumulation and protein oxidation. This study provides insights into the oxidative and antioxidant response of *P. algarbiensis* and *P. almogravensis* to low pH and Al stress contributing to elucidate the mechanisms of plant adaptation to acidic Al-rich soils. This knowledge could be useful for the selection of tolerant germplasm and for the development of plants with enhanced performance in these soils.

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CHAPTER 9

Changes on organic acid secretion and accumulation in *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. under aluminum stress

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Martins N, Gonçalves S, Andrade PB, Valentão P, Romano A (2013) Changes on organic acid secretion and accumulation in *Plantago almogravensis* Franco and *P. algarbiensis* Samp. under aluminum stress. Plant Science 198:1-6

9.1. Abstract

We investigated the effect of Al (400 μM) on organic acids secretion, accumulation and metabolism in *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. Al induced a significant reduction on root elongation only in *P. algarbiensis*. Both species accumulated considerable amounts of Al (> 120 μg g^{-1}) in their tissues, roots exhibiting the highest contents (> 900 μg g^{-1}). Al stimulated malonic acid secretion in *P. algarbiensis*, while citric, succinic and malic acids were secreted by *P. almogravensis*. Moreover, Al uptake was accompanied by substantial increases of citric, oxalic, malonic and fumaric acids contents in the plantlets of either species. Overall, the acid metabolizing enzymes were not directly involved in the Al induced organic acid secretion and accumulation. Our data suggest that Al detoxification in *P. almogravensis* implies both secretion of organic acids from roots and tolerance to high Al tissue concentrations, while in *P. algarbiensis* only the tolerance mechanism seems to be involved.

9.2. Introduction

Aluminum (Al) is the most abundant metal in the earth's crust where it remains mostly insoluble (Kochian et al. 1995). However, when soils become acidic as a result of natural processes or human activities, Al hydrolyses in a pH-dependent manner to form various complexes into soil solution with hydroxyl groups that show different biological impacts (Kochian et al. 1995). The most phytotoxic form of Al is Al^{3+}, which predominates in solutions below pH 4.5 (Kochian et al. 1995). The toxicity of this metal to plants is ascribed to the high affinity of Al^{3+} for cell walls, membranes and metabolites (Kochian et al. 2005). Some native plant species evolved, however, adaptations to avoid direct contact of Al^{3+} ions with vital structures and metabolic processes (Watanabe et al. 2002; Vardar et al. 2007) and can therefore grow well in acidic Al-rich soils. Indeed plant species greatly differ in their tolerance to Al stress being some plants inherently more tolerant than others and, thus, the concentration of Al used in Al tolerance studies should be chosen according to the plant and the purpose of the study.

The mechanisms that plants have evolved to cope with Al^{3+} stress can be divided into tolerance mechanisms and resistance or exclusion mechanisms. The first enable plants to safely accommodate Al^{3+} once it enters the symplast either by chelating it in the cytosol to form harmless complexes or by sequestering it to organelles where it cannot disrupt metabolism (Ryan et al. 2011). Resistance or exclusion mechanisms prevent Al from accumulating in the symplast through the root apices secretion of Al chelating compounds (Kochian et al. 1995). These mechanisms involve the accumulation and/or secretion of ligands with high affinity for Al cations. Stability constants for metal-ligand complexes used to theoretically evaluate the effectiveness of ligands in Al detoxification indicate that organic acids (OAs), namely citric, oxalic and malic, are the

most relevant ligands (Tolrà et al. 2005). Additionally, Al-induced disturbances in biochemical pathways of OAs metabolism have been observed in distinct plant species (Yang et al. 2004; Andrade et al. 2011). OAs metabolism involves an array of enzymes, such as citrate synthase (CS), phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), fumarase and NADP-isocitrate dehydrogenase (NADP-ICDH). These enzymes are interrelated, function in coordination with each other, and play important roles in OAs biosynthesis and degradation (Mariano et al. 2005).

Plantago almogravensis Franco is an endemic species that grows along the southwest coast of Portugal, considered to be an Al hyperaccumulator plant (Branquinho et al. 2007). Recently, results of our group showed that also *Plantago algarbiensis* Samp., another endemic species from Portugal (West-Central Algarve region), accumulates high amounts of Al (unpublished data). In this context, it seems interesting to understand the mechanisms of Al detoxification, based on the exclusion of Al from the symplast and/or intracellular chelation of Al by OAs in these *Plantago* species. This knowledge may provide an effective approach for illustrating fundamental aspects of plant physiology and allow new strategies of developing crop varieties with high tolerance to Al. Given that both species are protected under the European Habitats Directive and by Portuguese law and in order to have enough number of plants we established micropropagation protocols for these species (Gonçalves et al. 2009). We have demonstrated that they can grow in medium with low pH (Martins et al. 2011). We therefore set out to evaluate changes in OAs accumulation and secretion in *P. almogravensis* and *P. algarbiensis*, as well as in the activities of their related metabolic enzymes during Al stress.

9.3. Materials and methods

9.3.1. Plant material and Al treatment

P. algarbiensis shoots (~6 cm in length) and *P. almogravensis* shoots (~3 cm in length) were separated from *in vitro* axenic cultures proliferating for 6 weeks in MS medium (Murashige and Skoog 1962) containing 0.2 mg l^{-1} 6-benzyladenine as described by Gonçalves et al. (2009). Plantlets were obtained by cultivating the shoots for 3 weeks in rooting medium (½MS containing 0.5 mg l^{-1} indole-3-acetic acid). The shoots and plantlets were maintained at 25 ± 2 ºC with a 16 h photoperiod (cool white fluorescent lamps, 69 µmol m^{-2} s^{-1}).

Al stress was applied by aseptically transferring shoots and plantlets to autoclaved ¼MS liquid medium with the pH adjusted to 4.0 containing 0 or 400 µM $AlCl_3$, that correspond to 140 µM Al^{3+} activity, as estimated by Geochem-EZ (Shaff et al. 2010). The Al concentration used was selected based on preliminary assays with a range of Al concentrations. Shoots and plantlets were inoculated individually in 32 × 200 mm test tubes containing 20 ml liquid medium and using filter paper bridges as support. The bridges consisted of filter paper discs containing a small hole in the centre and molded to the shape of the tube. Cultures were incubated for 7 days under the conditions described above without changing the culture medium.

