UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Ecology of a rare and endemic *Plantago* species

Doutoramento em Biologia
Especialidade de Ecologia

Helena Cristina de Matos Serras Cadete Serrano

Tese orientada por:
Professora Doutora Maria Amélia Martins-Loução
Doutora Cristina Branquinho

Documento especialmente elaborado para a obtenção do grau de doutor

2015

ACKNOWLEDGEMENTS | AGRADECIMENTOS

Às minhas orientadoras, Professora Maria Amélia Martins-Loução e Cristina Branquinho, pela orientação, Paciência, empenho, amizade e sobretudo pelo entusiasmo – contagiante – com que vivem a ciência. Gostava muito de um dia conseguir chegar lá perto.

Ao Manuel João Pinto, que me guiou por montes e vales até aos *seus* plantagos, e partilhou o imenso conhecimento da natureza. Aos, Helena Cotrim, Cristina Máguas, Maria João Pereira e Manel Ribeiro, que partilharam comigo o seu tempo e conhecimentos, em áreas menos familiares mas muito interessantes. À Cristina Antunes, na compartilha do encanto pelos plantagos e a pela sua imensa energia positiva. À Professora Maria Salomé Pais, que me fez ganhar o gosto pelas aventuras na ciência.

Aos grupos do CBA/cE3c, (ESFE, PDC & eChanges), Jardim Botânico (MUNHAC) e CERENA (IST), em particular às pessoas que me acompanharam, apoiaram e ajudaram a crescer como bióloga e Ser Humano; mas também às instituições que possibilitaram o concretizar do trabalho. Também por isto à FCUL, ao ICNF, ao PNSACV e ao PNDonaña (em especial Enrique Sánchez Gullón). To Enrica Roccotiello in Genoa, Laura O'Connor in Barcelona, and their work groups, that welcomed me dearly and seeded new ideas.

À Lurdes Aires, por tanto, tanto trabalho mas também, principalmente, pela amizade. A todos os colegas e professores, que me auxiliaram nos trabalhos de campo, laboratório, conselhos e companheirismo: Sofia A, Pedro P, Cris A, Teresa M, Teresa D, Paula M, Rute V, Alice N, Silvana M, Sergio C, Patrícia C, Luis C, Graça O, Melanie B, Patricia F, Catarina C, Susana T, Mauro LC, Prof. Otília C, Prof. Cristina C, Prof. Ana Isabel C, Sara D, Joana B, Miguel P, Adelaide C, César G, Tila, e muitos *et al*....

Às restantes amigas e amigos, que me lembram que existe vida para além da Universidade.

Aos meus pais, pelas horas que passaram à minha espera enquanto eu fotografava umas plantinhas minúsculas no meio de nenhures. À família, em particular à minha mãe, avós, *terceira* avó e padrinhos, mas também a toda a família alargada, que tenho a sorte de me apoiar e abraçar com carinho.

The financial support for the development of this work is acknowledged to the Portuguese *Fundação para a Ciência e a Tecnologia* - FCT, for PhD grant SFRH/BD/38289/2007.

Parts of this work were also supported by the projects: (i) FCT-POCTI Project ECODILEMA (POCTI/BIA-BDE/60664/2004 & PPCDT/BIA-BDE/60664/2004) "New insights for understanding the causes of rarity in plant species". (ii) Transnational Cooperation Project, FCT-Portugal/CNR-Italy, "Biomonitoring of soil and atmospheric pollution at mine sites in Mediterranean areas: responses from cellular to ecosystem level". (iii) POA project *Estudo de referência de Plantago almogravensis 2004*, by *Parque Natural do Sudoeste Alentejano e Costa Vicentina* (PNSACV) and *Instituto Português para a Conservação da Natureza e Florestas* (ICNF), commissioned to the Botanical Garden of the *Museu Nacional de História Natural e Ciência da Universidade de Lisboa* (JB-MUHNAC).

GENERAL CONTENTS

RESUMO

A perda de biodiversidade é já aceite como incontornável e reconhece-se sobretudo a responsabilidade do Homem na aceleração da taxa de extinção natural. A nível de política internacional foram estabelecidos acordos para inventariar as espécies e decidir da sua vulnerabilidade à extinção. Esta inventariação e estatuto de conservação devem comportar conhecimentos sobre ecologia, ou seja, da relação dessas espécies com o ambiente e outras espécies coocorrentes. Somente com esses conhecimentos se podem desenvolver medidas de conservação adequadas.

Plantago almogravensis Franco, é uma planta incluída na lista de espécies com necessidade de medidas de conservação especial em Portugal (Diretiva Habitats). A planta tem o estatuto legal de *Muito Ameaçada*, o que implica um elevado risco de extinção. Os fatores que levaram a esta perceção são: (i) estar limitada a uma única população conhecida, na costa sudoeste de Portugal; (ii) registos antigos (ca. 100 anos) e mais recentes (década de 1990) sugerem a existência de outras populações, agora consideradas extintas; (iii) o tamanho da atual população está limitado a uma extensão de ocorrência de <3 ha. Recentemente, a sua classificação como espécie e endemicidade foram postas em causa, embora com base em critérios taxonómicos com lacunas e sem consideração pelo seu contexto ecológico particular. Até agora as medidas de conservação têm sido insuficientes, uma vez que a espécie mantém um mau estatuto de conservação (Diretiva Habitats 2013).

Neste enquadramento, esta tese surgiu com o objetivo de estudar a ecologia de *P. almogravensis*, em particular, para compreender as particularidades do seu nicho ecológico, nomeadamente no que respeita à sua relação com outras espécies e aos condicionantes ambientais do seu habitat, fundamentais para melhorar o estado de conservação da espécie. Procurou ainda avaliar-se a sua posição taxonómica. Especificamente, propôs-se identificar quais os fatores limitantes mais importantes que contribuem para a endemicidade e raridade desta espécie: (i) responsáveis pela amplitude do nicho realizado da espécie; (ii) diagnosticantes das fases críticas do ciclo de vida, explicativas da dinâmica populacional e fenologia; (iii) diferenciadores, por comparação, de espécies próximas, nomeadamente a *Plantago algarbiensis* Samp., com vista a compreender a sua filogenia e classificação taxonómica. Este trabalho pretendeu contribuir para o estudo teórico da raridade, o papel ecológico de *P. almogravensis* no seu ecossistema e a sua posição evolutiva. Finalmente, reconhecendo as limitações da espécie, contribuir para a melhoria das medidas de conservação.

O reconhecimento das limitações de uma espécie começa com a observação da sua morfologia e do seu habitat (**Capítulo 2**). O solo colonizado por *P. almogravensis* caracteriza-se por amplos gradientes de alumínio e carbono, destacando-se a sua presença em ilhas geoquímicas de Fe-Al rodeadas por podzol (solo franco-arenoso) mais profundo e mais rico, coberto maioritariamente por arbustos. Por comparação, o habitat de *P. algarbiensis* embora também rico em ferro, apresenta maior conteúdo em cálcio e partículas finas (argiloso). *Plantago almogravensis* coloniza preferencialmente as ilhas geoquímicas, que formam clareiras coluviais em zonas de sub-bosque de arbustos ou árvores. Como as áreas de clareira são muito expostas a diferentes stresses ambientais, os indivíduos presentes são geralmente mais jovens do que nas áreas de sub-bosque, onde podem atingir maiores tamanhos e/ou idades, mas onde raramente são encontradas plântulas. *Plantago almogravensis* é um acumulador de metais, particularmente alumínio, em tricomas glandulares de folhas e em zonas lenhosas. *Plantago almogravensis* e *P. algarbiensis* apresentaram diferenças significativas no padrão de crescimento e morfologia (número e comprimento de folhas, número de rosetas por planta; comprimento das espigas, pedúnculos ou brácteas; e peso das sementes). Estas espécies podem propagar-se vegetativamente e formar associações micorrízicas, relevantes para superar condições de stress ambiental. Para *P. radicata* Hoffmanns. & Link, apesar da comparação ter sido apenas feita com base em fotografias e descrições pré-existentes de floras, as diferenças com *P. almogravensis* são consideráveis, nomeadamente nas dimensões das espigas ou na morfologia da flor. As diferenças entre taxa foram observadas não só nas populações selvagens, mas também quando cultivadas nas mesmas condições ambientais (estufa e jardim), sugerindo uma forte separação entre os taxa (especiação), e não apenas uma adaptação às condições ambientais (plasticidade).

A distribuição de *P. almogravensis* no seu habitat reflete os limites do seu nicho realizado. No entanto, o desempenho fisiológico sugere uma amplitude diferente para o seu nicho fundamental. A espécie apresentou um melhor desempenho fisiológico em locais em que o seu desempenho ecológico foi menor, presumivelmente devido a compensações relacionados com restrições ambientais (em especial a toxicidade do alumínio no solo). Por outro lado, o desempenho ecológico estará mais ligado à competição com arbustos e assim, seguindo a Hipótese do Nicho Inclusivo (**Capítulo 3**). Este trabalho permitiu, pela primeira vez, a validação desta hipótese em plantas usando medições ecofisiológicas como ferramenta metodológica. Os componentes biótico (plantas vizinhas) e abiótico (edáfico) do nicho foram correlacionados com parâmetros de desempenho ecológico (densidade de plantas e cobertura) e fisiológico da planta (concentração de carbono e azoto e respetiva composição isotópica das folhas; parâmetros de crescimento). As limitações observadas contribuem para explicar o estatuto de raridade da espécie: pouca capacidade para competir mas, devido a uma maior tolerância ao alumínio ligada à capacidade de hiperacumular aquele metal, a espécie tem a capacidade de encontrar refúgio em ilhas geoquímicas cujas condições são demasiado agrestes para a maioria das outras espécies.

A capacidade de *P. almogravensis* para hiperacumular alumínio foi demonstrada na resposta ao gradiente no solo, em condições naturais (**Capítulo 4**), correspondendo a uma curva de saturação a 3,0 mg Al g^{-1}, nas folhas. Não houve redução da absorção de Ca e Mg, confirmando a elevada capacidade de tolerância interna ao metal.

Considerando que têm sido descritas origens genéticas para a hiperacumulação, a combinação de analise filogenética e de metais poderia proporcionar uma melhor resolução para a questão da distinção entre *P. almogravensis* e espécies próximas. A distribuição de hiperacumuladores na filogenia do género *Plantago* (**Capítulo 5**) mostrou uma alta frequência, compatível com uma origem filogenética, particularmente para o caso do alumínio. A distinção taxonómica não foi conseguida com os métodos moleculares utilizados (combinação de ITS + *trnLF*). Alguns autores sugerem uma ligação entre as características morfo-fisiológicas de plantas hiperacumuladoras e as de resistência a stress ambiental (nomeadamente à seca), que parecem ser compatíveis com os resultados deste trabalho.

A sobrevivência de uma planta pode depender da comunidade de micro-organismos no solo, quer contribuindo para a tomada de nutrientes, quer para a mitigação de toxicidade ou de ataques de agentes patogénicos. A estrutura espacial das comunidades microbianas do solo (**Capítulo 6**), foi relacionada com a distribuição de fatores bióticos (incluindo *P. almogravensis*) e de fatores edáficos no habitat. Os resultados mostraram que a abundância de *P. almogravensis* está associada a baixas atividades microbianas e a grupos funcionais específicos, que por sua vez podem afetar a capacidade de resiliência das plantas nas ilhas geoquímicas.

Acompanhou-se a dinâmica de uma subpopulação de *P. almogravensis* ao longo de três anos (**Capítulo 7**), observando-se um saldo ligeiramente negativo para a sobrevivência (em média -5% ao ano), o que corrobora o perigo de extinção a médio prazo. As maiores taxas de mortalidade foram encontrados para plântulas e juvenis durante o estabelecimento. No campo, a observação das fenofases sugere algum desfasamento entre as espécies *P. almogravensis* e *P. algarbiensis*. Estimou-se a importância de potenciais fatores limitantes às várias fases do ciclo de vida, nomeadamente (i) a necessidade de luz: necessária ao desenvolvimento; (ii) a toxicidade por alumínio: não afeta a germinação apenas o desenvolvimento da raiz, embora com grande resiliência por parte da planta; (iii) a curta persistência do banco de sementes e (iv) micro-organismos nativos no solo: mais importantes para a sobrevivência que para a germinação.

Finalmente, propuseram-se formas de abordar a conservação de *P. almogravensis* (**Capítulo 8**), tendo em conta os conhecimentos adquiridos. Identificam-se três grandes áreas prioritárias: (i) Reduzir a fragmentação das subpopulações, limpando áreas (corredores) que permitam fazer a sua conexão; isto poderia não só aumentar a polinização e a diversidade genética mas também facultar uma área menos restrita para expansão. (ii) Identificar novos habitats adequados para reintroduzir a espécie. Idealmente, locais que conjuguem baixa competição com menor stress ambiental (ex. em zonas metalíferas), poderiam melhorar o desempenho fisiológico global da nova população. A colonização assistida em áreas similares na costa sudoeste tem sido difícil, mas possível. Mais populações implicam uma redução do risco de extinção devida a um evento catastrófico. (iii) Reprodução assistida: A polinização assistida, germinação e transplantes, são opções a considerar devido às limitações observadas. O uso de solo nativo pode ser uma fonte de inóculo para microrganismos benéficos para as plantas. Para aumentar a taxa de sucesso, a sementeira direta no campo e o transplante de juvenis com menos de um ano, devido às altas taxas de mortalidade, não devem ser aconselhados.

Os esforços de conservação poderiam ser direcionados principalmente para continuar a reintrodução assistida e a monitorização, na costa Sudoeste, com a criação de novas populações. Isto, para além de manter ou melhorar as condições ecológicas da população atual. É importante considerar que qualquer projeto ou medida de conservação pode também incluir estudos ecológicos e taxonómicos, incorporando os atributos evolutivos únicos de cada espécie e as suas características fisiológicas.

Palavras chave: alumínio, competição, conservação, hiperacumulação, nicho

ABSTRACT

Biodiversity loss and the acceleration on the natural extinction rate are driven by anthropogenic factors. Governments are obliged to elaborate checklists of conservation status, where the knowledge about species' ecology is implied. That is to say, there should be information about the species' relation with the environment and with co-occurring species, for predicting suited conservation measures.

The *Critically Endangered* plant *Plantago almogravensis* Franco has been included in the Habitats Directive, in Portugal. It is limited to one known population (within an extent of occurrence of less than 3 ha) in the Southwest coast. Its taxonomic position and endemicity have been challenged, though based on incomplete taxonomic criteria and disregard for its particular ecological context. So far, the conservation measures have been insufficient and it has a *Bad* conservation status (Habitats Directive 2013).

The aims of this work were to study the ecology of *P. almogravensis*, contributing to the theoretical study of rarity and to envisage suited guidelines to improve its conservation status. Also it intends to better understand its taxonomic position among similar taxa.

Several morphologic and ecological differences were observed between *P. almogravensis* and its sister species (**Chapters 2** and 7) though not at the molecular level (**Chapter 5**). The description of *P. almogravensis* basic morphology and edaphic habitat (**Chapter 2**) was accompanied by the observation of limitations in pollination, production of seeds and dispersion. The ecological niche was characterized (**Chapter 3**), establishing the importance of competition and environmental stress (namely Al-toxicity) for the width of the realized niche. The enhanced Al-tolerance (Al-hyperaccumulation) and its refuge in geochemical islands were confirmed (**Chapter 4**). The hyperaccumulation of aluminium is likely to have a phylogenetic origin (**Chapter 5**), though the *Plantago* genus hyper-accumulates also other metals. The ecological niche was associated with specific soil microbial functional groups (**Chapter 6**), which influence germination and mostly plant survival (**Chapter 7**). For the life cycle of *Plantago* spp., light and aluminium impact plant development, in spite of the high aluminium plant resilience (**Chapter 7**). Limitations in germination and juvenile survival during establishment are critical. Overall, a decrease in the population is envisaged from field population dynamics (**Chapter 7**).

Three conservation priorities were identified (**Chapter 8**): (i) reduce fragmentation; (ii) identify new suitable habitats and colonize; (iii) assisted reproduction. Conservation efforts could be primarily directed to continue the assisted reintroduction and monitoring, on the Southwest coast, creating new sub-populations, independently of maintaining or improving the ecological conditions of the current population. This work shows that ecological and species evolutionary uniqueness should be incorporated in conservation measures.

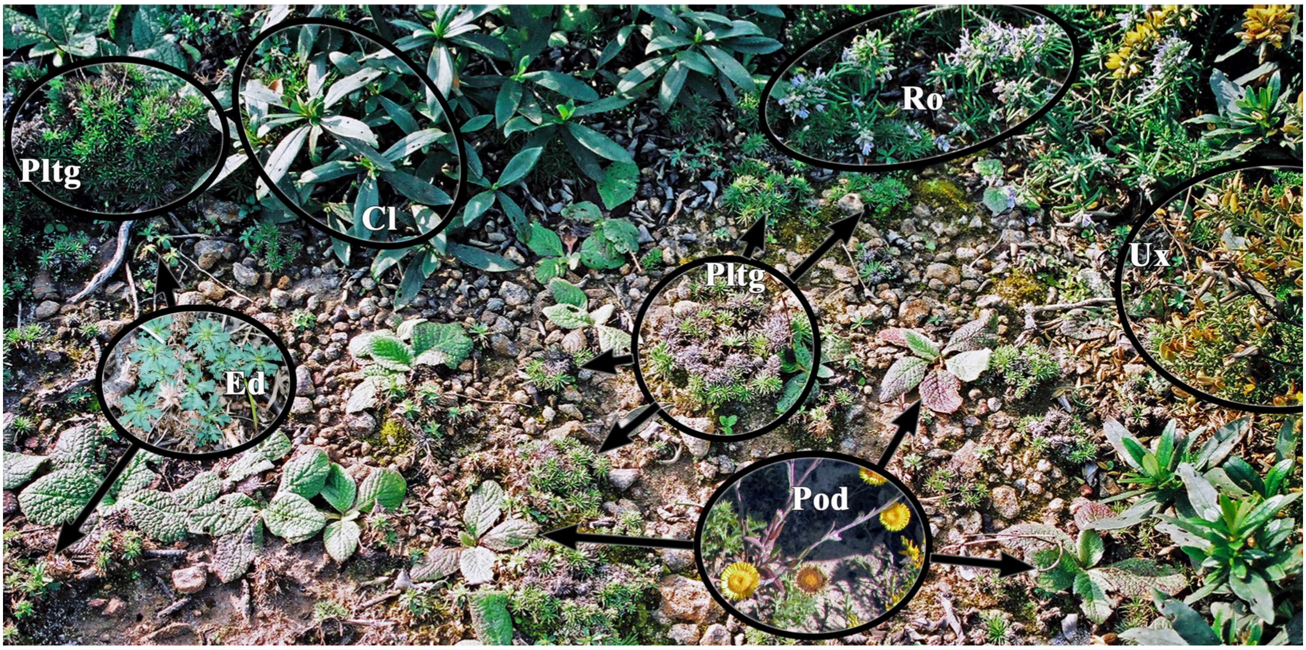
Key-words: aluminium, competition, conservation, hyperaccumulation, niche

ACRONYMS AND ABBREVIATIONS TABLE

AA	Amino Acid
AAS	Atomic Absorption Spectrometry
ACD	Area under the Curve of optical Density
aka	also known as
AM	Arbuscular Mycorrhizae
AM	Amine/amide
ANOVA	ANalysis Of VAriance
APG	Angiosperm Phylogeny Group system
bp	base pairs
BP	Bootstrap Percentage
ca.	circa; approximately
CA	Carboxylic Acid
CBA	Centro de Biologia Ambiental (now cE3c)
CBD	Convention on Biological Diversity
CEC	Cation Exchange Capacity (aka ECEC, in this work)
CERENA	Centro de Recursos Naturais e Ambiente, IST-UTL
Ch.	Chapter
CH	Carbohydrate
cont.	continued
cE3c	Centre for Ecology, Evolution and Environmental Changes
CEE	Comunidade Económica Europeia (aka EEC, EU)
CLPP	Community Level Physiological Profiling
DH	Directive Habitats
DNA	DeoxyriboNucleic Acid
DW	Dry Weight
E	Evenness (diversity index)
ECEC	Effective Cation Exchange Capacity (aka CEC, in this work)
eChanges	Ecology of Environmental Change, group of CBA/cE3c
ECM	Ectomycorrhiza
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Community (aka CEE, EU)
ENCNB	Estratégia Nacional de Conservação da Natureza e da Biodiversidade
ESFE	Environmental Stress and Functional Ecology, group of CBA/cE3c
ESPC	European Strategy of Plant Conservation
EU	European Union (aka Community, CEE, EEC)
FCT	Fundação para a Ciência e a Tecnologia
Fig.	Figure
GSPC	Global Strategy of Plant Conservation
H	Shannon's diversity index
HSD	Tukey's Honest Significant Difference, post hoc tests
ICN	Instituto de Conservação da Natureza (aka ICNB, ICNF)
ICNB	Instituto de Conservação da Natureza e da Biodiversidade (aka ICN, ICNF)
ICNF	Instituto de Conservação da Natureza e das Florestas (aka ICN, ICNB)
IST-UTL	Instituto Superior Técnico da Universidade Técnica de Lisboa (now IST-UL)
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservation of Nature
JB	Jardim Botânico
LOI	Loss On Ignition, method
max	Maximum
MGRS	Military Grid Reference System
min	Minimum
MUHNAC	Museu Nacional de História Natural e de Ciência
n	Number of replicates
NADH	Nicotinamide Adenine Dinucleotide
OM	Organic matter
p	Statistical probability significance, *p*-value
PCA	Principal Components Analysis
PDC	Plant Diversity and Conservation, group of CBA
PH	Phenol
PL	Polymer
PNDoñana	Parque Natural de Doñana, Spain
PNSACV	Parque Natural do Sudoeste Alentejano e Costa Vicentina, Portugal
PSRN2000	Plano Sectorial da Rede Natura 2000
PT	Portugal
r	Correlation coefficient
R^2	Coefficient of determination
RCM	Resolução do Conselho de Ministros
RELM	REsidual Maximum Likelihood
RH	Relative Humidity
RSD	Relative Standard Deviation (usually in %)
S	Richness (diversity index)
SD	Standard Deviation
SDS	Summer Drought Stress
sect.	section
SE	Standard Error (SD per square root of *n*) (aka SEM)
SEM	Standard Error of the Mean (aka SE)
ST	Standardized
subgen.	subgenus / subgenera
subsp.	subspecies
syn.	synonym
Tab.	Table
TBO	Toluidine blue O
TC	Total Concentration
trnLF	Plastid trnL intron, trnL 3' exon and trnL-F spacer regions
UL	Universidade de Lisboa
$\tilde{X}$	Median
v/v	volume per volume (e.g. cm^3/cm^3)
WCS	Winter Cold Stress
w/v	weight per volume (e.g. g/ml)

CHAPTER 1

GENERAL INTRODUCTION



Plant community at *Sudoeste Alentejano e Costa Vicentina* natural park (PNSACV). **Cl:** *Cistus ladanifer*; **Ed:** *Eryngium dilatatum*; **Pltg:** *Plantago almogravensis*; **Pod:** *Pulicaria odora*; **Ux:** *Ulex* sp..

CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

GENERAL INTRODUCTION

ENDEMICITY, RARITY & EXTINCTION: THE NEED FOR CONSERVATION

During our planet evolution, there have been several mass extinctions. Those most frequently acknowledged (End Ordovician, Late Devonian, End Permian, End Triassic, and End Cretaceous) ended with ca. 70% to 95% of the species. The main drivers were pointed to continental drift, environment and climate changes, modifications in atmospheric composition and other geological catastrophic events (Hallam and Wignall 1997; Póvoas 2011). Being extinction a natural event caused by environmental, ecological and evolutionary factors, the processes that lead to it depend on interactions of several phenomena, with natural selection, population genetics and habitat conditionings, over time (Stebbins 1980). Nowadays, anthropogenic factors are the most important catalysts accelerating those processes (e.g. pollution, deforestation, CO_2 rise, agriculture, etc.; Rockstrom et al. 2009; Steffen et al. 2007). At a smaller scale, everyday pressures occur over individuals, populations, species and ecosystems, which can lead them to extinction. The loss of biodiversity not only represents the loss of genetic capital, it seriously unbalances the ecosystem's resilience (Rockstrom et al. 2009; Walker 1995).

There are different factors, either isolated or in interaction, that may lead to the sequence of decline in numbers (depletion), rarity and eventually, extinction of a species. Rare species are more prone to isolation and have a higher probability of becoming extinct in a near future (Gaston and Fuller 2008; Kruckeberg and Rabinowitz 1985). The factors most commonly associated with species rarity (Ferreira and Boldrini 2011; Fiedler 1986; Gaston 2011; Klironomos 2002; Kruckeberg and Rabinowitz 1985; Stebbins 1980) are the following:

i) **Evolutive age and history**: new and old species (evolutionarily) are more prone to extinction. All new species start as rare, existing in small numbers. On the opposite extreme, old species that have been through biotype[1] depletion and/or speciation events may be reduced to small relictual populations.

ii) **Genetic variability**: a decrease in genetic variability (e.g. through endogamy, genetic drift or deleterious mutations) has potential to limit the population's capacity to adapt to habitat changes, and to decrease survival and reproduction fitness.

iii) **Population biology**: sympatric populations of close species may hybridize and reduce the size of the original parental populations. Also, the fragmentation of populations, either partial (metapopulation, in equilibrium between colonisations and extinctions) or total (isolation) hinders migrations and makes populations more vulnerable to genetic problems and stochastic events.

iv) **Reproduction**: reproductive success can be altered at many stages. Changes of sexual ratios, infertility or incompatibility and deficient pollination (e.g. absence of compatible pollinators), all may prevent fertilization. Inefficient seed dispersal and failure in germination or establishment prevent the recruitment of new individuals.

v) **Environment**: an abnormal rate of climatic change or habitat modification, faster or more severe than the species' capacity to adjust to the shift,

---

[1] **Biotype**: group of organisms having an identical genetic constitution. Oxford Dictionaries. Oxford University Press, s. v. "biotype". [http://www.oxforddictionaries.com, accessed 01-2015].

Table 1. Categories of rarity [a]: from *** not rare, to ^{RRR} extremely rare.

Geographic range:		Wide (*)		Limited (R)	
Habitat specialization:		Generalist (*)	Specialist (R)	Generalist (*)	Specialist (R)
Size of local populations:	Large, abundant (*)	***	*R*	R**	RR*
	Small, sparse (R)	**R	*RR	R*R	RRR

[a] adapted from Kruckeberg and Rabinowitz (1985) and Rabinowitz (1981).

reduces its fitness. The populations living in ecologic-islands (abruptly separated from contiguous habitats, e.g. by water, geographic discontinuities or geochemical abnormalities), being specialized into those particular habitats, become impaired from expanding outside the islands (isolated).

vi) **Biotic interactions**: the interactions with other organisms, beneficial or harmful, might be critical for the population survival. Mutualistic networks established when interactions of species are mutually beneficial are important examples of cooperation shaped by evolution (Suweis et al. 2013). Symbiotic mutualistic associations (e.g. pollinators, mycorrhizae, rhizospheric bacteria) offer better fitness, or even survival, to their partners. On the contrary, competition is a negative form of interaction; the struggle for nutrients, light or water determines which individuals establish successfully and those who do not. Another form of negative biotic interaction is represented by herbivory and pathogenicity. The frequency and number or virulence of their agents, determines the amount of fitness loss and ultimately, of survival for the population.

vii) **Anthropogenic factors**: The historical use of soil by man and recent human activities are responsible for an increased destruction of populations and habitats (e.g. urbanization and agriculture), contamination of habitats and changes in the balance among species (e.g. invasive species).

Apparently, the concepts of endemicity and rarity are often found together, but have different meanings and are both relative concepts. The expression ***endemic species*** refers generally to species native to a certain location (e.g. location limited by natural geologic barriers or by geopolitical frontiers, like countries). Nevertheless, the distribution of endemics is better-described combining isolation, geographic area and ecological breadth (Carlquist and Cole 1974; Ferreira and Boldrini 2011; Kruckeberg and Rabinowitz 1985). An endemic species can (but may not) be rare. In the 80's, authors (Kruckeberg and Rabinowitz 1985; Rabinowitz 1981) have presented an interesting framework to measure rarity more objectively. It considers the interaction of size of the local populations with the geographic range of the species and the habitat range (specialization). A species can be considered rare when local populations are small or sparse, when the geographic range is narrow or when it depends on a very particular type of habitat (Tab. 1). Not only the combination of these factors can give rise to seven different types of rarity, these can also be related to the risk of extinction (Kunin et al. 1997). Thus, a rare species is more prone to extinction as more rarity factors are found cumulatively (Tab. 1).

Conservation awareness

The importance of the problem of biodiversity loss, namely due to the extinction of rare species, is a present-day topic. Since the fifties last century, international organisations were established to gather knowledge to safeguard threatened species and prevent avoidable extinctions. The *International Union for Conservation of Nature* (IUCN) manages the *Red*

Table 2. Species in need of special conservation measures. For Portugal (**PSRN2000**) and in comparison with other status, namely EU priority, IUCN Red List status (**risk**) and Directive Habitats 2013 PT report (**DH 2013**).

PSRN2000	**EU priority** [a]	**IUCN risk** [b]	**DH 2013** [c]
Portuguese endemic species, highly compromised, occurring in only one or two sites:			
Bryoerythrophyllum campylocarpum	Yes	-	Inadequate
Linaria ricardoi	Yes	Near threatened	Inadequate
Omphalodes kuzinskyanae	-	Vulnerable	Inadequate
Plantago algarbiensis	-	Endangered	Bad
Plantago almogravensis	-	Critically endangered	Bad
Linaria coutinhoi	-	Data deficient	Inadequate
Portuguese endemics, highly susceptible, occurring in just one know site:			
Asphodelus bento-rainhae	Yes	Vulnerable	Inadequate
Convolvulus fernandesii	Yes	Vulnerable	Unknown
Silene rothmaleri	Yes	Near threatened	Favourable
Armeria berlengensis	-	Critically endangered	Inadequate
Armeria pseudarmeria	-	Endangered	Inadequate
Avenula hackelii	-	Vulnerable	Unknown
Biscutella vicentina	-	Vulnerable	Unknown
Chaenorhinum serpyllifolium subsp. *lusitanicum*	-	-	Inadequate
Cistus palhinhae	-	Data deficient	Unknown
Dianthus cintranus	-	-	Inadequate
Diplotaxis vicentina	-	Critically endangered	Inadequate
Festuca brigantina	-	Vulnerable	Inadequate
Festuca henriquesii	-	Least concern	Favourable
Herniaria algarvica	-	Vulnerable	Inadequate
Herniaria berlengiana	-	-	Inadequate

[a] Endangered species of Community interest, with particular responsibility in view of the proportion of their natural range within EU (Directive 92/43/CEE); **[b]** IUCN Red List categories (Bello et al. 2002; Bilz et al. 2011; Greuter 1989);
[c] Overall Conservation Status, update 2007-2012 of *Directive Habitats* in Portugal (2013) [http://bd.eionet.europa.eu/activities/Reporting/Article_17/Reports_2013/, accessed 24-07-2014].

List of Endangered Species[2], since 1964. The *Global Strategy of Plant Conservation* (GSPC), launched in 1999 within the *Convention on Biological Diversity*[3,4] (CBD) aims at the evaluation of the conservation status of all known plants. Portugal is involved in those initiatives since 1982, when signed and ratified the *Convention on the Conservation of European Wildlife and Natural Habitats*[5] (Berne Convention). Portugal has also enforced the European Union Directive Habitats[6] (Directive 92/43/CEE), responsible for the creation of *Natura 2000* network[4,6] of protected sites

---

[2] The IUCN Red List of Threatened Species [http://www.iucnredlist.org, accessed 24-07-2014].

[3] Convention on Biological Diversity. The Convention > CBD Convention text [http://www.cbd.int, accessed 24-07-2014].

[4] Diretivas, Convenções, Acordos [http://www.icnf.pt/portal/icnf/legisl/conv-dir-ac, accessed 24-07-2014].

[5] Treaties of the Council of Europe > Full list > n.104 Convention on the Conservation of European Wildlife and Natural Habitats [http://conventions.coe.int, accessed 24-07-2014].

[6] EU Nature Legislation [http://ec.europa.eu/environment/nature, accessed 24-07-2014].

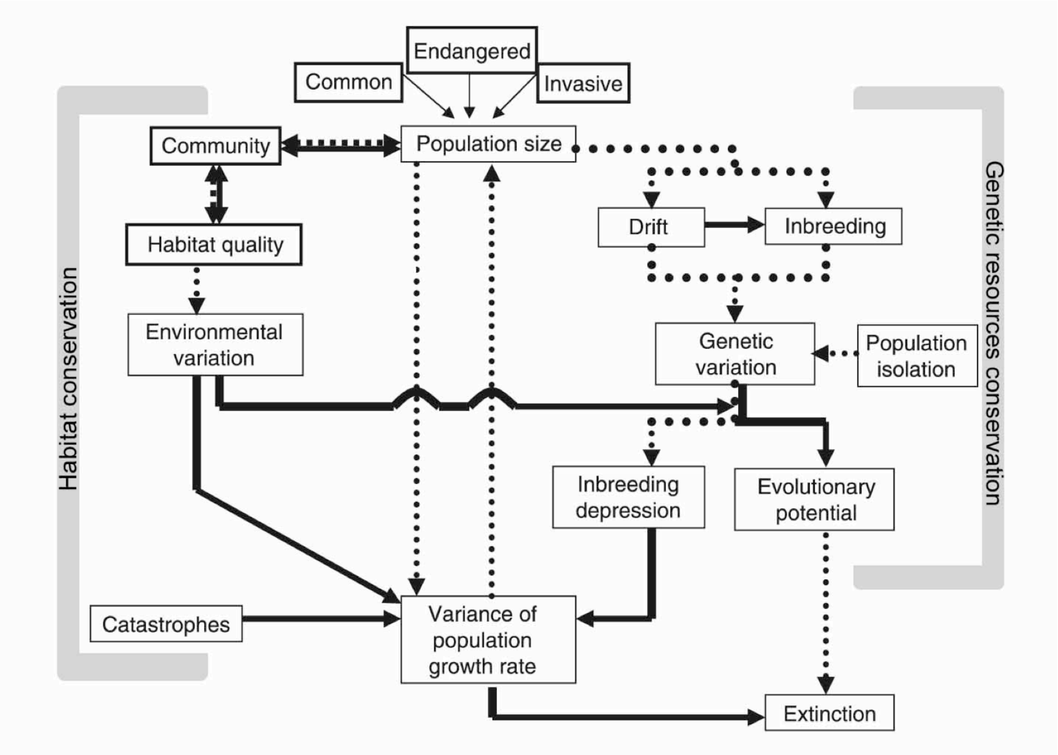


Figure 1. Two views on Biodiversity Conservation according to Ouborg et al. (2006) and Streit and Stumm (1993). Habitat Conservation (**left**) and Genetic Resources Conservation (**right**). Negative correlations are represented by **dotted** lines and positive correlations by **solid** lines.

and species, and the *Sectorial Plan for Natura 2000 Network*[4] (PSRN2000) that would implement those directives. Their goals are to help maintain biodiversity by defining a common framework for the conservation of wild plants, animals and habitats (following the strategic points of CBD). The annexes B-II and B-IV of the *Directive Habitats* and PSRN2000 highlighted species in need of special conservation measures, due to their endemicity, low presences and threatened status (Tab. 2).

Legislation efforts are more easily directed towards **habitat conservation**: to monitor and preserve habitat quality, through the creation of protected areas and natural parks. Another approach, less passive, is that of **genetic conservation**: it focus on increasing population sizes and reduce their isolation (Fig. 1). A combined approach of these two views is possible, desirable, and benefits from the thorough study of the species' ecology (Ouborg et al. 2006; Serrano et al. 2014; Smith et al. 2014). Only with scientific knowledge on the species' biology and ecology, adequate and efficient conservation measures can be planned.

THE ECOLOGICAL NICHE ASSOCIATED WITH GEOCHEMICAL ISLANDS

The **fundamental niche** of a species is traditionally defined as all the abiotic dimensions of environmental constrains where a species can maintain a viable population (Hutchinson 1957). The **realized niche** incorporates the contribution of biotic interactions to the distribution of the species and is often smaller than the previous (Colwell and Rangel 2009). Since that initial definition, the ecological niche concept has been further detailed, considering space, density dependence, dispersion, feedbacks or evolution, among others (e.g. Colwell and Fuentes 1975; Godsoe 2010; Holt 2009; Hubbell 1997; Sax et al. 2013).

Undisturbed metalliferous habitats (habitats rich in metals[7]; e.g. ore outcrops, serpentinic soils) are not frequent, especially since human mining activities begun; thus most metalliferous areas have anthropic influence (e.g. slag heaps; Baker et al. 2010). When restricted to zones with properties abruptly different from that of the surrounding background matrix, those areas exemplify an *inland ecological island* (Carlquist and Cole 1974; Kruckeberg and Rabinowitz 1985) and, particularly for metalliferous soils, a **geochemical island**. The plant communities in those *islands* (and metalliferous habitats in general) are well adapted, not only to high bioavailable metal concentrations in soil, but also to low nutrient availability (e.g. N- and P-deficiency), depletion of organic matter and drought (Baker et al. 2010). As a consequence, plant communities have low productivity, the vegetation is open, and succession is retarded (Baker et al. 2010).

The plants endemic from metalliferous habitats are often **obligate metallophytes** (e.g. *Alyssum pintodasilvae*; Dudley 1986 in Baker et al. 2010). They cannot maintain a viable population outside the metalliferous areas (Anacker 2011; Baker and Brooks 1989; Baker et al. 2010; Kruckeberg and Rabinowitz 1985; O'Dell and Rajakaruna 2011). **Facultative metallophytes** are species with viable populations either in metalliferous and non-metalliferous habitats (e.g. *Armeria maritima*, in Baumbach and Hellwig 2007; *Plantago erecta*, in O'Dell and Rajakaruna 2011; *Alyssoides utriculata*, in Roccotiello et al. 2015), sometimes recognized as ecotypes or subspecies endemic to those areas (Baker and Brooks 1989; Baker et al. 2010). Common species of wide ecological amplitude can be found in metalliferous areas, if tolerant to the soil composition, and are referred to as **pseudo-metallophytes**[8] (e.g. *Achillea millefolium*, *Phragmites australis*, *Plantago lanceolata;* Baker et al. 2010).

The dispersal of island-endemic recruits to the surrounding soil matrix, is not limited by physical barriers (like water in true islands). However, those recruits have often a pre-adaptation to the ecological island's stresses, which limit successful colonization outside that niche (O'Dell and Rajakaruna 2011). This limitation is the first step leading to ecotypes that, through isolation (gradual or catastrophic), may evolve to separate species (Kruckeberg and Rabinowitz 1985; O'Dell and Rajakaruna 2011). Considering the evolutionary history of these endemisms, they can be considered "old" or new" (Anacker 2014; Ferreira and Boldrini 2011; Kay et al. 2011; Stebbins 1980): **paleoendemics** are populations derived from former widespread species, after biotype depletion, occupying a small part of their former range; while **neoendemics** (also known as insular endemics) are younger lineages, originated after adaptive evolution. The latter are now considered the more common in the serpentine areas of California, a metalliferous environment (Mg, Ni) with a strong selective pressure and island-like nature (Anacker 2011; Anacker 2014).

---

[7] **Metal**: any of a class of substances characterized by high electrical and thermal conductivity as well as by malleability, ductility, and high reflectivity of light. Approximately three-quarters of all known chemical elements are metals. The most abundant varieties in the Earth's crust are aluminium, iron, calcium, sodium, potassium, and magnesium. *Encyclopædia Britannica Online*, s. v. "metal" [http://www.britannica.com/EBchecked/topic/377422/metal, accessed 22-07-2014].

[8] The terms *facultative metallophyte* and *pseudo-metallophyte* are often used as synonyms.

Resistance to metal toxicity

Metalliferous habitats are enriched in bioavailable toxic metals that cause stress to plants by affecting physiological functions, reducing fitness and, in extreme, inhibiting plant growth (Baker 1987). **Resistance** describes the response of the plant to that stress. It can be achieved through **exclusion** or **internal tolerance** to the stress factor (Baker 1987). The degree of tolerance to soil toxicity is independent from the type of resistance mechanism, though, generally, plants with high internal tolerance are more prone to higher soil-toxicity tolerance (Pollard et al. 2002). **Excluder plants** (Fig. 2) control high external concentrations producing ligands to chelate and detoxify the metal, immobilizing it in the rhizosphere or limiting its uptake beyond the root apoplast (Fig. 2; Baker 1987). Exclusion is the most common mechanism in metalliferous habitats (Krämer 2010; Pollard et al. 2014; van der Ent et al. 2013). **Accumulator plants** invest in tolerance mechanisms, to transport and compartmentalise the metal internally, in a safe non-toxic form (Baker 1987; Baker and Brooks 1989; Reeves and Baker 2000). Those compartments can be deciduous organs (e.g. leaves released by shedding, high turnover roots) or cell structures (e.g. chloroplasts, epidermis, cell walls, vacuoles) and ligands (e.g. organic acids, metal binding proteins) (de Andrade et al. 2011; Baker 1987; Jansen et al. 2002). Regarding the shoots, accumulator plants may respond linearly to the soil concentrations (Fig. 2 - **indicator plants**) or uptake abnormally high metal concentrations (Fig. 2 - **hyperaccumulators**).

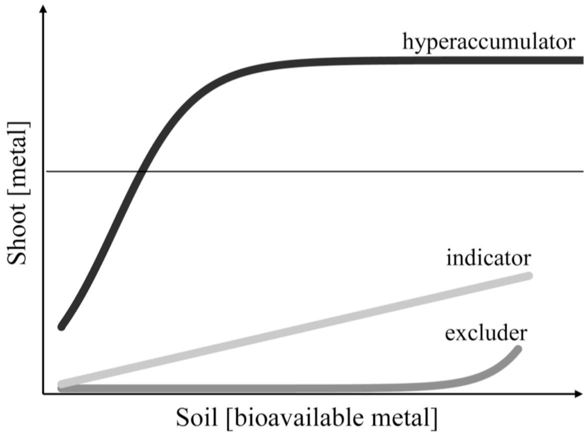


Figure 2. Hypothetical curves of metal exclusion or accumulation from soil to the shoots, adapted from Markert (1992b) and van der Ent et al. (2013). The horizontal line represents the hyperaccumulation threshold.

Metal hyperaccumulation

Hyperaccumulator plants, not only have the specific physiological mechanisms of accumulators to tolerate toxic-metals (Baker 1987), they have enhanced that trait to achieve abnormally high amounts of metals in their aboveground organs (leaves). The term was used initially for Ni accumulators (*hypernickelophores*, Jaffré and Schmid 1974) and plants found in serpentine environments (Brooks et al. 1977). Authors thereafter introduced a more objective definition of hyperaccumulation that included a **concentration threshold**, indicative of the hyperaccumulation ability (Tab. 3) and references for many other metals and metalliferous sites (Cu in Malaisse et al. 1978, reviewed by van der Ent et al. 2013; Co in Brooks et al. 1980; Pb in Reeves and Brooks 1983; Mn in Jaffré 1980; Zn in Baker and Brooks 1989; Si in Takahashi et al. 1990; Al in Jansen et al. 2002; Se in Reeves and Baker 2000; As in Bondada and Ma 2003). The plant aboveground metal concentrations must be, 10 to 100 times higher than the average for plants in metalliferous soils or, 100 to 1000 higher than the average in plants from non-metalliferous soils (van der Ent et al. 2013). Other criteria are sometimes added to better define the hyperaccumulation ability, namely (a) the bioconcentration or **enrichment factor** (shoot:soil ratio) > 1 or (b) the **translocation ratio** (shoot:root ratio) > 1 (Baker 1981; Branquinho et al. 2007).

Tolerance & hyperaccumulation

Most known plant hyperaccumulators are obligatory metallophytes, but some are facultative metallophytes, thus sometimes referred as **facultative hyperaccumulators** (Pollard et al. 2014). In these plants, if the metal hyperaccumulation capacity is present even in the non-metalliferous populations (though sometimes below the threshold, due to low soil burden), it has been described as **constitutive hyperaccumulation** or (to avoid confusion with the term from gene expression) a ***species-wide trait*** (Antonovics et al. 1971; Baker and Proctor 1990; Boyd and Martens 1998; Pollard et al. 2002). Also, most plants hyperaccumulate a single metal (Pollard et al. 2002) though a few others can hyperaccumulate several metals (e.g. *Noccaea caerulescens:* Zn, Ni, Cd, Pb and possibly Mn and Co, in Baker et al. 2000, Bert et al. 2000, Reeves and Baker 1984, Reeves and Baker 2000; *Gossia fragrantissima*: Mn & Zn, in Fernando et al. 2013). The capacity to maintain viable populations in metalliferous and non-metalliferous soils indicates also tolerance to soil toxicity, either in both soils (a **constitutive trait**) or just in the metallophyte populations (an **adaptive trait**). Though initially constitutive traits for metal tolerance were dismissed (Antonovics et al. 1971), subsequent works provided examples of innate tolerance (*Arabidopsis halleri*, Bert et al. 2002; *Phragmites australis*, Ye et al. 1997; *Thlaspi* spp., Reeves and Baker 1984, Meerts and Van Isacker 1997) or even constitutive and adaptive traits within the same species, depending on the metal considered (*Plantago arenaria* in Remon et al. 2007). This has led to the hypothesis that **constitutive tolerance** might be related to other wider ecological factors (than just metals) (Bert et al. 2000; Bert et al. 2002). One of those factors is water stress, since identified species were either wetland or xerophytic. As (adaptive) metal tolerance could result in an alleviation of metal-induced water stress (Schat et al. 1997), it was suggested that the capacity to alleviate water stress in dryland plants might also convey non-specific metal tolerance (Remon et al. 2007).

Table 3. Average metal concentrations[a] in plants and hyperaccumulation thresholds[b].

Metal	**Mean**[a] ($mg\ kg^{-1}$)	**Range**[a] ($mg\ kg^{-1}$)	**Hyper**[b] ($mg\ kg^{-1}$)
Al	80	90-530	1000
As	0.1	0.01-0.5	1000
Ca[d]	1 %	-	-
Cd	0.05	0.03-0.5	100
Co[d]	0.2	0.02-0.5	300 (1000)
Cr[d]	1	0.2-1	300 (1000)
Cu[d]	10	2-20	300 (1000)
Fe[d]	150	5-200	10 000 [c]
K[d]	1.9 %	0.5 - 3.4 %	-
Mg[d]	2000	0.1 - 0.9 %	-
Mn[d]	200	1-700	10 000
Ni	1.5	0.4-4	1000
Pb	1	0.1-5	1000
Se	0.02	0.01-2	100 (1000)
Si	0.1 %	0.02 - 0.8 %	10 000
Tl	0.05	0.03-0.3	100 (1000)
Zn[d]	50	15-150	3000 (10 000)

[a] *Reference plant* values by (Markert 1992a, b). [b] Former threshold in parenthesis (threshold references in the text). [c] value reported as hyperaccumulation for two species (Greuter 1989; Marhold 2011; Rodriguez et al. 2005; Streit and Stumm 1993). [d] Essential macro- and micro-nutrients.