9.3.2. Standards and reagents

The OAs standards were acquired from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Acetyl-CoA, isocitric acid, oxaloacetic acid, Bradford reagent, dithiothreitol (DTT), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), malic acid, polyvinylpolypyrrolidone (PVPP), and phosphoenolpyruvate (PEP) were obtained from Sigma-Aldrich (Steinheim, Germany). MDH was purchased from Calbiochem (San

Diego, USA) and NADH was acquired from Applichem (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

9.3.3. Determination of plant growth and Al content

Shoots and plantlets were harvested so their fresh weight and root elongation could be determined. The length of the longest root of each plantlet was measured before and after the treatment. Shoots and plantlets were then dried at 40 ºC until they reached a constant dry weight (DW). The dried material was powdered (mean particle size lower than 910 μm) using a blender and kept in a desiccator in the dark until they were subjected to OAs extraction and Al content determination.

The Al content in plant tissues (shoots, leaves and roots) was determined by atomic absorption spectrophotometry (GBC, Avanta-Sigma, USA) after the samples were dry ashed in a muffle furnace at 500 ºC and acid digested. The Al content was expressed in μg g^{-1} DW of tissue.

9.3.4. Extraction of OAs from plant tissues and root exudates

Each dried sample (0.1 g) was mixed with 0.01 N sulphuric acid (25 ml) for 30 min at 200 rpm and the resultant extract was filtered over a Büchner funnel. The extract was then evaporated to dryness under reduced pressure (40 ºC) and the residue obtained redissolved in 0.01 N sulphuric acid.

After 7 days of treatment, aliquots of culture medium were collected from test tubes with plantlets. The medium (25 ml) was dried under reduced pressure at 40 ºC (Rotavapor, Büchi) and the residue was redissolved in 0.01 N sulphuric acid.

9.3.5. Analysis of OAs by HPLC/UV

Aliquots (20 µl) of plant extracts (shoot, leaves and roots) and root exudates were analyzed on an analytical HPLC unit (Gilson, Villiers Le Bel, France), using an ion exclusion column Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device at 30 ºC. Elution was carried out isocratically at a solvent flow rate of 0.2 ml min^{-1}, with 0.01 N sulphuric acid as the mobile phase. Detection was performed with a UV detector at 214 nm. OAs quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, and the peaks in the chromatograms were integrated using a default baseline construction technique.

9.3.6. Measurements of acid-metabolizing enzyme activities

CS (EC 2.3.3.1), MDH (EC 1.1.1.37), NADP-IDH (EC 1.1.1.42), fumarase (EC 4.2.1.2) and PEPC (EC 4.1.1.31) activities were evaluated after shoots and plantlets had been cultured for 7 days. Fresh tissue (100 mg) was homogenized with a pre-chilled mortar and pestle in 100 mM Tris-HCl (pH 8.0) with 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1% (w/v) PVPP and 5 mM DTT. The homogenate was centrifuged at 20,000 *g* for 5 min at 4 ºC and the supernatant was used for subsequent enzymes assays. The specific enzyme activity for all enzymes was expressed as enzyme units per milligram of protein, based on the measurement of total soluble protein levels according to the method of Bradford (1976) using bovine serum albumin as standard.

CS activity was measured by the disappearance of acetyl-CoA at 412 nm using the method of Srere (1967). The reaction mixture comprised 100 mM Tris-HCl (pH 8.0), 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxaloacetic acid and 10 µl of the enzyme extract. PEPC activity was determined spectrophotometrically at 340 nm by coupling

the reaction to the oxidation of NADH in the presence of MDH as described by Lane et al. (1969). The reaction mixture comprised 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 10 units of MDH, 2 mM PEP and 20 µl of the enzyme extract. Fumarase activity was determined by the formation of fumarate at 240 nm using the method of Bergmeyer et al. (1974). The reaction mixture comprised 100 mM phosphate buffer (pH 7.6), 100 mM malic acid and 20 µl of the enzyme extract. MDH activity was determined by measuring the disappearance of NADH at 340 nm as described by Miller et al. (1998). The reaction mixture comprised 100 mM potassium phosphate buffer (pH 7.4), 0.15 mM NADH, 0.2 mM oxaloacetic acid and 10 µl of the enzyme extract. NADP-ICDH activity was determined by monitoring the formation of NADPH at 340 nm as described by Bergmeyer et al. (1974). The reaction mixture comprised 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM NADP, 4 mM isocitric acid and 20 µl of the enzyme extract.

9.3.7. Statistical analysis

The plant growth parameters were recorded using 15 shoots/plantlets, the acid metabolizing enzyme activities were analysed in 5 shoots/plantlets, whereas for the other experiments 3 replicates of at least 10 shoots/plantlets each were used. We carried out a Student's t-test on the results to assess treatment differences using the SPSS statistical package for Windows (release 19.0; SPSS Inc., Chicago, IL, USA).

9.4. Results and discussion

We observed that Al had no significant impact ($P \geq 0.05$) on fresh and dry weight of either *Plantago* species. Plant growth is known to be unaffected in species like *Rumex acetosa* L. and *Calluna vulgaris* (L.) Hull well adapted to acid soils with high Al

availability (De Graff et al. 1997; Tolrà et al. 2005), as observed in the present study. The root growth has proved to be a suitable criterion for assessing Al resistance of many plant species (Deng et al. 2009; Li et al. 2009). Therefore, the effect of Al on root elongation was used to evaluate the degree of Al resistance of *P. algarbiensis* and *P. almogravensis* and differences were observed between species. The presence of Al significantly reduced ($P < 0.05$) the root elongation by 32.6% in *P. algarbiensis*, whereas in *P. almogravensis* no significant changes ($P \geq 0.05$) were detected (Figure 9.1).

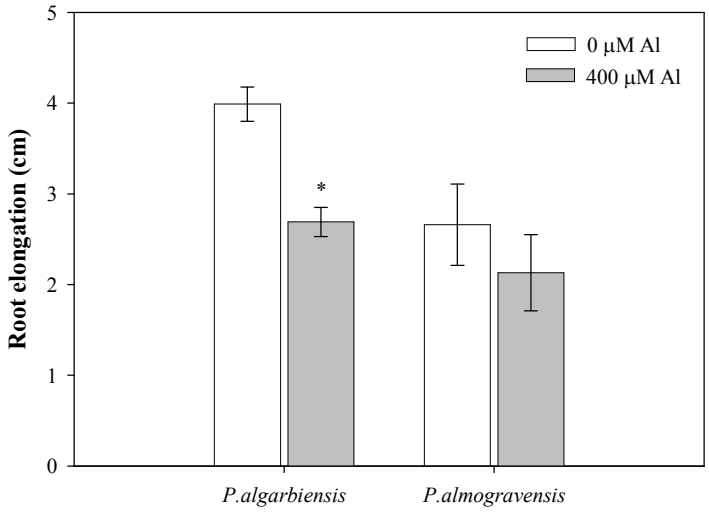

Figure 9.1. Effect of Al on root elongation of *Plantago algarbiensis* and *P. almogravensis* plantlets during 7 days of exposure. Values are expressed as the mean ± SE (n = 15). * indicates a significant difference ($P < 0.05$) between treatments.