Some hypothesis (not mutually exclusive) have prevailed about the origin and maintenance of the hyperaccumulation trait (Boyd and Martens 1992; Boyd 2004; Pollard et al. 2014):

i) **Inadvertent uptake:** hyperaccumulation could have arisen as a side effect from an over-efficient nutrient acquisition mechanism or as a by-product of other physiological functions. It would be a species-wide trait but hyperaccumulation would only be detectable in metalliferous soils;

ii) **Tolerance/resistance mechanisms**: the maintenance of the trait would rely on the accumulation and disposal of leaves to alleviate toxicity and induce allelopathic effects on neighbour plants, providing competitive advantages;

iii) **Elemental defence:** the metals accumulated would be toxic to pathogens and herbivores, thus provide defences for the plant; this is in agreement with the **incremental advantage** hypothesis, stating that in this case the hyperaccumulation physiology would be beneficial even for plants in non-metalliferous soils, thus conserving the trait.

iv) **Associated to drought resistance:** the xeromorphic characteristics of metallophytes or osmotic properties of metals could enhance drought resistance, providing advantages in dry environments.

v) **Phylogenetic conservation** is the hypothesis where facultative hyperaccumulators would descend from obligatory hyperaccumulators, that expanded their soil range and conserved the trait, though have lost its adaptive benefit.

Trace elements & major soil elements

The hyperaccumulation of major soil elements (Si, Al, Fe, Ca, Na, Mg, K) is traditionally considered apart from that of trace elements (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Tl, Zn), because of their ubiquity in soil. However, presence is not synonym of phytoavailability. Phytoavailability in exceptionally rich metalliferous areas enables the existence of particular metallophyte flora (e.g. Al in bauxite ores; Al and Fe in sesquioxide formations; Fe and other metals in pyritic ores or Mg and Fe in serpentine areas; Alloway 2013; Baker et al. 2010). The frequency of the concentrations measured in plants is (in general) log-normally distributed (Fig. 3) and hyperaccumulators are either at the skewed end of that distribution (Broadley et al. 2001; Watanabe et al. 2007) or, in some cases, they are part of a second cluster (in a bimodal frequency pattern; Fig. 3) that could be indicative of their distinct physiology (Metali et al. 2012; Pollard et al. 2002; Watanabe et al. 2007). Until now, more than 500 hyperaccumulators of trace elements have been identified, ca. 90% being Ni-hyperaccumulators (Cappa and Pilon-Smits 2014; van der Ent et al. 2013). For Al-hyperaccumulators we have found records of 174 to 1812 species (angiosperms and gymnosperms), depending on the authors (Metali et al. 2012; Chenery 1948, 1949). In the particular case of Al, it was suggested (Metali et al. 2012) that the threshold for the hyperaccumulation cluster was higher in tropical (2.7 mg Al g^{-1}) than in species from temperate regions (1.0 mg Al g^{-1}), justified with differences in soil, climate and time to evolve the tolerance to Al in soil. The tropics represent ancestral Al-rich environments, like podzolic soils, while temperate regions represent more recent soil acidification, often due to anthropogenic influence (Jansen et al. 2002; Metali et al. 2012).

Phylogeny of metal hyperaccumulation

The metal hyperaccumulation trait has appeared and disappeared many times in evolution at the species level (Reeves and Baker 2000). However, it is

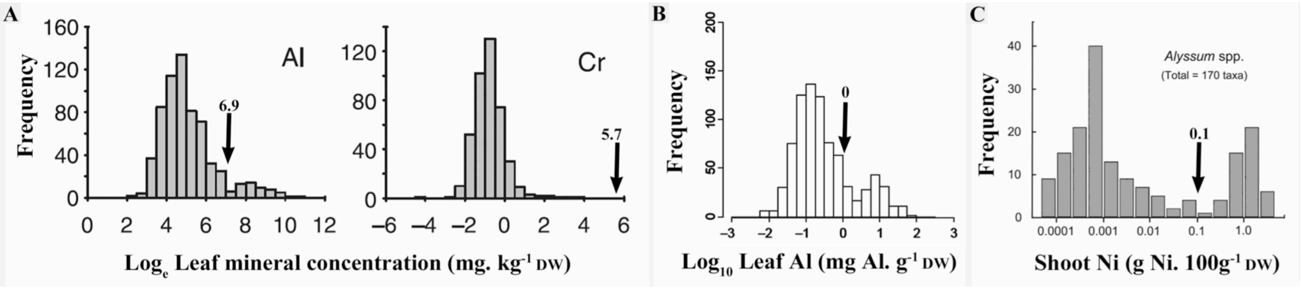


Figure 3. Frequency distribution of leaf mineral concentrations. A: adapted from Watanabe et al. (2007), *n*= 670 terrestrial plants; **B**: adapted from Metali et al. (2012), *n*= 815 angiosperms; **C**: adapted from Pollard et al. (2002), *n*= 170 *Alyssum* species. **Arrows** and **top value** indicate the hyperaccumulation threshold.

more frequent in endemics from metalliferous areas (e.g. Ni in serpentine soils) or in tropical acidic soils (e.g. Al in tropical podzolic soils), which often have a phylogenetic related flora (Baker et al. 2010; Jansen et al. 2002; Reeves and Baker 2000). For trace elements, the most significant variation in shoot metal content occurs at the classification level of order and above, suggesting an ancient evolution of traits. More occasionally, the existence of isolated hyperaccumulator species within a phylogeny suggests a recent evolutionary process (Broadley et al. 2001). The families with more Ni-hyperaccumulators (the most studied metal) are the Euphorbiaceae and Brassicaceae (Krämer 2010). The Al-hyperaccumulators are often associated with "primitive" groups, mostly woody, such as Rubiaceae, Melastomataceae or Vochysiaceae (Chenery 1948; Haridasan 1982; Jansen et al. 2002) but occasionally herbaceous, like the Lentibulariaceae (Jansen et al. 2002). According to Jansen et al. (2002) who studied the distribution of the Al-hyperaccumulation trait in 45 families, it could be used taxonomically to characterize 18 of those families. Thus, for these families where most member species were hyperaccumulators, the trait was suggested to have been inherited from an ancient ancestor and was usable as a taxonomic indicator. In the remaining 27 families the trait was restricted to species clustering as sub-families, tribes or genera, therefore implying a more recent evolution of the trait.

Al-toxicity

Soils in tropical regions are generally older, more leached and acidic than those in temperate regions (Jansen et al. 2002; Metali et al. 2012). The amount of nutrients present in soils (e.g. Ca, K Mg), as well as their Al-toxicity, is much dependant on soil pH. At low pH the nutrients are lixiviated. The ion Al^{3+} becomes bioavailable at $pH< 5$ and is considered the most phytotoxic Al-species (Kinraide 1991). Nevertheless, the presence of plants and microorganisms, the adsorption/complexation with organic matter and humic acids, the soil type and structure, or even climate, can alter the availability and form of Al, thus the Al-toxicity of a given soil (Marschner 1991; Tipping 2005). Toxicity is primarily characterized by inhibition of root elongation, swollen apices and poor root-hair development, limiting water and nutrient uptake (Bhatia et al. 2005; Kochian et al. 2005). Association of Al with apoplastic binding sites of root cell-walls may displace other cations (e.g. Ca^{2+}) necessary for the cell wall stability and elongation. Further displacement can occur in the plasma membrane, disturbing ion homeostasis (Barceló and Poschenrieder 2002; Kochian et al. 2005; Poschenrieder et al. 2008). Al-resistance is based on similar mechanisms as for trace elements (Fig. 2): exclusion and internal tolerance (accumulation) (Barceló and Poschenrieder 2002; Jansen et al. 2002; Kochian et al. 2005). Both are based mostly on the production of similar compounds, organic acids (e.g. citrate, malate, oxalate), either to immobilize externally the Al or to transport it, in an innocuous form, inside the plant to safe storage in wood, vacuoles, chloroplasts, cell walls or trichomes of leaves (Barceló and Poschenrieder 2002; de Andrade et al. 2011; Jansen et al. 2002; Kochian et al. 2005). Other suggested protection mechanisms include raising the rhizospheric pH through organic acid exudation or proton influx (Brunner and Sperisen 2013; Kochian et al. 2005); exudation of compounds like mucilage (e.g. pectin) or phenolic compounds, to help prevent Al-binding to root cell walls (Barceló and Poschenrieder 2002; Brunner and Sperisen 2013); and association with rhizospheric microorganisms like bacteria and fungi (Brunner and Sperisen 2013; Jansen et al. 2002).

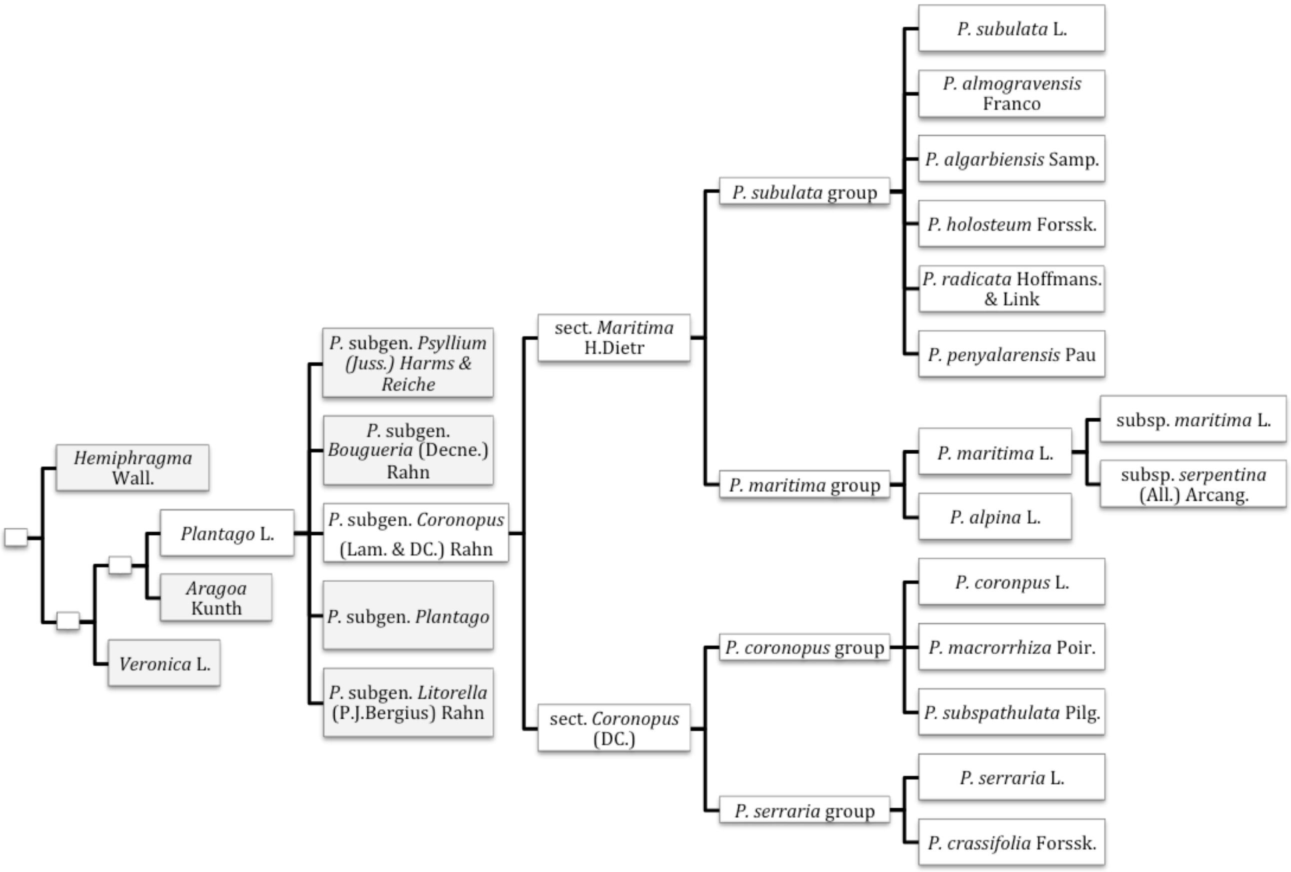


Figure 4. Detail of taxonomy and phylogenetic relations within *Plantago* subgen. *Coronopus*. After Bello et al. 2002; Greuter 1989; Marhold 2011; Rahn 1996 and Rønsted et al. 2002.

Microorganisms and metal toxicity

Bacteria and fungi in the rhizosphere are responsible for decomposing organic matter, cycling organic (C, N) and mineral nutrients (P) and for various mutualistic and pathogenic interactions (van der Heijden et al. 2008). Microorganisms can modify metal availability by producing exudates (e.g. siderophores, amino-acids, organic acids), by phosphatase-mediated solubilisation of metal-phosphates, redox changes, or by altering the rhizospheric pH (Alford et al. 2010). Mycorrhizal hyphae increase the root surface area and may compensate for nutritional unbalances (N, P) caused by excess Al (Marschner 1991). Also, although they would not be directly involved in the control of the Al-uptake to the plant, their function is quoted to be related with the protection of the root from direct contact with soil Al-species (Alford et al. 2010; Meharg 2003).

PLANTAGO ALMOGRAVENSIS – CASE STUDY

The *Plantago* genus

Plantago L. belongs to the family Plantaginaceae, order Lamiales, superorder Asteranae, subclass Magnoliidae (angiosperms) and class Equisetopsida (embryophytes), of the angiosperms in kingdom Plantae (Chase and Reveal 2009, according to APG III 2009). The genus *Plantago* appears monophyletic, supported by morphological, embryological, and chemical data (Rahn 1996) and molecular phylogenetic studies (nuclear ribosomal ITS[9] region; plastid *trnL- F* region[10], plastid *rps16* intron and plastid *matK-trnK* region; (Albach et al. 2005; Hoggard et al. 2003; Rønsted et al. 2002). According to those studies, the

---

[9] **ITS**: Internal Transcribed Spacer.

[10] ***trnL- F* region**: plastid *trnL* intron, *trnL* 3' exon and *trnL-F* spacer.

Table 4. Species within *Plantago* subgen. *Coronopus* sect. *Maritima*, present in Portugal, according to several sources (see text).

Taxa / Source	**Nova Flora de Portugal**[a]	**Flora Iberica**[b]	**Euro+Med Plantbase**[c]
P. algarbiensis	yes	yes	yes
P. almogravensis	yes	*syn. P. algarbiensis*	yes
P. holosteum	-	yes	-
P. penyalarensis	yes	*syn. P. alpina*	*syn. P. alpina*
P. radicata	yes	*syn. P. holosteum*	*syn. P. subulata*
P. subulata	-	-	yes
P. alpina	-	yes	yes
P. maritima	*subsp. marítima*	*subsp. maritima & subsp. serpentina*	*subsp. maritima & subsp. serpentina*

yes: present; (**-**): absent or not mentioned; **syn**: synonym of; **subsp**: subspecies. According to [a]Franco 1984; [b]Pedrol 2009 and [c]Marhold 2011.

genus is divided into four or five subgenera (*Coronopus*, *Littorella Plantago, Psyllium* and, possibly, *Bougueria*). Subgenus *Coronopus*, has two sections (sect.), *Maritima* and *Coronopus* (Rahn 1996; Rønsted et al. 2002) and authors further divide each section into two groups or aggregates (Fig. 4) (Greuter 1989; Rahn 1996).

Plantago is a worldwide genus of more than 200 species (Rahn 1996). Within Europe, at least 75 species (Marhold 2011) are described, of which 27 were mentioned for the Iberian Peninsula and Balearic Islands (Pedrol 2009). Within the subgenera, the species are not easily separated, thus the boundaries of the different species/subspecies are somewhat unresolved and changing according to the diverse authors. In Portugal (continental), 15 to 17 species have been described (ALFA 2011; Franco 1984; Greuter 1989; Marhold 2011; Pedrol 2009). In Tab. 4 some ambiguities in the taxonomy of *P.* subgen. *Coronopus* sect. *Maritima* in Portugal, are shown.

Within the *Directive Habitats* framework in Portugal (continental) only three *Plantago* taxa are mentioned:

i) ***P. radicata***, has no special protection regime but is mentioned to define two Portuguese sub-types of the habitat #6160 – Oro-Iberian *Festuca indigesta* grasslands (*Prados oro-ibéricos de Festuca indigesta*), where is considered a dominant species, bioindicator of the habitat types: (#6160pt2) *Matos rasteiros acidófilos temperados e mediterrânicos*, in mountain areas (Mountain ranges of *Peneda*, *Amarela*, *Gerês*, *Montemuro*, *Mourela* plateau, *Nogueira*, *Estrela* and *Açor*); and (#6160pt3) *Matos rasteiros silibasófilos*, in serpentinic areas (outcrops of *Bragança-Vinhais* and *Morais*, sectors *Orensano-Sanabriense* and *Lusitano-Duriense*).

ii) ***P. almogravensis*** (species #1743), one single known population included within a natural park, under protection by annexes B-II and B-IV, a *Critically Endangered* Portuguese endemic species (Tab. 2).

iii) ***P. algarbiensis*** (species #1742), under protection by annexes B-II and B-IV, though only some of the populations are located within protected areas (*Barrocal*), considered an *Endangered* Portuguese endemic species.

P. algarbiensis Samp. (Sampaio 1914) and ***P. almogravensis*** Franco (Franco 1984) were considered highly endangered species (Tab. 2) due to their limited distribution (one or two known populations in 2000) and low number of individuals (1000 to 10000). Their populations are allopatric (more than

60 km appart, Fig. 5). *Plantago almogravensis* is restricted to one known population within *Parque Natural do Sudoeste Alentejano e Costa Vicentina* (PNSACV Natural Park), with < 3 ha extent of occurrence[11]; the area of occupancy[12] should be much smaller, as most of the population is fragmented in small cohorts, with a total estimated of three to ten thousands individuals (*Fichas Flora*[13]; personal observations 2008-2011, included in *Directive Habitats 2013 report*[14]). In the 70's and in the 90's, when the species was rediscovered, other populations were accounted (meridional) that are no longer detectable today, probably extict (Pinto MJ, personal communication, 2008; Pinto et al. 2013). An artificial population was also created ca. 15 km south, from a project aiming at the conservation of the species, to where eight individuals where translocated and, of which, three are still alive and have a few descendants (Fonseca et al. 2004; Pinto et al. 2013).

Plantago algarbiensis has two main populations known in Portugal, Silves and Albufeira regions, plus a third small population located in the Faro region (*Almargem*[15] and Pinto MJ, personal communications, 2009; personal observations, 2008-2011). Their summed extent of occurrence is ca. 1.0 – 25.5 ha, but only with some areas within *Natura2000* protected zones (ca. 0.165 – 1.25 ha) (*Fichas Flora*[13]; personal observations 2008-2011 reported in *Directive Habitats 2013 report*[14]). Since the XIX century, known populations have disapeared, probably extinguished (e.g. Santiago-do-Cacém, Grândola, Odemira, Loulé and Portimão regions; Pinto MJ personal communication, 2008; *Fichas Flora*[13]).

Historical background

Hoffmannsegg and Link (1809) described the presence of *P. subulata* and *P. radicata* in Portugal, within the mountains of Estrela and near Bragança, respectively. But there was no reference to similar species in the south of Portugal. In 1870, there was a mention (Willkomm and Lange 1870) to *P. acanthophylla* Dsne. ß-*bracteosa* (Willk) in the south of Portugal, near Albufeira. Sampaio (1909) observed that same taxa in the south of the Mira river and Coutinho (1913) included it in *Flora de Portugal*, as a variety of the *P. acanthophylla* from the *Trás-os-Montes* region (North of Portugal), from the South and Southwest coasts but with longer curved bracts and harder leaves. Later, Sampaio (1914, 1947) introduced the name *P. algarbiensis* Samp. for those taxa. When revising the Portuguese flora, Franco (1984) separated *P. almogravensis* Franco (southwest coast), from *P. algarbiensis* Samp. (Algarve) considering their differences adequate to such distinction. Namely, he considered differences in extent of ramification, size or shape of leaves and reproductive structures, and colour after drying.

---

[11] **Extent of occurrence**: area of the smallest convex polygon containing all the known presences of a taxon (IUCN 2001).

[12] **Area of occupancy**: area effectively occupied by the taxon, measured within grid squares of appropriate size (IUCN 2001).

[13] ***Fichas flora***: Fichas de caracterização e gestão das espécies constantes no Anexo II da Directiva Habitats, [http://www.icnf.pt/portal/naturaclas/rn2000/p-set/psrn-flora, edition 17-03-2012].

[14] **Directive Habitats 2013 report**: [http://bd.eionet.europa.eu/activities/Reporting/Article_17/Reports_2013/, accessed 24-07-2014].

[15] ***Almargem***: Associação de Defesa do Património Cultural e Ambiental do Algarve [http://www.almargem.org © 2013].

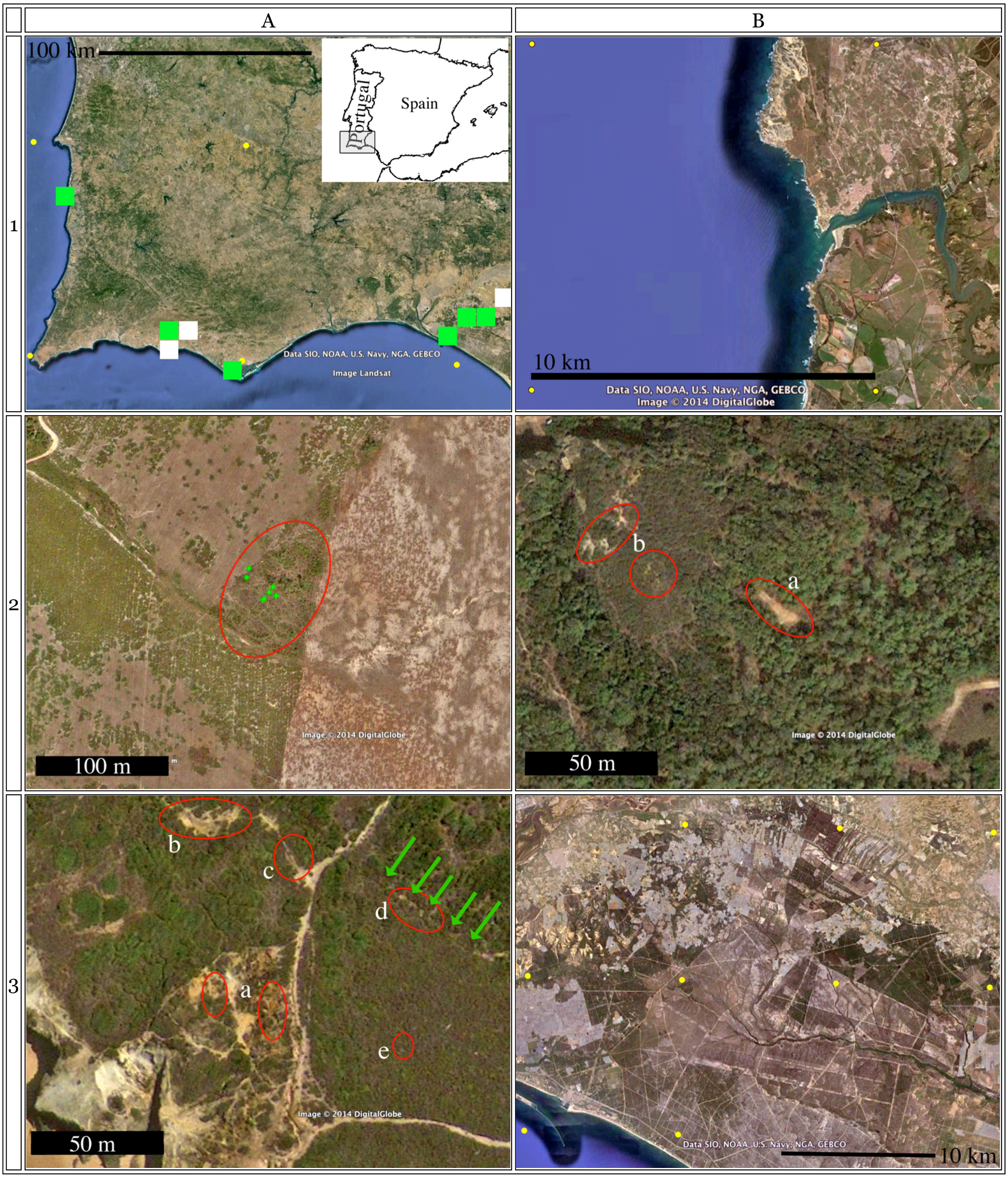


Figure 5. Location and habitat types for *P. almogravensis* and *P. algarbiensis*. Grid location-data, MGRS 10 km, from (**green squares**) personal observations (details bellow) or (**white squares**) locations from Flora-on[a] and Anthos[b]; corners of MGRS grid (100 km or 10 km) represented as **yellow dots**. Images ©Google Earth.

A1: Overall known locations.

***P. almogravensis*, B1:** overview of the 10 km green square (in A1); Details, **A2:** cohorts in low disturbance agricultural area (**green dots**); **B2:** cohorts in woodland gap (a) and shrubland gaps (b); **A3:** cohorts in gaps over cliffs (a), large gap (b), waterlogged gap (c), gaps (d) near the invasive Acacia sp. (**green arrows**), and shrubland cohort (e).

***P. algarbiensis* (Spain), B3:** overview of the 3x10 km green squares (in A1). (cont.)

---

[a] ***Flora-On***: Flora de Portugal Interactiva [http://www.flora-on.pt, accessed 13-12-2013]. [b] ***Anthos***: Sistema de información de las plantas de España [http://www.anthos.es, accessed 01-2014].

Figure 5. (cont.)

***P. algarbiensis* (Portugal):** Overview of the 10 km green squares (in A1) for **C1:** Silves-Albufeira and **D1:** Faro; Details, of **C2:** cohorts in low disturbance agricultural areas; **D2**: cohorts within woodland (a) and in water run-off valley (b); **D3:** cohorts near extractive industries: in ferruginous gaps (a), in shrubland (b), in water run-off areas (c), in waterlogged valley (d) and in colluvial zone (e).

***P. algarbiensis* (Spain)**, **C3:** cohorts (**green dots**) in waterlogged area (a) and open woodland (b).

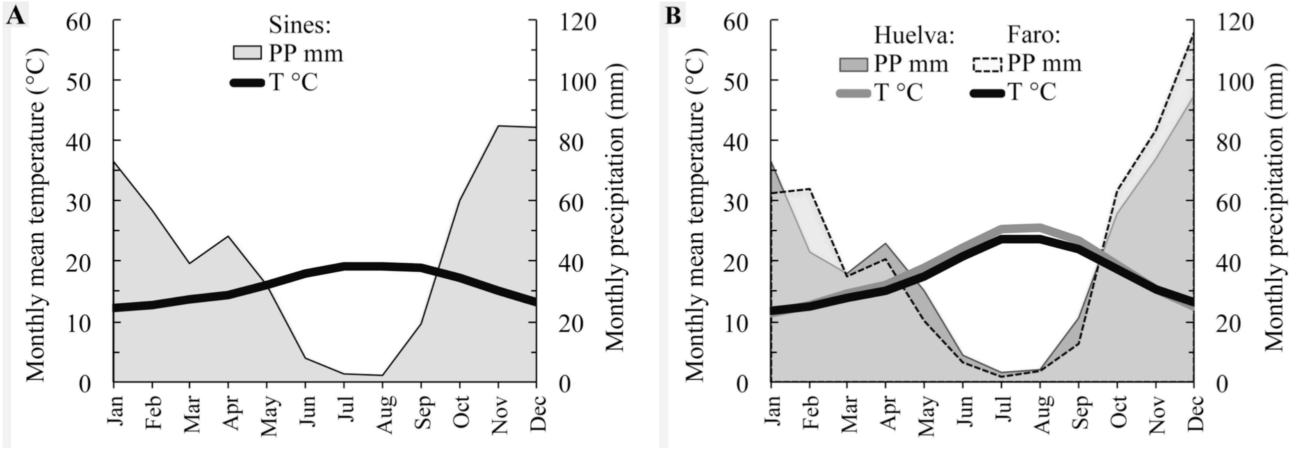


Figure 6. Ombrothermic curves from the climate normals 1971-2000. Air temperature (**T**) and precipitation (**PP**), from the stations closer to *P. almogravensis* population, **A**: Sines or *P. algarbiensis* populations, **B**: Faro and Huelva. Data from www.ipma.pt (Portugal) and www.aemet.es (Spain).

Species regional distribution and local habitat characterization

The species *P. almogravensis* and *P. algarbiensis* are present in the Mediterranean biogeographical region and occupy the Southwest coast of the Iberian Peninsula (Fig. 5). Both are coastal species (ca. <30 km from the sea) subject to maritime moist winds and sea mists, specially *P. almogravensis* that is found nearer the coast line. According to the Köppen-Geiger climate classification system (AEMET-IMP 2011) both species inhabit temperate climates with dry summers (Cs type). They are distinct in that *P. almogravensis* experiences temperate summers (Csb type, average temperature in the hotest month is <22°C) while for *P. algarbiensis* summers are hot (>22°C, Csa type). Accordingly, *P. almogravensis* is subject to slightly less winter-cold and summer-drought stresses, than *P. algarbiensis* (AEMET-IMP 2011). Also, the onset of the dry season is slightly earlyer for *P. algarbiensis* (April) than for *P. almogravensis* (May) (Fig. 6).

Both species occupy sandy to loamy soils with ferruginous outcrops, Haplic (orthic) Podzols[16], in the case of *P. almogravensis*, and Chromi-Calcaric Cambisols[17], for *P. algarbiensis* western populations, Calcaric-Eutric Cambisols for the Faro population (Cardoso 1965; EC-JRC 2005). Frequently, globular pisolitic concretions emerge at the surface. These are composed of complex silicon, iron and aluminium (hydr)oxides (Pimentel et al. 1996), also called sesquioxides. Sesquioxides can reach scorching temperatures under the sun due to their metal composition. Seasonal waterlogging is another feature common to both *P. almogravensis* and *P. algarbiensis* habitats. Frequently, the species are found in areas of water run-off and deposition, superficial water-table and or temporary shallow-lagoons or small-stream margins (summer dried) (*Fichas Flora*[13] and personal observations 2008-2011). Yet, during the summer, those soils are without water, sometimes with cracks at the surface, and plants may experience severe drought stress (AEMET-IMP 2011).

The soil in *P. almogravensis* habitat is a sandy dark-grey podzol (Cardoso 1965), presenting rusty-red patches of superficial sesquioxide hardpan's outcrops. The patchiness of the soil is related to erosion processes that exposed different horizons of the podzol (Pimentel et al. 1996). A dense dwarf-sclerophyllous shrubland (mostly of *Ericaceae*, *Cistaceae* and *Fabaceae* species like *Calluna, Cistus,*

---

[16] Acid soils with a subsurface accumulation of iron-aluminium-organic compounds (FAO 2006).

[17] Weakly to moderately developed soils (FAO 2006).

Corema, Erica, Stauracanthus, Ulex, etc.) or trees (*Juniperus, Quercus*), comprises the dominant vegetation; while on open areas *P. almogravensis* is the dominant species besides other herbaceous plants (annuals and hemicryptophytes). Besides the large open vegetation areas, smaller cohorts (group of individuals) or isolated individuals can also be found in areas of open shrubland or woodland (sometimes in the understory of shrubs) and in agricultural areas with low disturbance due to the presence of stones or rocks (personal observations 2008-2011; Fig. 5). The larger open areas seem to be time-stable gaps, as they are visible in old aerial photographs of the area (Fig. 7), so that they existed at least, in the last six decades.

The habitat of *P. algarbiensis* is a more open shrubland, also with acidophyle shrubs (*Ericaceae*, *Cistaceae* and *Fabaceae*), often at the edge of open woods. It is also found on low disturbance agricultural areas (with stones or rocks) and on roadsides, cleared annually to prevent fire (*Fichas Flora*[13] and personal observations 2008-2011; Fig. 5). The soil has more clay in the Algarve populations, such that there is an important extractive industry surrounding its populations.

The case study

Plantago algarbiensis can no longer be considered a Portuguese endemic species, since it was reported in Spain (Cabezudo et al. 2005; Sánchez Gullón and Rubio Garcia 2002; Valdés et al. 2007). In 2011, the *European Red List of Vascular Plants* (Bilz et al. 2011) considered *P. algarbiensis* an endangered endemic from the Iberian Peninsula. In 2012, the update of the *Catálogo Andaluz de Especies Amenazadas* (Decreto 23/2012 after Ley 8/2003, Comunidad Autónoma de Andalucía) included *P. algarbiensis* in a Spanish legal frame (vulnerable species). The Spanish populations have been described at the Huelva region, in areas of Luvic-Arenosols[18] (FAO 2006), both outside and within Natura 2000 protected areas (*Parque Natural de Doñana*, *LIC Dehesa del Estero Y Montes* and *LIC Doñana Norte Y Oeste*) (Fig. 5).

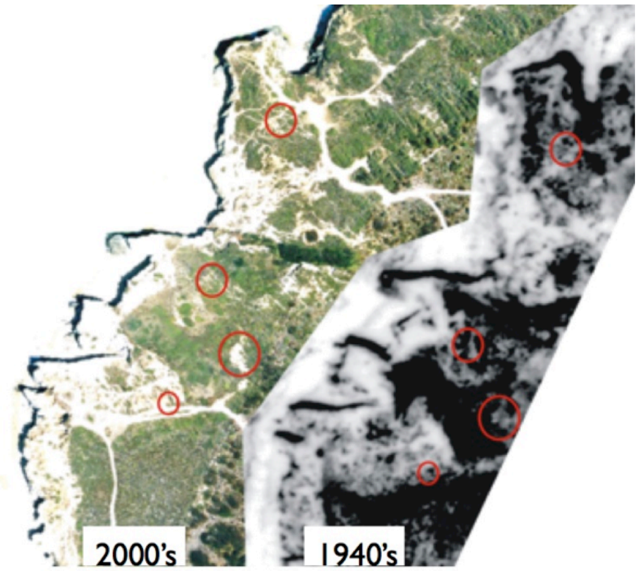


Figure 7. Comparison of images from *P. almogra-vensis* habitat, 60 years apart, showing the stability of the gaps where the species is more abundant (**circles**) (Pinto MJ, personal communication 2008). Images: (left) Google Earth© DigitalGlobe 2014; (right) map IGEOE, RAF1946(7).

In 2009, the XIII volume of *Flora Iberica* containing the family Plantaginaceae (Pedrol 2009), based on the examination of a few herbarium specimens, considered that *P. algarbiensis* and *P. almogravensis* should be merged into one single species (*P. algarbiensis*) maybe worthy of separation (upon further studies) but only at sub-specific level. In Portugal, ICNF continues to maintain *P. almogravensis* as a separate endemic species for the reports of *Directive Habitats* (e.g. see *DH 2013*, in Tab. 2) while botanical societies have mostly adopted the Pedrol (2009) nomenclature (e.g. *Flora-On*[19] or ALFA 2011).

---

[18] Sandy soils featuring very weak or no soil development (FAO 2006).

[19] **Flora-On**: Flora de Portugal Interactiva. Sociedade Portuguesa de Botânica [www.flora-on.pt, accessed 31-7-2014].

Therefore, the endemic and critically endangered *P. almogravensis* was chosen as the case study for this thesis because of the following reasons:

i) The **taxonomic position is unresolved** and based on herbarium specimens, lacking some of the identification traits (Pedrol 2009).

ii) The population is **critically endangered** and in need of special conservation measures in Portugal (*Directive Habitats*), still with a *Bad* conservation status (*Directive Habitats 2013 report*[20]).

iii) The **rarity** of the population: it is limited to one population, with scattered cohorts of < 25 individuals and estimated in 3000-4000 individuals; its habitat seems distinct in soil composition and vegetation cover from its surroundings suggesting a specialization (*Fichas Flora*[13]; Branquinho et al. 2007; Pinto et al. 2004). Considering all the three rarity factors (Tab. 1), it is expected that *P. almogravensis* is at a high risk of extinction, supporting the *Critically Endangered* status.

iv) The **endemicity** of the population: it is isolated (allopatric) from *P. algarbiensis* populations and is only known to exist in < 3 ha (extent of occurrence) in the Portuguese Southwest coast (Branquinho et al. 2007; Pinto et al. 2004).

We decided to consider the two sister taxa *P. almogravensis* and *P. algarbiensis* as separate species, thus our focus was *P. almogravensis* but with comparisons with *P. algarbiensis* whenever possible.

The main sources of knowledge about these species were, the National Floras (Franco 1984; Sampaio 1947) and the few studies conducted in preparation to the *Directive Habitats* (e.g. *Fichas Flora*[13]; Fonseca et al. 2004). In one of those projects aiming at perceiving the constraints towards the conservation of *P. almogravensis*, "Estudo de referência das populações de *Plantago almogravensis*"[21], we have found that the plant accumulates abnormal amounts of aluminium (Al). This work gave origin to an article where we (Branquinho et al. 2007) confirmed that those abnormal concentrations were in the range of what has been called metal hyperaccumulation (Jansen et al. 2002). A second project, "New insights for understanding the causes of rarity in plant species – ECODILEMA"[22] provided us with clues to the importance of microorganisms in the life cycle of *Plantago* species, namely at germination and survival stages. Furthermore, an MSc project, "Estudo de factores condicionantes da distribuição da planta rara *Plantago almogravensis* Franco"[23], suggested the connection of the vegetation (including *Plantago*) pattern to the chemo-edaphic characteristics of the habitat and to the accumulation of Al. Now, this thesis further explores that hypothesis, relating it to the ecological limiting factors that may have been/are driving the species to a rarity status near extinction.

The study of the ecological factors driving species rarity is a step towards understanding the best procedures for maintaining biodiversity. Thus, the subject of study was a highly endangered species of the genus *Plantago*, namely the Portuguese endemic *P. almogravensis*. We briefly surveyed its morphological and edaphic similarities and differences with sister species (Chapter 2); then we detailed the characterization of the ecological niche for *P. almogravensis* (Serrano et al. 2015)[24] to determine that the species inhabits geochemical islands with low competition and high Al-toxicity in the soil. The physiological limits of the metal tolerance were further examined by the characterization of the hyperaccumulation trait in

---

[20] Directive Habitats 2013 report. [http://bd.eionet.europa.eu/activities/Reporting/Article_17/Reports_2013/, accessed 24-07-2014].

[21] Pinto et al. 2004, project 2003-2004, MUHNAC, Universidade de Lisboa & ICN.

[22] POCTI/BIA- BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004.

[23] Antunes C. 2007. Tese de Mestrado, Universidade de Lisboa.

[24] Chapter 3

the field (Serrano et al. 2011)[25]. Because not only Al, but also other metals were found to accumulate highly in *P. almogravensis* (Chapter 2) we examined data on the accumulation of Al and other metals in *Plantago* phylogeny (Chapter 5), signalling a possible phylogenetic link for those traits. The hyperaccumulation results also suggested the importance of microorganisms for the survival in the field conditions thus we analysed soil microorganisms, considering their spatial distribution and the relation to the presence of *P. almogravensis* (Chapter 6). The importance of microorganisms, light and of Al-toxicity was further examined in the life cycle of the plants and related to conservation measures (Chapter 7). The overview of all these ecological factors allows a better understanding of the rarity factors and limitations associated with *P. almogravensis* and suggest directions towards conservation measures (Chapter 8). We expect to contribute to the theoretical study of rarity, the ecological role of *P. almogravensis* in its ecosystem, and its evolutionary position among the other members of the genus.

REFERENCES

AEMET-IMP. 2011. Iberian Climate Atlas, Air temperature and precipitation (1971-2000). ed. Ma IMP: Ministerio de Médio Ambiente y Médio Rural y Marino (España) and Instituto de Meteorologia (Portugal)

Albach DC, Meudt HM, Oxelman B. 2005. Piecing together the “new” Plantaginaceae. *American Journal of Botany* 92: 297-315

ALFA. 2011. Checklist da Flora de Portugal (Continental, Açores e Madeira). ed. M Menezes de Sequeira, MD Espírito-Santo, C Aguiar, J Capelo, J Honrado, pp. 74. Lisboa, Portugal: Associação Lusitana de Fitossociologia

Alford ER, Pilon-Smits EAH, Paschke MW. 2010. Metallophytes-a view from the rhizosphere. *Plant and Soil* 337: 33-50

Alloway B. 2013. Bioavailability of Elements in Soil In *Essentials of Medical Geology*, ed. O Selinus, pp. 351-73: Springer Netherlands

Anacker BL. 2011. Phylogenetic patterns of endemism and diversity In *Serpentine: the evolution and ecology of a model system. University of California Press, Berkeley*, ed. SP Harrison, N Rajakaruna, pp. 49-70. Berkeley, CA: Univ. of California Press

Anacker BL. 2014. The nature of serpentine endemism. *American Journal of Botany* 101: 219-24

Antonovics J, Bradshaw AD, Turner RG, Cragg JB. 1971. Heavy Metal Tolerance in Plants. In *Advances in Ecological Research*, pp. 1-85: Academic Press

Baker AJM. 1981. Accumulators and excluders -strategies in the response of plants to heavy metals. *Journal of Plant Nutrition* 3: 643-54

Baker AJM. 1987. Metal tolerance. *New Phytologist* 106: 93-111

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81–126

Baker AJM, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In *Ecology of Industrial Pollution*, ed. LC Batty, KB Hallberg, pp. 7-40. Cambridge, U. K.: Cambridge University Press

Baker AJM, Proctor J. 1990. The influence of cadmium, copper, lead, and zinc on the distribution and evolution of metallophytes in the British-Isles. *Plant Systematics and Evolution* 173: 91-108

Barceló J, Poschenrieder C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environmental and Experimental Botany* 48: 75-92

Baumbach H, Hellwig FH. 2007. Genetic differentiation of metallicolous and non-metallicolous *Armeria maritima* (Mill.) Willd. taxa (Plumbaginaceae) in Central Europe. *Plant Systematics and Evolution* 269: 245-58

Bello MA, Chase MW, Olmstead R, Rønsted N, Albach D. 2002. The paramo endemic *Aragoa* is the sister genus of *Plantago* (Plantaginaceae; Lamiales): evidence from plastid *rbcL* and nuclear ribosomal ITS sequence data. *Kew Bulletin* 57: 585-97

Bert V, Bonnin I, Saumitou-Laprade P, de Laguerie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* 155: 47-57

Bert V, MacNair MR, DeLaguerie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* 146: 225-33

Bhatia NP, Baker AJM, Walsh KB, Midmore DJ. 2005. A role for nickel in osmotic adjustment in drought-stressed plants of the nickel hyperaccumulator *Stackhousia tryonii* bailey. *Planta* 223: 134-39

Bilz M, Kell SP, Maxted N, Lansdown RV. 2011. European Red List of Vascular Plants. pp. 130. Luxembourg: Publications Office of the European Union

Bondada B, Ma L. 2003. Tolerance of Heavy Metals in Vascular Plants: Arsenic Hyperaccumulation by Chinese Brake Fern (*Pteris vittata* L.) In *Pteridology in the New Millennium*, ed. S Chandra, M Srivastava, pp. 397-420. Netherlands: Kluwer Academic Publishers

Boyd RS. 2004. Ecology of metal hyperaccumulation. *New Phytologist* 162: 563-67

Boyd RS, Martens SN. 1992. The raison d'être for metal hyperaccumulation by plants In *The Vegetation of Ultramafic (Serpentine) Soils*, ed. AJM Baker, J Proctor, RD Reeves, pp. 279–89. Andover: Intercept Ltd.

Boyd RS, Martens SN. 1998. The significance of metal hyperaccumulation for biotic interactions. *Chemoecology* 8: 1-7

---

[25] Chapter 4

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Broadley MR, Willey NJ, Wilkins JC, Baker AJM, Mead A, White PJ. 2001. Phylogenetic variation in heavy metal accumulation in angiosperms. *New Phytologist* 152: 9-27

Brooks R, Lee J, Reeves R, Jaffre T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration* 7: 49-57

Brooks R, Reeves R, Morrison R, Malaisse F. 1980. Hyperaccumulation of copper and cobalt - A review. *Bulletin De La Societe Royale De Botanique De Belgique* 113: 166-72

Brunner I, Sperisen C. 2013. Aluminum exclusion and aluminum tolerance in woody plants. *Frontiers in Plant Science* 4: 172

Cabezudo B, Talavera S, Blanca G, Salazar C, Cueto M, et al. 2005. Lista Roja de la Flora Vascular de Andalucía. ed. CM Ambiente, Jd Andalucía, pp. 126. Sevilla, España

Cappa JJ, Pilon-Smits EAH. 2014. Evolutionary aspects of elemental hyperaccumulation. *Planta* 239: 267-75

Cardoso JC. 1965. *Os solos de Portugal, sua classificação, caracterização e génese. 1 - A sul do Rio Tejo*. Portugal: Secretaria de Estado da Agricultura.

Carlquist SJ, Cole MJ. 1974. *Island Biology*. New York: Columbia University Press.

Chase M, Reveal J. 2009. A phylogenetic classification of the land plants to accompany APG III. *Botanical Journal of the Linnean Society* 161: 122-27

Chenery EM. 1948. Aluminium in the Plant World - Part I. General Survey in Dicotyledons. *Kew Bulletin* 3: 173-83

Chenery EM. 1949. Aluminium in the Plant World - Part II. Monocotyledons and gymnosperms. *Kew Bulletin* 4: 463-73

Colwell RK, Fuentes ER. 1975. Experimental studies of niche. *Annual Review of Ecology and Systematics* 6: 281-310

Colwell RK, Rangel TF. 2009. Hutchinson's duality: The once and future niche. *Proceedings of the National Academy of Sciences of the United States of America* 106: 19651-58

Coutinho A. 1913. *A Flora de Portugal (plantas vasculares) disposta em chaves dichotomicas*. pp. 773. Lisboa, Portugal: Aillaud, Albes & Cia.

de Andrade LRM, Barros LMG, Echevarria GF, do Amaral LIV, Cotta MG, et al. 2011. Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. *Environmental and Experimental Botany* 70: 37-42

EC-JRC. 2005. *Soil Atlas of Europe*. pp. 128. Luxembourg: Office for Official Publications of the European Communities.

FAO. 2006. *World Reference Base for Soil Resources 2006: A Framework for International Classification, Correlation and Communication*. pp. 144. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).

Fernando DR, Marshall AT, Forster PI, Hoebee SE, Siegele R. 2013. Multiple metal accumulation within a manganese-specific genus. *American Journal of Botany* 100: 690-700

Ferreira PMA, Boldrini, II. 2011. Potential Reflection of Distinct Ecological Units in Plant Endemism Categories. *Conservation Biology* 25: 672-79

Fiedler PL. 1986. Concepts of rarity in vascular plant-species, with special reference to the genus *Calochortus* Pursh (Liliaceae). *Taxon* 35: 502-18

Fonseca JP, Chozas-Vinuesa S, Sequeira C. 2004. Plano Nacional de Conservação da Flora em Perigo. Relatório de progresso de 2003. Projecto LIFE – Natureza III P\8048., Instituto de Conservação da Natureza, Portugal

Franco JA. 1984. *Nova Flora de Portugal (Continente e Açores)*. Lisboa, Portugal.

Gaston KJ. 2011. Common Ecology. *Bioscience* 61: 354-62

Gaston KJ, Fuller RA. 2008. Commonness, population depletion and conservation biology. *Trends in Ecology & Evolution* 23: 14-19

Godsoe W. 2010. I can't define the niche but I know it when I see it: a formal link between statistical theory and the ecological niche. *Oikos* 119: 53-60

Greuter W. 1989. Plantaginaceae. In *Med-Checklist - A critical inventory of vascular plants of the circum-mediterranean countries*. Published on the Internet http://ww2.bgbm.org/mcl/, accessed at 01-08-2014

Hallam A, Wignall PB. 1997. *Mass Extinctions and Their Aftermath*. Oxford University Press, UK.

Haridasan M. 1982. Aluminum accumulation by some cerrado native species of central Brazil. *Plant and Soil* 65: 265-73

Hoffmannsegg JC, Link HF. 1809. *Flore Portugaise*. Berlin.

Hoggard RK, Kores PJ, Molvray M, Hoggard GD, Broughton DA. 2003. Molecular systematics and biogeography of the amphibious genus Littorella (Plantaginaceae). *American Journal of Botany* 90: 429-35

Holt RD. 2009. Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary perspectives. *Proceedings of the National Academy of Sciences of the United States of America* 106: 19659-65

Hubbell S. 1997. A unified theory of biogeography and relative species abundance and its application to tropical rain forests and coral reefs. *Coral Reefs* 16: S9-S21

Hutchinson GE. 1957. Population studies - animal ecology and demography - concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology* 22: 415-27

IUCN. 2001. *IUCN Red List Categories and Criteria: Version 3.1*. ed. ISS Commission, pp. 30. Gland, Switzerland and Cambridge, UK: IUCN

Jaffré T. 1980. *Etude écologique du peuplement végétal des sols dérivés de roches ultrabasiques en Nouvelle Calédonie*. ORSTOM, Paris. 273 pp.

Jaffré T, Schmid M. 1974. Accumulation du nickel par une Rubiacée de Nouvelle-Calédonie, *Psychotria douarrei* (G. Beauvisage) Däniker. *Comptes Rendus de l'Académie des Sciences.Série D : Sciences Naturelles* 278: 1727-30

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Kay KM, Ward KL, Watt LR, Schemske DW. 2011. Plant Speciation. In *Serpentine: The Evolution and Ecology of a Model System*, ed. SP Harrison, N Rajakaruna, pp. 71-95. Berkeley, CA: Univ. of California Press

Kinraide TB. 1991. Identity of the rhizotoxic aluminum species. *Plant and Soil* 134: 167-78

Klironomos J. 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* 417: 67-70

Kochian LV, Pineros MA, Hoekenga OA. 2005. The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. *Plant and Soil* 274: 175-95

Kruckeberg AR, Rabinowitz D. 1985. Biological aspects of endemism in higher-plants. *Annual Review of Ecology and Systematics* 16: 447-79

Krämer U. 2010. Metal hyperaccumulation in plants. *Annu Rev Plant Biol* 61: 517-34

Kunin W, Gaston K, Mace G, Kershaw M. 1997. Extinction risk and rarity on an ecological timescale In *The Biology of Rarity*, pp. 130-49: Springer Netherlands

Malaisse F, Gregoire J, Brooks RR, Morrison RS, Reeves RD. 1978. *Aeolanthus biformifolius* De Wild.: A Hyperaccumulator of Copper from Zaïre. *Science* 199: 887-88

Marhold K. 2011. Plantaginaceae. In *Euro+Med Plantbase - the information resource for Euro-Mediterranean plant diversity*. Published on the internet http://ww2.bgbm.org/EuroPlusMed/, accessed at 01-08-2014

Markert B. 1992a. Establishing of reference plant for inorganic characterization of different plant-species by chemical fingerprinting. *Water Air and Soil Pollution* 64: 533-38

Markert B. 1992b. Presence and significance of naturally-occurring chemical-elements of the periodic system in the plant organism and consequences for future investigations on inorganic environmental chemistry in ecosystems. *Vegetatio* 103: 1-30

Marschner H. 1991. Mechanisms of adaptation of plants to acid soils. *Plant and Soil* 134: 1-20

Meerts P, Van Isacker N. 1997. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* 133: 221-31

Meharg AA. 2003. The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycological Research* 107: 1253-65

Metali F, Salim KA, Burslem D. 2012. Evidence of foliar aluminium accumulation in local, regional and global datasets of wild plants. *New Phytologist* 193: 637-49

Ouborg N, Vergeer P, Mix C. 2006. The rough edges of the conservation genetics paradigm for plants. *Journal of Ecology* 94: 1233-48

O'Dell RE, Rajakaruna N. 2011. Intraspecific variation, adaptation, and evolution. In *Serpentine: Evolution and ecology in a model system*, ed. SP Harrison, N Rajakaruna, pp. 97-137. Berkeley, CA: Univ. of California Press

Pedrol J. 2009. *Plantago* L. In *Flora Iberica*, ed. C Benedi, E Rico, J Güemes, A Herrero, pp. 4–38. Madrid, Spain: Real Jardín Botánico CSIC

Pimentel NL, Wright VP, Azevedo TM. 1996. Distinguishing early groundwater alteration effects from pedogenesis in ancient alluvial basins: Examples from the Palaeogene of southern Portugal. *Sedimentary Geology* 105: 1-10

Pinto MJ, Serrano H, Branquinho C, Martins-Loução MA. 2004. Estudo de referência de *Plantago almogravensis*, Jardim Botânico MUHNAC, Universidade de Lisboa & Instituto de Conservação da Natureza, Portugal

Pinto MJ, Serrano HC, Branquinho C, Martins-Loução MA. 2013. Éxito en la translocación de una planta con restricciones dispersivas. *Conservation Vegetal* 17: 8-9

Pollard AJ, Powell KD, Harper FA, Smith JAC. 2002. The genetic basis of metal hyperaccumulation in plants. *Critical Reviews in Plant Sciences* 21: 539-66

Pollard AJ, Reeves RD, Baker AJM. 2014. Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* 217–218: 8-17

Poschenrieder C, Gunse B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* 400: 356-68

Póvoas L. 2011. Terra, vida e extinções em massa In *A aventura da Terra - um planeta em evolução*, ed. MA Martins-Loução, pp. 123-34. Lisboa, Portugal: Esfera do Caos

Rabinowitz D. 1981. Seven forms of rarity In *The Biological Aspects of Rare Plant Conservation*, ed. H Synge, pp. 205- 17. New York: John Wiley and Sons

Rahn K. 1996. A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society* 120: 145-98

Reeves R, Baker A. 1984. Studies on metal uptake by plants from serpentine and non-serpentine populations of *Thlaspi goesingense* Hálácsy (Cruciferae). *New Phytologist* 98: 191-204

Reeves RD, Baker AJM. 2000. Metal-accumulating plants In *Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment*, ed. I Raskin, BD Ensley, pp. 193–229. New York: Wiley

Reeves RD, Brooks RR. 1983. Hyperaccumulation of lead and zinc by two metallophytes from mining areas of Central Europe. *Environmental Pollution Series A, Ecological and Biological* 31: 277-85

Remon E, Bouchardon JL, Faure O. 2007. Multi-tolerance to heavy metals in Plantago arenaria Waldst. & Kit.: Adaptative versus constitutive characters. *Chemosphere* 69: 41-47

Roccotiello E, Serrano HC, Mariotti MG, Branquinho C. 2015. Nickel phytoremediation potential of the Mediterranean *Alyssoides utriculata* (L.) Medik. *Chemosphere* 119: 1372-8

Rockstrom J, Steffen W, Noone K, Persson A, Chapin FS, III, et al. 2009. Planetary Boundaries: Exploring the Safe Operating Space for Humanity. *Ecology and Society* 14

Rodriguez N, Menendez N, Tornero J, Amils R, de la Fuente V. 2005. Internal iron biomineralization in *Imperata cylindrica,* a perennial grass: chemical composition, speciation and plant localization. *New Phytologist* 165: 781-89

Rønsted N, Chase MW, Albach DC, Bello MA. 2002. Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid *trnL-F* sequence data. *Botanical Journal of the Linnean Society* 139: 323-38

Sampaio G. 1947. *Flora portuguesa*. Porto, Portugal: Impr. Moderna.

Sampaio GASF. 1909. *Flora vascular de Odemira*. Coimbra (Portugal): Imprensa da Universidade de Coimbra. 128 pp.

Sampaio GASF. 1914. *Apêndice à lista das espécies representadas no Herbário português*. Porto, Portugal.

Sax DF, Early R, Bellemare J. 2013. Niche syndromes, species extinction risks, and management under climate change. *Trends in Ecology & Evolution* 28: 517-23

Schat H, Sharma SS, Vooijs R. 1997. Heavy metal-induced accumulation of free proline in a metal-tolerant and a nontolerant ecotype of *Silene vulgaris*. *Physiologia Plantarum* 101: 477-82

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Cotrim H, Branquinho C, Martins-Loução MA. 2014. A investigação ecológica como base de estratégias de conservação. In *O/El Botanico, Revista anual da/de la AIMJB*, pp. 37-39

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Smith TB, Kinnison MT, Strauss SY, Fuller TL, Carroll SP. 2014. Prescriptive Evolution to Conserve and Manage Biodiversity. *Annual Review of Ecology, Evolution, and Systematics* 45: 1-22

Stebbins GL. 1980. Rarity of plant-species - synthetic viewpoint. *Rhodora* 82: 77-86

Steffen W, Crutzen PJ, McNeill JR. 2007. The Anthropocene: Are humans now overwhelming the great forces of nature. *Ambio* 36: 614-21

Streit B, Stumm W. 1993. Chemical properties of metals and the process of bioaccumulation in terrestrial plants In *Plants as biomonitors: indicators for heavy metals in the terrestrial environment*, ed. B Markert, pp. 31–60. Weinheim- Cambridge

Suweis S, Simini F, Banavar J, Maritan A. 2013. Emergence of structural and dynamical properties of ecological mutualistic networks. *Nature* 500: 449-52

Sánchez Gullón E, Rubio Garcia JC. 2002. Novedades florísticas para el litoral de Huelva y provincia. II. *Lagascalia*: 7-19

Takahashi E, Ma J, Miyake Y. 1990. The possibility of silicon as an essential element for higher plants. *Comments on Agricultural and Food Chemistry* 2: 99-102

Tipping E. 2005. Modelling Al competition for heavy metal binding by dissolved organic matter in soil and surface waters of acid and neutral pH. *Geoderma* 127: 293-304

Valdés B, Girón V, Sánchez Gullón E, Carmona I. 2007. Catálogo florístico del Espacio Natural de Doñana (SO de España). Plantas vasculares. *Lagascalia* 27: 73-362

van der Ent A, Baker AJM, Reeves RD, pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil* 362: 319-34

van der Heijden MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310

Walker B. 1995. Conserving biological diversity through ecosystem resilience. *Conservation Biology* 9: 747-52

Watanabe T, Broadley MR, Jansen S, White PJ, Takada J, et al. 2007. Evolutionary control of leaf element composition in plants. *New Phytologist* 174: 516-23

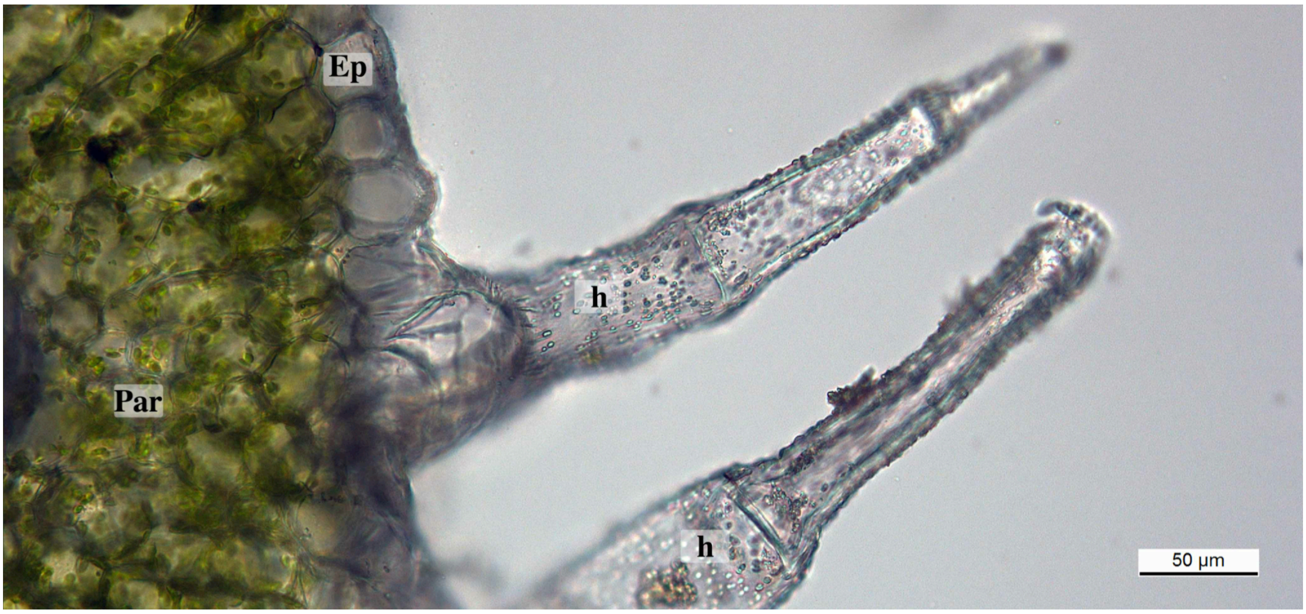
Willkomm M, Lange J. 1870. *Prodromus Florae Hispanicae seu, Synopsis methodica omnium plantarum in Hispania sponte nascentium vel frequentius cultarum quae innotuerunt*. pp. 680. Stuttgart: E. Schweizerbart.

Ye ZH, Baker AJM, Wong MH, Willis AJ. 1997. Zinc, lead and cadmium tolerance, uptake and accumulation by the common reed, *Phragmites australis* (Cav) Trin ex Steudel. *Annals of Botany* 80: 363-70

CHAPTER 2

PLANTAGO ALMOGRAVENSIS:

BASIC MORPHOLOGY AND EDAPHIC HABITAT



Fresh hand-section of the leaf, from optical microscopy. Epidermis (**Ep**); parenchyma with chloroplasts (**Par**); uniseriate hairs (**h**).

CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

PLANTAGO ALMOGRAVENSIS:
BASIC MORPHOLOGY AND EDAPHIC HABITAT

ABSTRACT

The rare and endemic species *Plantago almogravensis* is restricted to one population. To understand its adaptation to the local habitat conditions and the properties that contribute to its survival, we determined its local patterns and habitat edaphic properties, and observed its growth and morphology, comparing with its closer (geographically) sister taxa: *P. algarbiensis* and *P. radicata*.

The pattern of presence/absence of the species was analysed from *in loco* photographs stitched into orthophotomaps. The species was detected in colluvial gap areas and gap edges, suggesting wind and water as major dispersion forces. These gaps were known as geochemical gaps, rich in Fe and Al, which the plants accumulate among other metals. The presence of Al in the plant was detected in the wood and in leaf glandular trichomes, after histochemical staining. *Plantago almogravensis* and *P. algarbiensis* can propagate vegetatively and seem able to form mycorrhizal associations; both factors can be helpful in overcoming stressful environmental conditions. The species *P. almogravensis* has more rosettes (and leaves) and shorter leaves than *P. algarbiensis*. It also presents shorter spikes, peduncles or bracts and lighter seeds. The morphology of *P. almogravensis*, *P. algarbiensis* and *P. radicata* seems to be somewhat different among them, all of which suggests a taxonomic separation.

INTRODUCTION

The last Portuguese Flora published (Franco 1984) considers that within the country exist three species from the *Plantago subulata* group, *P.* subgenus *Coronopus* sector *Maritima* (Rahn 1996). Namely, *P. algarbiensis, P. almogravensis* and *P. radicata*. Recently, this taxonomy has been contested (Pedrol 2009) and in 2011 the Portuguese plant checklist (ALFA 2011) refers only two species from this group: *P. algarbiensis* (with *P. almogravensis* as a synonym) and *P. holosteum* (syn. for *P. radicata*). The taxon *P. almogravensis* is a rare endangered endemic with only one known population; while *P. algarbiensis* is restricted to two areas in Portugal and the Huelva region in Spain, thus being also considered a rare Iberian endemic. There has been no conclusive answer about their level of speciation and even Pedrol (2009) suggests they could eventually be separated at the infraspecific level. Due to the similarity of *P. algarbiensis* and *P. almogravensis*, they are often considered sister taxa or sister species. There has been no works so far comparing populations in their natural habitat or in garden conditions (milder environment). Geographically, *P. radicata* (Portuguese populations of *P. holosteum*) is the closest taxon. Neither *P. radicata* is protected in Portugal nor *P. holosteum* (Iberian taxon) is considered endangered.

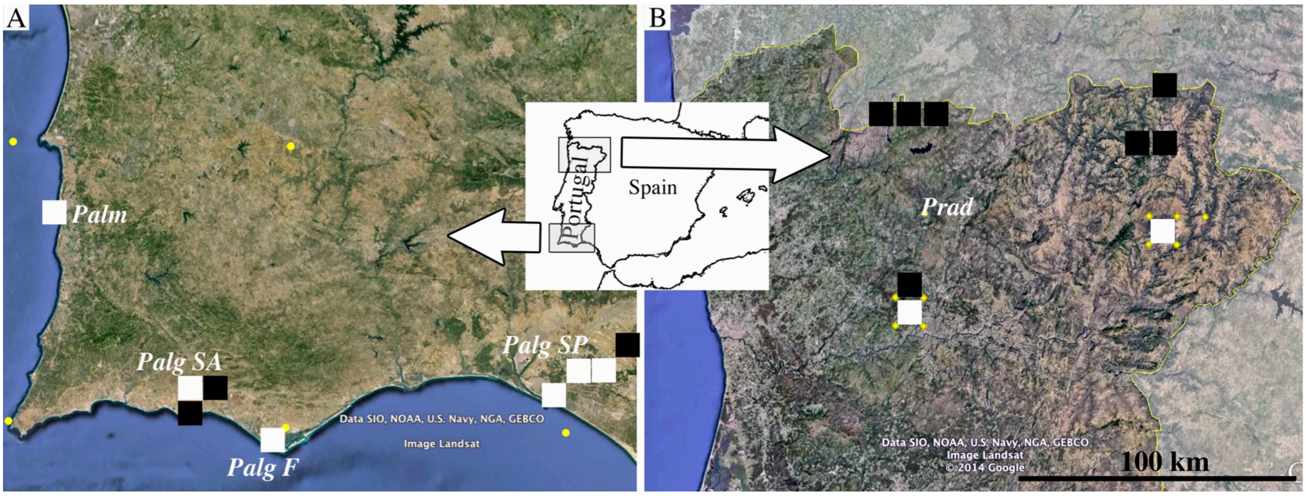


Figure 1. Map of the study areas. **A:** Southwest Portugal and Spain, with *P. almogravensis* (***Palm***) and *P. algarbiensis* (***Palg***); Locations: Silves-Albufeira region (**SA**), Faro (**F**), Spain (**SP**). **B:** *P. radicata* (***Prad***) in the North of Portugal (Macedo de Cavaleiros, Amarante-Vila Real). Sampled (**white squares**), or otherwise known locations (**black squares**), in a 10 km UTM grid. Data: personal observations and Aguiar et al. (2015). Images: © Google Earth 2014.

Most *P. almogravensis* cohorts are located in an unusual edaphic environment, geochemical-islands rich in metals (namely Al and Fe), in which this species has the ability to thrive and hyperaccumulate Al (Branquinho et al. 2007). The pattern of the *P. almogravensis* individuals within the cohorts was investigated. The distinctiveness of *P. almogravensis* in metal accumulation has been recognized in previous works (Branquinho et al. 2007). Here, we expanded that sampling, in a wider range of metals and soil compositions where the species is able to grow, to determine its accumulation ranges. In the particular case of Al, histochemical tests were used to determine the location of the major Al pools, within the plant leaves and branches.

The objectives of this work were, to (i) realize the distribution of *P. almogravensis* cohorts in the field and then compare the edaphic characteristics of its habitat with that of *P. algarbiensis*. (ii) Approach the relation between soil and plant composition and identify morphologic structures where metal accumulation was present. (iii) Compare the development and the morphologic features that may distinguish *P. almogravensis* from its sister species *P. algarbiensis* and *P. radicata*.

MATERIAL & METHODS

Locations and species studied

The main subject of this article is the extant *Plantago almogravensis* Franco population (Fig. 1A; Fig. 2A–D), with comparisons to the sister species (according to Franco 1984) *P. algarbiensis* Samp. (Fig. 1A; Fig. 2E–I). Besides those two species, individuals of *P. radicata* Hoffmanns. & Link (Franco 1984; synonym of *P. holosteum* Scop. according to Pedrol 2009) were also observed due to their taxonomic (*P. subulata* group) and geographic proximity (Fig. 1B; Fig. 2J,K). The experimental work (seeding and growing plants) was conducted at the Botanic Garden of the University of Lisbon (MUHNAC).

Distribution of *P. almogravensis* individuals

This part of the study was conducted in two large cohorts of *P. almogravensis*, encompassing areas of geochemical-island gaps and surrounding shrubland. Cohort is defined here as, all the *P. almogravensis* individuals located within a group, framed by shrub vegetation or geomorphic discontinuities.

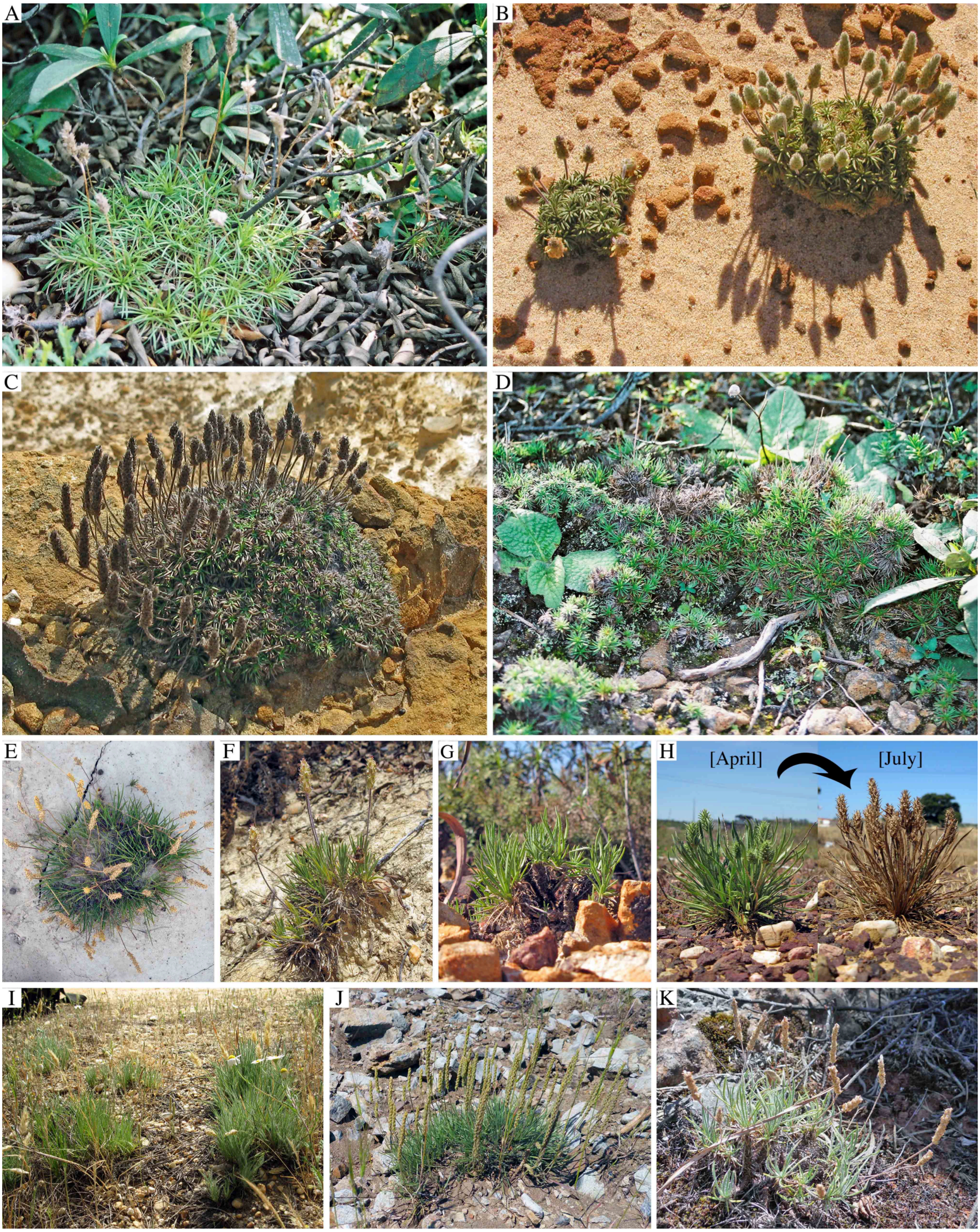


Figure 2. Plants in their natural habitat. **A–D:** *P. almogravensis*; **E–I:** *P. algarbiensis*; **J–K:** *P. radicata*. Population origin: Odemira (**A–D**), Silves-Albufeira (**E, G–H**), Faro (**F**), Spain/Huelva (**I**), Macedo de Cavaleiros (**J–K**). Photographs J–K: © E Roccotiello 2011.

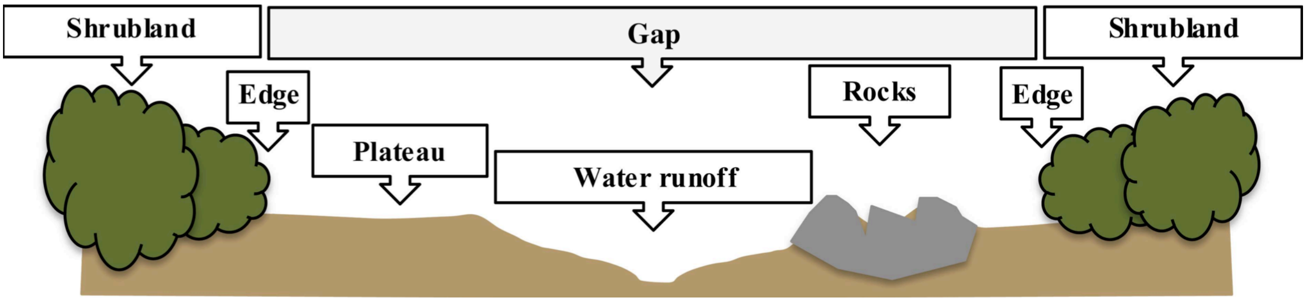


Figure 3. Sketch profile, with gap and shrubland microhabitats.

Cartography of the two cohorts was obtained through geo-referencing 2560 photographs taken at 1.35 m above the soil surface, stitched together to form orthophotographic maps (pixel resolution of 1 mm), covering total areas of 99 m^2 and 190 m^2, respectively. The maps were then used to define the different microhabitats observed (e.g. gap with or without runoff, shrubland and marginal edge areas; Fig. 3) and to measure the number of *Plantago* individuals and their cover (for details see Serrano et al. 2015)[1]. Other analyses (see next in methods) were sampled in these two, and in different cohorts, attempting to maximize the variety of habitat conditions and capture their maximum range heterogeneity (e.g. within microhabitats, Fig. 3; within soils, Fig. 4).

Edaphic characterization

Soil from cohorts of *P. almogravensis* (n = 12) and *P. algarbiensis* (n = 20) was sampled for the analyses below described.

Soil texture: The mass percentage of soil (2-mm fraction, 25g dry weight) passing a 75-µm sieve (wet sieving) was determined as the clay+silt fraction, while the retained fraction was the sand fraction. Sand was further divided into classes: < 2 mm < 1 mm < 500 µm < 250 µm < 125 µm. Method adapted from NRCS (2004). The texture classes (mass percentage) were obtained with a mean precision of 12% relative standard deviation (RSD).

Soil pH: The soil solution was prepared (1:5 w/v) with distilled water (pHw) or potassium chloride (KCl 1M, pHk) and measured with a pH electrode 91-02sc, ORION RESEARCH. The precision of the analyses was 2.6% RSD for pHw and 1.7% RSD for pHk. Knowing the soil weight (DW, gram), the volume of the KCl solution (V, millilitre) and the number of equivalent ions (Q = 1 for H_3O^+), the pHk was converted in $[H_3O^+]$ (mEq.100g^{-1}), to calculate ECEC:

Equation 1: $[H_3O^+]\ \mathrm{mEq.100g^{-1}} =$

$= 10^{-pHk}\ \mathrm{mmol.ml^{-1}} * V\ \mathrm{ml} * Q\ \mathrm{mEq} * 100/DW\ \mathrm{g}$

Effective Cation Exchange capacity (ECEC): The ECEC estimates the availability of chemical ions (nutrients and acidity) to the plant, at field conditions. The method used was adapted from NRCS (2004). The extraction of Ca, K, Mg and Na was per-

Figure 4. Colour diversity of topsoil (0-10 cm) from *P. almogravensis* habitat. Different colours were extracted from photographs of the original topsoils (inside plastic bags), and homogenized within 100x100 pixels.

---

[1] Chapter 3

formed using ammonium acetate (CH_3COONH_3, 1M, 1:20 w/v) and the extraction of Al with potassium chloride (KCl, 1M, 1:20 w/v). The concentration of those elements in the solutions was determined by Atomic Absorption Spectrometry (AAS), following standard protocols. Calculation of ECEC as the summation of base cations (Ca^{2+}, K^+, Mg^{2+}, Na^+) and exchangeable acidity (Al^{3+}, H^+) was done following equation 1:

Equation 2: $[\text{cation}]\ \text{mEq.100g}^{-1} =$

$= [\text{cation}]\ \text{mmol.ml}^{-1} * V\ \text{ml} * Q\ \text{mEq} * 100/DW\ \text{g}$

Total organic matter and carbonates estimated by Loss on Ignition (LOI): Organic matter (OM) was estimated by the LOI weight at 550°C, that should be proportional to the amount of organic carbon contained in the sample. Subsequently, the LOI weight at 950°C, multiplied by 1.36, should equal the weight of the carbonate (CO_3^{2-}) in the original sample (assuming a weight of 44 $g.mol^{-1}$ for carbon dioxide and 60 $g.mol^{-1}$ for carbonate). The method used followed Heiri et al. (2001), and the results (mass percentage) showed a mean precision of 25% RSD.

Elemental Nitrogen and Carbon: total content of C and N in ground-dry soil (*P. almogravensis* and *P. algarbiensis*) and in dry ground leaves (n = 10, *P. almogravensis* only) were obtained from an elemental analyser (EURO VECTOR) and expressed as mass percentage, with a mean precision of 19% RSD for N and 4.9% RSD for C.

Uptake of soil phytoavailable metals in *P. almogravensis*

Plants and respective soil samples were collected in 16 cohorts (n = 16), in a compromise between maximizing the heterogeneity of edaphic conditions (Fig. 4) and minimizing the amount of plants collected, due to its endangered status (see Serrano et al. 2011)[2].

Leaf metals: Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn were determined in dry leaf samples (mg kg^{-1} leaf DW) by Atomic Absorption Spectrometry (AAS) (for details see Branquinho et al. 2007).

Soil metals: Phytoavailable metals (Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Zn) were estimated (mg kg^{-1} soil DW) from a soil elution with Na_2EDTA (disodium ethylenediaminetetraacetic acid), and Na estimated from an aqueous elution (for details see Branquinho et al. 2007).

Soil Nitrate (NO_3^-): The pH and NO_3^- (mg kg^{-1} soil DW) were measured sequentially in a (1:2 w/v) water solution, with the respective electrodes - pH electrode 91-02sc, ORION RESEARCH (mean precision of 1.9% RSD); Nitrate Combination ISE/NBC, SENTEK 311-75 (mean precision, 5.0% RSD).

Histological staining of *P. almogravensis*

Samples (leaf and stem) from three year-old *P. almogravensis* plants grown in the botanic garden from seed, in a sand:native soil mixture (1:1) were transplanted to pots with washed sand and spiked with $Al(NO_3)_3$ during eight weeks (control, 0.05 mM and 0.5 mM Al), before staining. Samples were fixed in 10% buffered formalin (Pearse 1985) for 24 h, dehydrated in an ethanol series and embedded in *Technovit 7100 resin* (Heraeus Kulzer).

General anatomy: Cross sections were cut at 7 μm intervals, mounted, and stained with 0.05% toluidine blue O (TBO) in acetate buffer, pH4.4, for 1 min, as a metachromatic stain (O'Brien and McCully 1981). The stain TBO has the advantage of being a polychromatic dye, i.e. it reacts with different chemical components of cells differently and results in a multicoloured specimen.

Al-staining: For Al^{3+} localization, leaves and stems of *P. almogravensis* were sampled in Al-treated and

---

[2] Chapter 4

control plants. To avoid artifacts a variation of the classical method reported by Pearse (1985) was performed, as suggested by Rinino et al (2005). This consists into staining samples in 0.2% Solochrome Azurine B (pH 5; 15-20 min) before dehydration and then embedding the samples; fresh hand sections were also processed the same way. Al^{3+} ions stain deep blue. Observations were made using a Leica DM2000 optical microscope, equipped with Leica IM500 image processing software 4.0.

Development and morphology of *P. almogravensis* and sister taxa

To document development and morphology, photographs were taken on different phases of the plants life cycle (seeds, seedlings, juveniles and adults) and for different organs (roots, leaves, flowers and fruits), using scales whenever possible. Direct measurements were made on the length of spikes, peduncles, leaves and bracts; on the number of leaves, rosettes, flowers per spike, viable seeds per spike; and the weight of seeds. Data was gathered from field and garden-grown plants, see details within the respective captions. To account for the presence of mycorrhiza, roots were washed and either not treated, cleared with 10% KOH, or coloured with trypan-blue (0.05%), and observed at an optical microscope.

Data analysis

The *Independent-Samples Mann-Whitney U Test* (SPSS*statistics*, IBM) was used to compare data between species, because most of the variables had non-normal distributions (Shapiro-Wilk test, $P < 0.05$). Significance level was set at $p \leq 0.05$, unless stated otherwise. In the box-plots, hinges represent the 25th and 75th percentiles; a dash, the median (50th percentile); and whiskers indicate minimum and maximum values.

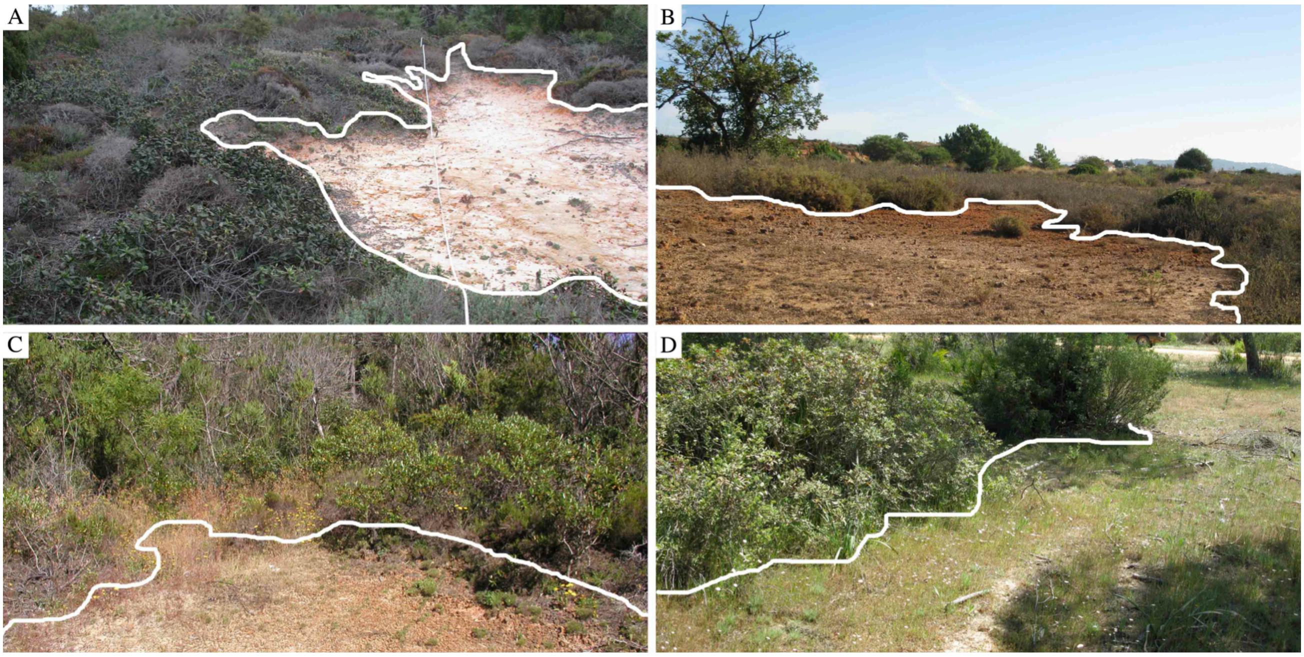


Figure 5. *Plantago* habitats highlighting gap areas (white lines). **A, C:** *P. almogravensis*; **B, D:** *P. algarbiensis*. **A–B:** gaps in dense shrubland; **C–D:** Edge of pinewoods.

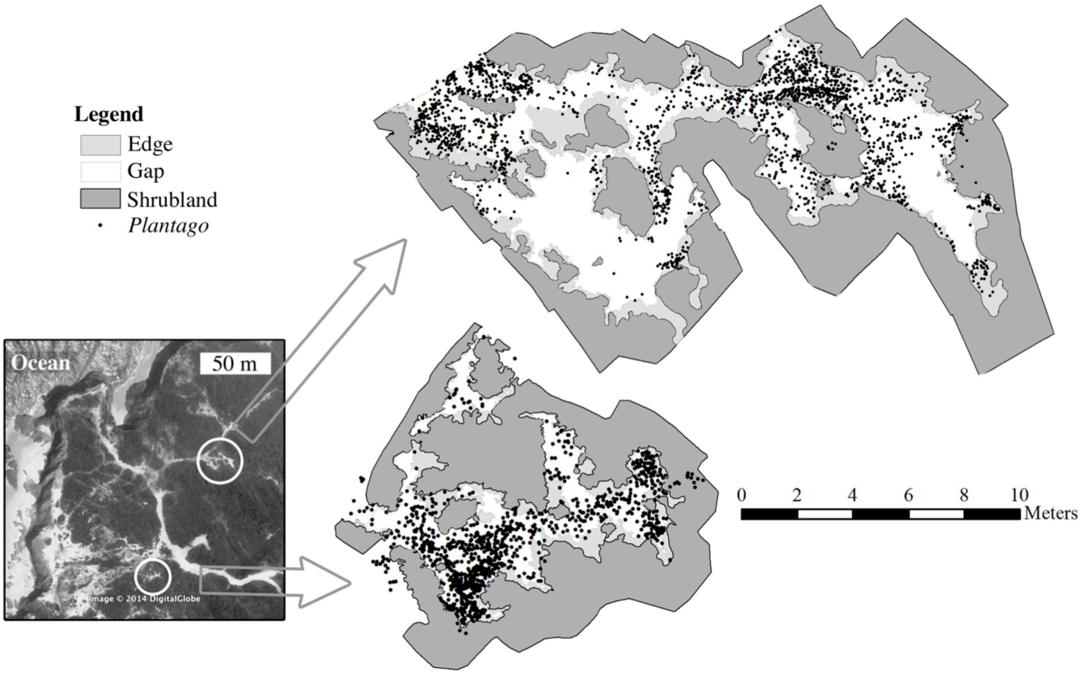


Figure 6. Patterns of *P. almogravensis* (dots) within two sample areas. Analysis of two large cohorts with a total of 2930 individuals. Image: © Google Earth 2014.

RESULTS & DISCUSSION

Habitat and distribution

The most representative cohorts of *P. almogravensis* were found in gaps without trees or shrub cover (Fig. 3; Fig. 5). The *P. almogravensis* individuals were established mainly in the core of the gap, in water run-off areas or in the plateaus (non-runoff) and hardly within the shrubland (Fig. 6; Fig. 7A). The total mean cover density in the measured areas was 3.2% in the gaps and 0.15% in the shrubland (Fig. 7B). The abundance was not homogeneous (Fig. 6). Rather, it showed an aggregation pattern, suggesting limitations in the dispersion or establishment of the seeds in other areas of the gap. Some of those limitations could have been due to physical barriers (e.g. slope, rocky substrate or hard crust, micro relief) or other environmental factors (Baskin and Baskin

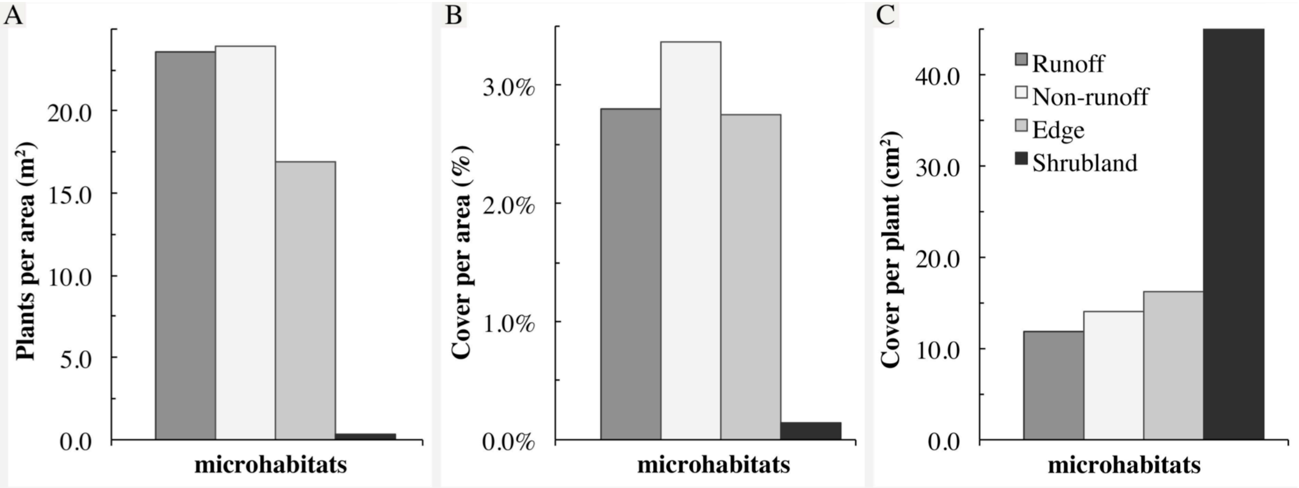


Figure 7. Distribution of *P. almogravensis* in the microhabitats. **A:** abundance, number of individuals per microhabitat area; **B:** density, total cover area of the individuals per microhabitat area; **C:** *P. almogravensis* average size (total individuals cover area, per number of individuals); results from the analysis of Fig. 6.

2014; Fenner and Thompson 2005; Harper et al. 1965; Zaady et al. 1997). The incidence pattern also suggests a water or wind seed dispersal mode, with individuals germinating in the colluvial areas, or in areas of entrapment, either biological (e.g. other plants and soil lichens) or edaphic (topography) (Baskin and Baskin 2014; Zaady et al. 1997). The individuals present in the gap areas showed a wide variety of sizes, thus their mean cover size was smaller than that of the individuals in the shrubland (Fig. 7C). Size is related to ramification and thus, to some extent, to age of the individuals. Shrubland cohorts were composed of adult individuals, but juveniles were hardly found (Fig. 7C; personal observations). This age/size pattern indicates a high turn-over of generations in the gap, compatible with an area of higher environmental stress; while in the shrubland, the lack of juveniles suggests impairment of new establishments after the initial colonization (by the older individuals), maybe due to competition with the surrounding vegetation.

Edaphic characterization

Both *P. almogravensis* and *P. algarbiensis* soils (Tab. 1) were within the sandy-loam or sandy-clay-loam types (NRCS 2004) due to the amount of sand present (≥ 53%). The sampled soils of *P. almogravensis* were coarser, predominating the sand 500-250 µm fraction, and overall richer in sand (75%). The soils of *P. algarbiensis* were richer in clay and silt (47%) (Tab. 1). These also showed more carbonates, probably of Ca, since this element was also enriched in soil. Yet, the total amount of soil carbon was greater in the sampled *P. almogravensis* soils, connected with their higher organic matter content (Tab. 1). The mean soil Al is similar in both soils, but the range of

Table 1. Soil properties compared between *P. almogravensis* and *P. algarbiensis* locations.

	P. almogravensis						*P. algarbiensis*					
Properties	n	Mean	SD	Min.	Med.	Max.	n	Mean	SD	Min.	Med.	Max.
Texture classes (%)												
2 - 1 mm***	12	0.75	0.45	0.27	0.63	1.95	20	3.2	2.0	0.8	3.0	6.5
1 - 0.5 mm**	12	6.6	2.1	3.1	6.9	10.0	20	3.7	1.8	1.1	3.6	7.9
500-250 µm***	12	41.3	7.9	25.8	42.2	54.8	20	9.5	4.9	2.5	8.6	22.3
250-125 µm	12	21.4	6.6	9.6	23.4	29.5	20	22.0	5.8	11.7	21.4	35.2
125-75 µm***	12	4.9	1.3	2.8	4.8	7.3	20	14.6	5.8	6.2	14.5	27.2
< 75 µm***	12	25	12	12	21	49	20	46.8	9.5	33.3	47.8	66.5
Chemical:												
pHw	12	5.60	0.48	4.87	5.60	6.44	20	5.85	0.67	4.96	5.78	7.78
pHk	12	4.63	0.67	3.89	4.78	5.94	20	5.13	0.96	3.67	5.15	7.08
ECEC *	12	3.9	1.4	1.8	4.0	6.3	20	4.8	1.0	3.3	4.7	7.0
Al mg.kg^{-1}	12	5.9	8.6	0	0.9	25.5	20	1.7	2.4	0	0.4	8.4
Ca mg.kg^{-1} **	12	330	152	146	282	589	20	456	126	248	470	695
K mg.kg^{-1}	12	72	34	26	63	139	20	64	13	42	61	94
Mg mg.kg^{-1}	12	171	82	75	177	317	20	214	84	51	196	410
Na mg.kg^{-1}	12	129	151	40	61	557	20	120	116	17	67	408
OM % **	7	3.67	0.67	2.43	3.68	4.42	20	2.96	0.61	2.05	2.95	4.49
CO$_3$ % ***	7	0.36	0.25	0.13	0.26	0.83	20	0.91	0.30	0.41	0.91	1.36
Total N %	10	0.030	0.024	0.001	0.027	0.083	14	0.031	0.019	0.001	0.030	0.061
Total C % **	12	1.32	0.70	0.35	1.45	2.30	20	0.70	0.26	0.35	0.66	1.34

SD: Standard deviation; **Min:** minimum; **Med:** median; **Max:** maximum.
Significance: * $p \le 0.10$; ** $p \le 0.05$; *** $p \le 0.001$.

values found in *P. almogravensis* soil is much wider, representing the existence of a wider gradient of Al-bioavailability. The acidic soil pH measured for most samples, namely pHk < 5.5 suggests also the presence of significant amounts of bioavailable Al (NRCS 2004). The ECEC was slightly lower for the soils of *P. almogravensis*, indicating a lower availability of nutrients to the plant, namely Ca (Tab. 1). The population of *P. almogravensis* is located in soils classified as *Orthic Podzols* while the populations of *P. algarbiensis* (Silves-Albufeira) are located in *Calcic-chromic Cambisols* (Cardoso 1965; FAO 2006).

Metal uptake in *P. almogravensis*

The leaves of *P. almogravensis* accumulated metals (Fig. 8), namely showing an enrichment factor (median shoot:soil ratio) above 10 to the phytoavailable soil fraction, for the following metals: Cu > Zn > Mg > K > Na > Ca > Mn > Al > Fe. Their median carbon content was 42% (12% range) and 1.0% nitrogen (0.5% range). Aluminium is a non-essential toxic metal. Its presence in such high concentrations in the plant shoot is considered extraordinary (Jansen et al. 2002a), thus the species must have developed strategies to manage such putative threat, some of which may be revealed histochemically.

Morphology and Al-accumulation

To detect Al accumulation within the plant organs, adult plants were subject to Al spiking over almost two months, before histochemical staining. Though there were no direct measurements of the amount of metal accumulated, there were visible effects of the Al-toxicity. Namely, mortality of 1/3 of the 0.5 mM spiked plants (no mortality on the 0.05 mM or control treatments); and rosette mortality during the experiment, of 16% (control), 19% (0.05 mM Al) or 55% (0.5 mM Al), per plant. These results suggest that, though the species can resist Al (Branquinho et al. 2007), as expected it has physiological limits to the concentrations tolerated.