Neither species accumulated detectable levels of Al in the shoots and plantlets when cultivated on unsupplemented MS medium because this does not contain an Al source. However, when cultivated in medium supplemented with Al both species accumulated

considerable amounts of the metal (120 - 1,200 μg g^{-1}) in their tissues (Figure 9.2), without showing visible toxic effects, such as chlorotic leaves and stubby, brittle and brown roots. Each analyzed tissue (shoots, leaves and roots) of both species accumulated similar ($P \geq 0.05$) Al contents (Figure 9.2). The Al amount detected in the aboveground parts (120 - 220 μg g^{-1}) of both species were clearly higher than the critical toxic Al concentrations (below 100 μg g^{-1}) reported for Al sensitive plants (Silva et al. 2010; Ali et al. 2011). The Al content was significantly higher in roots ($P < 0.05$) than in leaves of either species (Figure 9.2), indicating that most of the Al absorbed from the medium remains in the roots with only a small proportion being remobilized and translocated into aboveground plant parts. The restriction of Al translocation from roots to leaves may provide a means of protecting the shoot from the damaging effects of Al (Silva et al. 2004). The Al contents detected in *P. almogravensis* plantlets produced *in vitro* were lower than those previously observed in field-grown plants (Branquinho et al. 2007). This is not surprising since the controlled axenic *in vitro* conditions have a specific Al concentration in medium and Al exposure time. Moreover, other factors such as plant age and plant genotype could explain the discrepancies between the Al contents accumulated by *in vitro* produced plantlets and field-grown plants.

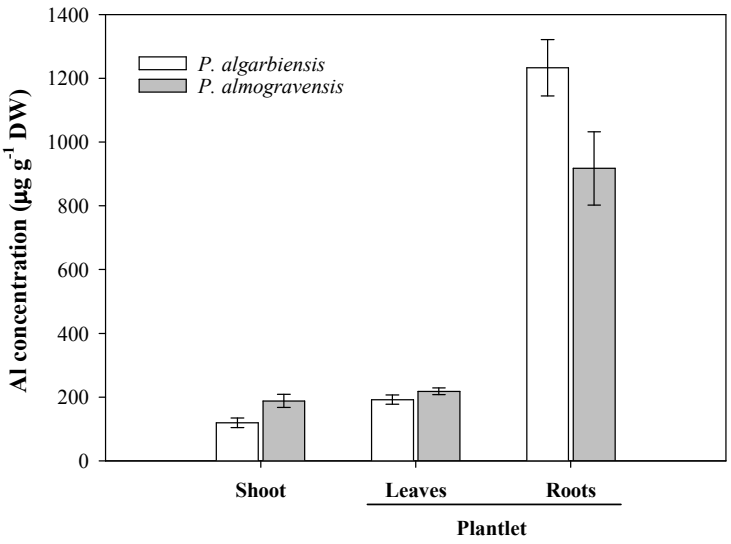

Figure 9.2. Aluminum accumulation in *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets (leaves and roots) after 7 days of culture in medium containing 400 µM Al. Values are expressed as the mean ± SE (n = 3).

Although many mechanisms of Al resistance have been proposed, secretion of OAs from the roots has been shown to be the major and most well documented one for avoiding Al toxicity in plants (Ryan et al. 2011; Inostroza-Blancheteau et al. 2012). Al chelation by OAs exudates reduces the activity of free Al^{3+} ions and, consequently, their binding to the root cell wall and/or plasma membrane. Therefore, in this work we evaluated the Al-induced secretion of OAs anions from the roots of *P. almogravensis* and *P. algarbiensis* (Table 9.1). Al induced a significant increase ($P < 0.05$, ~four-fold) on the secretion of malonic acid in *P. algarbiensis* (Table 9.1). However, because malonic acid is a relatively poor Al chelator, it is unlikely that the secretion of this acid is associated with Al exclusion. In *P. almogravensis*, Al significantly increased ($P < 0.05$) the secretion of citric, succinic and malic acids. It is well known that citric and malic acids are some of the commonly released OA anions and can form strong complexes with Al^{3+} (Ma et al. 2001). Although these OAs are released by *P. almogravensis* roots, Al was found to be accumulated in this species. These results

suggest the existence of additional internal Al detoxification mechanisms as observed in the Al accumulator buckwheat that secretes oxalic acid from the roots and accumulates Al in the form of nonphytotoxic Al-oxalate complexes (Ma et al. 1998; Zheng et al. 1998). Thus, we investigated the OAs profile of shoots and plantlets of *P. almogravensis* and *P. algarbiensis* under control conditions and Al stress.

Table 9.1. Effect of Al on the secretion of organic acids from *Plantago algarbiensis* and *P. almogravensis* roots.

Organic acids (μmol g^{-1} DW)[a]	Al (μM)	*P. algarbiensis*	*P. almogravensis*
Citric	0	41.36 ± 1.54	7.21 ± 0.12
	400	13.99 ± 0.14 *	15.74 ± 1.93 *
Malonic	0	68.12 ± 0.47	89.39 ± 3.36
	400	325.37 ± 16.54 *	68.42 ± 6.11
Succinic	0	29.96 ± 3.86	−
	400	−	7.28 ± 2.07 *
Malic	0	−	−
	400	−	179.23 ± 35.00 *
Σ	0	139.44	96.60
	400	339.36	270.68

Values are expressed as the mean ± SD (n = 3); −, not present; Σ, sum of the determined organic acids. * indicates a significant difference ($P < 0.05$) between treatments, for each organic acid. [a] The organic acids exudation in the culture medium is expressed per gram of root dry weight.

The OAs profile is composed by oxalic, citric, fumaric, succinic and malonic acids, malic acid being also present in the shoots (Table 9.2). The total OAs contents under control conditions varied from 118 to 213 μmol g^{-1} DW in *P. algarbiensis* samples and 35 to 343 μmol g^{-1} DW in *P. almogravensis*. Malonic acid was the main compound in all matrices of both species, representing 44 - 99% of the total OAs content, whereas succinic acid was detected only in vestigial amounts. This is the first report describing the OAs composition of *P. algarbiensis* and *P. almogravensis*. In addition, few chemical studies concerning the OAs profiles of *Plantago* genus have been developed. Several OAs have been reported in *Plantago major*, the most well studied species of this genus (Pailer and Haschke-Hofmeister 1969; Olennikov et al. 2005). *P. algarbiensis* and *P. almogravensis* OAs composition exhibits fumaric, citric, malonic and succinic acids in common with *P. major*.

In our study Al uptake in both species was accompanied by substantial changes in the quantitative OAs profile (Table 9.2). Al induced a significant increase ($P < 0.05$) in the contents of citric, oxalic, fumaric, malonic and malic acids. However, the effect on OAs accumulation was more pronounced in the plantlets than in the shoots (Table 9.2), probably because the whole plantlets accumulate more Al. This suggests the accumulation of OAs as part of a mechanism that binds and detoxifies Al taken up in these *Plantago* species. OAs also played a central role in internal Al detoxification in others Al accumulating plant species such as buckwheat intermittently exposed to 50 μM Al for 10 days (Ma et al. 1998), hydrangea grown for 2 weeks under 100 μM Al (Ma et al. 1997), melastoma grown under 1 mM Al for more than 2 months (Watanabe et al. 1998) and field tea plants (Morita et al. 2004).

Table 9.2. Effect of Al on organic acid contents in *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets.

Organic acids (μmol g^{-1} DW)	Al (μM)	*P. algarbiensis*			*P. almogravensis*		
		Shoot	Plantlet		Shoot	Plantlet	
			Leaves	Roots		Leaves	Roots
Oxalic	0	nq	nq	nq	23.49 ± 2.56	11.33 ± 0.96	0.52 ± 0.09
	400	nq	3.92 ± 0.11 *	5.40 ± 0.29 *	26.37 ± 2.92	16.73 ± 0.31 *	9.97 ± 0.37 *
Citric	0	1.95 ± 0.17	nq	2.73 ± 0.17	36.24 ± 7.60	5.24 ± 0.11	1.17 ± 0.02
	400	2.84 ± 0.25	4.47 ± 0.43*	19.16 ± 1.25 *	33.04 ± 6.71	7.40 ± 0.40 *	21.09 ± 1.58 *
Malonic	0	87.96 ± 5.24	211.81 ± 18.27	118.12 ± 2.13	159.24 ± 9.84	17.63 ± 0.10	341.01 ± 8.04
	400	79.53 ± 1.21	52.40 ± 1.76 *	191.41 ± 15.35 *	113.18 ± 29.87	36.01 ± 1.97 *	317.01 ± 17.24
Succinic	0	nq	nq	nq	nq	nq	nq
	400	nq	nq	nq	nq	nq	nq
Fumaric	0	0.61 ± 0.07	0.91 ± 0.10	0.08 ± 0.01	0.71 ± 0.06	0.80 ± 0.02	–
	400	0.74 ± 0.12	1.02 ± 0.02	0.54 ± 0.01 *	0.99 ± 0.06 *	1.64 ± 0.06 *	1.28 ± 0.14 *
Malic	0	27.30 ± 5.00	–	–	90.56 ± 12.66	–	–
	400	62.03 ± 4.29 *	–	–	113.02 ± 11.15	–	–
Σ	0	117.83	212.73	120.94	310.24	35.00	342.70
	400	145.14	61.81	216.52	286.59	61.78	349.34

Values are expressed as the mean ± SD (n = 3); –, not present; nq, not quantified; Σ, sum of the determined organic acids. * indicates a significant difference ($P < 0.05$) between treatments, for each organic acid.

Our results demonstrated that the Al detoxification in *P. almogravensis* involves both the secretion of OAs from roots and internal detoxification through the formation of nontoxic Al complexes with OAs. The apparent contradiction between the simultaneous Al-induced OAs secretion and Al accumulation has led to many investigations in buckwheat. Although this contradiction is not fully understood, Klug and Horst (2010) recently concluded that oxalate exudation into the root-tip water free space confers protection from Al toxicity and allows Al accumulation in the symplast. These authors presented a hypothesis to explain the transport of Al from the external solution to the xylem that involves an Al oxalate plasma-membrane transporter in the root cortex and a xylem-loading Al citrate transporter in the xylem parenchyma cells.

An increase in the activities of several acid metabolizing enzymes has been linked to the secretion and accumulation of OAs in response to Al stress (Yang et al. 2004; Mariano et al. 2005). Therefore, we investigated the effect of Al on the activities of five enzymes involved in OAs metabolism in shoots and plantlets of *P. almogravensis* and *P. algarbiensis*. Overall, we observed that Al had no significant ($P \geq 0.05$) impact on the activities of the acid metabolizing enzymes investigated in the shoots (Figures 9.3, 9.4), the exception being a significant ($P < 0.05$) two-fold decrease in PEPC activity in *P. algarbiensis* (Figure 9.3 A). Conversely, we observed significant changes in MDH, PEPC, fumarase, NADH-ICDH and CS activities in the plantlets of either species in response to Al (Figure 9.3, 9.4), which agrees with the higher accumulation of OAs. We observed a significant ($P < 0.05$) three-fold increase in CS activity in *P. algarbiensis* Al treated roots (Figure 9.3 C). Similarly, Al-induced accumulation of citric acid and increase of CS activity was also previously observed in *Cassia tora* L. grown under 10 - 50 μM Al up to 12 h and in *Citrus junos* Sieb ex Tanaka exposed to 100 μM Al for 24 h (Yang et al. 2004; Deng et al. 2009). Al significantly ($P < 0.05$) increased the

NADP-ICDH activity in *P. algarbiensis* leaves and *P. almogravensis* roots (Figure 9.3 E, F). In both species the activities of MDH and fumarase increased in roots and leaves, respectively (Figure 9.4). On the other hand, Al induced a significant ($P < 0.05$) four-fold decrease in PEPC activity in *P. algarbiensis* roots (Figure 9.3 A).