Considering the histochemical results, the cuts revealed the typical leaf structures (Fig. 9A–B) of the members of *P. subulata* aggregate (triquetrae leaves, at least in some part of their limb, having a triangular shape). We detected at least two different types of hairs (trichomes): long and acute uniseriate hairs, one to three cells long (Fig. 9A, D–E) and obtuse morel-like glandular hairs, with a stalk and a head of about four to seven cells (Fig. 9C, F–G). Both types have been described before for other member of the section *Maritima* (Andrzejewska-Golec and Świetos-

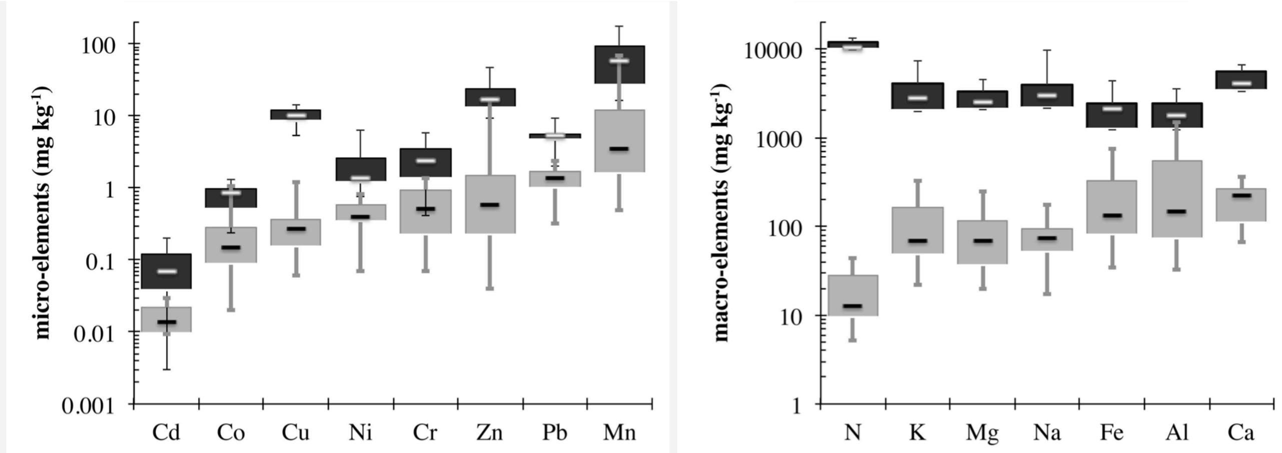


Figure 8. Chemical characterization of soil-phytoavailable and leaf elemental concentrations, for *P. almogravensis*. **Light-grey:** soil; **dark-grey:** leaves. For N, soil-N represents only the NO_3 extract results.

lawski 1998; Andrzejewska-Golec 1992; Rahn 1996).

We have detected a positive reaction to the presence of Al (blue colour) within the leaf glandular hairs (headed) of *P. almogravensis*, though not in the uniseriate hairs (Fig. 10A-C). Considering that leaves can hyperaccumulate Al, it is possible that other Al-sinks exist (besides the glandular hairs) that we did not detect with the histological tests. As an example, in the sclerophyllous Iberian endemic *Erica andevalensis,* from pyritic areas, Al (1745 mg Al kg^{-1}) is accumulated not only in glandular hairs, but also likewise in the cell walls of epidermal cells (Turnau et al. 2007). We have also detected Al positive reactions within the xylem of woody branches of *P. almogravensis* (Fig. 10E–F). In some species of the Rubiaceaea family, Al is more strongly accumulated in the outermost part of the secondary xylem, near the bark (Jansen et al. 2000). Authors that compared Al-accumulation in tropical rain forest trees (Jansen et al. 2002b; Masunaga et al. 1998) determined that generally Al-leaves > Al-wood or Al-bark (though a wider variation coefficient in bark). A faint reaction (blue colour) was detected in the xylem of control plants (Fig. 10D) probably due to their growth in native soil, naturally enriched in Al, prior to the onset of the experiment. The blue colour in the bark of control plants seems to indicate this is also a sink for the accumulated Al. Nevertheless, the intensity of the reaction in the bark of Al- treated plants could be overestimated due to contamination from the application of the Al-spiking solution or soil-Al. In Al-accumulators the metal has been associated with the epidermis and vascular pit of roots (Gonzalez-Santana et al. 2012); wood (Jansen et al. 2002b; Masunaga et al. 1998); leaf epidermal cell walls, mesophil cells, chloroplasts, vacuoles and glandular hairs (Carr et al. 2003; Cuenca et al. 1991; de Andrade et al. 2011; Gonzalez-Santana et al. 2012; Maejima et al. 2014; Tolra et al. 2011).

Plant development and morphology

As the seeds of *Plantago* spp. get hydrated, a mucilaginous layer forms around them (Fig. 11A, C) and the radicle begins to develop (Fig. 11A). The seedlings and juveniles of *P. almogravensis* and *P. algarbiensis* can be quite alike (Fig. 11B, D–F) though soon their differences start to be noticeable. The *P. almogravensis* plants develop more leaves in each rosette (Fig. 11E–F; Fig. 12A). Their rosettes are more compacted and flatten, while those of *P. algarbiensis* are more lax and with upward leaves, giving a different overall appearance to the plants (Fig. 11 top-views G–H; Fig. 13).

As *P. almogravensis* develop, plants are more prone to form branches (tips with rosettes) while *P. algarbiensis* can maintain just one rosette for longer time (Fig. 12A) thus being described as having only one or two woody branches (Franco 1984). We detected individuals of *P. almogravensis* with six orders of ramification and more than 100 rosettes, but older plants may have even more (Fig. 11K–L; Fig. 12B). The strategy of *P. algarbiensis* is different with less investment in rosettes (Fig. 11M–N; Fig. 12). Both species have a resprouting strategy, most likely to withstand drought stress (Zeppel et al. 2014). Their leaves dry during the summer drought and grow back (from apical and axial meristems) after the first rains. Resprouting has associated costs due to the need for maintain carbon and nutrients reserves during the summer, namely lower seed production, higher root to shoot ratio, longer recruitment time and formation of thicker bark to protect the gems (Zeppel et al. 2014). The dry leaves do not shed, instead they remain attached while decomposing.

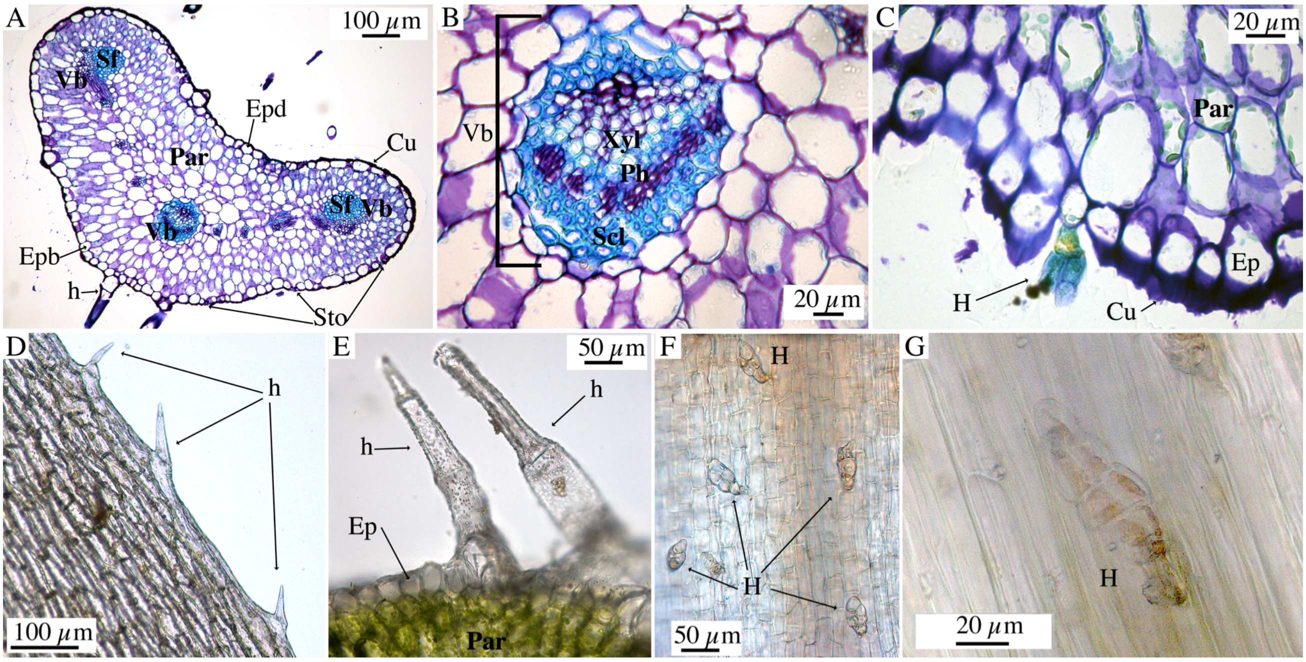


Figure 9. Anatomy details of *P. almogravensis* leaves. Embedded leaf cross-section with TBO staining (**A–C**) or without TBO staining (**D–G**). **Cu:** cuticle; **Sto:** stomata; **Epd, Epb:** adaxial and abaxial epidermis (**Ep**); **Par:** parenchyma with chloroplasts; **Vb:** vascular bundle; **Xyl:** xylem; **Ph:** phloem; **Scl:** sclerenchyma; **Sf:** sclerenchymatous fibers; **h:** headless hairs (uniseriate); **H:** headed hairs (glandular). Photographs & editing credits: © E Roccotielo, HC Serrano.

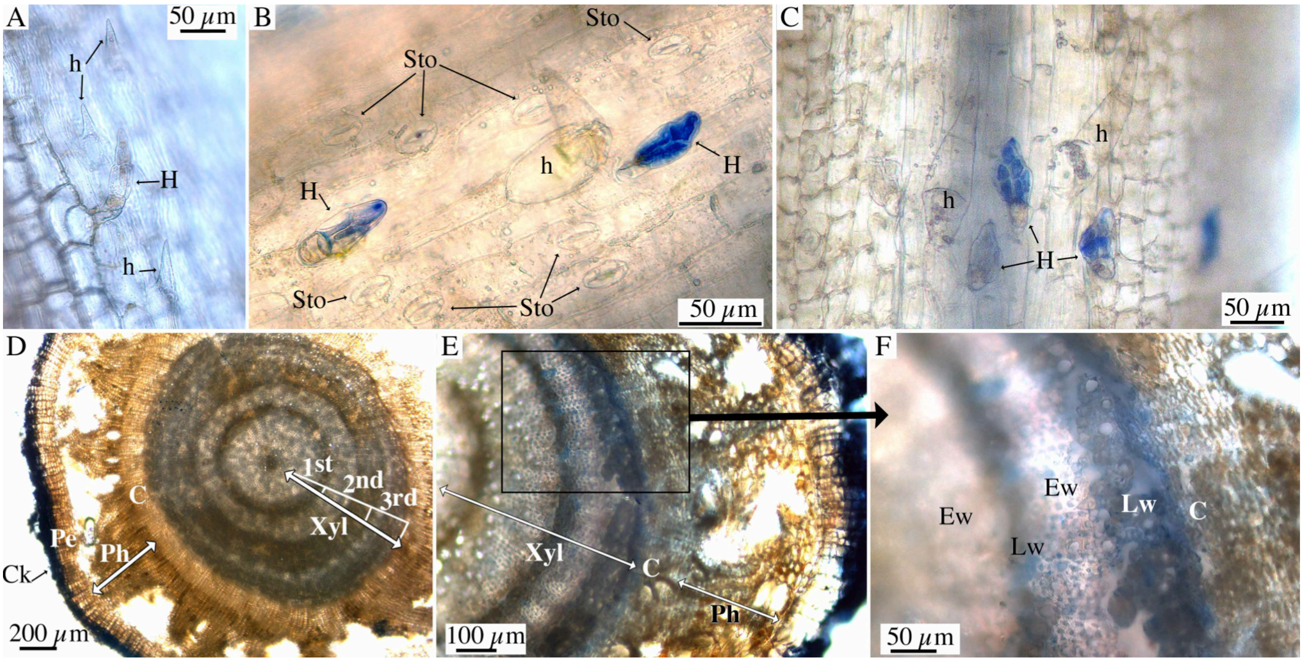


Figure 10. Solochrome-azurine-B staining in *P. almogravensis* leaves and stems. Leaf peeling (**A–C**) and stem cross-sections (**D–F**) of control (**A, D**) and Al-spiked plants (**B–C, E–F**). A blue colour develops in the presence of Al. **Sto:** stomata; **Xyl:** xylem (1st, 2nd and 3rd year growth rings; **Lw:** late wood; **Ew:** early wood); **Ph:** phloem; **h:** headless hairs (uniseriate); **H:** headed hairs (glandular); **Pe:** periderm; **C:** cambium; **Ck:** cork (bark). Photographs: © E Roccotielo & HC Serrano 2009.

Figure 11. General aspects of plant development in *P. almogravensis* (**A–B, E, G, I, K–L**) and *P. algarbiensis* (**C–D, F, H, J, M–N**). **A, C:** hydrated seeds, with mucilage and beginning of root development; **B, D:** seedlings ca. 1-2 weeks after germination, showing cotyledons, radicle and leaf primordia; **E–F:** juveniles ca. 2 months after germination; **G–H:** Adult plants, exposing woody roots and with top-view detail; **I–J:** adult plants with roots forming adventitious buds (zoom); **K–L:** adult *P. almogravensis* plant fragmented into its 103 rosettes and branches (primary trunk with 2 branches; 2nd, 3rd, 4th, etc. order branches); **M–N:** adult *P. algarbiensis* plant fragmented into its 21 rosettes and branches (primary trunk with two branches, five 2nd order branches, fifteen 3rd order branches with rosettes, six 4th order branches with rosettes).

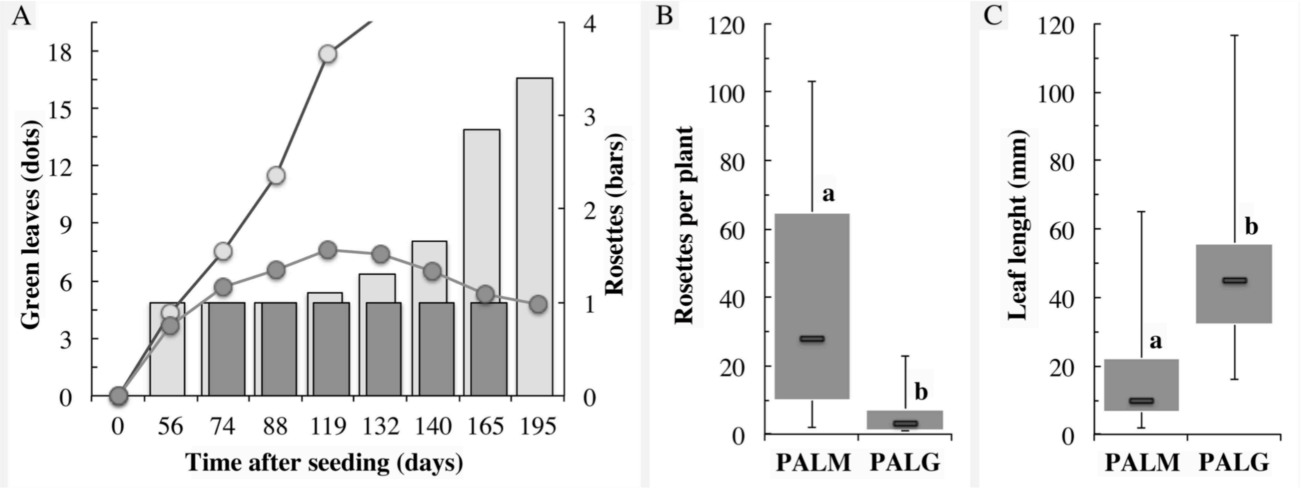


Figure 12. Number of leaves (**A**), rosettes (**A–B**) and maximum leaf length (**C**) in *P. almogravensis* and *P. algarbiensis*. **A:** Mean number of green leaves and rosettes per plant, in seedlings grown on indigenous soil; seeding was made in December; leaves > 20 were not counted; **light grey:** *P. almogravensis* (*n* = 20); **dark grey:** *P. algarbiensis* (*n* = 42). **B:** Number of rosettes in adult field plants; **PALM:** *P. almogravensis* (*n* = 22); **PALG:** *P. algarbiensis* (*n* = 28). **C:** Maximum leaf length, from field and pot-grown plants; **PALM:** *P. almogravensis* (*n* = 362); **PALG:** *P. algarbiensis* (*n* = 133). Different letters indicate significant differences in the distribution ($p \leq 0.05$).

The leaves of *P. almogravensis* are shorter (to the extreme of less than 2 mm) than those of *P. algarbiensis* (Fig. 12C), except in environmentaly protected zones and deep soils, where they can reach similar leaf length sizes. Our results show a wider range, and lower means, for the leaf length than that of Franco (1984) (1.5–5 cm for *P. almogravensis* and 3–9 cm for *P. algarbiensis*), probably due to a larger sample surveyed. The leaf phenotypes of *P. almogravensis* observed were diverse and showed a quick response to soil conditions (Fig. 13A), suggesting a good capacity to adapt to different edaphic environments. The leaves were linear to oblanceolate, with acute to acuminate tips (Fig. 13B), while that of *P. algarbiensis* were linear and acute (Fig. 13C) (Franco 1984).

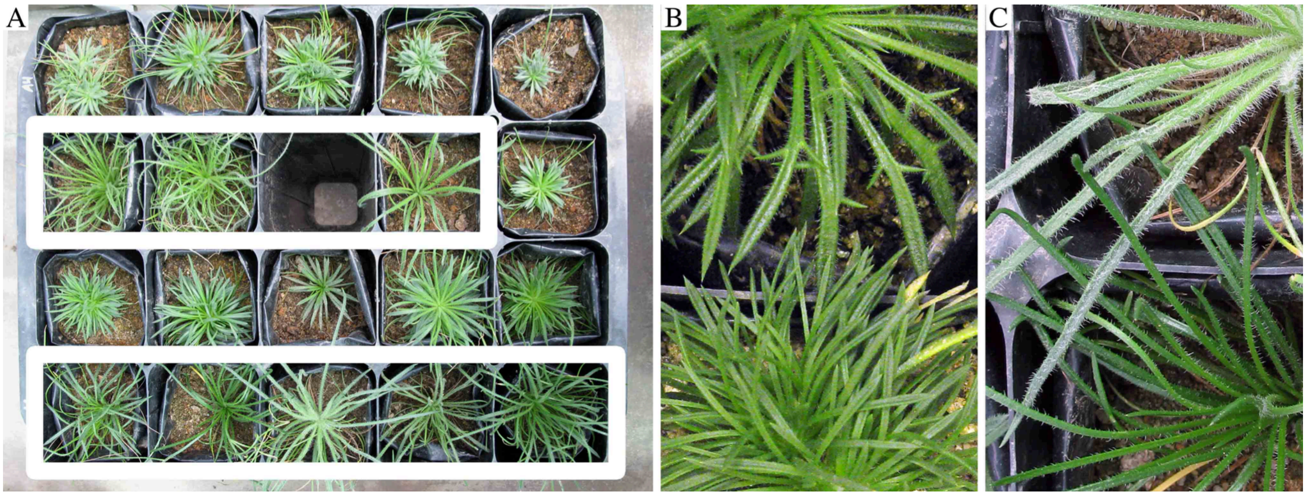


Figure 13. Juveniles of *P. almogravensis* and *P. algarbiensis*. **A:** Plants with ca. 1.5 years; **white highlight**: *P. algarbiensis*; for *P. almogravensis* (e.g. top row) a change in leaf morphology is visible, from thin long leaves to shorter and wider, two months after a substrate change (from a 1:1:1 peat:sand:mineral soil to 10:5:2 garden soil:sand:mineral soil). **B:** *P. almogravensis* plants, ca. 2.5 years, showing two extremes of leaf morphology, the thin leaves without perceived hairs (bottom) to a wider blade, with hairs clearly visible and occasionally one pair of teeth (top). **C:** *P. algarbiensis* plants, ca. 1.5 years, showing contrasting leaf morphologies, from extremely hairy (whitish) and wider blade (top) to thinner blade, less hairy (bottom).

Both of these *Plantago* spp. were observed to spread vegetatively, developing new gems from the woody roots, when exposed out of the soil (Fig. 11 I–J, zoom views). In the roots we could also find symbiotic associations with fungi (mycorrhizae). A preliminary observation of field collected *P. almogravensis* and *P. algarbiensis* detected the presence of ecto- (ECM) and endo- (AM) mycorrhizae (Fig. 14).

The *Plantago* genus is considered AM dependent (Koide and Mooney 1987; Wang and Qiu 2006) but regarding ECM we have found no references for *Plantago* species (Wang and Qiu 2006). Though this is probably because there are few woody species, (more susceptible to have ECM), care must be taken, as roots from other species may have contaminated field-collected samples. Nevertheless, this result is worthy of further studies.

In the studied *Plantago* spp. the flowers are grouped in spikes. The flower spikes of *P. almogravensis* were generally shorter (Fig. 15A; Fig. 16) and more compact (Fig. 15B; Fig. 17A) than that of *P. algarbiensis* (Fig. 18) and probably even *P. radicata* (Fig. 19), for which we measured 10-96 mm (minimum and maximum values out of four plants, or 36 spikes). This is not in line with what Franco (1984) described, as he considered that the spikes of *P. almogravensis* would be longer (20–40 mm) than that of *P. algarbiensis* (15–33 mm), while *P. radicata* would have the higher variability (10–45 mm). The width of the spikes of *P. almogravensis* seemed larger, as indicated by Franco (1984), than that of *P. algarbiensis* or *P. radicata*. Though we have not measured thickness of the spike, its perception is influenced by the presence or absence of a dense layer of hairs; consequently, not only *P. almogravensis* appeared to have wider spikes (Fig. 16), the phenotypes of *P. algarbiensis* with more hairs (e.g. Huelva population; Fig. 18B, G–H, P) seemed likewise wider. The peduncle that supports the spike (Fig. 15C) was shorter in the *P. almogravensis* plants sampled than in those of *P. algarbiensis*, in agreement with Franco (1984) comparison. Considering garden-grown and wild *P. almogravensis* plants, the latter showed a wider range in peduncle and spike lengths, probably reflecting their wider variety of microhabitats and genetic pool than the garden plants (Fig. 15A, C). In the wild plants, the spike is more compact than in the garden grown plants (Fig. 15B), suggesting that garden conditions stimulate the growth of the flower's internodes while in the more stressful field-conditions that biomass investment is reduced.

The bracts under each flower were shorter for *P.*

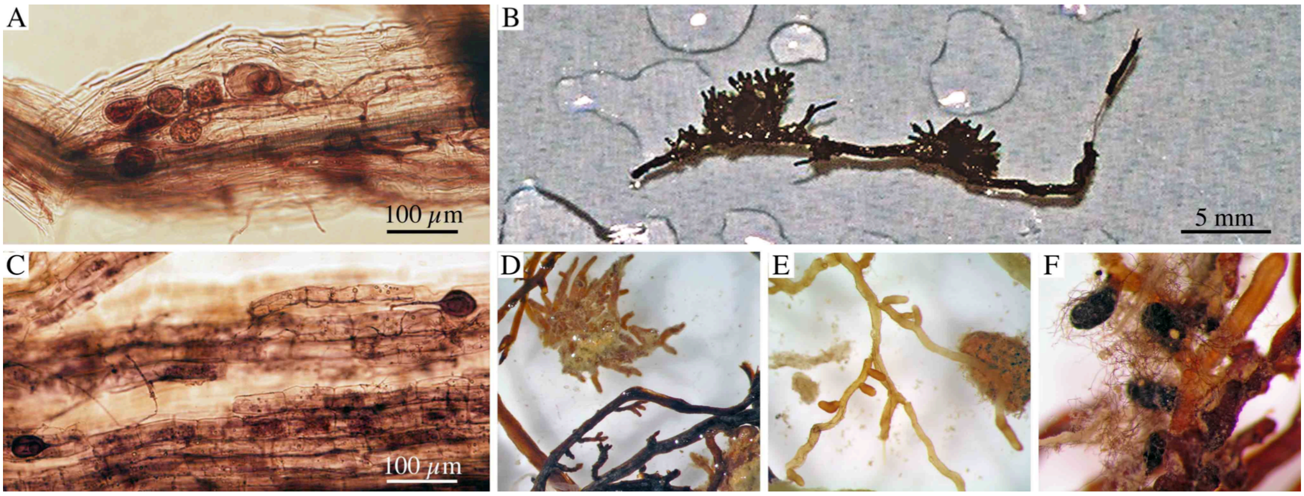


Figure 14. Mycorrhizae in putative roots from *P. almogravensis* (**A–B**) and *P. algarbiensis* (**C–F**). **A, C:** endomycorrhizae (AM); **B, D–F:** ectomycorrhizal root tips (ECM). The AM roots were coloured with trypan-blue (0.05%) and ECM were observed from untreated washed roots (B) or cleared roots (D-F; 10% KOH).

almogravesis than for of *P. algarbiensis*, in particular considering the lower bracts, more distinctive. The lower bracts were generally longer in both species (Fig. 15D; Fig. 17; Fig. 18), though in some *P. algarbiensis* phenotypes the difference in lengths gives the spike a conic appearance, rather than cylindrical (Fig. 18E, I). Bracts in *P. almogravensis* ranged from the same size as the calix, inconspicuous in the spike (Fig. 16C, G; Fig. 17A–B) to clearly visible and protruding (Fig. 16F,H; Fig. 17A), ca. two times the calix (Franco 1984). For *P. algarbiensis* the bracts were always longer than the calix (2–2.5 times) while in *P. radicata* the bracts (Fig. 19A–E) are usually subequal to the calix (Franco 1984). Though all three species showed hairs in the bracts, those of *P. almogravensis* are generally abundant and ciliate, while in *P. radicata* they are pubescent or puberulent and in *P. algarbiensis* usually glabrescent (except Huelva hairy phenotypes, Fig. 18P–Q) (Franco 1984). The sepals are similarly hairy as the bracts, with the anteriors being more symmetrical in *P. radicata* > *P. almogravensis* > *P. algarbiensis* (Franco 1984); Fig. 17C; Fig. 18M,R; Fig. 19F–J).

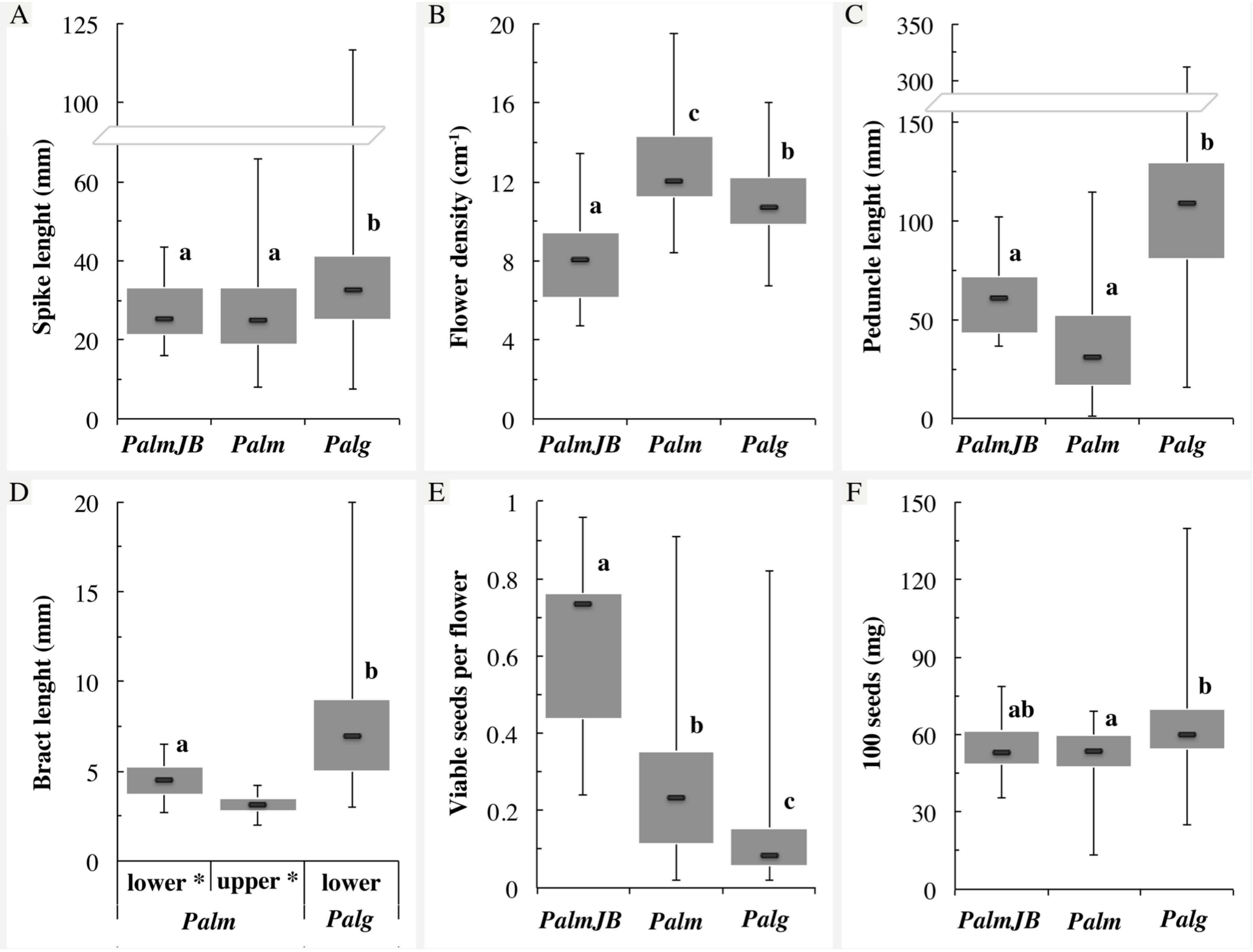


Figure 15. Reproductive features compared between *P. almogravensis* (***PalmJB***) grown in the garden or (***Palm***) from wild populations, and (***Palg***) *P. algarbiensis* from wild populations. Garden conditions differed from the wild, mainly in richer soil (native deep podzol), milder temperatures and regular water provision. **A:** spike length (n = 17, 89 and 381 spikes, respectively); **B:** flower density (number of flowers per centimetre of spike; n = 17, 60 and 109); **C:** peduncle length, without spike (n = 17, 31 and 93); **D:** average length of the lower or upper bracts in the flower spike (n = 22, 22 and 357); **E:** viable seeds per flower, from the ratio between seeds per spike and flowers per spike (viability estimated from visual assessment of the seeds; n = 14, 53 and 68 spikes); **F:** estimated weight of 100 seeds (n = 15, 34, 200 spikes); different letters indicate significant differences in the distribution ($p \leq 0.05$); significant differences (*) also between the size of upper and lower *P. almogravensis* bracts ($p < 0.05$).

The fruit of *Plantago* spp. is a pyxidium. Though these pyxidia are putatively capable of harbouring two seeds (Franco 1984; Pedrol 2009), two viable seeds were hardly found (Fig. 15E). For *P. almogravensis*, in the field, we accounted for only 0.27±0.21 viable seeds per flower, corresponding to (14±11)% of the maximum possible (2 seeds per flower) but in the milder garden conditions (JB in Fig. 15) the number of viable seeds was higher (0.70±0.32), corresponding to (35±16)%. For *P. algarbiensis*, this is even more pronounced with only 0.14±0.16 viable seeds per flower, thus (7±16)% of the maximum possible (Fig. 15E). In most cases a large proportion of flowers did not produce viable seeds at all. Possibly causes are lack of pollination or impaired development after pollination (personal observations). Nevertheless, seeds collected from field spikes might be underestimated due to early dehiscence caused by wind or summer rain, as garden grown plants seemed to produce more viable seeds (Fig. 15E). The viable seeds of *P. almogravensis* were lighter than those of *P. algarbiensis* (Fig. 15F; Fig. 17F; Fig. 18U), similarly to what was determined by Martins et al (2012).

Conclusions

I—distribution and soil type: *P. almogravensis* prefers to inhabit open gaps, not the understory of trees or shrubs. It colonizes those areas probably after seed dispersion by wind or water, not showing much mobility. The turnover of individuals seems to be greater in the gaps than in more protected areas (shrubland) where individuals can reach larger sizes/ages.

The soil where *P. almogravensis* inhabits is a sandy-loam with less clay than that of *P. algarbiensis*. The wider gradient of Al and carbon in the soil of *P. almogravensis* reflects the heterogeneity of its habitat with Fe-Al geochemical islands surrounded by the deeper richer podzol, mostly covered by shrubland.

II—Metal-accumulation: *P. almogravensis* is a strong accumulator of metals, particularly of Al. The metal is accumulated in wood and in leaves, where it was detected in leaf headed trichomes. Though the plants tolerate Al, at high concentrations the plants show toxicity symptoms and die.

III—Development & Morphological comparisons: *P. almogravensis* and *P. algarbiensis* can propagate vegetatively and seem able to form mycorrhizal associations; both factors can be helpful in overcoming stressful conditions. From the three species compared, *P. almogravensis*, *P. algarbiensis* or *P. radicata*, the first two showed significant differences in growth pattern and morphology (number and length of leaves; number of rosettes per plant; spike and peduncle or bract length; and seed weight). For *P. radicata* the comparison is only from photographs, but together with previous descriptions (e.g. (Franco 1984) the differences to *P. almogravensis* are also significant, namely in spike dimensions or flower morphology. The differences were not only verified in the wild but some also when grown in the same environmental conditions (garden conditions) suggesting a strong separation between the taxa (speciation) rather than just an adaptation to environmental conditions (plasticity).

Legend Fig. 17: A: bract (**Br**) morphotypes (short and long); **B:** calix with four sepals (**Se**; **Se-a:** anterior; **Se-p:** posterior) and corolla (**Co**) with four petals (**Pe:** free petals; **Co-t:** corolla tube); **C:** heteromorphic sepals; **D:** reproductive organs, pistil (**Sty:** style; **Ov:** ovary) and stamens (**Ant:** anther; **Fil:** filament); **E:** fructification pyxidium (**Px**); **F:** seeds; background 1 mm squares.

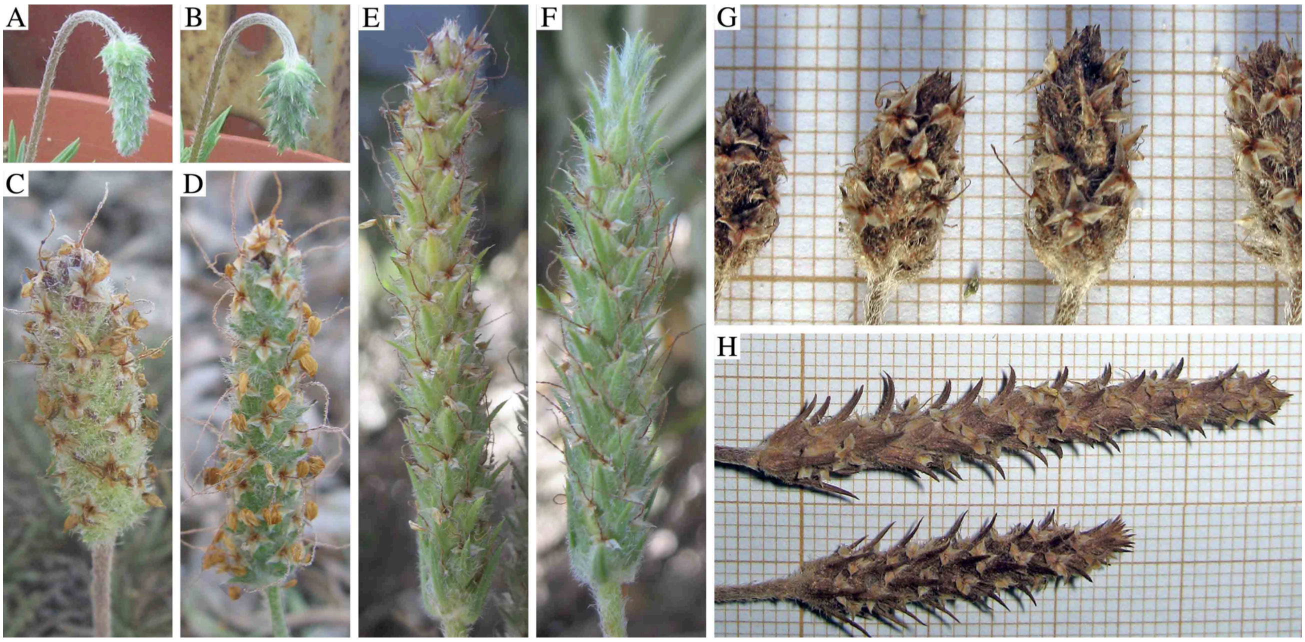


Figure 16. Stages of flowering and spike morphotypes in *P. almogravensis*. **A–B:** immature spikes; **C–F:** mature spikes; **G–H:** dry spikes; background 1 mm squares. Extra photographs C-F © MJ Pinto 2008.

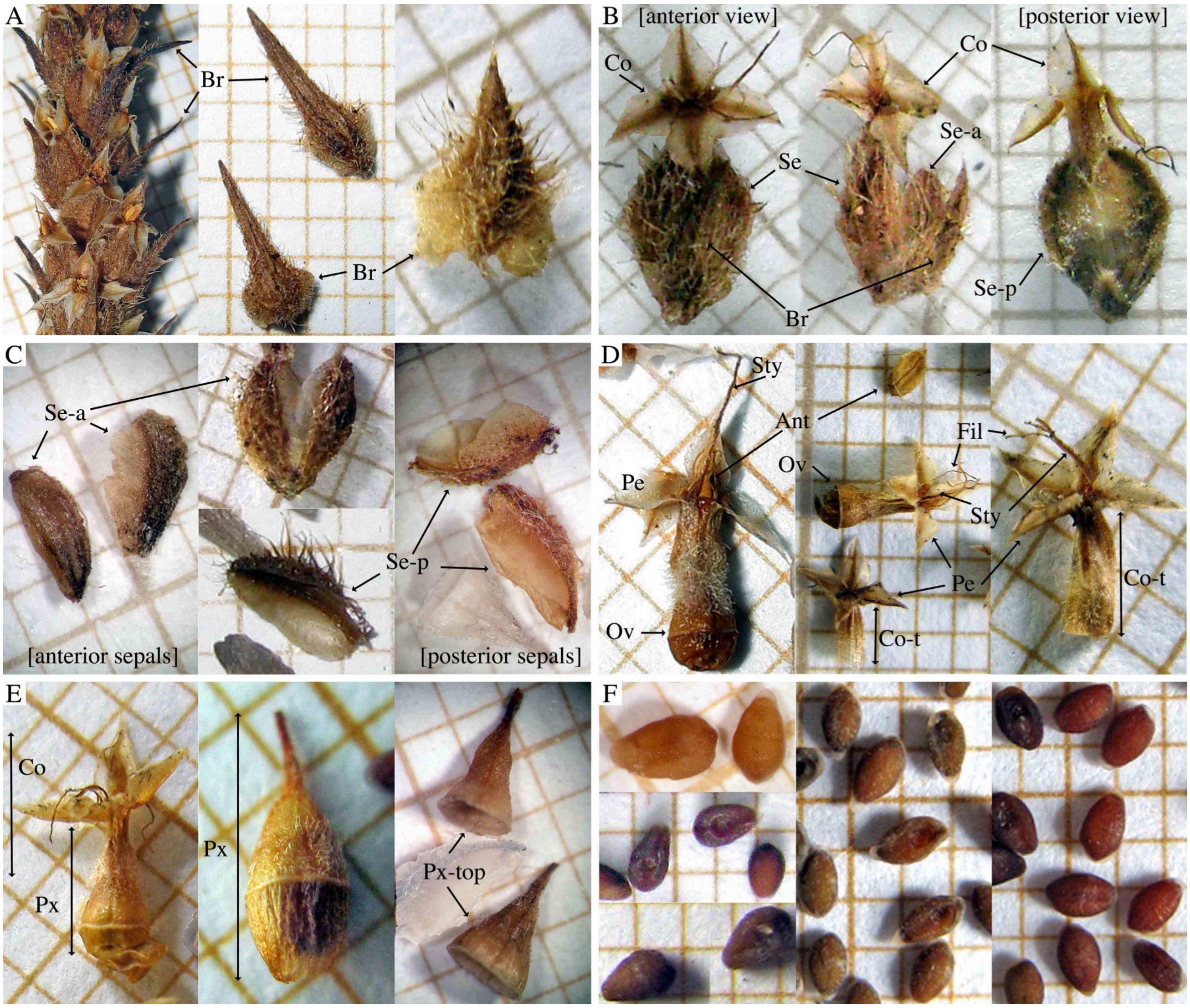


Figure 17. Flower and fruit components in *P. almogravensis*. See legend on previous page.

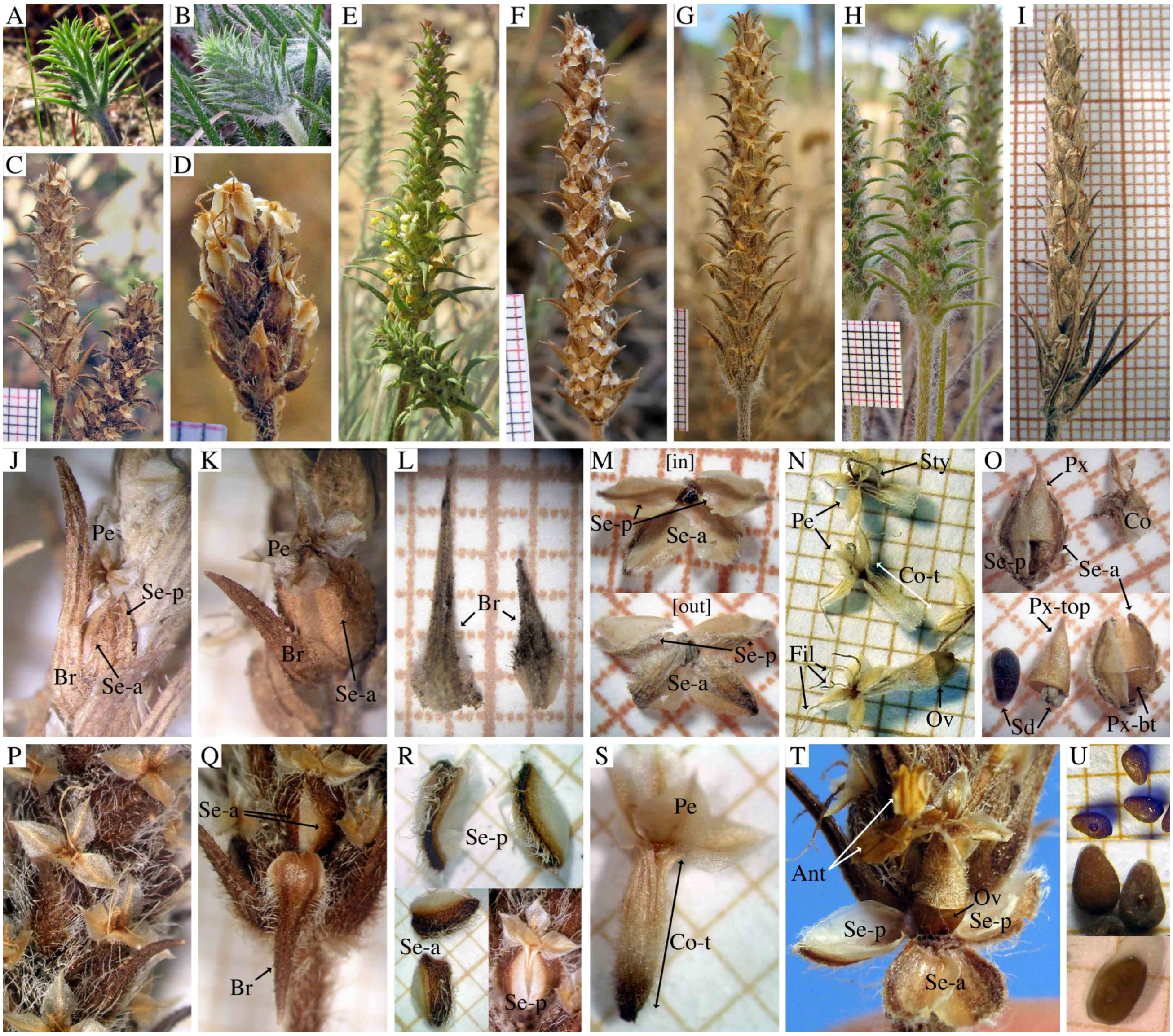


Figure 18. Spike morphotypes and flower components in *P. algarbiensis*. **A–B:** immature spikes; **C–H:** mature spikes; **I:** dry spikes; **J–K, P–Q:** complete flowers; **L, Q:** bract morphotypes; **M, R:** calix; **N, S–T:** corolla and reproductive organs, the pistil and stamens; **O, U:** fructification, pyxidium and seeds. See legend on next page.

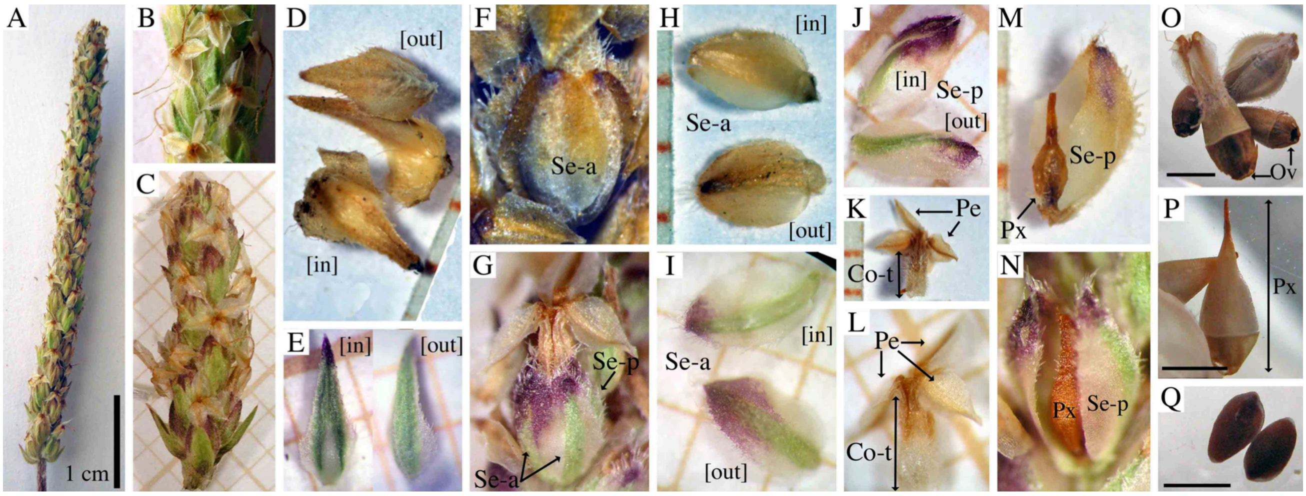


Figure 19. Spike morphotypes and flower components in *P. radicata*. **A–C:** spikes; **D–E:** bracts; **F–J:** calix; **K–L:** corolla; **M–P:** fructification; **Q:** seeds. See legend on next page.

ACKNOWLEDGEMENTS

This work was supported by *Fundação para a Ciência e a Tecnologia* [grant SFRH/BD/38289/2007 to HC Serrano; *Investigador FCT* contract to C Branquinho] by projects [POCTI/BIA- BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004]; [Transnational Cooperation Project] FCT-Portugal/CNR-Italy 2009; and *Parque Natural do Sudoeste Alentejano e Costa Vicentina* and *Instituto de Conservação da Natureza e das Florestas* (ICNF) by project [*Estudo de referência de Plantago almogravensis* 2004] at *Museu Nacional de História Natural e da Ciência, da Universidade de Lisboa* (MUHNAC). Special thanks to Antunes C, Branquinho C, Martins-Loução MA and Pinto MJ. The authorizations regarding *P. almogravensis* and *P. algarbiensis* populations were provided by ICNF (Portugal) and *Parque Natural Donãna* (Spain). Extra photographs (see captions): E Roccotiello and MJ Pinto.