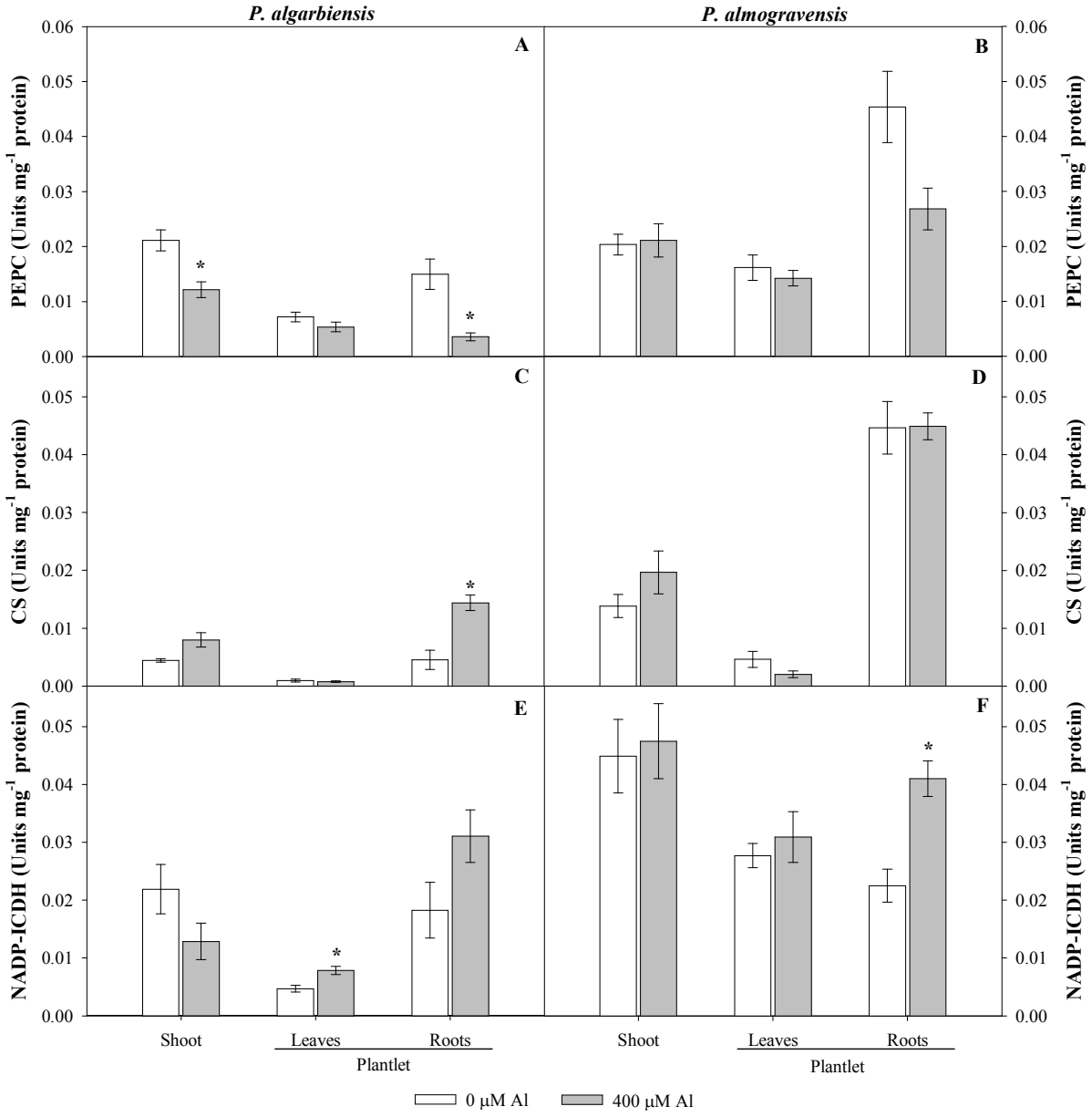

Figure 9.3. Effect of Al on the PEPC (A, B), CS (C, D) and NADP-ICDH (E, F) activities of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets. Values are expressed as the mean ± SE (n = 5). * indicates a significant difference ($P < 0.05$) between treatments.

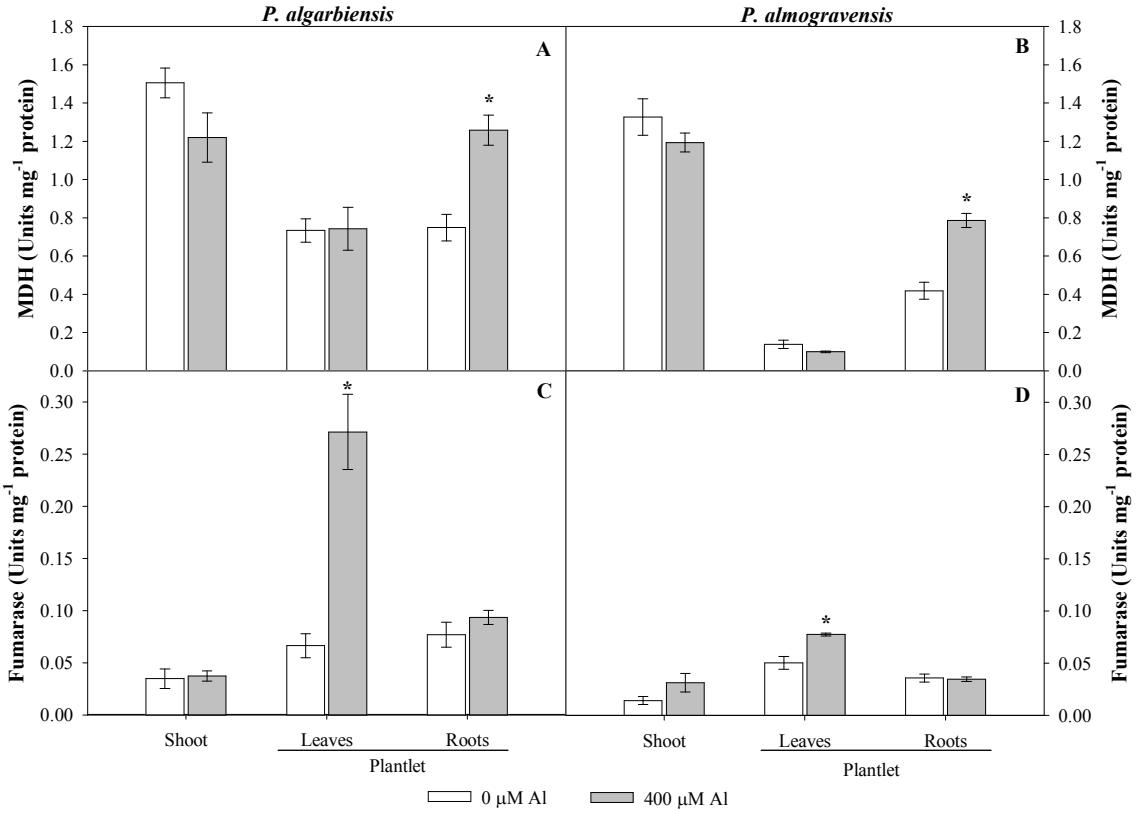

Figure 9.4. Effect of Al on the MDH (A, B) and fumarase (C, D) activities of *Plantago algarbiensis* and *P. almogravensis* shoots and plantlets. Values are expressed as the mean ± SE (n = 5). * indicates a significant difference ($P < 0.05$) between treatments.