REFERENCES

Aguiar C, Carapeto A, Araújo PV, Silva A, Porto M, et al. 2015. *Plantago* (várias espécies) - mapa de distribuição. In *Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica*. Published on the internet http://www.flora-on.pt/#wid21862190, accessed at 25-02-2015

ALFA. 2011. Checklist da Flora de Portugal (Continental, Açores e Madeira). ed. M Menezes de Sequeira, MD Espírito-Santo, C Aguiar, J Capelo, J Honrado, pp. 74. Lisboa, Portugal: Associação Lusitana de Fitossociologia

Andrzejewska-Golec E. 1992. Hair morphology in *Plantago* sect *Coronopus* (Plantaginaceae). *Plant Systematics and Evolution* 179: 107-13

Andrzejewska-Golec E, Świetoslawski J. 1998. Microhairs of *Plantago maritima* L. under electron scanning microscopy. *Feddes Repertorium* 109: 225-29

Baskin CC, Baskin JM. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Elsevier Science.

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Cardoso JC. 1965. *Os solos de Portugal, sua classificação, caracterização e génese. 1 - A sul do Rio Tejo*. Portugal: Secretaria de Estado da Agricultura.

Carr HP, Lombi E, Kupper H, McGrath SP, Wong MH. 2003. Accumulation and distribution of aluminium and other elements in tea (*Camellia sinensis*) leaves. *Agronomie* 23: 705-10

Cuenca G, Herrera R, Mérida T. 1991. Distribution of aluminium in accumulator plants by X-ray microanalysis in *Richeria grandis* Vahl leaves from a cloud forest in Venezuela. *Plant, Cell & Environment* 14: 437--41

de Andrade LRM, Barros LMG, Echevarria GF, do Amaral LIV, Cotta MG, et al. 2011. Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. *Environmental and Experimental Botany* 70: 37-42

FAO. 2006. *World Reference Base for Soil Resources 2006: A Framework for International Classification, Correlation and Communication*. pp. 144. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).

Fenner M, Thompson K. 2005. *The Ecology of Seeds*. Cambridge University Press.

Franco JA. 1984. *Nova Flora de Portugal (Continente e Açores)*. Lisboa, Portugal.

Gonzalez-Santana IH, Marquez-Guzman J, Cram-Heydrich S, Cruz-Ortega R. 2012. *Conostegia xalapensis* (Melastomataceae): an aluminum accumulator plant. *Physiologia Plantarum* 144: 134-45

Harper JL, Williams JT, Sagar GR. 1965. The behavior of seeds in soil I. The heterogeneity of soil surfaces and its role in determining the establishment of plants from seed. *Journal of Ecology* 53: 273-86

Heiri O, Lotter A, Lemcke G. 2001. Loss on ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results. *Journal of Paleolimnology* 25: 101-10

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002a. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Jansen S, Robbrecht E, Beeckman H, Smets E. 2000. Aluminium accumulation in Rubiaceae: An additional character for the delimitation of the subfamily Rubioideae? *Iawa Journal* 21: 197-212

Jansen S, Robbrecht E, Beeckman H, Smets E. 2002b. A survey of the systematic wood anatomy of the Rubiaceae. *Iawa Journal* 23: 1-67

Koide RT, Mooney HA. 1987. Spatial variation in inoculum potential of vesicular arbuscular mycorrhizal fungi caused by formation of gopher mounds. *New Phytologist* 107: 173-82

Maejima E, Hiradate S, Jansen S, Osaki M, Watanabe T. 2014. Comparative analysis of aluminum accumulation in leaves of three angiosperm species. *Botany* 92: 327-31

Martins N, Goncalves 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: 144-49

Legends Fig. 18 & Fig. 19: Bracts (**Br**); calix with 2+2 sepals (**Se:** sepals; **Se-a:** anterior; **Se-p:** posterior); corolla with 4 petals (**Pe:** free petals; **Co-t:** corolla tube); reproductive organs, the pistil (**Sty:** style; **Ov:** ovary) and stamens (**Ant:** anther; **Fil:** filament); fructification, pyxidium (**Px**; divided in **Px-top** and **Px-bt:** bottom) and seeds (**Sd**); internal view (**in**) and external view (**out**). Background squares and bars, 1 mm.

Population origin, ***P. algarbiensis*:** Silves-Albufeira (**A, D–F, I, J–O, U**), Faro (**C**), Spain/Huelva (**B, G–H, P–U**).

Population origin, ***P. radicata*:** Macedo de Cavaleiros (**A–B, D, F, H, K–M, O–Q**); Amarante-Vila Real (**C, E, G, I–J, N**).

Masunaga T, Kubota D, Hotta M, Wakatsuki T. 1998. Mineral composition of leaves and bark in aluminum accumulators in a tropical rain forest in Indonesia. *Soil Science and Plant Nutrition* 44: 347-58

NRCS. 2004. Soil Survey Laboratory, Methods Manual, United States Department of Agriculture, Natural Resources Conservation Service

O'Brien TP, McCully ME. 1981. *The study of plant structure : principles and selected methods*. Melbourne, Australia: Termarcarphi.

Pearse AGE. 1985. Histochemistry : theoretical and applied In *Analytical Technology*, ed. AGE Pearse, pp. 1000-23. Edinburgh, UK: Churchill Livingstone Inc

Pedrol J. 2009. *Plantago* L. In *Flora Iberica*, ed. C Benedi, E Rico, J Güemes, A Herrero, pp. 4–38. Madrid, Spain: Real Jardín Botánico CSIC

Rahn K. 1996. A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society* 120: 145-98

Rinino S, Bombardi V, Giordani P, Tretiach M, Crisafulli P, et al. 2005. New histochemical techniques for the localization of metal ions in the lichen thallus. *Lichenologist* 37: 463-66

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Tolra R, Vogel-Mikus K, Hajiboland R, Kump P, Pongrac P, et al. 2011. Localization of aluminium in tea (*Camellia sinensis*) leaves using low energy X-ray fluorescence spectro-microscopy. *Journal of Plant Research* 124: 165-72

Turnau K, Henriques FS, Anielska T, Renker C, Buscot F. 2007. Metal uptake and detoxification mechanisms in *Erica andevalensis* growing in a pyrite mine tailing. *Environmental and Experimental Botany* 61: 117-23

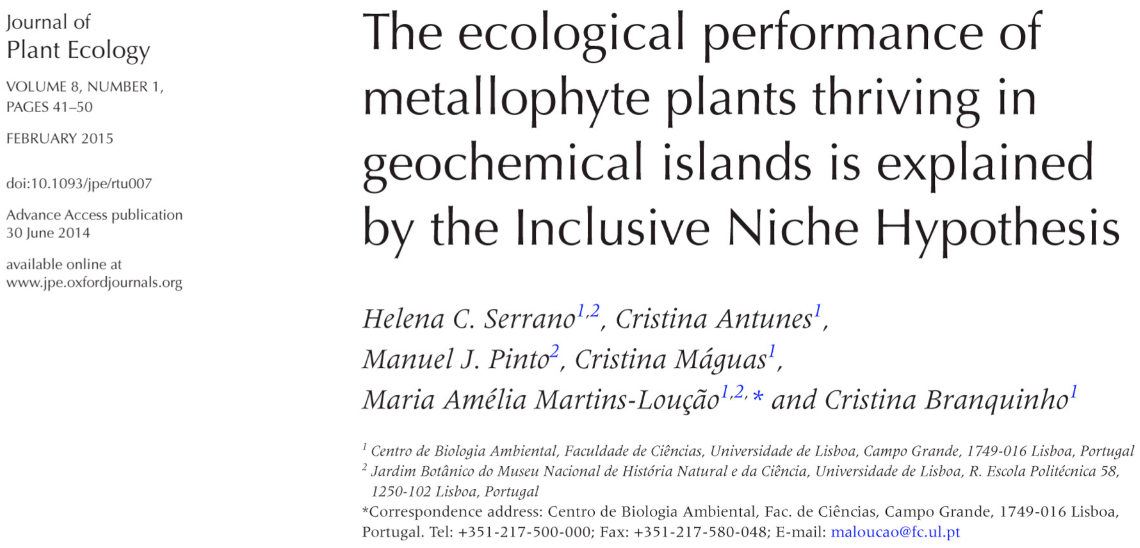
Wang B, Qiu YL. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16: 299-363

Zaady E, Gutterman Y, Boeken B. 1997. The germination of mucilaginous seeds of *Plantago coronopus*, *Reboudia pinnata,* and *Carrichtera annua* on cyanobacterial soil crust from the Negev Desert. *Plant and Soil* 190: 247-52

Zeppel MJB, Harrison SP, Adams HD, Kelley DI, Li G, et al. 2014. Drought and resprouting plants. *New Phytologist* (early view)

CHAPTER 3

THE ECOLOGICAL PERFORMANCE OF METALLOPHYTE PLANTS THRIVING IN GEOCHEMICAL ISLANDS IS EXPLAINED BY THE INCLUSIVE NICHE HYPOTHESIS



Journal of
Plant Ecology
VOLUME 8, NUMBER 1,
PAGES 41–50
FEBRUARY 2015
doi:10.1093/jpe/rtu007
Advance Access publication
30 June 2014
available online at
www.jpe.oxfordjournals.org

The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis

Helena C. Serrano[1,2], *Cristina Antunes*[1],
Manuel J. Pinto[2], *Cristina Máguas*[1],
Maria Amélia Martins-Loução[1,2,*] *and Cristina Branquinho*[1]

[1] *Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal*
[2] *Jardim Botânico do Museu Nacional de História Natural e da Ciência, Universidade de Lisboa, R. Escola Politécnica 58, 1250-102 Lisboa, Portugal*
*Correspondence address: Centro de Biologia Ambiental, Fac. de Ciências, Campo Grande, 1749-016 Lisboa, Portugal. Tel: +351-217-500-000; Fax: +351-217-580-048; E-mail: maloucao@fc.ul.pt

De acordo com o disposto na alínea a, do número 2, do artigo 25, do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa (Despacho n.º 2950/2015 da Reitoria da Universidade de Lisboa), a autora desta tese declara que participou activamente no planeamento e execução do trabalho experimental, na interpretação dos resultados obtidos e na elaboração do seguinte manuscrito, publicado no *Journal of Plant Ecology*, 2015, 8(1):41-50. doi: 10.1093/jpe/rtu007.

CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

THE ECOLOGICAL PERFORMANCE OF METALLOPHYTE PLANTS THRIVING IN GEOCHEMICAL ISLANDS IS EXPLAINED BY THE INCLUSIVE NICHE HYPOTHESIS

ABSTRACT

The Inclusive Niche Hypothesis has not been validated for plants using ecophysiological performance. The few experiments have measured growth and competition but not the physiological response of plants. A metallophyte plant that hyperaccumulates aluminium (Al), *Plantago almogravensis*, showed a defined spatial distribution by occurring mostly on vegetation gaps associated with metalliferous areas (geochemical islands). This case was used to determine, *in situ*, whether the Inclusive Niche Hypothesis was suitable to explain the extent of the species realized niche.

The vegetation associated with *P. almogravensis* geochemical islands in the SW coast of Portugal was mapped. The biotic (neighbouring plants) and abiotic (edaphic) components of the niche were correlated with parameters of the plant's ecological and physiological performances (plant density and cover; leaf C and N concentration and isotopic composition; growth). The results were obtained using image analysis, abundance and morphological measures, isotopic signatures and chemical composition.

The species showed better physiological performance where its ecological performance was lower due to trade-offs with environmental constraints. The species' realized niche was mostly limited by shrub competition and soil Al-toxicity. These limits contribute to explain the rarity status of the species: the species has a poor capacity to compete but, due to an enhanced Al-tolerance and Al-hyperaccumulator trait, has the ability to find refuge in geochemical islands that are too harsh for most other species. This work, based on ecophysiological field studies, provides support for the Inclusive Niche Hypothesis relating to plant species.

INTRODUCTION

The realized niche concept (Colwell and Rangel 2009) revisited after Hutchinson (1957), takes into account all the direct ties between abiotic environment and species interactions. In conservation biology of rare/endemic species, the niche concept offers novel approaches to classical questions (Harrison et al. 2010; Gaston 2012). Population stability and distribution depend on both genetic and environmental-based variation in functional traits and thus, on their ecological performance. Indeed, a species' ecological performance can be measured by its abundance (cover and/or density), which will reflect the range of biotic and abiotic factors that determine its distribution. Thus, there is the need to measure those environmental conditions that individuals are experiencing, and determine how their performance is affected, to get an estimate for the width of their realized niche (Devictor et al. 2010).

However, the ecological performance of a species does not always match its physiological performance. This is observed in plant populations where negative correlations between plant physiological performance and its abundance are obtained (McGill 2012). The application of stable isotopes as integrated tools to evaluate plant physiological performance is widely accepted (e.g. Handley and Raven 1992; Robinson 2001; Dawson *et al.* 2002; Werner *et al.* 2012). Carbon isotope composition (∂^{13}C) integrates physiological (e.g. photosynthesis), seasonal (e.g. phenology) and morphological changes (e.g. leaf structure) (Werner and Máguas 2010; Máguas *et al.* 2011). During C3 photosynthesis, in harsh habitat conditions, when water conservation or light/temperature protection is required, stomata closure leads to an higher incorporation of the heavier ^{13}C isotopes into plant biomass, raising ∂^{13}C values (e.g. Brugnoli and Farquhar 2000). Another useful isotopic species is the ^{15}N (Handley and Raven 1992), and its fractionation in plant leaves (∂^{15}N) reflects not only the integration of soil nitrogen availability and processes of uptake but also assimilation and mobilization within plants (Martins-Loução *et al.* 2000). The ∂^{15}N values measured in the leaves depend on N source available for plant assimilation. Nitrogen can be taken up directly from soil, from atmosphere (originated from symbiotic microorganisms) or from internal remobilization (Martins-Loução *et al.* 2000). As a whole, physiological changes are related to C and N metabolism and, thus, regulated by leaf C/N ratio that may function as a physiological proxy (Loveless 1961; Correia and Martins-Loução 1997; Martins-Loução and Lips 2000). Accumulation of C products, in conjunction with small and dense leaves, can be evaluated using leaf sclerophylly, an ecophysiological indicator of environmental stress, particularly in Mediterranean plants (Correia *et al.* 1992, Anacker *et al.* 2011a).

According to Colwell and Fuentes (1975), the Inclusive Niche Hypothesis states that all species have optimal performance under optimal environmental conditions. The stronger competitors occupy the optimal areas. The weaker competitor species are cast to suboptimal zones, where they do not perform so well. Their fundamental niche is reduced to a smaller realized space, in a trade-off between competitive ability and environmental tolerance (Colwell and Fuentes 1975; Anacker *et al.* 2011b). That is the case for plants capable of surviving and reproducing in soils with high content of toxic metals, the metallophytes (Kruckeberg 1954; Boyd 2004; Baker *et al.* 2010) and particularly the case of serpentinophytes, found in isolated island-like rocky outcrops (Kruckeberg and Rabinowitz 1985; Anacker *et al.* 2011b; Harrison and Rajakaruna 2011). Plants with smaller realized niches are more vulnerable to fragmentation and isolation, further contributing to their rarity (Kruckeberg and Rabinowitz 1985; Baker *et al.* 2010). *Plantago almogravensis* Franco (Plantaginaceae) is a critically endangered species endemic to Portugal (Franco 1984; Bilz *et al.* 2011) and able to hyperaccumulate aluminium (Al). To our knowledge, it is the only reported Al-hyperaccumulator from a Mediterranean climate (Jansen et al. 2002; Branquinho et al. 2007; Serrano et al. 2011[1]; Metali et al. 2012). The extant population of *P. almogravensis* is limited to <4 ha in the *Sudoeste Alentejano e Costa Vicentina* Natural Park (PNSACV) located on the SW coast of Portugal. These plants occur mostly over hardpan outcrops (a cemented sesquioxide layer), rich in Al and Fe, that form a mosaic landscape with the surrounding podzolized sand deposits (loamy sand podzol; Cardoso 1965). The natural sclerophyllous vegetation is absent in these hardpan areas with shallow soils (gaps).

Al-toxicity is highly deleterious for plants, therefore not many Al-resistant species are found in nature (Jansen *et al.* 2002; Metali *et al.* 2012). Hyperaccumulators not only create special allocation patterns for the sequestration of metals to prevent toxicity, but often tolerate otherwise toxic soil metal concentrations (de Andrade *et al.* 2011; Brunner and Sperisen 2013). The extra expenditure of energy for tolerance results in phenotypic plasticity due to trade-offs between traits and environment (Cuenca and Herrera 1987; Maestri *et al.* 2010). It is common for metallophytes to have low productivity (low shoot/root mass ratios, short internodes, short leaves), a stout root

---

[1] Chapter 4

system, increased sclerophylly, tolerance to N- and P-deficiency and resistance to drought (Brady *et al.* 2005; Sambatti and Rice 2007; Baker *et al.* 2010). These features are thought to evolve as adaptations to the particular habitat conditions (Kruckeberg 1954; Boyd 2004; O'Dell and Rajakaruna 2011). Other important modulators of plasticity, reflected by the phenotype, are light, nutrients and drought (Correia *et al.* 1992; Read and Stokes 2006).

Considering that *P. almogravensis* has an unusual internal tolerance to Al and a critically endangered status, we hypothesized that the Inclusive Niche Hypothesis would explain the ecological niche of this species. In particular, *P. almogravensis* would have a small realized-niche (gap hardpans) because of less competitive ability (assuming a cost to tolerance). In addition, the optimal edaphic conditions for *P. almogravensis* would be in areas of competitor dominance. This implies that the species' realized niche would not necessarily reflect the optimal physiological performance for the individuals. This kind of framework is unusual in analysis of plant communities (McGill 2012) but more frequently applied in animal systems (Colwell and Fuentes 1975). It has never been used in the context of a metallophyte hyperaccumulator of a major soil element (Al). The study was conducted on a small geographic scale, yet encompassing large edaphic and biotic gradients, allowing efficient and reliable ecophysiological field measurements. This is generally only possible with the traditional (time consuming) transplant experiments (e.g. Kruckeberg 1967; Harrison and Rajakaruna 2011; Bieger *et al.* 2012) and has the benefits of capturing the natural *in situ* constraints.

To test our hypothesis, we studied the ecological performance of the plant and its distribution along a gradient of soil Al. Specifically: (i) identifying ecological limiting factors; (ii) evaluating plant's physiological status in areas of the niche differing in the species' ecological performance and (iii) determining whether impact on physiological performance was detectable in growth patterns.

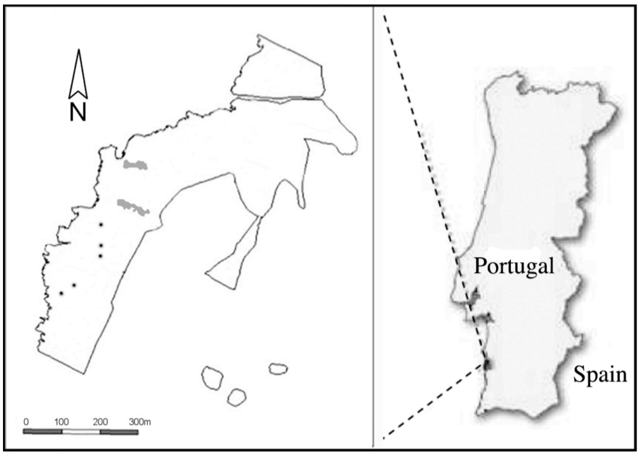


Figure 1. Location (right) and limits of the *P. almogravensis* population (left), with sampling sites. Grey areas: first sampling (ecological performance); black dots: second sampling (physiological performance).

MATERIALS AND METHODS

Study area

We conducted this study at PNSACV Natural Park, habitat of the only known *P. almogravensis* population (Fig. 1). Located within the Mediterranean biogeographic region, local climate is temperate with dry summers (Köppen-Geiger climatic classification Csb-type, temperate-mesothermal; AEMET-IMP 2011) characterized by lower temperatures during the rainy winters and warm temperatures in the dry summers. Annual average air temperature is 16°C (0-34°C) and total precipitation is <600 mm per year, with dry periods of 2-4 months (varying among years) and almost permanent maritime moist winds (mean 87% moisture ranging from 29 to 100%), at an annual average velocity of 10 km h^{-1} (AEMET-IMP 2011; APA 2011). The average number of sunlight hours is 3000 per year with 155-160 Kcal cm^{-2} of solar radiation (APA 2011). Temperature and air moisture were determined from *in situ* measurements (2009/10) with climatic loggers (DS1923 iButton, Hygrochron).

Ecological performance

The study species is a cushion hemicryptophyte, with linear leaves forming rosettes at the tip of woody branches. It produces spikes with wind-pollinated hermaphroditic flowers developing into pyxidia. On rare occasions, vegetative reproduction has been observed. We selected the two largest cohorts of *P. almogravensis* for our survey. Cohort is defined here as all the individuals located within a group, framed by shrub vegetation or geomorphologic discontinuities. The two gap areas were located at ca. 70 m from each other (Fig. 1), populated with scattered hemicryptophytes (*P. almogravensis, Eryngium dilatatum* and *Pulicaria odora*) and large areas of bare soil without any vegetation. The limits of the sampled areas included at least 1 m beyond the vegetation gaps into the surrounding native shrub vegetation (sclerophyllous *Ericaceae* and *Cistaceae* species).

Cartography of the two cohorts was obtained through the geo-referencing of 2560 photographs taken at 1.35 m above the soil surface, stitched together to form ortho-photographic maps (pixel resolution of 1 mm), covering total areas of 99 and 190 m^2, respectively. We used this methodology to develop a stratified sampling scheme for the different microhabitats observed (e.g. gap with runoff, gap without runoff, shrub vegetation and marginal debris areas) based on 110 random sampling points (generated by Hawth's Analysis Tools © 2002-06, version 3.27 for ArcGIS®; see Annex I: Table T1 and Annex II: Figure F1). Using these maps, we calculated proxies for plant ecological performance (cover and density). The biotic cover in the sample area was determined by considering the percentage of occupation by leaf debris (*litter cover*) or plants (*P. almogravensis cover*, *hemicryptophyte cover* and *shrub cover*). Cover measures the total crown projected area; it was calculated within a circle around each of the 110 sample points (30 cm radius buffer) and expressed in percentage of the total buffer area. The density of *P. almogravensis* (number of individuals per square meter) was calculated within the same buffer.

Soil characterization

To evaluate soil resistance to penetration we used a thin, 0.50 m length, steel probe (penetration force of ca. 25 kg cm^{-2}), estimating the depth of soil layer above the impermeable hardpan (*soil depth*) where moisture could be retained. We then collected the surface soil in each of the 110 sampling points, at ca. 5 cm depth (100 cm^3). The samples were stored in closed plastic bags at 4.0°C.

To evaluate soil relative humidity (RH) at the time of collection (g g^{-1} soil), fresh soil was homogenized inside the collection bags and aliquots were weighed fresh and dried (60°C) to calculate the proportion of water. We determined soil pH in sieved (2 mm) air-dried soil, with deionized water (1:10, w/v; 370 r.p.m., 1 h), and measured with a pH electrode. Concentration of soil phytoavailable elements (Ca, Mg, K, Fe, Na, Al) was estimated by an ethylenediaminetetraacetic acid (EDTA) elution (5 mM, adapted from Branquinho *et al.* 1997, 2007). For this, air-dried sieved soil eluted in a 1:20 (w/v) soil: extractant mixture was agitated (2 h, 175 r.p.m.) and let settle. The supernatant was analysed for Ca, Fe, K, Mg and Na, by Flame Atomic Absorption Spectrometry (SpectrAA50, VARIAN), and for Al by Graphite Atomic Absorption Spectrometry (932 plus, GBC), following standard procedures (see Annex I: Table T1).

Physiological performance

We collected *P. almogravensis* plants together with the soil core surrounding the main root (350-1000 cm^3, 10 cm deep). This sampling (independent from the previous) was made in five different cohorts (Fig. 1 and see Annex I: Table T5) to target the maximum microhabitat heterogeneity: from typical shallow gaps to areas of deeper podzol (shrubs understory). Within each location (cohort), samples were collected from the gap, margin and shrub's understory; however, being a protected species, the number of samples was kept to a minimum (16 plants).

Plant and soil processing and soil chemical analyses, for phytoavailable Al and Ca (EDTA-extractable elements), followed Branquinho *et al.* (2007).

We examined the physiological performance (n = 10) by measuring C and N leaf isotopic composition (‰) and elemental concentration (%). Leaf concentration should reflect nutritional status (N) and photosynthetic performance (C and N) while higher $\partial^{13}C$ or lower $\partial^{15}N$ are indicative of physiological stress (Martins-Loução *et al.* 2000; Werner and Máguas 2010). Dried ground leaves of *P. almogravensis* were processed in an elemental analyser (EURO VECTOR) interfaced with a continuous flow stable isotope ratio mass spectrometer (SIRA II, VG ISOGAS for C and ISOPRIME, MICROMASS for N). Isotope ratios were calibrated against international standards, namely IAEA CH6 (sucrose) and IAEA CH7 (polyethylene) for carbon isotope ratio and IAEA N1 (ammonium sulphate) for nitrogen isotope ratio. Isotopic composition is expressed in ∂ notation (‰). The $\partial^{13}C$ results were standardized against Vienna Pee Dee Belemnite (VPDB), as the international standard, and $\partial^{15}N$ reported relative to $\partial^{15}N$ of atmospheric air (Hellmann *et al.* 2011; Máguas *et al.* 2011). The precision of the repeated measurement was 0.06 ‰ for $\partial^{13}C$ and 0.09 ‰ for $\partial^{15}N$.

Growth performance

The species *P. almogravensis* is a woody hemicryptophyte (rarely more than 12 cm high), cushion-like from a branched stock with multiple terminal rosettes of needle-like leaves (Appendix S2: Figures F2 and F3). We evaluated its growth performance by linking morphological traits (n = 16) to physiological performance proxies. Considering this species' morphology, the chosen traits were as follows:

i) Number of rosettes per plant (*rosettes*);

ii) Mean leaf length (*leaf length*, mm). For this parameter, at least 25% of the plant rosettes were assessed. In each selected rosette, the length of one mature leaf was measured to obtain the plant's average leaf length;

iii) Highest ramification order (*max branching order*), per plant and

iv) Mean branch length (*branch length*, mm), calculated by a similar procedure as for the *leaf length*.

Plants under stress are expected to respond by diminishing their exposed area (smaller leaves, compact structure) while increasing the ramification due to dieback of apical meristems (Salguero-Gómez and Casper 2011).

Data analysis

To estimate soil Al-toxicity we used the index Al/Ca (concentration ratio), adapted from Cronan and Grigal (1995) by inverting the terms of their molar ratio, so that higher toxicity would correspond to a higher ratio.

We applied either parametric or non-parametric statistical methods according to the data type (normal, normal after log transformation or non-normal), using the programs SPSS® (IBM Corp.) and Statistica® (StatSoft Inc.). The multivariate results of the edaphic analyses were reduced to factor scores, using Principal Components Analysis (PCA) of the correlation matrix (Webster 2001). The factor scores were then correlated with the biotic cover components (*Plantago*, *shrubs*, *hemicryptophytes* or *litter*) using Spearman's rank order correlations.

To validate the discrimination of the biotic spatial niches, a simple analysis of variance (ANOVA) was performed using the samples with single or mixed cover of *P. almogravensis* and shrubs, as dependent variables, followed by Tukey's Honest Significant Difference (HSD) *post hoc* tests (see Annex I: Table T2). To test for possible competition effects between the groups of plants studied (biotic factors), Spearman's correlation analyses were performed on their cover and/or on *P. almogravensis* density. Pearson's correlations were used for plant physiological and growth performance evaluation, except for *branching order*, where Spearman's correlation was used (discrete data and with a small range). Correlation analyses were made between physiological performance indicators ($\partial^{15}N$, $\partial^{13}C$ and C/N) and metal concentrations (soil and plant) to assess the photosynthetic and nitrogen use performance of the plants. Similarly, the physiological performance of *P. almogravensis* was correlated with plant growth patterns to evaluate the impact of the environmental constraints on growth traits.

Unless otherwise indicated, statistical significance is less than the $P = 0.05$ level and average values are presented with standard errors (mean ± SE).

RESULTS

Ecological performance

Native shrub vegetation surrounds gap areas, with litter from shrubs accumulating in the gap's margins. In the inner part of the gap there are patches of *P. almogravensis*, other hemicryptophytic species and bare soil. Analysis of the plant cover, in buffer areas of 110 sample points, on the two major cohorts of *P. almogravensis* (grey areas, Fig. 1), produced a measure of ecological performance. Sampled areas showed from 0 to 16.4% of *P. almogravensis* cover and 131 plants m^{-2} of maximum density. The remaining percent cover consisted of other hemicryptophytic species (0-22.3%), litter (0-91%), shrubs (0-100%) or bare soil (0-100%). Considering the total *P. almogravensis* plants evaluated, only 3% of the individuals were found outside the gaps (in the shrub understory, within 1 m of the margin) and those were, on average, larger plants (47 cm^2 vs. 13 cm^2 inside the gaps). From personal observations, we know that isolated

Table 1. First three eigenvalues and eigenvectors, from the PCA analysis of the soil data ($n = 109$)

	Factor 1	Factor 2	Factor 3
Eigenvalue	3.59	2.06	1.02
Variance, %	44.9	25.7	12.7
Ca	**-0.908**	-0.207	-0.096
K	**-0.854**	0.254	0.088
Mg	**-0.832**	0.204	0.248
Fe	-0.461	**0.773**	-0.156
Al	-0.144	**0.895**	-0.078
Depth	-0.530	-0.563	-0.444
RH	**-0.854**	-0.357	-0.163
pH	0.318	0.254	**-0.829**

Numbers in **bold** denote strong factor loadings (eigenvectors)

Table 2. Spearman Rank Order Correlations between the biotic cover (and density) and the PCA's first three factor component scores ($n = 109$)

	Factor 1	Factor 2	Factor 3
Plantago density (plants m^{-2})	0.373	0.316	ns
Plantago cover (%)	0.331	0.256	ns
Shrub cover (%)	-0.535	-0.449	ns
Hemicryptophytic cover (%)	0.210	ns	ns
Litter cover (%)	ns	ns	ns
Bare soil (%)	0.517	0.515	ns

ns = non-significant correlations

Table 3. Spearman Rank Order Correlations between biotic cover, and *P. almogravensis* density or cover ($n = 110$)

	P. almogravensis	
Cover (%)	Density (plants m^{-2})	Cover (%)
Shrubs	-0.505	-0.423
Bare soil	0.380	0.257
Hemicryptophytic	0.338	0.263
Litter	ns	ns

ns = non-significant correlations

plants outside the large gaps produce bigger spikes with more seeds than those produced by gap plants. Nonetheless, those plants rarely have juveniles around them, contributing further to the larger size measured.

In order to identify limiting factors determining the distribution of *P. almogravensis* we extracted three factors from a PCA of the edaphic variables. The first 3 factors explained 83% of the variance observed (Tab. 1) and grouped variables related to soil nutrition (low Ca, K, Mg and Fe) and water availability (low RH and soil depth) in the first factor; variables related to the hardpan gaps (high Al and Fe, low soil depth) in the second factor; and a third factor dominated by the soil pH (Tab. 1, bold values). The scores from these extracted factors were then correlated with the biotic variables (plant cover and *P. almogravensis* density), considered as ecological performance proxies (Tab. 2). Both *P. almogravensis* cover and density showed significant positive correlations with the two first PCA factors extracted (Tab. 2). This indicated the highest ecological performance of *P. almogravensis* in areas with low nutritional and water status and higher levels of Al and Fe. Showing an opposite trend, shrub cover was negatively associated with the first and second factors of the PCA (Tab. 2), indicating increased cover in soils with better nutritional and water status, away from the hardpan gaps. The third group of plants, hemicryptophytes, showed a weak significant positive correlation with the first PCA factor only (Tab. 2). The third PCA factor extracted (related to pH) did not display significant correlations with any of the biotic variables considered (Tab. 2).

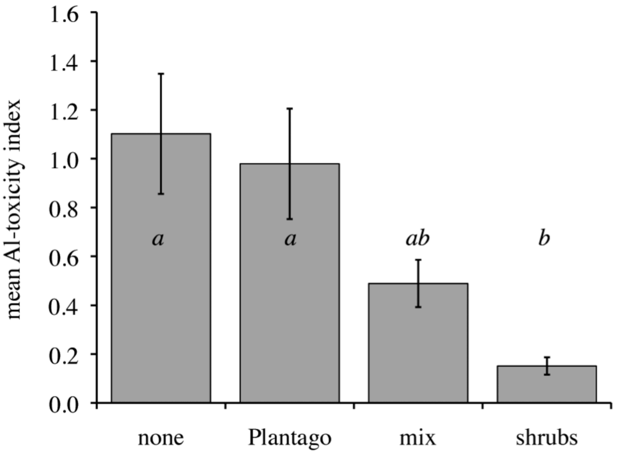


Figure 2. Discrimination of biotic spatial niches using the index of Al-toxicity (means from ANOVA with standard errors). None: samples without both *P. almogravensis* and shrubs, n = 8; *Plantago*: samples with *P. almogravensis* but no shrubs, n = 28; Shrubs: samples with shrubs but no *P. almogravensis* cover, n = 31; Mix: samples with both shrubs and *P. almogravensis* cover, n = 42. Different letters indicate significant differences among spatial biotic groups ($P \leq 0.01$).

To explore possible competition effects between *P. almogravensis* and its neighbours, we made correlation analyses between plant cover and *P. almogravensis* density. The highest (negative) significant correlation was obtained for shrub cover (Tab. 3). A positive correlation was found with hemicryptophyte cover (Tab. 3). From the two ecological performance proxies chosen, density displayed the strongest significant correlations, so that the number of *P. almogravensis* individuals was more affected by the studied factors than the cover produced by those individuals.

The soil variables, with the highest contribution for PCA factor 1 and PCA factor 2, were Ca and Al (respectively), elements traditionally involved in the estimation of soil Al-toxicity (e.g. Cronan and Grigal 1995). Analysis of the Al-toxicity index considering the spatial distribution of *P. almogravensis* and shrubs (Fig. 2: ANOVA, n = 109, F = 7.46, df = 3.105, $P < 0.001$) resolved the two main plant niches: higher Al-toxicity values associated with *P. almogravensis* and lower toxicity associated with the shrub niche. Areas of mixed cover showed intermediate values of Al-toxicity while the samples without both *P. almogravensis* and shrubs presented the highest mean toxicity values (Fig. 2). In general, *P. almogravensis* cover increased with increasing soil Al-toxicity, while the shrub cover decreased with increasing Al-toxicity (Fig. 3, see also Annex I: Tables T3 and T4). Both sets

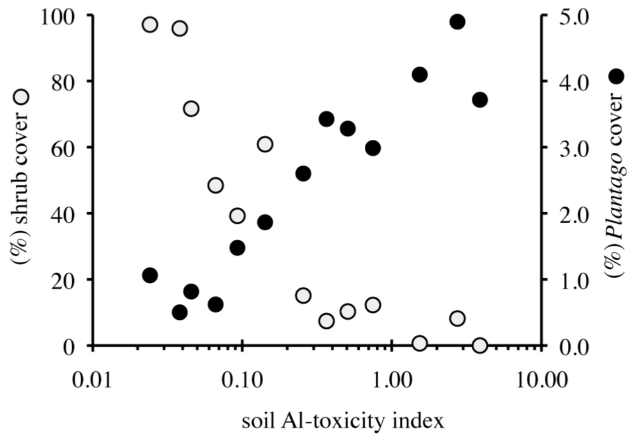


Figure 3. Mean *P. almogravensis* (*Plantago*) cover and mean shrub cover, along 13 classes of soil Al-toxicity index (logarithmic scale).

of data displayed significant Spearman's correlation coefficients with soil Al-toxicity (ρ>0.92); being positive with *P. almogravensis* cover and negative with shrub cover (Fig. 3). The soil Al (112 ± 29 μg g^{-1}) in *P. almogravensis*' spatial niche (defined as in Fig. 2) was significantly higher (ANOVA followed by Tukey HSD, P < 0.02) than that of the shrub's niche (39.4 ± 5.3 μg g^{-1}). On the other hand, soil Ca was significantly higher (ANOVA followed by Tukey HSD, P < 0.001) in the shrub's spatial niche (434 ± 56 μg g^{-1}) than in that of all others, including *P. almogravensis*' niche (116 ± 13 μg g^{-1}) (see Annex I: Table T2).

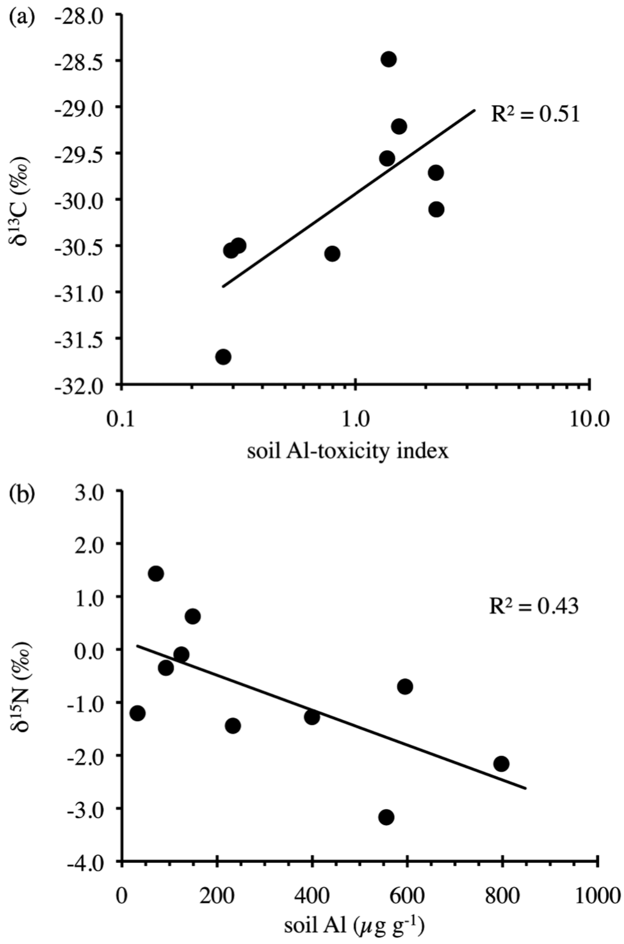


Figure 4. Significant variations in the leaf isotopic composition related to (a) soil Al-toxicity (logarithmic scale) and (b) soil Al-concentration (P < 0.05).

Physiological performance

The leaf C/N ratio (ranging from 34 to 56) was not significantly correlated with soil elements (see Annex I: Table T6). However, both soil Al-toxicity and Al-concentration showed significant correlations with leaf isotopic composition (Fig. 4). Within the studied area (black dots, Fig. 1), it was possible to observe a wide variation in leaf isotopic ∂^{13}C (from -31.7 to -28.5‰), resulting in a maximum range of 3.2‰, as well as a significant positive correlation with soil Al-toxicity (Fig. 4a). Concerning ∂^{15}N in the leaf material, the range was -3.17 to 1.43‰, and a significant negative correlation was observed with soil Al (Fig. 4b).

Growth performance

Branch length (6-28 mm), a trait related to biomass production, was significantly and positively correlated with soil Al (r > 0.69, P < 0.05). Number of rosettes (4-103 rosettes/plant) and average leaf length (4-33 mm) were both correlated with leaf C/N, showing that higher leaf C/N (lower N) was associated with plants having shorter leaves and more rosettes (Fig. 5a). A similar response was observed for ∂^{13}C. The number of rosettes and the maximum order of ramification per plant (third to sixth order branches) were both positively and significantly correlated with leaf ∂^{13}C (Fig. 5b), showing that *P. almogravensis* plants with more ramification (or rosettes) and shorter leaves were enriched in ^{13}C. Average branch length was negatively and significantly correlated with the leaf $\partial^{15}N$ (Fig. 5c), indicating lower $\partial^{15}N$ values in the leaves of plants with longer branches (see Annex I: Table T6).

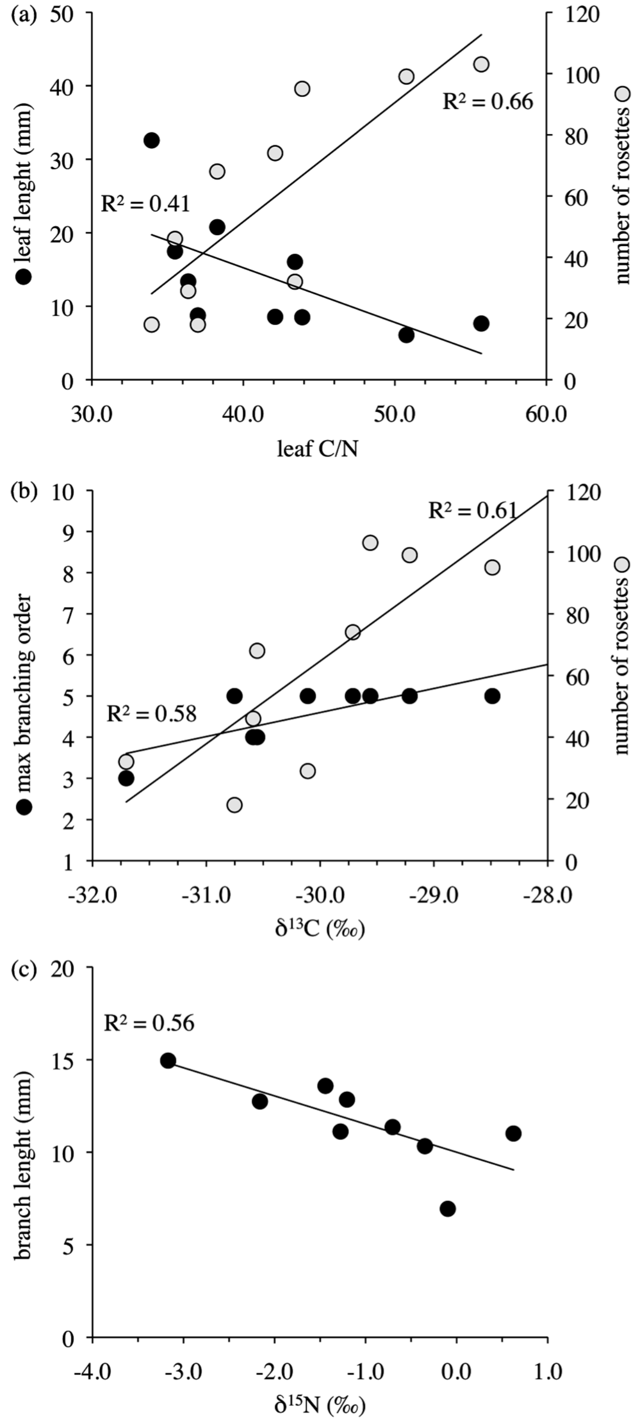


Figure 5. Significant variations in plant morphology (leaf and branch length, maximum branching order and number of rosettes) related to physiological performance traits (a) leaf C/N, (b) leaf $\partial^{13}C$ and (c) leaf $\partial^{15}N$.

DISCUSSION

Our results confirm the hypothesis that *P. almogravensis* has a small realized niche due to the trade-off between ecological tolerance and physiological performance. This is in agreement with the Inclusive Niche Hypothesis (Colwell and Fuentes 1975), since the plant shows better physiological performance and growth pattern where its ecological performance is lower and the plant is less abundant. Therefore, the fundamental niche of this rare species should be, apparently, much wider than the realized niche.

The realized niche of *P. almogravensis*

Our work showed that the ecological performance of *P. almogravensis* is limited by (i) the harsh gap conditions, namely soil Al-toxicity; and (ii) a competing shrub cover, evidenced by its negative correlation with *P. almogravensis* abundance. *Plantago almogravensis* presented higher abundance where Al-toxicity indices were higher, demonstrating better tolerance to Al-toxicity and capacity to survive and establish with success under those conditions. Those soils with higher Al-toxicity match the gaps in the shrub vegetation, where there are large areas of bare soil (highest toxicity) and a small cover of other hemicryptophytes. *Plantago almogravensis* is more abundant in the shallow, dry, nutritional poor, and Al- and Fe-enriched soil, implying that it is able to cope with these unfavourable edaphic conditions. Thus, its ecological performance is not limited by the gap edaphic conditions. All the chemical features we found in the gaps match typical metallophyte habitats, presenting metal-enriched islands in a sea of background concentrations of metals (Baker *et al.* 2010), i.e. to say, a “geochemical island”.

Acknowledging that the existing geochemical islands have maintained their boundaries at least in the last seven decades (based on a time-series sequence of decadal aerial photographs since 1947, not shown), edaphic factors are probably restricting shrub colonization. Most likely, shrubs are unable to cope with one or several of the following factors that exist in the gap: high Al-toxicity, low macronutrients, low soil depth and low soil RH. The Inclusive Niche Hypothesis (Colwell and Fuentes 1975; McGill 2012) states that the shared optimal location in niche space is dominated by the most competitive species, which in turn cannot tolerate poor environmental conditions (in this case, lower habitat resources and increased Al-toxicity).

The ability of *P. almogravensis* to tolerate levels of Al toxic to other plants seems to function as a survival strategy. In these harsh geochemically different sites, the species colonizes vegetation gaps without shrub competition. In fact, this plant is able to tolerate and accumulate such high concentrations of Al in its aboveground parts that was classified as an Al-hyperaccumulator plant (Branquinho *et al.* 2007; Serrano *et al.* 2011[2]). This tolerance would allow the less competitive species to persist in poorer areas, escaping from the competition, but narrowing its realized niche to the vegetation gaps. Given that there is a negative correlation between *P. almogravensis* and shrub cover, and that only a few (3%) older individuals are present outside the gaps, we suggest that in the studied habitat, the plant is not ecologically competitive outside the geochemical islands. Competition involves the ability of an organism to exploit a shared resource, namely water, nutrients, light or space. An inferior competitive ability of *Plantago* spp. in face of the higher growth rates and sizes of shrub vegetation has been documented by other authors (Blom 1992). *Plantago maritima*, a species with a similar habitat to *P. almogravensis*, is strongly limited by light competition from high shrub vegetation at the germination, survival and establishment stages (Blom 1992). The other hemicryptophytic species that occupy the gap area do not seem stronger competitors, as they exhibit a similar size and shorter longevity (annual plants). The apparent lack of competition might indicate that they explore different niches (resources or functions) in the gap and are, therefore, able to coexist.

Plantago almogravensis physiological performance

In the gap areas, where the shrub cover was lower, *P. almogravensis* faced stressful edaphic conditions. According to other similar experiences (Chen et al. 2010; Flanagan and Jefferies 1989) the wide range of leaf ∂^{13}C found in this work (-31.7 to -28.5‰) can be explained by a high degree of heterogeneity in a small scale (ca. 300 m), unusual to find within a single species, except in examples of high stress conditions, e.g. intensive induced saline stress in *P. maritima* (Flanagan and Jefferies 1989). Thus, the more enriched ∂^{13}C values (-28.5‰) can be explained by a stomatal closure and photosynthetic limitation in the geochemical islands (higher water stress and soil Al-concentrations), where the plants are more abundant. More depleted ∂^{13}C values (-31.7‰) in the shrubland are explained by a higher photosynthetic activity (higher nutrient content), where the plants are less abundant. Soil Al-toxicity has been related to the reduction in the CO_2 assimilation and stomatal closure, leading to a reduction in transpiration and higher water use efficiency (Chen et al. 2010) that would correspond to the increase in leaf ∂^{13}C.