Overall, the differences observed in OAs accumulation and secretion between control and Al treated samples cannot be directly explained by differences in acid metabolizing enzyme activities. In agreement with our results, an increased OAs accumulation and/or secretion were detected in wheat grown under 50 µM Al for 12 h and in triticale exposed to 50 µM Al for up to 24 h despite the acid metabolizing enzyme activities remained unaffected (Li et al. 2000; Hayes and Ma 2003). Increased activity of plasma membrane OAs transporters and increased availability of OAs ligands for transport can also be responsible for the high accumulation of OAs (Kochian et al. 2005). In addition, Mariano et al. (2005) suggested that the Al induced OAs accumulation may not

specifically result from the metabolic activity of the tissue where these changes were found.

Both *Plantago* species accumulate considerable and similar amounts of Al, although this metal induced reduction in root elongation only in *P. algarbiensis*. Our results suggest that OAs play an important role in Al detoxification in these species, mainly in *P. almogravensis*. In this species the Al detoxification may be achieved by the combination of OAs secretion from roots and Al intracellular chelation by OAs. However, the role of each mechanism as well as the process of Al uptake and translocation in this species deserves to be further investigated.

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GENERAL CONCLUSIONS

Aluminum (Al) toxicity is the most widespread problem affecting plant development on acid soils that comprise more than 40% of the arable land. Low soil pH tends to release large amounts of Al cations in soil solution, which bind to several targets in the root system, blocking cell division, decreasing water and nutrient uptake, and ultimately interrupting plant growth. Most acid soils are not completely infertile and some wild plant species can grow vigorously in such soils. Because of their agronomic importance most studies of Al toxicity and tolerance are performed in crops, although the Al tolerance levels in wild species native from acidic Al-rich soils are much higher when compared to those observed in crops. Therefore, wild plants may be much better resources to isolate high-tolerant genes and are excellent models to understand the mechanisms against this stress or to search for novel mechanisms that could be useful to increase tolerance in other species.

Plantago almogravensis Franco and *Plantago algarbiensis* Samp. are two Portuguese endemic species in risk of extinction, which grow in acid soils. *P. almogravensis* was classified as an Al hyperaccumulator plant in a study conducted using plants growing in field conditions. However, more studies to evaluate in detail the toxicity effects of Al and the tolerance mechanisms are required. Thus, the aim of this work was to study the Al bioaccumulation and the mechanisms of low pH and Al toxicity and tolerance in this species and also in its closely related species *P. algarbiensis*.

To overcome several difficulties of studying Al toxicity in field conditions, such as the requirement of several months for concluding the assays, the influence of environmental factors and the difficulty to study root systems, solution culture assays have been extensively used. However, to conduct these studies a number of factors must be considered, mainly the effects of pH and the solubility of Al and nutrients, otherwise results with reduced precision and confidence will be obtained. Programs such as Geochem-EZ have been reported as important tools to develop nutrient solutions for plant Al tolerance experiments since they can predict the solubility and speciation of Al and nutrients.

Almost all studies that select Al tolerant plants and evaluate their tolerance mechanisms have been conducted with seed-derived plants in hydroponic solutions requiring a high number of seeds. Since *P. almogravensis* and *P. algarbiensis* are in risk of global extinction and are legally protected the use of a reduced number of seeds was imperative. This constraint was overcome with the use of *in vitro* propagation techniques that allowed the production of a high number of shoots per initial explant. In this context, the first step of this work was to establish micropropagation protocols for both species using seedlings obtained from *in vitro* germinated seeds as explants (Chapter 2). Shoots of both species showed high shoot multiplication rates and rooting frequencies. The best multiplication results were obtained in MS medium supplemented with BA (8.5 ± 1.1 and 9.2 ± 1.2 shoots per explant in *P. algarbiensis* and *P. almogravensis*, respectively). High rooting frequencies were attained (100 and 80%, respectively) that were not significantly influenced by the concentration of MS macronutrients or auxins. *In vitro* produced plantlets were successfully acclimatized to *ex vitro* conditions with high survival rates (80 - 95%). Thus, fulfilling the first objective of this work, two effective micropropagation protocols were developed for

P. algarbiensis and *P. almogravensis*. Following these protocols it was possible to produce enough plant material (shoots and plantlets) in a relatively short time to use in Al toxicity studies.

Low pH can affect plant growth and development directly by high H^+ activity, or indirectly by high levels of minerals, particularly Al^{3+}. Much attention has been given to Al toxicity, but not much is known about how plants cope with high H^+ activity. To better understand H^+ toxicity, it is essential to evaluate the effects of low pH separately from the combination of low pH and Al. After having optimized the methodology to produce plants and having in mind that both *Plantago* species seem to thrive in acidic soils, the ability of *in vitro* cultures of both species to grow in low pH conditions was investigated in Chapter 3. In general it was found that medium pH did not affect *in vitro* multiplication and rooting of micropropagated shoots of both species. Moreover, the cultures developed normally in all the medium pH tested, without visually noticed damages caused by low pH. Interestingly, cultures of both species modified the initial values of medium pH (4.50, 5.00 and 5.75) to the same final value that was much more acidic during rooting, suggesting an active role of the plant roots in establishing an optimum pH environment. In this way, both *Plantago* species can be considered apt to grow *in vitro* in medium with pH values much lower than the commonly used in tissue culture (pH 5.70 - 5.80), which is in agreement with the fact that both species colonize acid soils.

It is known that metal tolerant species should express resistance at all developmental stages, particularly during germination when the young plants are most vulnerable. In fact, germination and root growth assays in seedlings have been widely used to evaluate the Al tolerance in several plant species. Before conducting such studies in *P. algarbiensis* and *P. almogravensis* it was essential to optimize the germination

frequencies obtained in Chapter 2, 80% for *P. algarbiensis* and 58% for *P. almogravensis*. Thus, in Chapter 4 the germination requirements were studied and results showed that germination frequencies of both species increased to 100% at 15 ºC under either light or darkness. This is in accordance with the optimal germination temperature ranges observed in others *Plantago* species. The seeds of these two species reached the maximum germination without any pre-treatment showing, therefore, no intrinsic dormancy.

In Chapter 5 the impact of several Al concentrations (0, 100, 200 or 400 μM) on seed germination and early development of *P. algarbiensis* and *P. almogravensis* seedlings was evaluated. To avoid the problem of Al precipitation in culture medium the seeds were germinated in a $CaCl_2$ solution solidified with bactoagar that allowed reproducible Al concentrations and has been widely used in Al studies performed with seedlings. However, this $CaCl_2$ solution can only be used to evaluate young seedlings, when the seed is still capable of providing all necessary mineral nutrients. The results showed that up to 400 μM Al had no impact on the germination percentage and mean germination time in either species or on the overall morphology of the seedlings, reflecting an Al-tolerant behavior. The Al accumulation in the root tips of both species was concentration dependent only up to 200 μM suggesting the lack of an effective Al exclusion mechanism. On the other hand the reduction of Al accumulation observed between 200 to 400 μM may reflect the induction of the Al exclusion under high Al concentrations, or the consequence of root apex damage. Al inhibited the root growth of both species in a concentration-dependent manner (more severely in *P. algarbiensis*). It was also observed a concentration-dependent induction of SOD activity but no changes in CAT activity, resulting in the accumulation of H_2O_2 in both species. However, the accumulation of H_2O_2 was not sufficient to induce membrane damage indicating that the

inhibition of root growth reflects the impact of the oxidative stress on the intracellular targets. The results demonstrated a correlation between Al uptake, H_2O_2 accumulation and root growth inhibition during early seedling development in both *Plantago* species, although *P. almogravensis* is more tolerant towards higher concentrations (400 μM) of the metal. The Al responses during the germination and early seedling growth were relevant for a better understanding of the Al stress process in all phases of plant development.

After knowing the effects of Al during seed germination stage it became essential to evaluate its toxicity at later stages of development. *In vitro* culture has shown an enormous potential to select Al-tolerant genotypes and to study Al tolerance mechanisms since it can provide a rapid mean of producing thousands of plants from a single explant, easy access to the root system, non-destructive measurements, controlled conditions, the possibility of eliminating the influence of other environmental effects and, therefore, evaluate stresses individually, reducing confounding variables and giving quantitative answers. As a first approach it was decided to initiate the studies with micropropagated shoots due to the facility of obtaining and handling this kind of plant material (Chapter 6). Several Al concentrations (0, 100, 200 or 400 μM) were tested to choose the most suitable for the subsequent studies. In these studies it was used MS medium at ¼ of strength with pH 4.0 to more closely match Al availability and toxicity in acid soils and to minimize the problems related with Al speciation, precipitation and polymerization. This medium was adequate to investigate Al tolerance in plants at all stages of development, conversely to the $CaCl_2$ solution used in the previous chapter. Moreover, as estimated by the chemical speciation Geochem-EZ program, the modified MS culture medium used allowed similar percentages of available Al to those of other nutrient solutions often used in Al tolerance studies.

In the first study the aluminon colorimetric method was used and large amounts of Al (1,000 - 4,500 μg g^{-1} DW) were quantified in *P. algarbiensis* and *P. almogravensis* shoots and a positive correlation was observed between the accumulation of Al *in planta* and the concentration of Al in the medium. However, some inconsistencies in the reproducibility of the results were observed, which may point out the requirement of a more sensitive and precise analytical method such as the atomic absorption spectrophotometry.

Furthermore, the impact of low pH and Al on the metabolism of the shoots was also investigated in Chapter 6. These *Plantago* species showed differences in terms of sensitivity to low pH and Al and in the specific responses. In the shoots of *P. almogravensis* no membrane damage was found, suggesting that this species was protected from oxidative damage. It is well known that all plants develop non-enzymatic and enzymatic antioxidant systems to mitigate oxidative stress. Therefore, the higher proline and carbohydrate contents and enhanced antioxidant enzyme activity observed in this species under stress may be responsible for the improved protection. In *P. algarbiensis* the high proline content and antioxidant enzyme activity induced by low pH and/or Al were not sufficient to suppress the oxidative membrane damage observed in the shoots. Overall, in this study the presence of H^+ and Al^{3+} at low concentration (medium supplied with 100 μM Al at pH 4.0) reduces the stress, suggesting that each ion alleviates the toxicity of the other to a certain extent, as already reported in other plant species. The highest Al concentration (400 μM) was selected to be used in the subsequent studies, since under this Al concentration no visible symptoms of toxicity were observed and a distinct biochemical response to Al stress was observed between both species.

Since root growth inhibition is the earliest and most dramatic symptom of Al toxicity, it was essential to evaluate the response to Al also in plantlets. Therefore, in Chapter 7 the accumulation of Al in shoots and plantlets (leaves and roots) of both species was determined through atomic absorption spectrophotometry, which provides higher sensitivity and precision to quantify Al in plant matrices than the aluminon method used in the previous chapter. Atomic absorption spectrophotometry is the technique most widely used in the determination of more than 60 metals and metalloids and their sensitivity typically reaches concentration ranges in the order of parts per million to parts per billion. This method presents other advantages such as speed and high selectivity.

The quantification of Al by atomic absorption spectrophotometry revealed that the shoots and plantlets leaves of both species accumulated similar amounts of Al (120 - 220 μg g^{-1} DW) that were higher than critical toxic Al concentrations (< 100 μg g^{-1} DW) reported in Al sensitive plants. The roots displayed higher Al contents (900 - 1,200 μg g^{-1} DW), suggesting the existence of a mechanism that immobilizes and sequesters Al preventing its translocation to the aboveground parts and, therefore, protecting these tissues from the damaging effects of Al. The Al accumulated in the shoots was clearly lower compared with the results previously obtained via the aluminon method, which may reveal the inadequacy of the aluminon method for this kind of analysis. With the development of more selective techniques such as atomic absorption spectrophotometry and inductively coupled plasma spectroscopy, colorimetric methods were only used as preliminary approaches. Considering the Al contents detected by atomic absorption spectrophotometry, the values obtained in plantlets produced *in vitro* were lower than the previously observed in field-grown plants of both species. This is not surprising considering the differences

between the controlled axenic *in vitro* conditions that have a specific Al concentration in medium and Al exposure time, and the natural field conditions. Other factors such as plant age and plant genotype could also explain the discrepancies between the Al contents accumulated by *in vitro* produced plantlets and field-grown plants. In the future it will be important to test higher Al concentrations in the medium in order to assess if the *in vitro* cultures can indeed accumulate Al amounts similar to field plants, and to evaluate the maximum Al amounts that these plants can tolerate without suffering severe growth damage. It is also important to understand how plants cope with Al soil toxicity in natural conditions, since studies performed *in vitro* disregard some natural conditions, such as realistic Al soil concentrations and natural climatic conditions. Using natural or semi-natural trials it is possible to confirm the key patterns and mechanisms that determine the plant response to Al toxicity under natural conditions. Therefore, in future works it is important to conduct Al studies with micropropagated plants already in *ex vitro* conditions and in acid soils with Al levels similar to the soil where these plants grow in order to mimic the field conditions.