To thrive in the geochemical islands, *P. almogravensis* shows trade-offs with the level of photosynthetic activity. The lower photosynthetic efficiency (∂^{13}C) was associated with plant morphological traits, namely higher level of ramification. This could suggest a survival strategy typical of species adapted to drought conditions (Schenk *et al.* 2008). Ramification and modular growth are traits adapted to more stressful conditions, like water stress, allowing partial canopy dieback (Davis *et al.* 2002; Zanne *et al.* 2006; Schenk *et al.* 2008; Salguero-Gómez and Casper

---

[2] Chapter 4

2011). The plants with more rosettes and shorter leaves showed a higher level of sclerophylly based on C/N ratio results. This is also an adaptation associated with stressful environments, namely drought and excessive light, because it decreases water loss and exposed leaf area (Correia *et al.* 1992). In the geochemical islands, the studied plants are under a combination of chemical stresses with low water availability and excessive sunlight. Due to combination of shallow soils and higher exposure to sun and wind, the water in the soil evaporates quickly, and plants get an exaggerated photochemical input from the lack of cover and light reflection on the bare soil.

Plantago almogravensis plants, due to several limitations of N uptake, particularly related to low levels of N in the soil, seem to have a conservative N use (Correia and Martins-Loução 1997; Craine 2009). The leaf N content was low (high C/N) especially in the plants with shorter leaves and with more rosettes. We found lower $\partial^{15}N$ in the geochemical islands and higher $\partial^{15}N$ in the shrub areas, with a large variation (-3.17 to 1.43‰) showing diverse N use strategies depending on site characteristics. These distinct isotopic signatures may arise from a diversity of N sources explored by the plants: uptake by mycorrhizas or proximity of rhizospheric bacteria, multiple assimilation events, organic loss of N and resorption and reallocation of N (Martins-Loução *et al.* 2000; Evans 2001; Robinson 2001; Dawson *et al.* 2002; Craine *et al.* 2009).

There was an association of $\partial^{15}N$ with soil Al and branch length. This means that under high soil Al-availability the increment of this growth morphological trait was made at a N mobilization cost, explained by the most negative $\partial^{15}N$. It is also likely that in this species, the branch length is associated with new leaf formation. One of the methods that some plants have to cope with internal metal toxicity is the sequestration of the metal in the leaves and elimination of the leaves when those levels are too high; in fact, some Al-accumulators have shown higher concentration of Al in older leaves (Watanabe and Osaki 2002). We suggest that the turnover of the leaves in the geochemical islands could be stimulated to cope with the high internal Al-levels. Species typical of gaps are also known to exhibit rapid leaf turnover (Valladares *et al.* 2000).

Conclusions

The results of this work are in agreement with the Inclusive Niche Hypothesis, and help to explain the species' rarity status. There is an automatic bias in adaptive evolution towards maintaining adaptation to this habitat, at the expense of adaptation outside the gaps. Apparently, this spatial-ecological pattern plays the primary role in promoting the species endemism/rarity. It seems likely that the scarceness and fragmentation of geochemical islands could be the main driver for the current rarity status of *P. almogravensis*. While taking refuge from competition in a stressful environment, the species has had to compromise between survival and best performance. The survival is only possible due to internal tolerance to soil Al (related to the Al-hyperaccumulation trait) and the ability to thrive under low resources, at the cost of lower physiological performance.

Future conservation efforts for *P. almogravensis* should reflect these findings: the species is a poor competitor but has the ability to find refuge in geochemical islands, too harsh for most other species.

ACKNOWLEDGEMENTS

Funding: Fundação para a Ciência e Tecnologia (FCT) through project ECODILEMA [POCTI/BIA- BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004]; CB acknowledges a *Investigador FCT* contract and HCS acknowledges the PhD grant [SFRH/BD/38289/2007] from FCT. Authors would like to thank L Aires, and R Maia for the elemental and isotopic analysis, and the two anonymous reviewers, for their constructive comments. Also to *Parque Natural do Sudoeste Alentejano e Costa Vicentina*; *Instituto de Conservação da Natureza e das Florestas*; and the study *Estudo de referência de Plantago almogravensis 2004*. This research has been in compliance with Portuguese laws.

REFERENCES

AEMET-IMP. 2011. Iberian Climate Atlas, Air temperature and precipitation (1971-2000). ed. M IMP: Ministerio de Médio Ambiente y Médio Rural y Marino (España) and Instituto de Meteorologia (Portugal). URL:http://www.ipma.pt/resources.www/docs_pontuais/ocorrencias/2011/atlas_clima_iberico.pdf

Anacker B, Rajakaruna N, Ackerly D, Harrison S, Keeley J, Vasey M. 2011a. Ecological strategies in California chaparral: interacting effects of soils, climate, and fire on specific leaf area. *Plant Ecology & Diversity* 4: 179-88

Anacker BL, Whittall JB, Goldberg EE, Harrison SP. 2011b. Origins and consequences of serpentine endemism in the California flora. *Evolution* 65: 365-76

APA. Version 15 Feb 2011. Sistema Nacional de Informação de Ambiente da Agência Portuguesa do Ambiente (SNIAmb). Portugal: Instituto do Ambiente, URL:http://sniamb.apambiente.pt/webatlas/topo.html

Baker AJM, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In *Ecology of Industrial Pollution*, ed. LC Batty, KB Hallberg, pp. 7-40. Cambridge, U. K.: Cambridge University Press

Bieger A, Rajakaruna N, Harrison S. 2014. Little evidence for local adaptation to soils or microclimate in the post-fire recruitment of three Californian shrubs. *Plant Ecology & Diversity* 7: 411-20

Bilz M, Kell SP, Maxted N, Lansdown RV. 2011. European Red List of Vascular Plants. pp. 130. Luxembourg: Publications Office of the European Union

Blom CWPM. 1992. Germination and Establishment. In *Plantago: A Multidisciplinary Study*, ed. PJC Kuiper, M Bos, pp. 88-98. Berlin, Germany; New York, New York, USA: Springer-Verlag

Boyd RS. 2004. Ecology of metal hyperaccumulation. *New Phytologist* 162: 563-67

Brady KU, Kruckeberg AR, Bradshaw HD. 2005. Evolutionary ecology of plant adaptation to serpentine soils In *Annual Review of Ecology Evolution and Systematics*, pp. 243-66. Palo Alto: Annual Reviews

Branquinho C, Brown DH, Catarino F. 1997. The cellular location of Cu in lichens and its effects on membrane integrity and chlorophyll fluorescence. *Environmental and Experimental Botany* 38: 165-79

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Brugnoli E, Farquhar G. 2000. Photosynthetic fractionation of carbon isotopes In *Photosynthesis: Physiology and Metabolism*, ed. RC Leegood, TD Sharkey, S von Caemmerer, pp. 399-434. Netherlands: Springer

Brunner I, Sperisen C. 2013. Aluminum exclusion and aluminum tolerance in woody plants. *Frontiers in Plant Science* 4: 172

Cardoso JC. 1965. Os solos de Portugal, sua classificação, caracterização e génese. 1 - A sul do Rio Tejo. Portugal: Secretaria de Estado da Agricultura.

Chen L-S, Qi Y-P, Jiang H-X, Yang L-T, Yang G-H. 2010. Photosynthesis and photoprotective systems of plants in response to aluminum toxicity. *African Journal of Biotechnology* 9: 9237-47

Colwell RK, Fuentes ER. 1975. Experimental studies of niche. *Annual Review of Ecology and Systematics* 6: 281-310

Colwell RK, Rangel TF. 2009. Hutchinson's duality: The once and future niche. *Proceedings of the National Academy of Sciences of the United States of America* 106: 19651-58

Correia OA, Martins AC, Catarino FM. 1992. Comparative phenology and seasonal foliar nitrogen variation in Mediterranean species of Portugal. *Ecologia Mediterranea* 18: 7-18

Correia PJ, Martins-Loução MA. 1997. Leaf nutrient variation in mature carob (*Ceratonia siliqua*) trees in response to irrigation and fertilization. *Tree Physiology* 17: 813-19

Craine JM. 2009. *Resource Strategies of Wild Plants*. Princeton University Press.

Craine JM, Elmore AJ, Aidar MPM, Bustamante M, Dawson TE, et al. 2009. Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist* 183: 980-92

Cronan CS, Grigal DF. 1995. Use of calcium aluminum ratios as indicators of stress in forest ecosystems. *Journal of Environmental Quality* 24: 209-26

Cuenca G, Herrera R. 1987. Ecophysiology of aluminum in terrestrial plants, growing in acid and aluminum-rich tropical soils. *Annales De La Societe Royale Zoologique De Belgique* 117: 57-73

Davis SD, Ewers FW, Sperry JS, Portwood KA, Crocker MC, Adams GC. 2002. Shoot dieback during prolonged drought in *Ceanothus* (Rhamnaceae) chaparral of california: A possible case of hydraulic failure. *American Journal of Botany* 89: 820-28

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. 2002. Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics* 33: 507-59

de Andrade LRM, Barros LMG, Echevarria GF, do Amaral LIV, Cotta MG, et al. 2011. Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. *Environmental and Experimental Botany* 70: 37-42

Devictor V, Clavel J, Julliard R, Lavergne S, Mouillot D, et al. 2010. Defining and measuring ecological specialization. *Journal of Applied Ecology* 47: 15-25

Evans RD. 2001. Physiological mechanisms influencing plant nitrogen isotope composition. *Trends in Plant Science* 6: 121-26

Flanagan LB, Jefferies RL. 1989. Effect of increased salinity on CO_2 assimilation, O^2 evolution and the delta-C-13 values of leaves of *Plantago maritima* L developed at low and high NaCl levels. *Planta* 178: 377-84

Franco JA. 1984. Nova Flora de Portugal (Continente e Açores). Lisboa, Portugal.

Gaston KJ. 2012. ECOLOGY The importance of being rare. *Nature* 487: 46-47

Handley LL, Raven JA. 1992. The use of natural abundance of nitrogen isotopes in plant physiology and ecology. *Plant Cell and Environment* 15: 965-85

Harrison S, Cornell H, Moore KA. 2010. Spatial niches and coexistence: testing theory with tarweeds. *Ecology* 91: 2141-50

Harrison S, Rajakaruna N. 2011. *Serpentine: The Evolution and Ecology of a Model System*. University of California Press.

Hellmann C, Sutter R, Rascher KG, Máguas C, Correia O, Werner C. 2011. Impact of an exotic N_2-fixing *Acacia* on composition and N status of a native Mediterranean community. *Acta Oecologica-International Journal of Ecology* 37: 43-50

Hutchinson GE. 1957. Population studies - animal ecology and demography - concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology* 22: 415-27

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Kruckeberg AR. 1954. The Ecology of Serpentine Soils: III. Plant species in relation to serpentine soils. *Ecology* 35: 267-74

Kruckeberg AR. 1967. Ecotypic response to ultramafic soils by some plant species of Northwestern United States. *Brittonia* 19: 133-&

Kruckeberg AR, Rabinowitz D. 1985. Biological aspects of endemism in higher-plants. *Annual Review of Ecology and Systematics* 16: 447-79

Loveless AR. 1961. Nutritional interpretation of sclerophylly based on differences in chemical composition of sclerophyllous and mesophytic leaves. *Annals of Botany* 25: 168-&

Maestri E, Marmiroli M, Visioli G, Marmiroli N. 2010. Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. *Environmental and Experimental Botany* 68: 1-13

Martins-Loução MA, Cruz C, Correia PM. 2000. New approaches to enhanced ammonium assimilation in plants In *Nitrogen in a sustainable ecosystem: from the cell to the plant*, ed. MA Martins-Loução, SH Lips, pp. 349-60: Backhuys Publishers

Martins-Loução MA, Lips SH. 2000. Nitrogen in a sustainable ecosystem: a matter of integration In *Nitrogen in a sustainable ecosystem: from the cell to the plant*, ed. MA Martins-Loução, SH Lips, pp. 415-19: Backhuys Publishers

McGill BJ. 2012. Trees are rarely most abundant where they grow best. *Journal of Plant Ecology* 5: 46-51

Metali F, Salim KA, Burslem D. 2012. Evidence of foliar aluminium accumulation in local, regional and global datasets of wild plants. *New Phytologist* 193: 637-49

Máguas C, Rascher KG, Martins-Loução A, Carvalho P, Pinho P, et al. 2011. Responses of woody species to spatial and temporal ground water changes in coastal sand dune systems. *Biogeosciences* 8: 3823-32

O'Dell RE, Rajakaruna N. 2011. Intraspecific variation, adaptation, and evolution. In *Serpentine: Evolution and ecology in a model system*, ed. SP Harrison, N Rajakaruna, pp. 97-137. Berkeley, CA: Univ. of California Press

Read J, Stokes A. 2006. Plant biomechanics in an ecological context. *American Journal of Botany* 93: 1546-65

Robinson D. 2001. delta N-15 as an integrator of the nitrogen cycle. *Trends in Ecology & Evolution* 16: 153-62

Salguero-Gomez R, Casper BB. 2011. A hydraulic explanation for size-specific plant shrinkage: developmental hydraulic sectoriality. *New Phytologist* 189: 229-40

Sambatti JBM, Rice KJ. 2007. Functional ecology of ecotypic differentiation in the Californian serpentine sunflower (Helianthus exilis). *New Phytologist* 175: 107-19

Schenk HJ, Espino S, Goedhart CM, Nordenstahl M, Cabrera HIM, Jones CS. 2008. Hydraulic integration and shrub growth form linked across continental aridity gradients. *Proceedings of the National Academy of Sciences of the United States of America* 105: 11248-53

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Valladares F, Martinez-Ferri E, Balaguer L, Perez-Corona E, Manrique E. 2000. Low leaf-level response to light and nutrients in Mediterranean evergreen oaks: a conservative resource-use strategy? *New Phytologist* 148: 79-91

Watanabe T, Osaki M. 2002. Mechanisms of adaptation to high aluminum condition in native plant species growing in acid soils: A review. *Communications in Soil Science and Plant Analysis* 33: 1247-60

Webster R. 2001. Statistics to support soil research and their presentation. *European Journal of Soil Science* 52: 331-40

Werner C, Máguas C. 2010. Carbon isotope discrimination as a tracer of functional traits in a mediterranean macchia plant community. *Functional Plant Biology* 37: 467-77

Werner C, Schnyder H, Cuntz M, Keitel C, Zeeman MJ, et al. 2012. Progress and challenges in using stable isotopes to trace plant carbon and water relations across scales. *Biogeosciences* 9: 3083-111

Zanne AE, Sweeney K, Sharma M, Orians CM. 2006. Patterns and consequences of differential vascular sectoriality in 18 temperate tree and shrub species. *Functional Ecology* 20: 200-06

ANNEX I

Table T1. Data for ecological performance. Abundance (cover and density) and average size were calculated within a 30 cm radius buffer, around each sampling point. (RH) relative humidity (grams per gram of soil).

	cover (%)					*Plantago*		soil							
sample	*Plantago*	shrubs	hemicryptophytes	litter	bare soil	density (plants m⁻²)	size (cm²)	depth (cm)	RH (g g⁻¹ of soil)	pH	Ca (µg g⁻¹)	K (µg g⁻¹)	Mg (µg g⁻¹)	Fe (µg g⁻¹)	Al (µg g⁻¹)
1	0	100	0	0	0	0	0	26.0	0.076	6.12	704.1	64.1	84.8	83.2	14.7
2	6.6	19.6	11.2	34.1	28.6	40.7	16.15	16.5	0.029	5.72	217.9	50.1	38.6	133.3	75.7
3	3.2	2.0	6.2	22.3	66.3	46.9	6.81	3.5	0.018	5.90	147.5	42.3	36.4	150.7	99.8
4	6.6	0	0.2	9.5	83.8	71.9	9.11	1.0	0.011	5.94	96.1	93.0	68.6	643.7	365.7
5	9.1	88.8	2.1	0	0	25.0	36.18	23.5	0.043	5.94	168.8	22.3	28.2	61.8	21.6
6	0.8	42.7	0.6	38.0	17.9	15.6	4.86	22.0	0.065	4.94	117.2	31.3	44.0	108.2	32.2
7	0	38.6	0.5	60.6	0.3	0	0	29.5	0.163	5.57	339.6	33.2	77.9	143.1	27.8
8	0.8	0	16.0	0.1	83.1	15.6	5.08	12.5	0.017	5.46	70.9	33.1	23.0	66.8	35.2
9	0	34.6	1.4	48.4	15.7	0	0	26.0	0.097	6.12	259.5	45.7	38.8	112.1	35.6
10	0.3	0	3.2	25.7	70.8	12.5	2.16	6.0	0.017	5.78	69.1	24.9	24.9	38.9	28.1
11	5.0	0	0	10.9	84.0	46.9	10.76	7.0	0.006	6.06	68.7	34.7	24.1	92.8	32.4
12	0.9	12.7	2.3	60.4	23.6	6.3	14.37	20.5	0.062	5.96	108.7	28.0	25.2	57.8	32.0
13	0.4	23.7	13.0	28.4	34.5	6.3	6.71	31.0	0.108	5.92	172.8	45.3	26.5	99.4	49.0
14	0.9	0	1.7	0	97.4	18.8	4.53	8.5	0.001	5.01	65.1	37.9	32.0	59.0	29.9
15	11.0	0	3.9	0	85.0	59.4	18.54	6.5	0.009	5.28	74.2	34.7	30.1	79.0	41.0
16	13.2	0	1.8	0	85.0	65.7	20.1	10.0	0.017	6.14	83.2	35.4	34.3	257.4	114.6
17	5.1	6.2	1.8	21.8	65.2	59.4	8.54	18.5	0.028	5.76	138.0	38.3	27.3	65.7	36.8
18	0.2	26.4	17.4	38.3	17.7	6.3	3.05	35.5	0.110	6.28	375.7	35.0	35.0	118.1	46.6
19	0	100	0	0	0	0	0	37.0	0.210	6.26	909.5	73.1	79.1	158.3	34.4
20	0	100	0	0	0	0	0	28.0	0.280	5.44	915.4	114.4	95.6	591.2	129.6
21	1.9	26.3	22.3	17.3	32.2	34.4	5.55	21.5	0.060	6.29	543.0	37.0	37.6	108.2	36.5
22	12.6	0.7	2.1	4.2	80.3	131.4	9.61	12.0	0.032	5.96	147.5	62.2	36.1	197.7	178.6
23	8.4	0	0	0	91.6	100.1	8.34	11.0	0.024	6.11	138.1	65.3	59.7	504.7	341.5
24	1.7	98.3	0	0	0	6.3	26.9	38.0	0.132	5.93	336.2	51.0	40.5	96.7	52.5
25	4.8	12.6	2.7	3.5	76.4	56.3	8.53	6.0	0.021	6.01	150.1	48.8	31.6	139.8	108.0
26	3.2	28.7	2.3	24.0	41.9	28.2	11.29	6.0	0.031	5.95	118.8	30.7	36.2	65.6	45.3
27	1.3	10.9	0.7	4.5	82.6	12.5	10.66	2.0	0.016	6.33	135.1	48.7	59.6	670.9	404.6
28	9.6	0	0	0	90.4	56.3	17	3.0	0.010	5.95	204.2	85.6	81.6	760.0	561.6
29	0.3	21.9	0	2.0	75.8	6.3	5.29	2.0	0.009	5.21	46.7	32.6	32.0	169.9	129.7
30	0	0	2.1	0	97.9	0	0	2.0	0.012	5.86	72.9	33.3	37.5	289.8	114.4
31	1.6	0	0.3	0	98.1	15.6	10.28	3.0	0.012	5.88	155.7	38.2	53.0	82.8	106.3
32	1.1	27.1	5.1	57.6	9.0	9.4	11.36	9.0	0.017	5.16	188.1	33.0	29.8	64.0	66.0
33	0	100	0	0	0	0	0	22.0	0.071	5.42	298.4	62.9	53.7	117.4	51.0
34	6.0	5.7	4.2	33.6	50.5	37.5	16	13.0	0.022	5.90	139.5	39.8	33.2	89.2	98.0
35	0	29.0	2.2	68.8	0	0	0	20.0	0.021	6.04	155.7	31.5	40.1	102.6	76.7
36	0	4.6	1.4	4.3	89.7	0	0	2.5	0.019	5.48	127.2	47.3	91.3	66.9	55.0
37	0	100	0	0	0	0	0	14.0	0.132	4.70	472.7	40.8	64.9	134.3	58.5
38	2.9	3.9	2.6	6.0	84.6	43.8	6.54	4.0	0.023	6.34	103.8	22.1	26.0	203.5	127.9
39	0.4	20.2	0.1	44.1	35.1	6.3	6.37	6.5	0.018	5.93	122.1	25.4	28.3	93.7	53.5
40	0	100	0	0	0	0	0	9.0	0.149	5.18	1017.7	146.2	151.2	333.6	120.7
41	0.2	23.4	0.4	30.3	45.7	3.1	5.29	9.0	0.010	6.33	168.4	18.5	36.9	32.9	33.4
42	0	35.2	0.1	64.7	0.1	0	0	17.0	0.031	5.95	348.5	30.8	81.4	79.9	21.3
43	0	9.5	0	3.3	87.2	0	0	6.0	0.014	5.94	76.2	31.1	25.0	148.0	78.0
44	1.9	12.5	1.0	77.0	7.7	6.3	30.48	9.0	0.007	5.65	75.7	18.3	24.5	72.1	43.6
45	0.1	75.2	0.1	18.8	5.7	3.1	3.06	9.0	0.011	6.06	175.3	18.8	37.5	55.4	15.5
46	6.7	6.7	0	0	86.6	78.9	8.51	3.0	0.008	6.63	83.6	36.6	31.3	92.6	59.7
47	6.2	0	0	0	93.8	103.9	6.01	3.0	0.014	6.32	164.7	27.5	36.5	83.1	102.5
48	1.8	9.1	0.3	88.8	0	10.8	16.92	18.0	0.077	5.60	596.0	77.6	61.9	110.5	38.1
49	0	48.8	0.5	29.9	20.8	0	0	10.0	0.033	6.00	307.6	28.2	46.2	64.6	20.1
50	0	71.9	0.2	17.1	10.7	0	0	9.0	0.057	6.08	323.0	46.8	52.9	87.4	35.0
51	0.1	65.0	0	7.4	27.4	3.6	3.73	8.0	0.030	5.73	157.3	27.7	34.8	74.4	154.8
52	3.0	81.3	0	13.4	2.3	7.2	42.31	12.0	0.040	5.72	166.6	33.6	32.6	93.7	76.7
53	0.6	0	0	51.1	48.2	3.6	18.04	2.0	0.018	6.37	152.5	46.1	56.8	560.7	535.2
54	0	0	0	0	100	0	0	0.5	0.013	5.64	104.5	56.1	78.6	12.5	27.4
55	0	35.0	0	35.9	29.1	0	0	6.0	0.029	5.98	298.7	47.7	52.9	100.2	39.8
56	0	0	0.9	0	99.1	0	0	3.0	0.014	6.38	78.0	19.9	29.8	70.3	39.1
57	0	28.5	0.2	0	71.3	0	0	4.5	0.027	6.09	151.8	38.1	36.5	39.8	16.8
58	0	90.8	0	0	9.2	0	0	50.0	0.268	5.28	1364.4	161.4	117.8	311.4	32.5
59	0	5.8	8.9	79.4	5.8	0	0	33.5	0.031	5.51	285.0	23.9	31.2	53.2	14.2
60	12.8	23.4	0	15.5	48.2	86.0	14.93	6.5	0.021	6.26	121.4	45.5	28.0	36.3	17.5
61	2.1	35.7	8.2	9.0	45.0	21.5	9.82	9.0	0.022	6.07	174.6	33.4	43.6	56.3	44.6
62	0	100	0	0	0	0	0	15.5	0.035	6.07	420.6	25.8	41.7	70.6	12.0
63	14.0	0	0	0.1	85.9	64.5	21.72	1.0	0.020	6.05	86.6	34.6	40.0	21.6	19.8
64	0	100	0	0	0	0	0	21.0	0.208	5.46	951.5	90.5	112.5	188.0	32.3
65	0.3	31.2	0	30.7	37.8	3.6	9.47	4.0	0.027	5.44	160.0	32.8	46.9	375.3	105.5
66	0.1	0	0	0.6	99.2	3.6	3.57	1.5	0.047	5.53	275.6	49.9	78.4	22.2	61.1
67	2.8	0	2.7	0	94.5	71.7	3.91	11.5	0.012	6.04	121.4	30.8	32.7	132.7	54.1
68	3.3	91.5	0	0	5.2	3.6	91.2	8.0	0.028	6.23	395.9	41.5	61.7	98.0	17.0
69	4.3	0	0.6	0	95.1	96.8	4.4	3.5	0.016	5.67	112.5	39.9	47.0	107.0	38.5
70	0	99.1	0	0.8	0.1	0	0	16.5	0.038	5.03	427.9	30.7	59.9	96.6	51.6
71	0.3	0	14.8	14.8	70.1	3.6	9.71	16.0	0.066	5.97	167.6	27.3	34.2	93.8	17.0
72	1.2	0	12.2	0	86.6	32.3	3.79	13.5	0.045	6.01	135.8	25.0	30.0	61.5	18.6
73	0.1	43.3	0	53.9	2.7	3.6	2.55	9.0	0.070	6.11	584.7	31.3	58.5	231.5	51.1
74	0	90.7	0	9.1	0.1	0	0	29.0	0.152	5.37	549.0	51.7	67.2	168.1	33.9
75	0.8	0	2.4	2.1	94.7	17.9	4.19	11.5	0.024	5.99	48.5	12.4	15.1	31.1	23.9
76	5.1	7.9	1.8	44.6	40.6	10.8	47.32	15.5	0.029	6.29	108.0	17.2	28.0	36.4	15.5
77	4.0	0	0.2	11.0	84.8	64.5	6.12	9.0	0.022	6.02	47.6	16.2	21.7	123.4	206.5
78	10.2	0	0	0.3	89.5	129.0	7.91	4.0	0.028	5.91	115.2	29.6	29.2	63.5	45.9
79	3.8	2.3	0	20.1	73.8	68.1	5.6	16.0	0.034	5.67	41.7	15.1	14.2	64.3	35.5
80	2.0	84.6	0	13.3	0	3.6	56.11	29.0	0.102	5.83	238.3	27.0	37.2	76.6	9.7
81	4.9	0	0.6	0.3	94.2	75.3	6.46	7.0	0.030	5.81	104.9	40.3	44.3	35.7	57.0
82	1.4	21.5	0	67.0	10.2	14.3	9.46	17.0	0.051	6.04	52.9	16.4	11.6	25.1	30.0
83	0	80.6	0	0	19.4	0	0	23.5	0.059	6.00	285.6	19.6	54.1	91.5	22.6
84	2.9	0	3.8	0	93.3	32.3	8.99	11.5	0.022	6.64	37.6	32.3	12.8	19.1	12.1
85	0	0	0	0	100	0	0	7.5	0.024	5.83	42.7	32.1	31.7	39.0	69.4
86	0	6.2	0	2.2	91.6	0	0	8.0	0.035	5.20	39.5	46.6	30.8	26.6	14.5
87	0	100	0	0	0	0	0	14.0	0.062	5.64	439.3	26.6	45.8	112.6	10.8
88	16.4	0	0	19.1	64.5	14.3	114.46	2.0	0.026	4.80	33.5	33.7	15.6	35.8	19.0
89	4.8	1.8	2.3	91.2	0	7.2	66.33	12.5	0.041	6.75	215.0	18.8	31.2	48.3	18.5
90	0	99.1	0	0.9	0	0	0	20.5	0.119	5.56	453.9	29.2	64.0	108.8	18.4
91	5.7	0	0	0	94.3	50.2	11.36	5.0	0.037	5.93	34.9	30.1	14.0	13.6	14.6
92	0	100	0	0	0	0	0	11.5	0.051	4.97	264.7	26.1	24.8	48.6	12.4
93	0	100	0	0	0	0	0	22.0	0.082	5.74	307.4	36.1	50.5	288.3	48.7
94	3.8	66.6	0	28.5	1.2	21.5	17.55	10.0	0.063	5.86	160.5	22.5	30.2	41.4	22.5
95	5.3	94.7	0	0	0	17.9	29.63	15.0	0.058	4.80	515.5	47.3	61.7	100.3	12.0
96	5.1	43.9	1.5	48.9	0.6	14.3	35.27	19.0	0.176	4.46	512.9	36.8	74.8	205.8	49.4
97	4.5	16.3	0	24.4	54.8	43.0	10.56	4.5	0.030	6.04	92.0	37.6	32.4	125.0	53.3
98	3.1	0	0.9	0	96.0	32.3	9.68	0.5	0.043	6.18					
99	0.5	45.3	0.6	11.2	42.4	7.2	7.21	9.0	0.043	6.45	97.6	38.9	37.1	57.8	20.2
100	2.7	40.2	0	10.2	46.9	17.9	15.34	5.5	0.045	5.98	141.2	21.0	30.0	55.8	16.6
101	0	89.2	0	0	10.8	0	0	24.0	0.134	5.88	346.7	52.8	71.7	108.2	14.9
102	2.8	0	0.3	0	96.9	64.5	4.37	1.5	0.016	5.33	218.9	34.7	69.8	118.7	74.6
103	5.3	0	0.1	0	94.6	46.6	11.33	3.0	0.026	6.31	298.1	59.1	86.9	1088.5	165.5
104	0	100	0	0	0	0	0	14.0	0.102	6.28	487.7	49.6	56.9	563.1	63.7
105	0	0	0	0	100	0	0	5.0	0.015	6.30	54.1	28.5	27.9	97.1	27.1
106	0.7	0	0	0	99.3	3.6	19.78	0.5	0.014	5.86	61.1	24.6	38.8	20.0	21.4
107	0	15.1	0.1	9.8	75.0	0	0	4.0	0.016	5.91	118.2	41.0	48.0	25.1	25.5
108	0	0	0	0	100	0	0	6.5	0.021	5.21	28.5	46.6	48.6	56.9	55.7
109	0	0	0	0	100	0	0	2.5	0.025	5.15	31.3	54.4	119.7	127.9	56.5
110	0	0	0	0	100	0	0	42.0	0.030	6.03	26.5	20.6	16.7	40.2	15.9

Table T2. Discrimination of biotic spatial niches (ANOVA) using the index of Al-toxicity (A), from figure 2, and (B) soil bioavailable Al and (C) soil bioavailable Ca. (none) samples without both *P. almogravensis* and shrubs; (*Plantago*) samples with *P. almogravensis* but no shrubs; (shrubs) samples with shrubs but no *P. almogravensis* cover; (mix) samples with both shrubs and *P. almogravensis cover.*

(A)

		Soil Al-toxicity index (Al/Ca, concentration ratio)			
Spatial niches	n	mean	SE	min	max
none	8	1.10	0.25	0.262	1.951
Plantago	28	0.98	0.23	0.101	4.339
shrubs	31	0.151	0.036	0.021	1.023
mix	42	0.489	0.097	0.023	2.994

ANOVA

	sum of squares	df	mean square	F	sig.
Between Groups	12.7	3	4.22	7.46	0.00
Within Groups	59.4	105	0.565		
Total	72.0	108			

Tukey HSD test

(I) spatial niches	(J) spatial niches	(I-J) mean difference	SE	sig.
none	*Plantago*	0.123	0.30	0.98
	shrubs	0.951	0.30	0.01
	mix	0.613	0.29	0.16
Plantago	none	-0.123	0.30	0.98
	shrubs	0.828	0.20	0.00
	mix	0.490	0.18	0.04
shrubs	none	-0.951	0.30	0.01
	Plantago	-0.828	0.20	0.00
	mix	-0.338	0.18	0.23
mix	none	-0.613	0.29	0.16
	Plantago	-0.490	0.18	0.04
	shrubs	0.338	0.18	0.23

(B)

		Soil Al (μg g^{-1})			
Spatial niches	n	mean	SE	min	max
none	8	51	11	15.9	114
Plantago	28	112	29	12.1	562
shrubs	31	39.4	5.3	10.8	130
mix	42	63	10	9.7	405

ANOVA

	sum of squares	df	mean square	F	sig.
Between Groups	83697	3	27899	3.44	0.02
Within Groups	851163	105	8106		
Total	934859	108			

Tukey HSD test

(I) spatial niches	(J) spatial niches	(I-J) mean difference	SE	sig.
none	*Plantago*	-61.6	36	0.33
	shrubs	11.2	36	0.99
	mix	-12.1	35	0.99
Plantago	none	61.6	36	0.33
	shrubs	72.8	23	0.01
	mix	49.5	22	0.12
shrubs	none	-11.2	36	0.99
	Plantago	-72.8	23	0.01
	mix	-23.3	21	0.70
mix	none	12.1	35	0.99
	Plantago	-49.5	22	0.12
	shrubs	23.3	21	0.70

(C)

		Soil Ca (μg g^{-1})			
spatial niches	n	mean	SE	min	max
none	8	55	10	26.5	105
Plantago	28	116	13	33.5	298
shrubs	31	434	56	39.5	1364
mix	42	203	23	41.7	596

ANOVA

	sum of squares	df	mean square	F	sig.
Between Groups	1926224	3	642075	17.2	0.00
Within Groups	3918379	105	37318		
Total	5844602	108			

Tukey HSD test

(I) spatial niches	(J) spatial niches	(I-J) mean difference	SE	sig.
none	*Plantago*	-61.0	77	0.86
	shrubs	-379	77	0.00
	mix	-148	75	0.20
Plantago	none	61.0	77	0.86
	shrubs	-318	50	0.00
	mix	-86.9	47	0.26
shrubs	none	379	77	0.00
	Plantago	318	50	0.00
	mix	231	46	0.00
mix	none	148	75	0.20
	Plantago	86.9	47	0.26
	shrubs	-231	46	0.00

Table T3. Classes for Al-toxicity index (Fig. 3, in main text). Calculated from the division of the range of Al/Ca, in 16 equal parts (logarithmic values). *We present 13 classes, as 3 are aggregated to the previous, due to the large proportion of *Plantago* absences (cover = 0%).

		soil Al-toxicity index (Al/Ca, concentration ratio)				***Plantago* cover (%)**				**Shrub cover (%)**			
Class	n	mean	SE	min	max	mean	SE	min	max	mean	SE	min	max
1	5	0.0242	0.0012	0.0208	0.0285	1.1	1.1	0	5.31	97.1	1.9	90.8	100
2	4	0.0382	0.0015	0.0340	0.0405	0.50	0.50	0	2.01	95.9	3.8	84.6	100
3	4	0.0456	0.0017	0.0429	0.0496	0.82	0.82	0	3.27	72	22	5.83	100
4	6	0.0664	0.0027	0.0610	0.0791	0.62	0.39	0	1.91	48	13	9.13	90.7
5	7	0.0928	0.0036	0.0818	0.108	1.48	0.89	0	5.06	39	11	0	75.2
6*	20	0.1421	0.0057	0.111	0.207	1.86	0.78	0	12.8	60.9	8.3	0	100
7	9	0.2558	0.0094	0.216	0.295	2.6	1.5	0	14.0	15.1	5.3	0	42.7
8	11	0.3657	0.0094	0.321	0.419	3.43	0.94	0	10.2	7.4	3.5	0	28.7
9	18	0.507	0.012	0.433	0.580	3.3	1.0	0	16.4	10.3	4.7	0	81.3
10*	11	0.749	0.043	0.601	1.02	2.99	0.81	0	6.71	12.3	5.9	0	65.0
11*	7	1.54	0.11	1.21	1.95	4.1	2.3	0	13.2	0.67	0.55	0	3.93
12	4	2.75	0.11	2.47	2.99	4.9	2.4	0.331	9.57	8.2	5.2	0	21.9
13	3	3.89	0.24	3.51	4.34	3.7	1.7	0.647	6.55	0	0	0	0

Table T4. Spearman's correlation coefficients between Al-toxicity and plant cover, within the 13 classes of the Al-toxicity index ($p < 0.001$).

Spearman's *rho* ($n = 13$)	Al-toxicity index	*Plantago* cover (%)
Plantago cover (%)	0.923	
Shrub cover (%)	-0.934	-0.896

Table T5. Data for plant physiological and morphological performance, and soil. (leaf length) average leaf length; (rosettes) number of rosettes per plant; (ramification order) maximum order of ramification found in the plant; (branch length) average branch length.

	Leaf			**Morphology**				**Soil**	
Sample	$\partial^{15}N$	$\partial^{13}C$	C/N	Leaf length (mm)	Rosettes	Branch length (mm)	Ramification order	Al (μg g^{-1})	Ca (μg g^{-1})
1	-3.17	-29.7	42.1	9	74	15	5	556	252
2				19	4	28	3	1477	261
3				7	65	10	4	1125	173
4	-1.28	-29.2	50.8	6	99	11	5	399	260
5	0.624	-29.6	55.7	8	103	11	5	149	109
6	-0.10	-28.5	43.9	9	95	7	5	125	90.0
7				13	15	9	3	200	92.9
8	-0.703	-30.8	37.0	9	18	11	5		
9	-1.44	-30.6	35.5	17	46	14	4	233	293
10	-2.16	-30.1	36.4	13	29	13	5	798	359
11	-0.349	-30.6	38.3	21	68	10	4	92.3	314
12	1.43	-30.5	34.0	33	18			71.3	226
13	-1.21	-31.7	43.4	16	32	13	3	32.7	120
14				4	34	6	3	34.1	66.7
15				9	27	34	5	33.5	222
16				5	31	11	4	39.8	89.1

Table T6. Significant Spearman's correlation coefficients between physiological, morphological and soil variables. (leaf length) average leaf length; (rosettes) number of rosettes per plant; (ramification order) maximum order of ramification found in the plant; (branch length) average branch length; soil available (Al) and (Ca). (ns) non-significant, $P > 0.05$.

Spearman's *rho* (n = 9-15)	**Physiological**			**Morphological**				**Soil**	
	$\partial^{15}N$	$\partial^{13}C$	C/N	Rosettes	Ramification order	Branch length	Leaf length	Al	Ca
$\partial^{13}C$	ns								
C/N	ns	ns							
Rosettes	ns	0.717	0.839						
Ramification order	ns	0.677	ns	ns					
Branch length	-0.833	ns	ns	ns	ns				
Leaf length	ns	-0.685	-0.818	ns	ns	0.532			
Al	-0.733	ns	ns	ns	ns	ns	ns		
Ca	ns	ns	ns	ns	ns	0.635	0.636	0.511	
Al-toxicity index	ns	0.667	ns	ns	ns	ns	ns	0.925	ns

ANNEX II

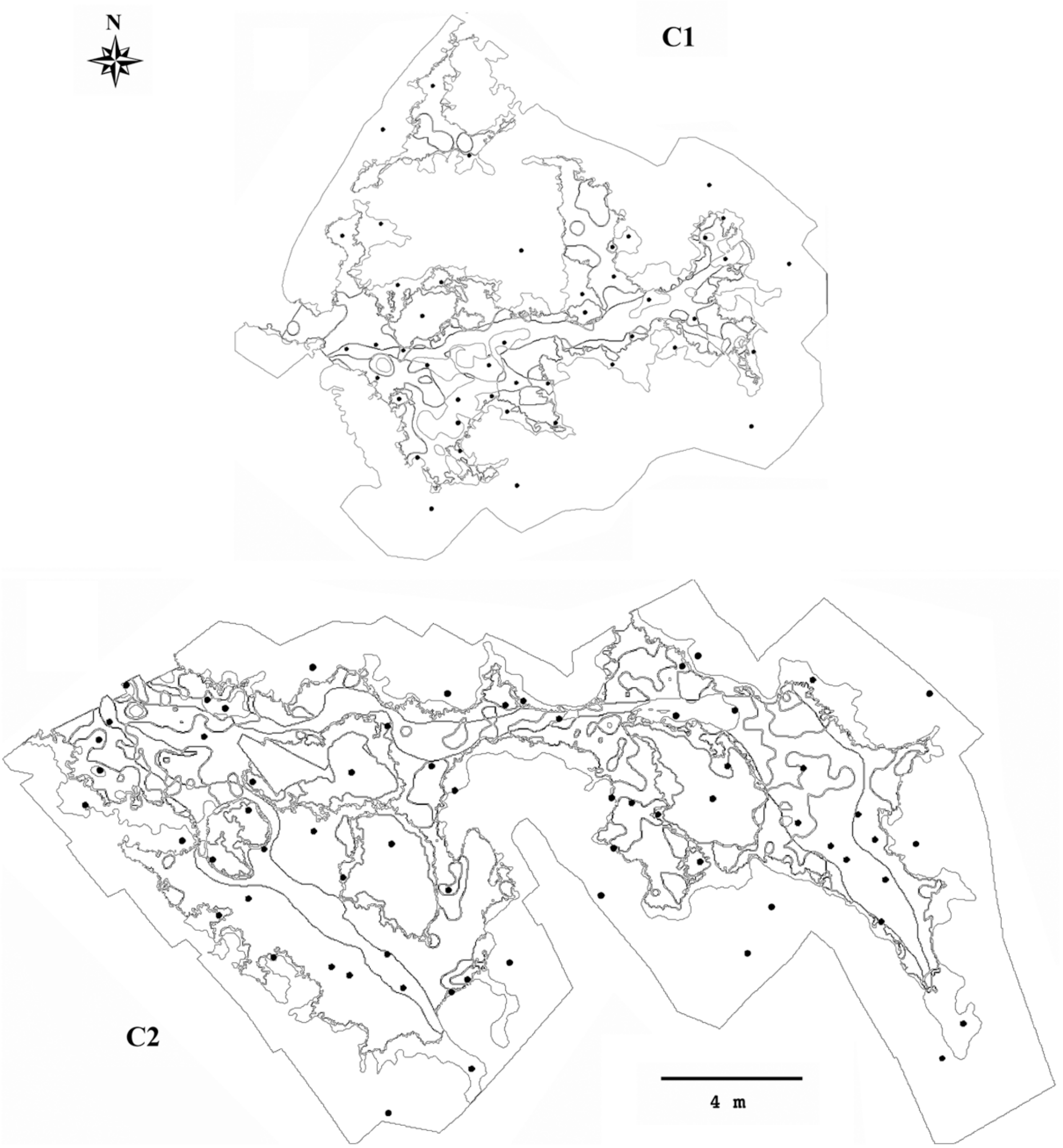


Figure F1. Limits and sampling points (filled circles) in the two areas (C1 and C2) of the ecological performance assessment.

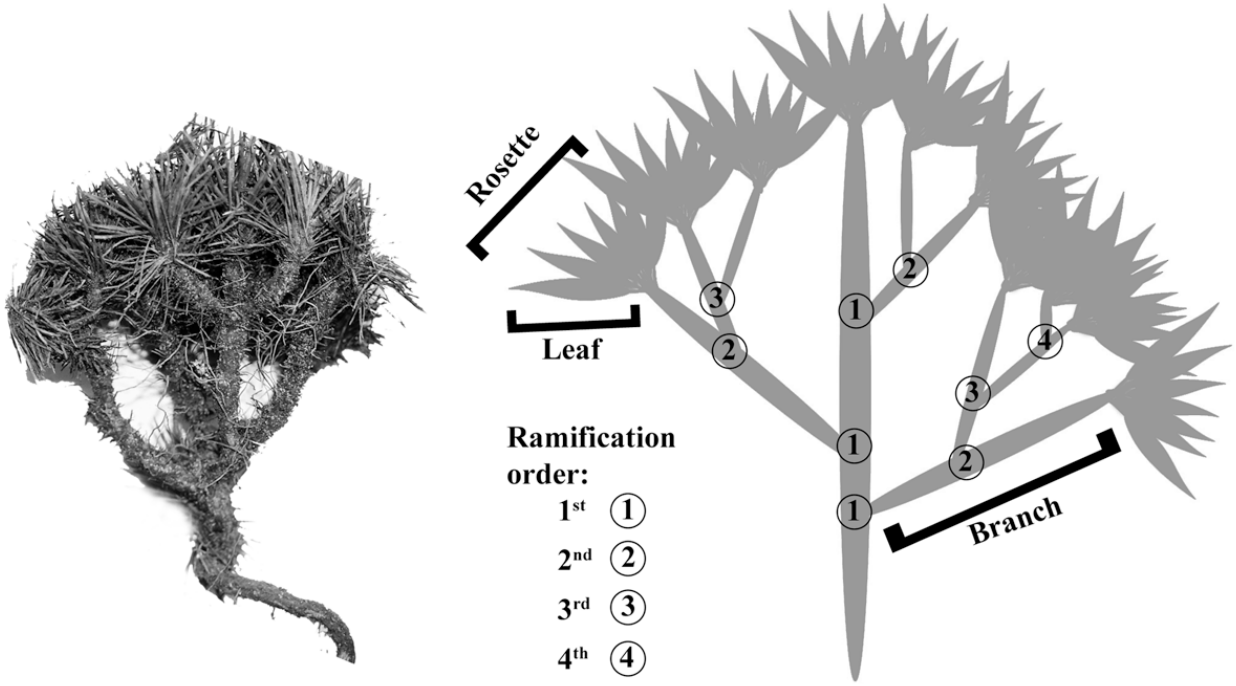


Figure F2. *Plantago almogravensis* photograph (left) and morphology scheme (right).