The physiological responses of the shoots and plantlets to low pH (4.0) and Al stress (400 μM) were investigated in Chapter 7. Overall, it was found that neither of the stress treatments influenced the growth of both species, the only exception being the Al induced reduction (~33%) on root elongation in *P. algarbiensis*. Since root growth inhibition is the most suitable criterion for assessing Al tolerance, *P. algarbiensis* can be considered less tolerant than *P. almogravensis*. This was also supported by the higher capacity of *P. almogravensis* for energy dissipation by regulatory mechanisms in the presence of Al as well as some internal adjustments such as carbohydrate accumulation and maintenance or increase of nutrient contents that, at least, partially enabled this species to cope with Al^{3+} and H^{+} toxicity. Overall, the results showed that both species

have a relatively adequate capacity to cope with Al^{3+} and H^{+} stress, but *P. almogravensis* appeared to be more adapted to maintain cellular physiology and growth under those conditions.

Taking into account that differences in Al and low pH tolerance can be explained through the presence of different mechanisms and many studies indicated that an efficient ROS scavenging system might be an important trait linked with Al tolerance, the impact of low pH and Al stress on the oxidative stress, energy partitioning and antioxidant responses in the plantlets of both *Plantago* species, was investigated in Chapter 8. It was found that low pH and Al stress did not affect the function of photosystem II and no energy was diverted to non-regulated energy dissipation processes in both species, indicating that these species are able to eliminate the harmful effects of ROS at the leaves. Indeed, it was shown that leaves and roots of *P. almogravensis* were able to minimize the accumulation of H_2O_2 and, therefore, limit the oxidative damage on cellular targets in response to low pH and Al stress. In *P. algarbiensis* the antioxidant system was unable to fully suppress the toxicity imposed by Al and so the balance between the ROS production and detoxification is lost, leading to protein oxidation in the roots. Since the Al stress is inevitably studied in combination with the low pH stress, sometimes a genotype more sensitive to one stress is classified as being tolerant to the other stress, therefore in the last three chapters the effect of low pH (medium at pH 4.0 without Al) was studied separately from the combined low pH and Al (medium at pH 4.0 containing 400 μM Al). Overall, it was found that Al triggered more changes on several parameters than low pH alone in both species, suggesting that the two stresses differ in their toxicity effects.

Roots encounter different Al concentrations through acid soils and, therefore, root elongation may not be uniform, occurring root growth inhibition and recovery

simultaneously. Thus, it is of outmost importance to explore the mechanisms underlying the Al toxicity recovery in the two species. In fact, some preliminary assays investigating how the *Plantago* roots, upon return to an Al-free condition, recover from the injury caused by the exposure to Al, were conducted. The results showed a clear difference between recovered roots and continuously Al treated roots. The recovered roots transferred from Al solution to Al free solution showed less root growth inhibition, Al accumulation and Al induced dead or injured cells, compared with the roots continuously treated with Al. The mechanisms of Al recovery are not simple, some may repair or reduce the injury caused by Al while others may lead to the exclusion of toxic Al, but both mechanisms can also operate simultaneously in some plant species. Physiological studies are being conducted to better elucidate the mechanisms involved during this process in *P. almogravensis* and *P. algarbiensis*.

Considering the Al tolerance demonstrated throughout this study by both species, particularly by *P. almogravensis*, understanding the mechanisms underlying Al detoxification was of outmost importance. Organic acids (OAs) play an important role in such mechanisms forming stable, non-phytotoxic complexes with Al either externally in the rhizosphere or internally in the symplasm, thus in Chapter 9 the changes in OAs secretion and accumulation in both *Plantago* species under Al stress (400 μM) were investigated, as well as the activities of their related metabolic enzymes. Although Al stimulated malonic acid secretion in *P. algarbiensis*, it is unlikely that its secretion is associated with Al exclusion since this acid is a relatively poor Al chelator. In *P. almogravensis*, Al increased the secretion of the strong Al chelators citric and malic acids. In addition, Al accumulation was accompanied by the increase in the contents of citric, oxalic, malonic and fumaric acids, in both species. Although Al induced the secretion and accumulation of OAs in both species, no related changes were observed in

the activities of the acid metabolizing enzymes. The results obtained in this chapter suggest that OAs play an important role in Al detoxification in both species, but mainly in *P. almogravensis*. In this species the Al detoxification may be achieved by the combination of OAs secretion from roots and Al intracellular chelation by OAs. In *P. algarbiensis* only the internal detoxification mechanism seems to be involved.

Different chemical forms of Al have been identified in distinct Al accumulating species and it has been reported that the chemical form depends on the localization and the concentration of Al within the plant. Therefore, to better understand the mechanisms by which both *Plantago* species tolerate Al it is essential to identify the chemical forms of Al. Moreover, it is also important to investigate the cellular localization of Al and if changes in the chemical form occur with the internal concentration of Al and during its uptake, translocation and accumulation in plants.

In conclusion, this work extended the knowledge on the endangered *P. algarbiensis* and *P. almogravensis* species regarding the Al bioaccumulation capacity as well as the mechanisms of toxicity and tolerance to Al. The *in vitro* produced shoots and plantlets allowed performing several Al toxicity and tolerance studies without compromising the populations of both species in risk of global extinction. Both species accumulated considerable amounts of Al and demonstrated to be moderately tolerant to low pH and Al. However *P. almogravensis* displayed more effective protective mechanisms to the stress imposed by Al than *P. algarbiensis* and, therefore, seems to be more adapted to grow under acidic Al-rich conditions. These results give new data to reinforce the distinctiveness of *P. algarbiensis* and *P. almogravensis* supporting their classification as independent species. This work also contributes to a better understanding of wild plants adaptation to acidic Al-rich soils and provides fundamental information that could be used to improve the Al-tolerance in other species. In addition, the knowledge acquired

in this work, in terms of seed germination and *in vitro* propagation requirements, is useful for the implementation of conservation strategies integrating different conservation approaches, e.g. the restoration of wild populations with individuals raised *in vitro* and the establishment of germplam banks, contributing for the preservation of two endangered species in risk of global extinction.