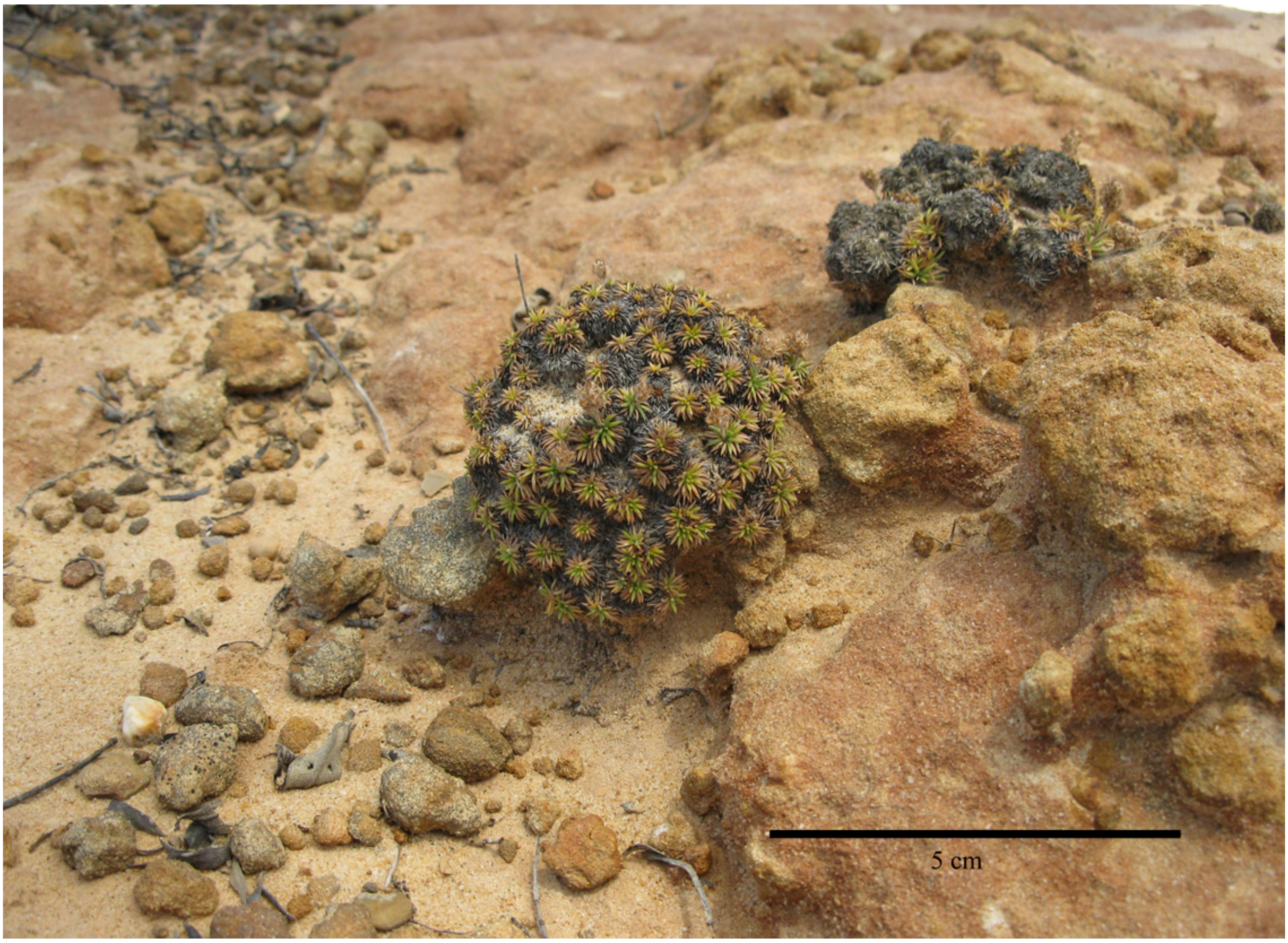
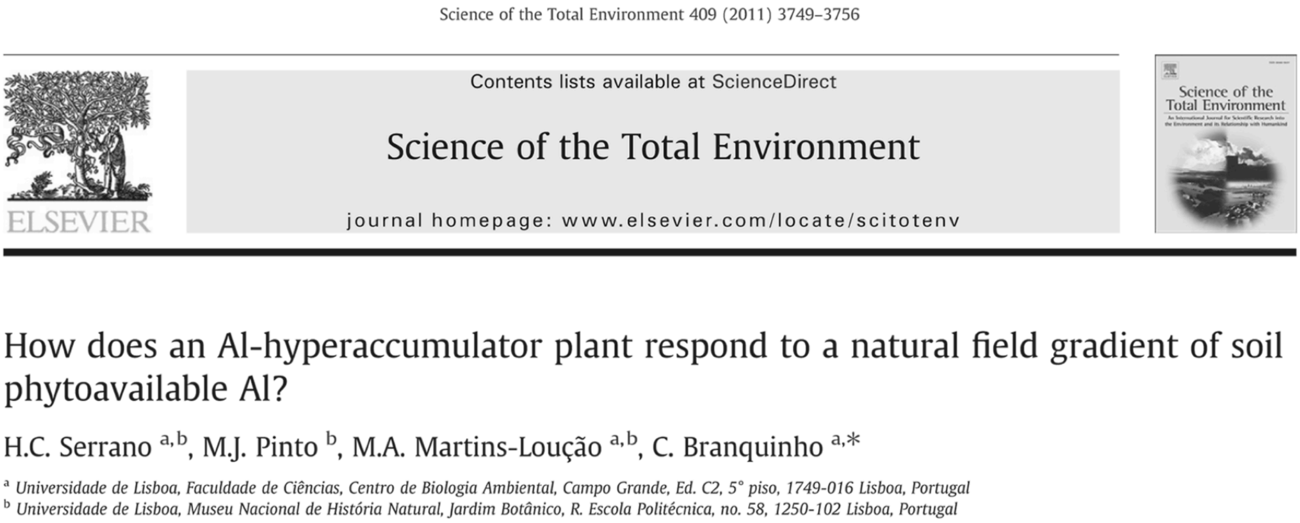


Figure F3. *Plantago almogravensis*.

CHAPTER 4

HOW DOES AN AL-HYPERACCUMULATOR PLANT RESPOND TO A NATURAL FIELD GRADIENT OF SOIL PHYTOAVAILABLE AL?



Science of the Total Environment 409 (2011) 3749–3756

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al?

H.C. Serrano [a,b], M.J. Pinto [b], M.A. Martins-Loução [a,b], C. Branquinho [a,*]

[a] *Universidade de Lisboa, Faculdade de Ciências, Centro de Biologia Ambiental, Campo Grande, Ed. C2, 5° piso, 1749-016 Lisboa, Portugal*

[b] *Universidade de Lisboa, Museu Nacional de História Natural, Jardim Botânico, R. Escola Politécnica, no. 58, 1250-102 Lisboa, Portugal*

De acordo com o disposto na alínea a, do número 2, do artigo 25, do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa (Despacho n.º 2950/2015 da Reitoria da Universidade de Lisboa), a autora desta tese declara que participou activamente no planeamento e execução do trabalho experimental, na interpretação dos resultados obtidos e na elaboração do seguinte manuscrito, publicado em *Science of the Total Environment* 409 (2011) 3749–3756. doi:10.1016/j.scitotenv.2011.06.036.

CONTENTS

HOW DOES AN AL-HYPERACCUMULATOR PLANT RESPOND TO A NATURAL FIELD GRADIENT OF SOIL PHYTOAVAILABLE AL?

ABSTRACT

The physiological ability of plants to cope with Al-toxicity has attracted considerable attention. In this study we used an endemic Al-hyperaccumulator plant, *Plantago almogravensis,* which is the only known representative of the *Plantaginaceae* with this trait growing under a field gradient of Al, to understand the root and shoot patterns of Al accumulation and tolerance in its natural environment. We analysed phytoavailable elements in the soil and their accumulation in the plant. For the first time under field conditions, the accumulation pattern of an Al-hyperaccumulator showed a saturation curve with a maximum accumulation capacity being reached (ca. 3.0 mg g^{-1}). The Al toxicity was not associated with the expected reduction in the Ca and Mg uptake by the plant. Iron was accumulated in a more linear pattern. The magnitude and the proportion of the elements found in the apoplastic fraction of the root, compared to the soil and plant internal fractions, suggested that the control of uptake occurs at the rhizospheric level. Unlike the majority of the Al-hyperaccumulator plants that are found in tropical humid areas, this plant is described from a sub-arid Mediterranean climate, subject to drought conditions which give it a unique status that deserves to be studied further.

INTRODUCTION

Aluminium is the third most abundant element of the Earth's crust (Markert 1992) and when available is toxic to plants. According to the IUSS (2006), soils with a pH below five and more than 60% Al saturation of the cation exchange capacity (CEC) may suffer from Al toxicity. This Al availability can also be caused by air pollution deposition from anthropogenic sources progressively leading to soil acidification and increasing the areas with soil Al toxic concentrations (Barcelo and Poschenrieder 2002; Wood et al. 2000). In acid soils Al is currently the major constraint for crop production and those soils represent more than 25% of the agricultural land around the world (Wood et al. 2000).

Podzols are examples of acid soils that are naturally enriched in bioavailable Al and Fe and plants that thrive and reproduce in those metal enriched soils are called metallophytes (Baker et al. 2010). In ecological terms, an obligate metallophyte only occurs in metal enriched areas, and several of them are endemic to those areas; by contrast, facultative metallophytes can also be found outside those metal rich areas (Baker et al. 2010). Aluminium hyperaccumulators, plants with more than 1.0 mg g^{-1} DW of Al in the aboveground parts (Jansen et al. 2002), are not common. It is vital that rare and endemic Al-hyperaccumulators are identified and preserved before they become extinct (Baker et al. 2010; Jansen et al. 2002). Nevertheless, these special plants occur in approximately 45 families (Jansen et al. 2002) and have a worldwide distribution, though they are most common in tropical forests (Chenery and Sporne 1976), which have acid soils and relatively high rainfall. Drier areas with more alkaline soils and relatively low rainfall have no aluminium tolerant plants (Jansen et al. 2002). Thus it is important to understand how plants cope with Al soil toxicity in natural conditions together with other environmental constraints. External mechanisms that control the uptake of Al to the hyperaccumulator plants might act at the soil-root interface (Jentschke and Goldbold 2000; Meharg 2003). Moreover, exclusion of aluminium from plant tissue is probably more important than internal mechanisms for aluminium tolerance, at least in some crop plants (Jansen et al. 2002). Studies performed in hydroponics ignore some of these exclusion processes at the soil-root interface, namely, realistic Al soil concentrations, natural climatic conditions and interactions with other living organisms. Only using natural or semi-natural trials we will be able to confirm what are the key patterns and mechanisms that determine the plant response to Al toxicity under natural conditions.

The cell wall of the root is the first site of Al binding, and to some extent, limits the movement of Al into the symplasm (e.g. Horst et al. 2010). Although not clear for all plants, results showed that some, with a high root cation-exchange capacity (CEC) were thought to be more sensitive to Al than plants with a low CEC (Blamey et al. 1990). Other mechanisms that might reduce the uptake of Al in plants are related to a plant-induced increase in rhizosphere pH (Dakora and Phillips 2002); plant release of organic acids with Al chelating capacity (Horst et al. 2010; Jansen et al. 2002); and the limitation of Al transport to plant roots through mycorrhizal associations (Jentschke and Goldbold 2000; Meharg 2003). The high concentration of Al in the shoot of Al-hyperaccumulators suggests that it is transported across the plasma membrane into the symplasm (Poschenrieder et al. 2008). A prerequisite for Al tolerance in Al-hyperaccumulators is the need for internal detoxification mechanisms, from the transport of Al through the xylem to its accumulation in the shoot vacuoles or apoplast and/or leaf epidermis and trichomes (Barcelo and Poschenrieder 2002; Kochian et al. 2005).

It is expected that Al inhibit Ca and Mg uptake, at least in non-accumulator crop plants (Boudot et al. 1994; Poschenrieder et al. 2008; Rengel and Zhang 2003) and forest ecosystems (Al-excluder trees) (Boudot et al. 1994; Vanguelova et al. 2007). Accordingly, the Al/Ca ratio has been used as a long-term indicator of the Al toxicity symptoms (Poschenrieder et al. 2008). However for Al-hyperaccumulator plants, authors (Haridasan 1982; Haridasan and De Araujo 1988; Jansen et al. 2003) have shown constant values of Ca and Mg with increasing Al concentrations. Masunaga et al. (1998) also noticed that for Al-hyperaccumulators (> 3.0 mg g^{-1}) the leaf Al/Ca was significantly higher than for the non-accumulator plants at the same site.

Most studies on the pattern of Al uptake, translocation and tolerance mechanisms on Al-accumulator plants are focused on short-term uptake and/or axenic conditions, which do not reflect the conditions experienced in the field (e.g. Guinel and Larue 1993; Watanabe et al. 2006). There are some studies concerning Al-accumulator plants which are developed under field conditions (e.g. de Andrade et al. 2011; Haridasan 1982; Mazora et al. 1987), but these lack the use of a clear gradient of bioavailable Al concentrations, for the same plant species, and in this way they cannot determine Al uptake under field conditions. We decided to study Al-hyperaccumulation under field conditions in a rare endemic and recently identified Al-hyperaccumulator plant *Plantago almogravensis* Franco (Branquinho et al. 2007). Moreover this plant has unique ecological behaviour and an uncommon location for an Al-hyperaccumulator plant: it is found in the SW of Portugal under Mediterranean climate (hot and dry summer with cold and humid winter). It grows in podzolic soils rich in bioavailable Al and Fe (Buurman and Jongmans 2005;

Pimentel et al. 1996). These enriched soils have a patchy spatial pattern forming geochemical islands.

Our objective was to understand how an Al-hyperaccumulator plant responds to a natural field gradient of soil phytoavailable Al, and for that we made the following questions:

i) Could we confirm the Al-hyperaccumulator nature of *P. almogravensis* measured under a gradient of soil Al concentrations?

ii) What is the pattern of Al uptake and accumulation in an Al-hyperaccumulator plant, under a field gradient of bioavailable Al and Fe, growing in geochemical islands under Mediterranean climate?

iii) Is there any control in the uptake of Al and if so, at which level of the plant does it occur?

iv) What is the impact of the Al uptake and accumulation in the uptake of macronutrients such as Ca and Mg?

MATERIALS AND METHODS

Site characterisation

The study area was located in the SW coast of Portugal, in the Natural Park of *Sudoeste Alentejano e Costa Vicentina*, nearby *Vila Nova de Milfontes*. The climate is of Mediterranean type, characterised by cold rainy winters and hot dry summers. The annual average air temperatures are about 15 °C; total precipitation is less than 600 mm per year, with dry periods of two to four months per year; and almost permanent maritime moist winds, at an annual average velocity of 10 km h^{-1}.

The soil is a sandy dark-grey podzol (Cardoso 1965; Jones et al. 2005), presenting rusty-red patches of superficial sesquioxide hardpans (rich in Al, Fe and Si), which are scarcely colonised by vegetation. The patchiness of the soil is related to erosion processes that exposed different horizons of the podzol and created a mosaic of soil chemical compositions and soil depths, forming geochemical islands. A dense dwarf sclerophyllous shrubland, mostly constituted by *Ericaceae* and *Cistaceae* species, forms the dominant vegetation on the deeper soils while on the shallow hardpans *P. almogravensis* is the dominant species.

Species characterisation

P. almogravensis Franco (*Plantaginaceae*) is an extremely rare Portuguese endemic Al-hyperaccumulator plant (Branquinho et al. 2007). The only known population in the world is found in a 7 ha area. It is a dwarf woody hemicryptophyte forming a cushion less than 15 cm wide and 7 cm high, characterised by an inconspicuous woody stem, covered by decomposing remains of leaf sheaths and with terminal leaf rosettes. The leaves are needle-like, 2 to 60 mm long by 0.5 mm to 0.7 mm wide. The main root is woody and long.

Sampling of plants and soil

Entire plants were collected (roots and shoots) together with the soil surrounding the roots (350-1000 cm^3, 10 cm deep) during February 2004, after heavy winter rains saturated the soil and washed the dust from the vegetation. Sampling was performed within a 2.5 ha area. Considering that *P. almogravensis* is at risk of global extinction being extremely localised, rare, threatened and strictly protected by Portuguese law, only 16 specimens were collected in total to reduce negative impacts.

Soil analysis

The soil was dried after cleaning of debris and sieving (2 mm mesh), to be used in pH measurements, analysis of total elemental concentrations and estimation of cation phytoavailability. For evaluation of potential soil particles contaminating plant samples, the total elemental composition of the soil was conducted. Two 0.3 g soil aliquots, per sample, were microwave acid-digested (65% HNO_3: 48% HF: 30% H_2O_2, 2:2:1) for atomic absorption spectrometry (AAS) analyses. Similar ratios of Al per lithophilic element both in plants and soil reflect possible particle contamination, according to Bargagli (1995). Therefore, for confirmation of the efficiency of the plants cleaning steps, their Al/Fe (lithophilic) ratios were compared to the ones from the soil total elemental composition.

To estimate phytoavailable cations in soils (Kabata-Pendias 2004; Ure 1996; Wang et al. 2004), two separate methods were used, from Branquinho et al. (2007): extraction with water, (1:20) for 6h, and extraction with ethylenediaminetetraacetic acid (EDTA) 20 mM, (1:40) for 3 h. Both solutions were analysed by atomic absorption spectrometry (AAS), to evaluate soluble and other easily exchangeable soil cation concentrations, respectively. Considering the EDTA solution also extracts the soluble cations, it was the difference between the two fractions that was used in the data analyses (EDTA-extract). Soil pH was measured in water (1:2) with a glass electrode (91-02sc, ORION RESEARCH).

Plant analysis

Once in the laboratory, *P. almogravensis* plants were thoroughly washed in running tap water followed by deionised water to remove any contamination from soil particles. From each plant two fractions were separated: the terminal rosettes of green leaves and the woody underground parts: main roots and stems (root fraction). The leaves were dried (70°C) until constant weight (dry weight, DW) and ground for analysis of the elemental concentration. The root fraction was further eluted in EDTA (see *External Root EDTA*) before a final wash in deionised water, drying (70°C) and grounding, for analysis of the elemental concentrations. Aliquots of 0.3 g (2 to 3 per sample) of dried roots or leaves were microwave-digested (65% HNO_3: 30% H_2O_2, 4:1) for AAS analyses, as detailed in Branquinho et al. (2007). Text mentions of “root concentration” refer to this (internal) root concentration unless stated otherwise.

External Root EDTA

In order to estimate the root external apoplastic bound cations, the root material was eluted as proposed by Azcue (1996) in EDTA. This chelating agent (20 mM EDTA) was found to be able to extract several metals without causing changes in membrane permeability in sensitive organisms, such as lichens (Branquinho et al. 1997). The solutions obtained were used to determine the concentration of root-desorbed cations, on a root dry weight base, by AAS. The procedure was to shake the roots for 1.5 h (150 rpm) in 20 mM EDTA (1:20) (Branquinho et al. 2007).

Elemental AAS analyses

The plant and soil digests, as well as the root EDTA extract, the soil soluble and the soil EDTA extract were all analysed by AAS, using standard operating procedures, for the elements Al, Ca, Fe, K and Mg.

The concentration was determined for Al by Graphite Furnace AAS (932 plus, GBC) and for Ca, Fe, K and Mg by Flame Atomic Absorption Spectrometry (SpectrAA50, VARIAN). The detection limits of the AAS analysis were (µg dm^{-3}): Al (6.2), Ca (30), Fe (150), K (80) and Mg (10). The accuracy of the results (Al: 94%, Ca: 74 to 96%, Fe: 94%, K: 84% and Mg: 89%) was checked by processing the reference material BCR-100, “beech leaves”. All elemental concentration results were expressed on a dry weight basis.

Data Analysis

Accumulation patterns of Al and Fe were obtained by plotting root Al and Fe concentrations against those found in the soil, within the soil gradient. For both the extracts of soil phytoavailable Al and Fe (soluble and EDTA-extractable), the pattern of accumulation to the root was fitted to an asymptotic (saturation) curve,

$$f(x) = (a * x) / (b + x) \qquad (1)$$

and to a linear fit. The plotted curves in the figures (Fig. 2) were considered the best fits since they showed: i) a higher correlation coefficient between observed and predicted values for the simplest fit using the parsimony principle; ii) a lower mean deviation and, iii) a lower standard-error-of-estimate.

If not stated otherwise, results are presented as mean ± standard error (SE) of n=16. Concentration ratios of different elements were calculated from the molar concentration (per gramme of dry weight) of those elements. Saturation (%) was calculated based on the total concentration (TC) of Al, Ca, Fe, K and Mg, in cmol$^{(+)}$ kg^{-1}. Statistical significance was $p \leq 0.05$ for the samples analysed (n=16). Level of significance was indicated by * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

RESULTS

Soil gradient characterisation

The results depicted in Fig. 1 show the composition of the soil samples analysed in relation to the phytoavailable concentrations (soluble and EDTA extractable) of Al, Ca, Fe, K and Mg. The gradient of Fig. 1 is ordered by the sum of Al and Fe concentrations (Fig. 1, in both extracts). However, the ratio between the macronutrients (K, Ca and Mg) and Al and Fe, decreased along the gradient (Fig. 1, secondary Y axis), showing a lower proportion of macronutrients in the Al and Fe naturally enriched soils.

The ratios between the highest and the lowest Al concentrations were 46 and 96 times, for EDTA and soluble extracts, respectively. Whereas for Fe the ratios were 20 and 86 for those same extracts.

The pH ranged from 4.91 to 6.79 (5.92 ± 0.14) and significant correlations were found between pH and soluble Al (r = 0.52*), EDTA-extractable Al (r = 0.68**) or soluble macronutrients (r > 0.52*).

Root accumulation

The concentrations of Al and Fe in the roots were taken as a measure of the accumulation of those metals in the plant. The accumulation patterns were obtained by plotting the phytoavailable Al or Fe concentrations in the soil versus the metal concentrations

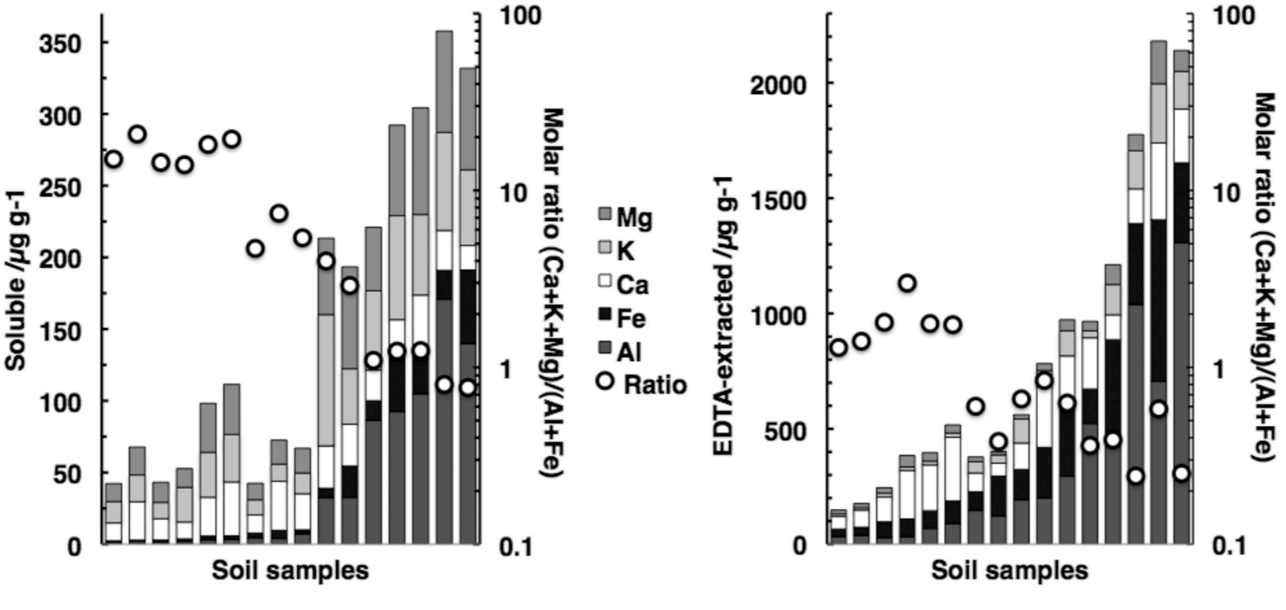


Figure 1. Soil sample compositions from the soluble and EDTA–extracted fractions; the samples are ordered by the (Al+Fe) concentration gradient. Secondary Y axis: molar ratio of the concentrations of (Ca, K and Mg) and (Al+Fe), on a logarithmic scale.

inside the root present in those soils (Fig. 2). The uptake of Al from soil to root (from both soil extracts) depicted a saturation curve (Fig. 2a and 2b). The pattern of accumulation can be divided into two types: in soils with lower availability of Al, the accumulation was proportional to the soil concentration; and in soils with higher availability of Al, the accumulation seems to be controlled. In this case, the maximum Al accumulation-capacity calculated from the equations obtained was, on average, 3.67± 0.42 mg g^{-1} for the root (Fig. 2a, b). The pattern of Fe accumulation from the EDTA-extract was similar to that of Al (Fig. 2d). In contrast, the pattern of soil-soluble Fe accumulation in these Al-hyperaccumulator plants revealed a different profile. Iron accumulation (Fig. 2c) did not evidence an asymptotic trend. Iron was found between 0.93 and 3.54 mg g^{-1} in the root, distributed linearly along increasing Fe concentrations in the soil.

Al and Fe Translocation

The concentrations of Al and Fe in the root and in the leaves were taken as a measure of their translocation between those organs (Fig. 3). By plotting the metal concentrations in the roots against the con-

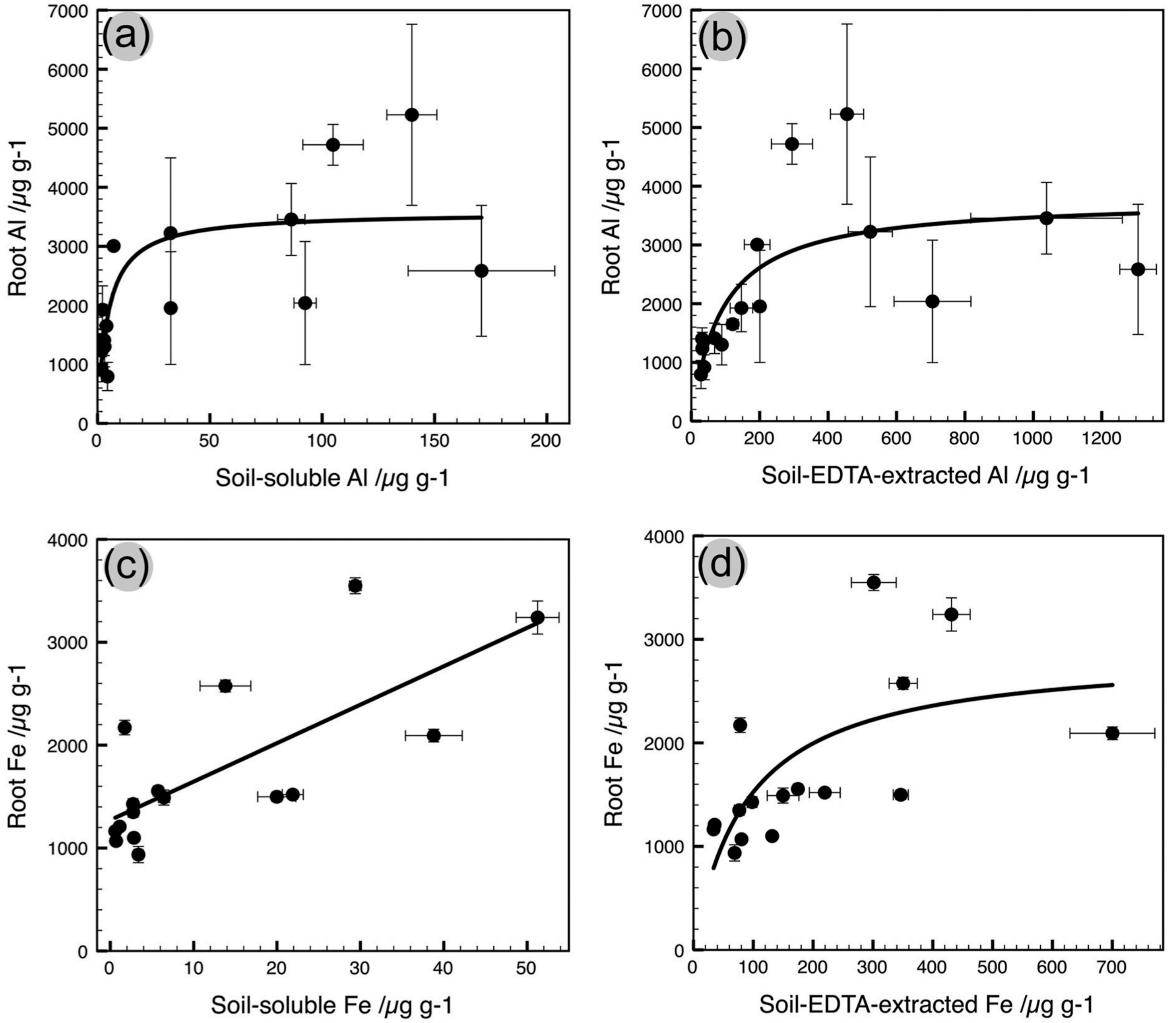


Figure 2. Root uptake of phytoavailable Al and Fe. (a, b) Al and (c, d) Fe uptake, from the soil (a, c) soluble and (b, d) EDTA-extracted metal concentrations. Error bars are SE of the chemical analysis (soil, *n*= 2; root, *n*= 3). Estimated line fits: (a) f(x)= (3575*x)/(4.35+x), R^2= 0.565; (b) f(x)= (3778*x)/(89.2+x), R^2= 0.537; (c) f(x)= (37.3*x)+1272; R^2= 0.552; (d) f(x)=(2885*x)/(89.2+x), R^2= 0.400.

Table 1. Total concentration and saturation (%) of measured elements.

	Soil soluble extract	Soil EDTA extract	Ext. root EDTA	Roots	Leaves
Al (%)	27.8 ± 22.6 $^{ab\,A}$	50.6 ± 19.1 $^{a\,B}$	5.0 ± 3.8 $^{a\,C}$	36.9 ± 9.7 $^{a\,AB}$	26.0 ± 8.8 $^{a\,A}$
Fe (%)	3.4 ± 2.1 $^{c\,A}$	13.9 ± 5.0 $^{bc\,B}$	6.7 ± 5.3 $^{a\,AC}$	9.6 ± 2.4 $^{b\,C}$	8.9 ± 4.0 $^{b\,C}$
Ca (%)	20.4 ± 12.6 $^{ad\,A}$	23.3 ± 14.9 $^{b\,A}$	52.2 ± 14.1 $^{c\,B}$	21.8 ± 5.0 $^{c\,A}$	27.6 ± 5.6 $^{a\,A}$
K (%)	11.6 ± 5.2 $^{cd\,A}$	3.3 ± 1.6 $^{c\,B}$	5.9 ± 3.8 $^{a\,B}$	11.6 ± 4.6 $^{b\,A}$	10.0 ± 4.6 $^{b\,A}$
Mg (%)	36.8 ± 10.3 $^{b\,A}$	9.0 ± 4.9 $^{c\,B}$	30.2 ± 10.6 $^{b\,AC}$	20.1 ± 4.1 $^{c\,D}$	27.5 ± 6.3 $^{a\,CD}$
TC cmol$^{(+)}$ kg^{-1}	1.06 ± 0.92 ^{A}	5.84 ± 5.38 ^{A}	21.5 ± 9.75 ^{B}	65.3 ± 21.8 ^{C}	80.2 ± 17.8 ^{D}

TC: total cation concentration±SD; %: saturation (±SD) of the measured ions in relation to TC; same capital letters in line or same lower-case letters in one column, indicate no significant differences (ANOVA and Tukey post hoc tests).

centration in the leaves, significant correlations for both Al (Fig. 3a) and Fe (Fig. 3b) were observed, and the linear fit showed an even allocation (circa 1:1) between leaves and roots. From these results, the accumulation pattern of Al from the soil into the leaves, is also a saturation curve, similar to the one depicted to the root (Fig. 2), with an average maximum Al accumulating capacity calculated as 2.7±0.2 mg g^{-1} DW.

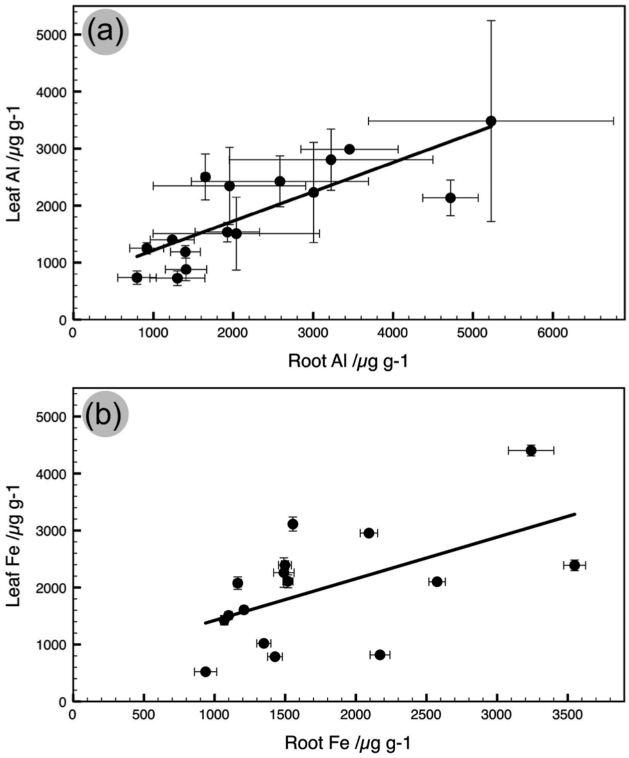


Figure 3. Translocation of (a) Al and (b) Fe between root and leaves. Error bars are SE of the chemical analysis (n=3). Significant linear fits (p< 0.05): (a) f(x)= (0.513*x)+701, R^2= 0.634; (b) f(x)= (0.73*x)+692, R^2= 0.322.

Impact of Al in the uptake of Ca and Mg

In order to evaluate the presence of such stressful conditions (Al toxicity) we estimated the contribution of each element analysed (% saturation) to the total cation concentration (TC, including Al, Ca, Fe, K and Mg) of each plant fraction or soil fraction (Tab. 1). In soil, Al represented 28% to 51% of the total TC, depending on the soil extract, either similar or dominating over Ca and Mg. The external fraction of the root, eluted with EDTA, was dominated by Ca (52% saturation) followed by Mg (30% saturation), while Al only represented 5%. In the root, Al saturation was 37%, followed by Ca and Mg with similar amounts. In the leaves, Al was slightly lower (26%) and not significantly different from Ca or Mg. The contribution of K (3–12%) or Fe (3–14%) to TC was generally of lower importance than Al, Ca or Mg.

By comparing Al and Ca, along the studied gradient, there was no significant relation between the increase of Al in the soil and the amount of Ca (or Mg) inside the plant (data not shown). In fact, from Tab. 2, we see that there was a significant positive correla-

Table 2. Aluminium/Calcium and Aluminium/Magnesium ratios of the plants.

	Rt-edta	Roots	Leaves
Al:Ca			
molar ratio:	0.10 ±0.17 ^{a}	1.23 ±0.57 ^{c}	0.68 ±0.35 ^{b}
r=	0.152	0.502*	0.049
Al:Mg			
molar ratio:	0.12 ±0.09 ^{a}	1.31 ±0.54 ^{c}	0.70 ±0.33 ^{b}
r=	0.468	0.754**	-0.209

RT-edta: external root EDTA; Roots: internal root concentration; Leaves: leaf concentration; molar ratio: average molar ratio (n = 16) ± SD; r: Pearson's correlation coefficient with significance of, * p <0.05; ** p <0.01, *** p < 0.001; molar ratios followed by different letters are significantly different (p<0.01).

tion between Al and Ca and Al and Mg in the root and no correlation at all in the leaves.

DISCUSSION

The studied soils represent a natural and wide (86 to 96 times more concentrated) gradient of Al and Fe concentrations where an endemic plant, *P. almogravensis* naturally grows. This is not only a soil Al and Fe concentration gradient but it is also a toxicity gradient, since the proportion of Ca, K and Mg in soil decreases with increasing Al concentrations (Boudot et al. 1994; Kinraide and Parker 1987). This plant only occurs in an area of podzolic soils. Specifically, the plant dominates in geochemical islands of Al and Fe enriched hardpans. Surrounding these geochemical islands, sclerophyllous shrub vegetation dominates. However, these surrounding areas have lower bioavailable Al and Fe concentrations.

Confirming the hyperaccumulator nature

Most of the Al concentrations determined in the leaves were above 1.0 mgg^{-1}, the common used threshold for Al-hyperaccumulation proposed by Jansen et al. (2002). These results confirmed the hyperaccumulator nature of this plant, suggested in Branquinho et al. (2007) but here in a wider range of soil phytoavailable Al concentrations and in a large number of samples of the same species. Major Al-hyperaccumulators can be found in the following families: *Ebenaceae* (e.g. 41 000 mg kg^{-1}), *Euphorbiaceae* (e.g. 42 000 mg kg^{-1}), *Hydrangeaceae* (e.g.3000 mg kg^{-1}), *Melastomataceae* (e.g.66 000 mg kg^{-1}), *Myrtaceae* (e.g. 2550 mg kg^{-1}), *Rubiaceae* (e.g.36 000 mg kg^{-1}), *Theaceae* (e.g.30 000 mg kg^{-1}), or *Vochysiaceae* (e.g.25 000 mg kg^{-1}) among the various described as Al-hyperaccumulators (Jansen et al. 2002; Watanabe and Osaki 2002). For the first time we reported Al-hyperaccumulation for the *Plantaginaceae* family (Branquinho et al. 2007) and our contribution was also an important addition for the order Lamiales, where Al-hyperaccumulation has only been scarcely reported (Jansen et al. 2002). Despite being the only report of an Al-hyperaccumulator species, other high metal accumulations or tolerances have been described for this family, e.g. Cu, Cd (Djingova and Kuleff 1993; Remon et al. 2007; Zu et al. 2005). The woody nature of *P. almogravensis* is also a common trait shared with other Al-hyperaccumulators (Chenery and Sporne 1976; Jansen et al. 2002). It is likely that, in this case, Al-hyperaccumulation is a trait that has been selected due to the species isolation in a very limited specific geochemical area (Branquinho et al. 2007). Unlike the majority of the Al-hyperaccumulator plants that are found in tropical humid areas this plant is described in a sub-arid Mediterranean climate. The fact that we have in the same plant the ability to simultaneously tolerate Al toxicity and drought conditions gives to this plant species a unique status that deserves priority for studying it and for its conservation.

Most of the plant distribution is found in naturally Al rich soils. This suggests that ecologically *P. almogravensis* is an obligate metallophyte (Baker et al. 2010) since outside these soil Al rich areas, the plant is not able to compete with the surrounding sclerophyllous vegetation. However, we suggest that, physiologically, it can rather be considered a facultative metallophyte since outside the metal rich areas, and without competition, better physiological performance is observed (data not shown).

Fe being a micronutrient, there is no consensus in the literature concerning hyperaccumulation thresholds. A few Fe hyperaccumulators have been reported accumulating above 10 mg g^{-1} (*Imperata cylindrica*, by Rodriguez et al. 2005; *Eichhornia crassipes* by Streit and Stumm, 1993). In contrast to Al, Fe is an essential element used regularly by the plant, but with low mobility, which leads to the need for being continuously supplied for the plant to grow normally (Ma 2005). The average normal Fe concentration in plants is ca. 0.10 mg g^{-1} (Ma 2005; Markert 1992). In this context, *P. almogravensis* is an unusually high Fe-accumulator because it has 30 times more Fe than the amount quoted for *normal* plants. Fe content in leaf tissues of wetland plants was shown to be toxic for lower levels (between 1.10 and 1.60 mg g^{-1}), than the ones found in our plants (Marschner 1995). Therefore, *P. almogravensis* might be a Fe-tolerant and an unusual Fe accumulator.

Accumulation pattern in a hyperaccumulator plant

This work is one of the few studies that deals with Al-hyperaccumulation, under a natural gradient of soil conditions, and that relates these factors within the same plant species (e.g. Dong et al. 1999; Xie et al. 2001). Dong et al. (1999), showed a curve of Al uptake under field conditions for the tea plant (*Camellia sinensis*) where he fitted a linear regression. By revisiting that data we could re-interpret it as having a saturation plateau, although not as clear as the one presented here.

Thus our work represents the first time it was possible to draw a clear accumulation pattern in an Al-hyperaccumulator plant over a large gradient of Al phytoavailability in natural conditions.

We found that, for both soil-soluble and EDTA-extractable Al, a saturation curve was obtained (Fig. 2). This could be expected from theoretical uptake (Callahan et al. 2006; Watanabe et al. 2001) or hydroponic studies (Guinel and Larue 1993) but has been seldom recorded under field conditions for Al-hyperaccumulator plants.

The maximum accumulation capacity for *P. almogravensis* was ca. 3.0 mg g^{-1} DW and this maximum level can be fairly characteristic of a species (Kovacs et al. 1993). The plant, at its maximum accumulation capacity (three times above the hyperaccumulation threshold), was able to cope with a wide range (four-fold) of increasing soil Al phytoavailable concentrations without significantly changing its root Al concentration (Fig. 2). The plant is not only able to control the internal root Al levels in a wide range of soil concentrations but also to cope with these high levels at the aboveground part of the plant. On the other hand iron showed a linear pattern of accumulation, in accordance with results produced by other authors that reported concentration dependent Fe-uptake (e.g. Batty and Younger 2003). The lower percentage of explanation of the relation between Fe in the soil and Fe in the plant shows that the environmental availability of Fe explains only a small amount of the variance. The remaining variance can be explained by the nutritional role that this element has on the physiological processes.

Soil-Root interactions

From the high concentrations of soluble Al measured in the soils in this study, we would expect lower soil pHs (namely below 5) than the ones observed here (4.9–6.8). Although infrequent, the complexity of Al soil chemistry has shown similar behaviour in other works (Branquinho et al. 2007; Poschenrieder et al. 2008; Tipping 2005) where several mechanism that can alter Al availability were suggested: mineral solubilisation reactions; presence of organic matter and associated complexation/adsorption reactions; presence of rhizospheric microorganisms or mycorrhizal fungi; activity of competing divalent cations (like Mg^{2+} and Ca^{2+}) or activity of Fe^{3+}. This result is also in accordance to what Dong et al. (2001) found: exchangeable Al and sorbed Al were not correlated with pH measurements made in water solution from soil. In this case it seems that pH and available Al should be measured in the same elution to be comparable. In our work we measured the pH in water and the available Al in EDTA and in water but at a constant pH. Finally, this result is also compatible with plant-induced increase in rhizospheric pH in order to limit the Al uptake to the plant (Dakora and Phillips 2002).

The external root fraction showed a decrease in the Al saturation at the same time that the Ca saturation increased at this location. Unlike most plants (Jansen et al. 2002), *P. almogravensis* cell walls were not the major site of Al binding; the proportion of elements that bound the outer surface of the root was only 33% of the elements that entered the root. These results are in accordance to what have been suggested by Blamey et al. (1990), that the CEC in accumulator plants is lower than in non-accumulator plants. The different pattern of Al saturation of the root external fraction seems to corroborate the results by other authors (Horst et al. 2010) that in Al accumulator plants the Al concentration is kept lower in the apoplast. These results also suggest that the rhizospheric microenvironment is subjected to a different proportion of Al, Fe, Ca and Mg compared to that of the surrounding soil (Tab. 1). These elements in the external root fraction are probably apoplastic bound metals plus some eventual ones present in soil colloids, strongly adhering at the root surface. The Al decrease in the external root fraction can be a result of several processes described in the literature such as: the release of organic acid anions that complex Al and can protect the root apoplast from Al binding either in Al-tolerant or Al-accumulating plants (Horst et al. 2010); the existence of symbiotic associations with bacteria that produce substances, with special metal affinity to sequester metals, such as siderophores (Alford et al. 2010); or the restriction of soil metal movement to the roots by means of absorption to the hyphal sheath and external mycelium of mycorrhiza (Gadd 1993; Hall 2002). Most of the studies that concern Al hyperaccumulation are developed under controlled conditions (e.g. in hydroponics; Watanabe et al. 2001) which do not put in evidence the effect of rhizospheric mechanisms on plant Al tolerance and uptake. The results here reported point out the ecological relevance of field studies to fully understand the ecophysiological mechanisms of Al hyperaccumulation and tolerance in plants. Mycorrhizas have been observed in putative roots of this species and the existence of some particular associations of rhizospheric bacteria to *P. almogravensis* roots has also been observed (data not shown).

The impact of Al on Ca and Mg uptake

Under field conditions it is almost impossible to ascertain direct cause–effect events. Nevertheless, we observed that *P. almogravensis* plants did not reduce the uptake of Ca and Mg along the Al gradient unlike the response of most plants to Al toxicity (Boudot et al. 1994; Poschenrieder et al. 2008). The concentrations of Ca and Mg in the aboveground part were maintained along the Al gradient. Interestingly, the average Al/Ca ratio was in the same order of magnitude of the ratios determined for other Al-hyperaccumulator plants, such as the ones quoted by Haridasan and De Araujo (1988); Jansen et al. (2003) or Masunaga et al. (1998). It seems that the ability to control the internal Ca and Mg levels is a trait related with Al-hyperaccumulation. This is supported by this work and by the results from Haridasan, (1982, 1987), Haridasan and de Araújo (1988), Jansen et al. (2003), Masunaga et al. (1998), Osaki et al. (1998) or Watanabe and Osaki (2002), that showed that plants adapted to acid soils or soils with high available Al are able to maintain the levels of Ca and Mg independently of the Al concentration in the leaves. On the contrary the traditional laboratorial studies have shown that Al sensitive crops (maize, wheat) evidence a reduction of the uptake of Ca and Mg.

This plant seems able to exclude part of the aluminium through low CEC, increasing rhizospheric pH and biotic associations (as likely candidates) to balance the entrance of Al into the plant root. This suggests that through all these processes the plant is able to control Al uptake explaining the non-linear accumulation pattern within the root.

Conclusions

P. almogravensis is the only Al-hyperaccumulator member of its family described so far growing in sub-arid Mediterranean conditions. This species grows within a toxic and wide gradient of soil phytoavailable Al and Fe in its natural habitat and seems to be an obligate metallophyte since its distributions are confined to geochemical islands of Al and Fe enriched hardpans.

For the first time under field conditions, the accumulation pattern of an Al-hyperaccumulator showed a saturation curve after the maximum accumulation capacity was reached (ca. 3.0 mg g^{-1} DW). The Al toxicity was not evidenced in the reduction in the Ca and Mg uptake. The distribution of Al in the plant, suggested that the interactions between soil and rhizosphere are a key step in the accumulation process. All these facts reinforce the interest for the study of hyperaccumulator plants under field conditions. Though these have some limitations they provide clues about interactions that cannot be established in minimal, axenic, and restricted laboratory trials, pointing to the ecological relevance of field studies to fully understand the ecophysiological mechanisms of Al-hyperaccumulation and tolerance in hyperaccumulator plants.

ACKNOWLEDGEMENTS

This research has been supported by the projects "Estudo de referência de *Plantago almogravensis*" funded by *Parque Natural do Sudoeste Alentejano e Costa Vicentina* (POA), Portuguese Institute of Natural Conservation (ICN); project "ECODILEMA" POCTI/BIA-BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004 from FCT (Portuguese Foundation for Science and Technology); we thank FCT/CNR bilateral for supporting "Biomonitoring of soil and atmospheric pollution at mine sites in Mediterranean areas: responses from cellular to ecosystem level"; and HC Serrano PhD grant SFRH/BD/38289/2007 from FCT. Authors also thank Mrs. Lurdes Aires for her technical support at the laboratory.

REFERENCES

Alford ER, Pilon-Smits EAH, Paschke MW. 2010. Metallophytes-a view from the rhizosphere. *Plant and Soil* 337: 33-50

Azcue JM. 1996. Comparison of different cleaning procedures of root material for analysis of trace elements. *International Journal of Environmental Analytical Chemistry* 62: 137-45

Baker AJM, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In *Ecology of Industrial Pollution*, ed. LC Batty, KB Hallberg, pp. 7-40. Cambridge, U. K.: Cambridge University Press

Barceló J, Poschenrieder C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environmental and Experimental Botany* 48: 75-92

Bargagli R. 1995. The elemental composition of vegetation and the possible incidence of soil contamination of samples. *Science of the Total Environment* 176: 121-28

Batty LC, Younger PL. 2003. Effects of external iron concentration upon seedling growth and uptake of Fe and phosphate by the common reed, *Phragmites australis* (Cav.) Trin ex. Steudel. *Annals of Botany* 92: 801-06

Blamey FPC, Edmeades DC, Wheeler DM. 1990. Role of root cation-exchange capacity in differential aluminum tolerance of lotus species. *Journal of Plant Nutrition* 13: 729-44

Boudot JP, Becquer T, Merlet D, Rouiller J. 1994. Aluminum toxicity in declining forests - a general overview with a seasonal assessment in a Silver Fir forest in the Vosges Mountains (France). *Annales Des Sciences Forestieres* 51: 27-51

Branquinho C, Brown DH, Catarino F. 1997. The cellular location of Cu in lichens and its effects on membrane integrity and chlorophyll fluorescence. *Environmental and Experimental Botany* 38: 165-79

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Buurman P, Jongmans AG. 2005. Podzolisation and soil organic matter dynamics. *Geoderma* 125: 71-83

Callahan DL, Baker AJM, Kolev SD, Wedd AG. 2006. Metal ion ligands in hyperaccumulating plants. *Journal of Biological Inorganic Chemistry* 11: 2-12

Cardoso JC. 1965. *Os solos de Portugal, sua classificação, caracterização e génese. 1 - A sul do Rio Tejo*. Portugal: Secretaria de Estado da Agricultura.

Chenery EM, Sporne KR. 1976. Note on evolutionary status of aluminum accumulators among dicotyledons. *New Phytologist* 76: 551-54

Cronan CS, Grigal DF. 1995. Use of calcium aluminum ratios as indicators of stress in forest ecosystems. *Journal of Environmental Quality* 24: 209-26

Dakora FD, Phillips DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* 245: 35-47

de Andrade LRM, Barros LMG, Echevarria GF, do Amaral LIV, Cotta MG, et al. 2011. Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. *Environmental and Experimental Botany* 70: 37-42

Djingova R, Kuleff I. 1993. Monitoring of heavy metal pollution by *Taraxacum officinale*. In *Plants as Biomonitors: Indicators for Heavy Metals in the Terrestrial Environment*, ed. B Markert, pp. 435-60: VCH Weinheim

Dong DM, Xie ZL, Du YG. 2001. The bioavailability of Al in soils to tea plants. *Applied Geochemistry* 16: 1413-18

Dong DM, Xie ZL, Du YG, Lin CM, Wang ST. 1999. Influence of soil pH on aluminum availability in the soil and aluminum in tea leaves. *Communications in Soil Science and Plant Analysis* 30: 873-83

EC-JRC. 2005. *Soil Atlas of Europe*. pp. 128. Luxembourg: Office for Official Publications of the European Communities.

FAO. 2006. *World Reference Base for Soil Resources 2006: A Framework for International Classification, Correlation and Communication*. pp. 144. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).

Gadd GM. 1993. Interactions of fungi with toxic metals. *New Phytologist* 124: 25-60

Guinel FC, Larue TA. 1993. Excessive aluminum accumulation in the pea mutant E107 (BRZ). *Plant and Soil* 157: 75-82

Hall JL. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* 53: 1-11

Haridasan M. 1982. Aluminum accumulation by some cerrado native species of central Brazil. *Plant and Soil* 65: 265-73

Haridasan M. 1987. Distribution and mineral nutrition of aluminium accumulating species in different plant communities of the cerrado region of central Brazil In *La capacidad bioproductiva de sabanas*, ed. JJS José, R Montes, CIdE Tropical, IVdI Científicas, pp. 309–48: Centro Internacional de Ecología Tropical (UNESCO/CIET)

Haridasan M, Araújo GM. 1988. Aluminium-accumulating species in 2 forest communities in the Cerrado region of central Brazil. *Forest Ecology and Management* 24: 15-26

Horst WJ, Wang YX, Eticha D. 2010. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. *Annals of Botany* 106: 185-97

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Jansen S, Watanabe T, Dessein S, Smets E, Robbrecht E. 2003. A comparative study of metal levels in leaves of some Al-accumulating Rubiaceae. *Annals of Botany* 91: 657-63

Jentschke G, Goldbold DL. 2000. Metal toxicity and ectomycorrhizas. *Physiologia Plantarum* 109: 107-16

Kabata-Pendias A. 2004. Soil-plant transfer of trace elements - an environmental issue. *Geoderma* 122: 143-49

Kinraide TB, Parker DR. 1987. Cation amelioration of aluminum toxicity in wheat. *Plant Physiology* 83: 546-51

Kochian LV, Pineros MA, Hoekenga OA. 2005. The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. *Plant and Soil* 274: 175-95

Kovacs M, Turcsanyi G, Penksza K, Kaszab L, Szoke P. 1993. Heavy metal accumulation by ruderal and cultivated plants in a heavily polluted district of Budapest In *Plants as Biomonitors: Indicators for Heavy Metals in the Terrestrial Environment*, ed. B Markert, pp. 495–505: VCH Weinheim

Ma JF. 2005. Plant root responses to three abundant soil minerals: Silicon, aluminum and iron. *Critical Reviews in Plant Sciences* 24: 267-81

Markert B. 1992. Presence and significance of naturally-occurring chemical-elements of the periodic system in the plant organism and consequences for future investigations on inorganic environmental chemistry in ecosystems. *Vegetatio* 103: 1-30

Marschner H. 1995. *Mineral Nutrition of Higher Plants*. Academic Press.

Masunaga T, Kubota D, Hotta M, Wakatsuki T. 1998. Mineral composition of leaves and bark in aluminum accumulators in a tropical rain forest in Indonesia. *Soil Science and Plant Nutrition* 44: 347-58

Mazorra MA, Jose JJS, Montes R, Miragaya JG, Haridasan M. 1987. Aluminum concentration in the biomass of native species of the Morichals (swamp palm community) at the Orinoco-Llanos, Venezuela. *Plant and Soil* 102: 275-77

Meharg AA. 2003. The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycological Research* 107: 1253-65

Osaki M, Watanabe T, Ishizawa T, Nilnond C, Nuyim T, et al. 1998. Nutritional characteristics in leaves of native plants grown in acid sulfate, peat, sandy podzolic, and saline soils distributed in Peninsular Thailand. *Plant and Soil* 201: 175-82

Pimentel NL, Wright VP, Azevedo TM. 1996. Distinguishing early groundwater alteration effects from pedogenesis in ancient alluvial basins: Examples from the Palaeogene of southern Portugal. *Sedimentary Geology* 105: 1-10

Poschenrieder C, Gunse B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* 400: 356-68

Remon E, Bouchardon JL, Faure O. 2007. Multi-tolerance to heavy metals in *Plantago arenaria* Waldst. & Kit.: Adaptative versus constitutive characters. *Chemosphere* 69: 41-47

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

Rodriguez N, Menendez N, Tornero J, Amils R, de la Fuente V. 2005. Internal iron biomineralization in *Imperata cylindrica,* a perennial grass: chemical composition, speciation and plant localization. *New Phytologist* 165: 781-89

Ryan PR, Kochian LV. 1993. Interaction between aluminum toxicity and calcium-uptake at the root apex in near-isogenic lines of wheat (*Triticum-aestivum* L) differing in aluminum tolerance. *Plant Physiology* 102: 975-82

Streit B, Stumm W. 1993. Chemical properties of metals and the process of bioaccumulation in terrestrial plants In *Plants as biomonitors: indicators for heavy metals in the terrestrial environment*, ed. B Markert, pp. 31–60. Weinheim- Cambridge

Tipping E. 2005. Modelling Al competition for heavy metal binding by dissolved organic matter in soil and surface waters of acid and neutral pH. *Geoderma* 127: 293-304

Ure AM. 1996. Single extraction schemes for soil analysis and related applications. *Science of the Total Environment* 178: 3-10

Vanguelova EI, Hirano Y, Eldhuset TD, Sas-Paszt L, Bakker MR, et al. 2007. Tree fine root Ca/Al molar ratio - Indicator of Al and acidity stress. *Plant Biosystems* 141: 460-80

Wang XP, Shan XQ, Zhang SZ, Wen B. 2004. A model for evaluation of the phytoavailability of trace elements to vegetables under the field conditions. *Chemosphere* 55: 811-22

Watanabe T, Jansen S, Osaki M. 2006. Al-Fe interactions and growth enhancement in *Melastoma malabathricum* and *Miscanthus sinensis* dominating acid sulphate soils. *Plant Cell and Environment* 29: 2124-32

Watanabe T, Osaki M. 2002. Mechanisms of adaptation to high aluminum condition in native plant species growing in acid soils: A review. *Communications in Soil Science and Plant Analysis* 33: 1247-60

Watanabe T, Osaki M, Tadano T. 2001. Al uptake kinetics in roots of *Melastoma malabathricum* L. - an Al accumulator plant. *Plant and Soil* 231: 283-91

Wood S, Sebastian KL, Scherr SJ, Institute WR. 2000. *Pilot analysis of global ecosystems: agroecosystems*. World Resources Institute.

Xie ZM, Ye ZH, Wong MH. 2001. Distribution characteristics of fluoride and aluminum in soil profiles of an abandoned tea plantation and their uptake by six woody species. *Environment International* 26: 341-46

Zu YQ, Li Y, Chen JJ, Chen HY, Qin L, Schvartz C. 2005. Hyperaccumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China. *Environment International* 31: 755-62

CHAPTER 5

METAL HYPERACCUMULATION TRENDS WITHIN *PLANTAGO* PHYLOGENY (PLANTAGINACEAE)

Plantago holosteum Scop.

CONTENTS

METAL HYPERACCUMULATION TRENDS WITHIN *PLANTAGO* PHYLOGENY (PLANTAGINACEAE)

ABSTRACT

The phylogeny of metal accumulation (Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn), particularly hyperaccumulation, has been poorly studied. We focus our study on metal accumulation and hyperaccumulation within genus *Plantago*, in particular in the Mediterranean subgenus *Coronopus* in face of the high number of species reporting metal accumulation and hyperaccumulation. We analysed metal accumulation and hyperaccumulation data from direct and indirect (bibliographic sources) measures, together with species autoecology combined with phylogenetic analysis. Our hypothesis stated that metal accumulation, namely the hyperaccumulation trait, was associated with the phylogeny of genus *Plantago*, in particular for *P.* subgenus *Coronopus*.

To complete the *Plantago* phylogeny, the taxa *P. algarbiensis*, *P. almogravensis* and *P. holosteum* were included in the molecular (ITS+*trnLF*) phylogeny of *Plantago*, as part of *P.* subgen. *Coronopus* sect. *Maritima*, forming a separate clade (80 BP) together with *P. subulata*.

Regarding metal accumulation, our results indicate a high frequency of hyperaccumulation in *Plantago* (9/15 species). The hyperaccumulation trait was acknowledged for Al, Cu, Zn and Pb, while abnormally high values were also recorded for Fe. For *P.* subgen. *Coronopus*, hyperaccumulation was perceived in 5/7 species (4/7 for Al, 1/6 for Cu and 2/7 for Zn); *P.* subgen. *Plantago* showed 2/3 species with hyperaccumulation (1/1 Al, ½ Pb and ½ Zn) and in *P.* subgen. *Psyllium*, apparently 3/5 species hyperaccumulate Al. In the case of Al-hyperaccumulation, the high frequency (8/12) and dispersion among *Plantago* sub-ranks is compatible with a phylogenetic link.

The correlation found between Al-hyperaccumulation and environmental stresses (Mitrakos index for Mediterranean cold and drought stresses) calculated from the species distribution in the Iberian Peninsula, together with the variety of other metals accumulated (Fe, Cu and Zn) suggests that the maintenance of *P.* subgen. *Coronopus* hyperaccumulation traits may have a connection with the Mediterranean climate.

Our work shows that the Al-hyperaccumulation trait in *Plantago* and particularly in *P.* subgen. *Coronopus* has a phylogenetic link and contributes to the scarce knowledge of that uncommon trait, namely in Mediterranean plants.

INTRODUCTION

The accumulation trait in plants refers to the ability of concentrate an element (usually metals) in the shoot, above the normal concentration for plants (Markert 1992b; McGrath et al. 2000; van der Ent et al. 2013). One extreme of this trait is hyperaccumulation, where that concentration reaches over 10 to 1000 times the normal range for plants (Brooks et al. 1977; van der Ent et al. 2013). From the current knowledge it is not yet clear if accumulation and hyperaccumulation are part of the same continuum or are indeed two separate phenomena associated to different traits (Broadley et al. 2001; van der Ent et al. 2013; Watanabe et al. 2007). A species is considered a hyperaccumulator when the shoot metal concentration is above a defined threshold, measured in plants (at least one) from natural populations (Reeves 1992). The concept of being from natural populations is important because those particular plants should be able to thrive in metalliferous environments, that is to say reproduce and maintain a viable population. The hyperaccumulation trait is restricted to a small number of plant species. About 0.2% of angiosperm species are known to hyperaccumulate metals/metalloids (Mostly Ni but also Zn, Cd, Mn, As, Se and, in less or doubtful cases, Pb, Cu, Co, Cr and Tl; (Cappa and Pilon-Smits 2014; Krämer 2010; Pollard et al. 2014; van der Ent et al. 2013; Verbruggen et al. 2009) while only ca. 0.06% have been recorded to hyperaccumulate Al (Chenery 1948; Metali et al. 2012; Jansen et al. 2002; Watanabe et al. 2007).

Though initially most hyperaccumulator species where envisaged as obligate metallophyte plants, it is now accepted that there can also exist facultative metallophytes (Baker et al. 2010; Pollard et al. 2014; van der Ent et al. 2013) with populations in both metalliferous and non-metalliferous areas (Roccotiello et al. 2015). In this case, the hyperaccumulation trait might be only detected in the populations of the metalliferous sites, and the term facultative hyperaccumulation has been adopted in parallel to facultative metallophyte (Reeves 2006; van der Ent et al. 2013). Considering that plant metal concentrations varies a lot with local soil availability, a positive result for high metal accumulation from natural populations, would confirm the presence of the trait in the population while a negative result would not imply absence of the trait.

As a survival strategy, the hyperaccumulator plant invests, not only in (a) enhanced metal uptake, but also in (b) restraining the internal ionic activity of the metal, in (c) converting the metal to an innocuous form and/or (d) storing and discarding it (Jansen et al. 2002; Krämer 2010; Poschenrieder et al. 2011). All these processes are energy-expensive and, therefore, plants that accumulate metals are often weak competitors (Baker et al. 2010; Brady et al. 2005). The evolutionary origin of the metal accumulation trait or hyperaccumulation is not yet clear and has been related to hyper-tolerance; elemental defence against herbivory or diseases; environmental stress tolerance, namely drought; inadvertent uptake or allelopathy (Boyd 2007; Boyd and Martens 1992).

Despite many studies on the metal hyper- and accumulation trait, regarding possible applications (e.g.) in phytoremediation or ore detection, only in 2001 the relation to phylogeny was approached to quantify the phylogenetic influence on plant metal accumulation (Cd, Cr, Cu, Ni, Pb, Zn; Broadley et al. 2001). By analysing the phylogenetic lineage, one can find clues to the origin of a trait and to the context where it was selected, as traits can be maintained or modified according to selective pressures and chance. Yet, close species should resemble more than species drawn at random from the same phylogenetic tree (Münkemüller et al. 2012). Most attempts to assess the metal hyper- and accumulation trait in a phylogeny are difficult because metallophytes are often associated with severe geo-localized selection pressures (Baker et al. 2010). Thus, accumulation is often related to only some populations (ecotypes) among species. In general, metal hyperaccumulation (Ni, Zn, Cd, As, Se, Cu, Co, Mn, Pb, Cr) is found isolated in one or a few species within a family or even within an order, suggesting numerous independent events (Broadley et al. 2001; Cappa and Pilon-Smits 2014; Reeves and Baker 2000; White 2012). Significant correlations of the metal accumulation trait with phylogeny have been found at the classification level of order and above (Broadley et al. 2001). The main exception is Ni in the Family Brassicaceae, for the genera *Alyssum* (Ni) and *Thlaspi* (Ni and Zn) with most species being hyperaccumulators (Verbruggen et al. 2009). Notwithstanding, the aluminium (Al) hyperaccumulation trait was related to primitive (basal) branches of fairly advanced clades (e.g. Rosids and Asterids) corresponding to a stronger phylogenetic link (Jansen et al. 2002). Works thereafter (e.g. Metali et al. 2012; Watanabe et al. 2007), have shown Al-accumulation to be phylogenetically related above the species level, with higher predominance in some families than in others. The main differences between Al and other metals, which may account for the presence of the phylogenetic signal, are the ubiquity (Al is the 3rd most abundant element on the Earth's crust) and the strong relation with soil pH (acid soils have more available toxic Al). Therefore, most of Al-hyperaccumulator families (also the highest hyperaccumulators) are found in tropical climates in acidic soils, where Al is easily soluble (Haridasan and Araújo 1988; Jansen et al. 2002; Jansen et al. 2004; Metali et al. 2012). In temperate climates, the proportion of Al-hyperaccumulators is believed to be less than half that of tropical climates (Metali et al. 2012), but the information is scarce on Al-hyperaccumulation for Mediterranean angiosperms (e.g. Batista 2003; Branquinho et al. 2007; Serrano et al. 2011[1]; Turnau et al. 2007). In fact no study has so far focused on Al-hyperaccumulation in Mediterranean angiosperms (Chenery 1948; Jansen et al. 2002; Metali et al. 2012; Watanabe et al. 2007).

The Portuguese endangered endemic *Plantago almogravensis* Franco is an Al-hyperaccumulator (Branquinho et al. 2007). The species belongs to *P.* subgenus *Coronopus*, which is endemic or distributed in the Iberian Peninsula and Mediterranean area (Pedrol 2009; Rahn 1996; Rønsted et al. 2002). Other species of *Plantago* have also been reported having high metal concentrations (e.g. Broadley et al. 2001; Reeves and Baker 2000). Yet, before Branquinho et al. (2007) the family was not referenced for Al-accumulation (Jansen et al. 2002). In 2002, Rønsted et al. using nuclear ribosomal ITS and plastid *trnL-F* sequence data, produced a molecular phylogeny for *Plantago*. It confirmed most of previous morphological, embryological and chemical results (Rahn 1996) for the infra-classification of the genus, thus providing a fair phylogeny for *Plantago*.

In the present work, we proposed to evaluate the presence of the metal hyperaccumulation trait in the phylogeny of *Plantago* with emphasis on the species of *P.* subgen. *Coronopus*. Considering that *P. almogravensis* is an Al-hyperaccumulator and that phylogenetic links have been described for Al-hyperaccumulation, we hypothesized that we would find more *Plantago* taxa with this accumulation trait. We also wanted to evaluate if the distribution of other metals accumulated by the genus (Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn), follow a similar accumulation trend as Al. Since that *Plantago* phylogeny is still unclear for some Iberian species, we revisited the ITS+*trnLF* molecular phylogeny (Rønsted et al. 2002) and added three more Iberian taxa, expecting to disentangle their taxonomic proximity and metal accumulation properties, and contributing to strengthen the knowledge about the genus.

MATERIALS AND METHODS

Plant sampling and data collection

For the species identification, we followed *Nova Flora de Portugal* (Franco 1984), Flora of the Canary Islands (Bramwell 1997) and *Flora Iberica* (Pedrol 2009). For the taxonomic ranks within genus *Plantago* we adopted the classification of Rønsted et al. (2002) after Rahn (1996) that considers five subgenera divided further into sections (Tab. 1). The classification of subgenus *Coronopus* has not been a simple task in the Euro-Mediterranean area, with many taxa changing name and taxonomic rank (e.g. see the long synonyms lists in Pedrol 2009), based mostly on the

---

[1] Chapter 4

comparison of morphologic characters from herbarium specimens alone. As such, for subgenus *Coronopus* we considered the taxa *sensu* Pedrol (2009) except for *P. algarbiensis* Samp. and *P. almogravensis* Franco, considered independent species *sensu* Franco (1984) and by our own observations (Chapter 2). Conversely, we collected material identified as *P. radicata* by Franco (1984), that more recently it is considered a synonym of *P. holosteum* (*sensu* Pedrol 2009), but we adopted the latter name, as we did not have any other base for comparison.

Concerning the data extraction from bibliographic sources (references in Tab. 1), we searched for metals analysed in the shoots of *Plantago* from natural populations or, at least, from soil grown plants originating in seeds of natural populations in metalliferous or contaminated sites. If individual sample values were not available, minimum, maximum, median or mean values (from text or estimated from charts) were acknowledged. Concentration values of zero or below the detection limit were replaced by the detection limit. In the bibliographic search we noticed references to As (Bech et al. 2012; Chopin and Alloway 2007; Orłowska et al. 2012) and Se (Mężyk and Więckowski 1999) but with low values and for less than three species, thus we did not include them. We also did not include species results with only a single

Table 1. Species collected in the field and bibliographic references of metal accumulation.

Species	**Distribution**	**Origin[a] (MGRS)**	**DNA sequencing**	**Metal analysis**	**Bibliographic data (metals)**
Subgen. Coronopus, sect. Maritima					
P. algarbiensis Samp.	Iberian Peninsula	Pt: Ag (29SNB61)	+*	+*	-
P. almogravensis Franco	Portugal	Pt: Bal (29SNB17)	+*	+	4,18
P. holosteum Scop.[bc]	Mediterranean	Pt: TM[b],TM[b]; Hs: Le[c] (29T-NF96, -PF89, -QG47)	+[b]*/+[b]*/-	-/+/+	8,9,11,19
P. alpina L.	Europe	Hs: O (29TQH37)	-	+*	-
P. maritima L.[d]	Cosmopolite	Hs: O (29TPJ92)	+[d]*	+	7
Subgen. Coronopus, sect. Coronopus					
P. coronopus L.	Euro-Mediterranean	Pt: Ag, R, Aal, E (29S-NB60, -ND36, -NC86, -MC86)	-/-/-/-	+/+/+/+	5
P. serraria L.	Mediterranean	Pt: E (29SMC79)	-	+*	-
Subgen. Plantago sect. Plantago					
P. asiatica L.[e]	S & E Asia				20,21
P. major L.	Cosmopolite	Pt: E (29SMC88)	-	+	1,6,10,22
Subgen. Plantago sect. Virginica					
P. australis Lam.	America				3
Subgen. Psyllium sect. Psyllium					
P. afra L.	Mediterranean	Pt: E (29SMD91)	-	+*	-
P. arborescens Poir.	Macaronesia	Pt: Md (28SCB)	+*	-	-
P. arenaria Waldst. & Kit.	Mediterranean		-	-	16,17
Subgen. Psyllium sect. Hymenopsyllium					
P. bellardii All.	Mediterranean	Pt: Ag (29SPB01)	-	+*	-
Subgen. Psyllium sect. Lanceifolia					
P. lagopus L.	Mediterranean	Pt: Aal, E (29S-PC13, -MC86)	-/-	(+/+)*	-
P. lanceolata L.	Cosmopolite	Pt: R, E, E (29S-ND69, -MC99, -MC86)	-/-/-	+/+/+	2,6,8,12,13,14,15,19

[a] **Origin:** Portugal (Pt): (Ag) Algarve, (AAl) Alto-Alentejo, (BAl) Baixo-Alentejo, (E) Estremadura, (Md) Madeira Islands, (R) Ribatejo, (TM) Trás-os-Montes; Spain (Hs): (Le) León, (O) Asturias. [b] Synonym *P. radicata* Hoffms. & Link.; [c] syn. *P. subulata* L. subsp. *radicata*. [d] *P. maritima maritima* L. (the subspecies used was unknown in Rønsted et al. 2002 work). [e] Syn. *P. orbignyana* Steinheil. **MGRS:** Military Grid Reference System coordinates (10x10 km). Plant material used (+) or not used (-) in sequencing or metal analysis. *Novelty data (previously unreported DNA sequences or species metal analysis). **Bibliographic data:** 1- Babalonas et al. 1987; 2- Barrutia et al. 2011; 3- Bech et al. 2012; 4- Branquinho et al. 2007; 5- Chopin and Alloway 2007; 6- Djingova and Kuleff 1999; 7- Fitzgerald et al. 2003; 8- Freitas et al. 2004; 9- Güleryüz et al. 2002; 10- Kabata-Pendias 2010; 11- Lazaro et al. 2006; 12- Lestan et al. 2003; 13- Mężyk and Więckowski 1999; 14- Nadgórska-Socha et al. 2013; 15- Orłowska et al. 2012; 16- Remon et al. 2007; 17- Remon et al. 2013; 18- Serrano et al. 2011[1]; 19- Shallari et al. 1998; 20- Zhou and Wei 2010; 21- Ye et al. 2009; 22- Yoon et al. 2006. The list of references for the DNA sequencing is on Annex I.

metal (e.g. Cd in *P. depressa*; Zhou and Wei 2010).

To complement these results of metal accumulation, we collected *Plantago* spp. leaves from the three terrestrial *Plantago* subgenera present in the Iberian Peninsula (*Coronopus, Plantago* and *Psyllium*), and used them for metal analysis (Tab. 1). For at least six species these were the first known results concerning metal accumulation in natural populations. The entire green leaf rosettes were used, with no discrimination on leaf age. For most species *n* = 1 to 4, each comprising bulked samples of leafs from several plants, from the same location; for *P. algarbiensis n* = 4 to 35 samples and *n* = 16 samples for *P. almogravensis*.

The fresh *Plantago* spp. leaves were cleaned (Branquinho et al. 2007) and dried at 70 °C, before grinding in a ball mill. The resulting material was digested with HNO_3 (65% HNO_3: 30% H_2O_2, 4:1) and analysed by Atomic Absorption Spectrometry according to Branquinho et al. (2007) and Serrano et al. (2011[2]) for the metals Al, Co, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn. The results were expressed per leaf dry weight.

Data analysis: metal accumulation

Considering a theoretical curve for metal concentration in the shoot (Fig. 1), and the fact that accumulation is considered extraordinary, we defined levels of accumulation (hyper, very high, high, low or no-accumulation) from the range between “normal” plant concentrations and the hyperaccumulation thresholds, simply by dividing it in thirds (33%, 67%; Fig. 1). As such, in the calculations we adopted:

(i) “Normal” reference plant concentration values for each metal, obtained from the *Worldwide Standard Reference Plant* project (Markert 1992a), such as (mg kg^{-1}) for Al (80), Cd (0.05), Co (0.2), Cr (1.5), Cu (10), Fe (150), Mn (200), Ni (1.5), Pb (1) and Zn (50), and

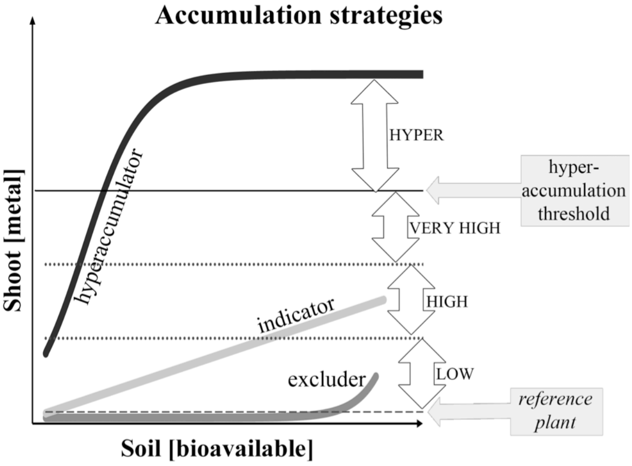


Figure 1. Conceptual accumulation strategies (adapted from Markert 1992b, McGrath et al. 2000 and van der Ent et al. 2013). The hypothetical shoot metal-accumulation strategies (exclusion, indication and hyperaccumulation) are represented in relation to soil availability. To define levels of accumulation (hyper, very high, high, low or no-accumulation) the range between normal concentration (*reference plant*; horizontal dashed line) and the hyper-accumulation threshold (horizontal filled line) was divided in thirds (33% and 67%; dotted lines).

(ii) Hyperaccumulation thresholds suggested for each metal (mg kg^{-1}) for Al (1000), Cd (100), Co (300), Cr (300), Cu (300), Fe (3000), Mn (10000), Ni (1000), Pb (1000) and Zn (3000) (Jansen et al. 2002; Reeves 2006; van der Ent et al. 2013). For Fe (no threshold defined in literature), we considered the same threshold as Zn because of similar plant concentration’s range and micronutrient function.

We made the following assumptions: (i) the shoot concentrations within the *very high* to *hyper* levels (Fig. 1), are probably of plants that have the ability to hyperaccumulate (the hyperaccumulation trait) in the presence of bioavailable metals in the soil; (ii) the plants that present a *high* level of metals are probably facultative hyperaccumulators collected in soils with low availability, or indicator plants from richer soils; (iii) the plants with a *low* level of accumulation, have a higher probability of being excluder plants from metal rich areas or indicator plants from metal poor

---

[2] Chapter 4

areas; (iv) and finally, most *non-accumulator metal-excluder plants* would have metal concentrations below those of the *reference plant* (Fig. 1).

Because of the diversity of ranges among the different elements, and to homogenize the values to comparable ones, when required they were standardized (eq. 1) such that, Y = 0 would be equal to the *reference plant* concentration; and Y = 1 the same value as the hyperaccumulation threshold.

$$Y = (x_i - x_n) / (x_H - x_n) \qquad \text{eq.1}$$

For each metal, x_n is the *reference plant* concentration, x_H is the hyperaccumulation threshold and x_i is the sample value to standardize (from analytical results or bibliography). In the text, these standardized values were identified by a ST tag (e.g. $\tilde{X}_{ST} = 1.37$).

The non-parametric Independent-Samples Kruskal-Wallis Test was used to compare between the groups of data, with significant differences in the distribution considered at the $p = 0.05$ level. One-Sample Wilcoxon Signed Rank Tests were used to compare the data median ($\tilde{X}$) to hypothetical median values. Due to the low number of *Plantago* species ($n = 15$) to which we obtained metal concentrations results, we could not make a statistically robust quantification of phylogenetic signal (e.g. Broadley et al. 2001 or Münkemüller et al. 2012) and decided not to include it.

DNA extraction, amplification and sequencing

For the DNA analysis, the plants collected comprised (Tab. 1 and Annex I):

(i) taxa not included in any previous molecular phylogenetic analysis: *P. algarbiensis, P. almogravensis* and *P. holosteum* (syn. *P. radicata sensu* Franco 1984); and

(ii) taxa with only one genomic region previously analysed (*P. arborescens,* ITS sequence replaced and *trnL-F* sequence added) or, that had no information about the origin in the work of Rønsted et al. (2002) (*P. maritima* sequences replaced by *P. maritima maritima*).

(iii) from the databases we also added sequences missing in Rønsted et al. (2002) original work and now available: ITS for *P. nubicola* (Hoggard et al. 2003).

DNA from the collected plants (n = 1 plant per species or location) was extracted from leaf material (0.3 g silica-gel-dried or 1.0 g fresh) with a version of the 2 % (w/v) CTAB extraction method modified from that of Doyle and Doyle (1987). Some of the samples were further cleaned from polysaccharides with one half volume of 7.5 M ammonium acetate (Weising et al. 1994).

Amplification of the nuclear ribosomal internal transcribed spacer (ITS) region was carried out with primers AB101 and AB102 (Sun et al. 1994), DMSO (2%), 1.5 mM $MgCl_2$ and 0.1 mg/mL BSA. Thermocycler conditions employed for ITS amplification were an initial cycle of 1 min at 97°C followed by 27 cycles of: 1 min denaturation (97°C), 1 min annealing (50°C) and 3 min extension (72°C). After the last cycle, the temperature was kept at 72°C for a final 6 min extension.

The plastidial *trnL-F* region was amplified with ‘c’ and ‘f ’ primers (Taberlet et al. 1991). The PCR mix included 1.5 mM $MgCl_2$ and 0.1 mg/mL BSA. Amplification conditions consisted of 1min at 95°C followed by 29 cycles of: 1 min denaturation (95°C), 1 min annealing (50°C) and 1 min extension (72°C). After the last cycle, the temperature was kept at 72°C for a 7 min extension.

Amplification products were purified with Exo-AP Clean-up method using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) according to the manufacturer protocol. The cleaned products were used in sequencing reactions according to the protocol for BigDye Terminator v3.1

Cycle Sequencing Kit from Applied Biosystems (USA). Both DNA strands were sequenced in an ABI 3730 XL Genetic Analyzer apparatus from Applied Biosystems (USA). The chromatograms were edited and assembled with Bioedit v.7.1.3.0 software (Hall 1999).

Phylogenetic analyses

Sequences alignments were performed in ClustalX v.2.0.10 (Thompson et al. 1994) using default parameters and subsequently adjusted visually. The new sequences reported in this study were submitted to GenBank (accession no. KJ579140 to KJ579149, Annex I).

In order to position the new sequences in the *Plantago* phylogeny a second dataset was compiled from GenBank. This consisted in ITS and *trnL-F* sequences from *Aragoa*, *Plantago* and *Veronica* (Annex I). Data were analysed as separate ITS and *trnL-F* matrices and as a combined data set obtained using the software Concatenator v1.1.0 (Pina-Martins and Paulo 2008).

Phylogenetic analyses were performed using PAUP® v4.b10 for MAC (Swofford 2003). Gaps were inserted at positions where indels occurred and, these areas, were treated as missing data in the nucleotide matrix (Swofford 2003); multistate taxa interpreted as uncertainty. Trees were obtained using random sequence addition (1000 replicates), searched using equally weighted maximum parsimony (Fitch 1971), with tree bisection-reconnection (TBR) branch swapping for best trees and no tree limit (auto-increase). Strict and majority rule (50%) consensus trees were produced and were rooted using *Veronica spp.* as outgroup (Rønsted et al. 2002). Internal support was assessed by bootstrapping, computed after resampling followed by a parsimony reconstruction (500 replicates of heuristic search; random sequence addition, 1000 replicates), TBR branch swapping and no tree limit (Harrison and Langdale 2006). We report only scores greater than 50% bootstrap (50 BP) for clades present in the strict consensus tree.

Ecology of *P.* subgen. *Coronopus* in the Iberian Peninsula

Data from the distribution, habit and habitat of subgenus *Coronopus* species within the Iberian Peninsula (Tab. 2) were gathered from field observations, floras (Franco 1984; Pedrol 2009) and dedicated websites (Anthos 2012; Araújo et al. 2013). When there were conflicting synonyms, the taxa from Anthos (2012) and Araújo et al. (2013) were modified *sensu* Pedrol (2009) (e.g. if *P. subulata* was referenced outside the Gerona region then it was considered as *P. holosteum sensu* Pedrol (2009).

Mitrakos stress indexes: The locations of 16 Iberian *Plantago* species from subgenera *Coronopus*, *Plantago* and *Psyllium* (2179 points in the 10x10 km MGRS grid corresponding to 4643 species locations; the list of points can be obtained from the author) were crossed with meteorological data for the Iberian Peninsula (Hijmans et al. 2005) to extract the Mediterranean plant stress indexes of Mitrakos (Mitrakos 1981): the Summer Drought Stress (SDS) and the Winter Cold Stress (WCS).

The SDS is calculated as the sum of the stresses (D, eq.2) from June, July and August, based on the monthly precipitation (P, in mm):

$$D = 2*(50-P) \qquad \text{eq.2}$$

The WCS is calculated as the sum of the stresses (C, eq.3) from December, January and February, based on the mean minimum monthly air temperature (t, in °C):

$$C = 8*(10-t) \qquad \text{eq.3}$$

The indexes range between 0 (no stress) and 100 (extreme stress), thus the SDS and WCD range between 0 and 300. These indexes reflect the view that Mediterranean plants are under stress when experiencing less than 50 mm of precipitation in the sum-

Table 2. Habit, habitat and occurrence of *Plantago* subgenus *Coronopus* species, of the Iberian Peninsula.

Sections	Species	Endemicity[1]	IBP Biogeographic regions[1]	IBP MGRS[3] (%)	IBP Regions[2] (%)	EUR + MED + MAC Regions[2] (%)	Altitude (m)	Habit
Maritima	*P. algarbiensis* [4]	IBP	MED (Coast)	0.4%	3%	3%	20-100	Dwarf shrub, branched stock / cushion like, with terminal rosettes; leaves somewhat rigid, triquetrae.
	P. almogravensis [5]	Lu	MED (Coast)	0.09%	2%	1%	0-50	
	P. holosteum	MED	MED (Mountain) & ALP	16%	65%	24%	700-2000	
	P. subulata	MED	MED (Coast)	0.3%	2%	15%	0-100	
	P. maritima	EUR + MED + others	ATL (Coast) to MED & ALP	17%	65%	64%	0-50 & 400-1800	Perennial forbs with basal rosettes, sometimes a branched stem/stock; leaves flat to fleshy.
	P. alpina	EUR + MED	MED (Mountain) & ALP	6%	28%	19%	1350-2800	
Coronopus	*P. coronopus*	EUR + MAC + others	MED & ATL	37%	97%	73%	0-1100	Annual, biannual or occasionally perennial forbs, basal rosettes.
	P. macrorhiza	IBP + MED	MED (Coast)	0.6%	5%	16%	0-50	Perennial forbs with basal rosettes, sometimes a branched stem/stock; leaves flat to fleshy.
	P. serraria	IBP + MED	MED	4%	22%	25%	50-660	
	P. crassifolia	IBP + MED	MED (Coast)	3%	15%	42%	0-200	

[1] **Occurrence:** Alpine (ALP), Atlantic (ATL), Europe (EUR), Iberian Peninsula (IBP), Mediterranean (MED), Macaronesia (MAC), Portugal (Lu). [2] **Regions:** percentage of occurrence in the Iberian provinces (out of 60 total; Pedrol 2009) or in the Europe+Mediterranean regions (67 total; Marhold 2011). [3] **MGRS:** percentage of occurrences acknowledged in the MGRS 10x10 km grid (2179 total data squares; Anthos 2012, Araújo et al. 2013 and personal observations). [4] Less than 10 populations known. [5] Only one population known (ca. 3 ha).

mer months or less than 10˚C in the winter months. Student's t-Test was used to compare locations /species.

RESULTS

Update of the ITS-*trnLF* *Plantago* phylogeny

The former *Plantago* phylogeny (Rønsted et al. 2002) included the *P.* subgen. *Coronopus* sect. *Maritima* species *P. alpina, P. maritima* and *P. subulata*, but left out taxa that have been difficult to discriminate. We added three of those Iberian taxa (*P. algarbiensis, P. almogravensis* and *P. holosteum* syn. *P. radicata*) to *Plantago* combined ITS+*trnLF* molecular phylogeny. From the analysis, the three species were included in subgenus *Coronopus*, in accordance to the classification by classic taxonomic methods (Franco 1984; Pedrol 2009) (Tab. 1). These three taxa formed their own clade (74 BP), paraphyletic with *P. subulata* and together creating a subgroup (80 BP) in section *Maritima* (Fig. 2).

We also analysed a sample of *P. algarbiensis* from Huelva (Spain), distant about 110-140 km from the Portuguese ones, and a putative *P. holosteum* (or *P. alpina*) from the Pyrenees (Spain), both clustering

Table 3. Data for the final aligned matrices and parsimony analysis results (heuristic search).

Region	Taxa (*n*)	Matrix length (bp)	Variable characters (%)	PI characters (%)	Trees produced (n)	Length ST (steps)	CI	RI
ITS	65	783	41	32	113	955	0.534	0.825
trnL-F	60	1082	28	19	536	451	0.783	0.921
Combined	65	1865	34	24	831	1424	0.606	0.852

Parsimony informative (PI); shortest tree (ST); consistency index (CI); retention index (RI).

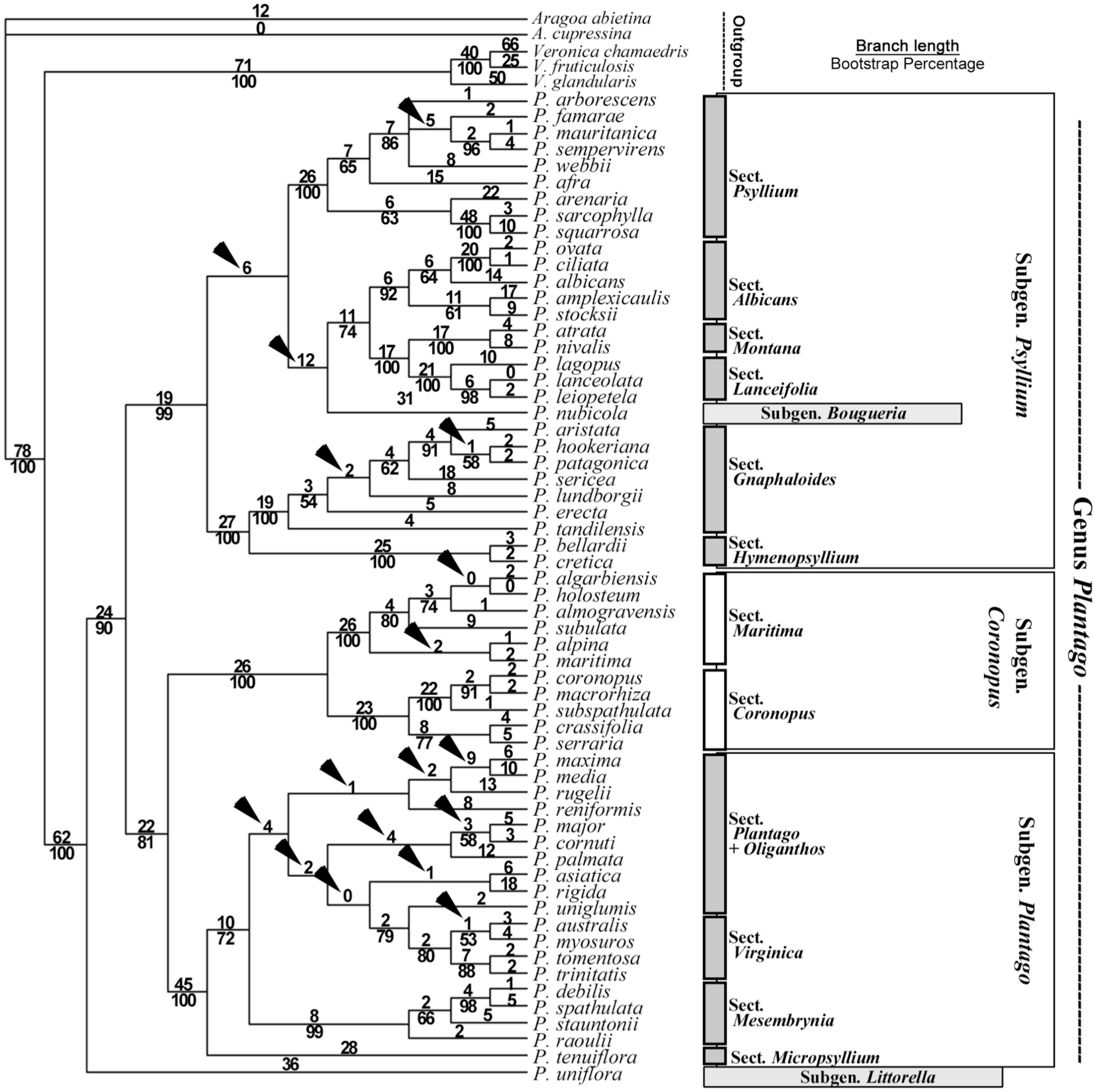


Figure 2. Combined (ITS+*trnL-F*) tree. One of the most parsimonious trees obtained from the combined ITS and *trnL-F* regions analysis. Branch length (bp) and bootstrap percentages (BP) are shown above and below the branches, respectively. Consistency index, CI = 0.606; Retention index, RI = 0.852. Branches not present in the strict consensus tree are marked with an arrow. The subgenera and sections after Rønsted et al. (2002) are indicated.

within the same clade (70 BP) as the other samples, thus were considered redundant for this analysis and were not included in the results (data not shown). As a curiosity, the *P. maritima* sequences from the original (Rønsted et al. 2002) work were paraphyletic with those we used (*P. maritima maritima*), and the latter was monophyletic with *P. alpina* (53 BP), suggesting that the original *P. maritima* was a different subspecies (origin unknown at Rønsted et al. 2002).

Regarding the remaining species within the updated phylogeny, the results obtained were similar to those of Rønsted et al. (2002), as expected. In the combined analysis of the two genomic regions (Fig. 2; Tab. 3), *P.* subgen. *Coronopus* is sister to *P.* subgen. *Plantago* (81 BP), and paraphyletic with the remaining subgenera. The two sections of *P.* subgen. *Coronopus* (*Maritima* and *Coronopus*) are monophyletic and have high bootstrap support (100 BP each). The separate results for the ITS and *trnL-F* phylogenies, are presented in the Annexes II & III.

Environmental range of *P.* subgen. *Coronopus* within the Iberian Peninsula

Following the phylogeny in Fig. 2 for the species found in the Iberian Peninsula we related them to their distribution and potential environmental stress (Fig. 3).

Section *Coronopus* of *P.* subgen. *Coronopus* is composed mostly of perennial forbs with basal rosettes. Iberian species (Tab. 2) are found ca. 0–1000 m, in ruderal pastures or saline soils, on both Mediterranean and Atlantic regions of temperate to semi-arid climates, experiencing more drought stress in the summer than cold stress in winter (Fig. 3).

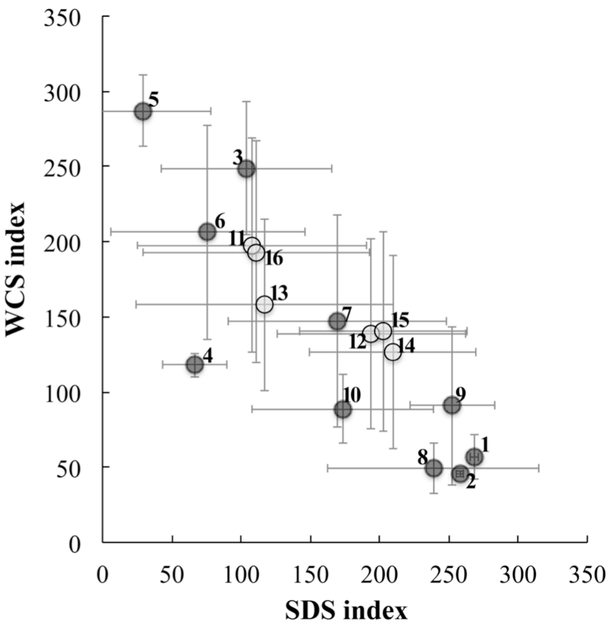


Figure 3. Mitrakos Index for Winter (WCS) and Summer (SDS) stress, for *Plantago* species in the Iberian Peninsula. WCS and SDS calculated from the species distribution in the Iberian Peninsula (mean ± SD). Circle colour (subgenus): grey, *Coronopus*; white, other subgenera. *P.* subgen. *Coronopus* sect. *Maritima:* 1- *P. algarbiensis*; 2- *P. almogravensis*; 3- *P. holosteum*; 4- *P. subulata*; 5- *P. alpina*; 6- *P. maritima*; *P.* subgen. *Coronopus* sect. *Coronopus:* 7- *P. coronopus*; 8- *P. macrorhiza*; 9- *P. serraria*; 10- *P. crassifolia*; *P.* subgen. *Plantago:* 11- P. major; *P.* subgen. *Psyllium* sect. *Psyllium:* 12- *P. afra*; 13- *P. arenaria*; *P.* subgen. *Psyllium* sect. *Hymenopsyllium*: 14- *P. bellardii*; *P.* subgen. *Psyllium* sect. *Lanceifolia*: 15- *P. lagopus*; 16- *P. lanceolata*; n = 8, 2, 346, 6, 124, 373, 814, 12, 83, 75, 618, 385, 32, 209, 511 and 1045, respectively.

Section *Maritima* of *P.* subgen. *Coronopus* is composed of perennial forbs (*P. maritima, P. alpina*) and woody-branched stock plants (*P. subulata* clade). Most of these Iberian species are challenged with extreme environmental stresses: either cold stress in winter (*P. alpina, P. holosteum* and *P. maritima serpentina*) or drought stress in the summer (*P. algarbiensis* and *P. almogravensis*) (Tab. 2 and Fig. 3).

Only the worldwide distributed *P. maritima* (the subspecies *P. maritima maritima* from the north Atlantic coast of the Iberian Peninsula) and *P. subulata* (Gulf of Leon and Genoa) inhabit milder environments. The three species added to the *Plantago* molecular phylogeny showed no overlap in their SDS range ($p < 0.001$) but regarding the WCS, *P. algarbiensis* and *P. almogravensis* experience similar cold stress ranges ($t_{(8)} = 1.023$; $p = 0.336$). The species *P. holosteum* and *P. subulata* also did not show significant differences in SDS ($t_{(350)} = 1.479$; $p = 0.140$).

The Iberian *Plantago* plants from the other subgenera (*Plantago* and *Psyllium*) were found in milder environments, with less cold or drought stresses (Fig. 3), though never without stress, according to Mitrakos index (Mitrakos 1981).

Metal accumulation

The hyperaccumulation trait was observed in the genus *Plantago* for at least five metals (Fig. 4; values above 1), considering the Reeves (1992) definition of hyperaccumulation values found at least in one plant from natural populations, and assuming that all the values collected originated from correctly decontaminated leaves. All the three subgenera presented hyperaccumulators of Al and Fe; some subgenera also showed hyperaccumulation of Zn (*P.* subgen. *Coro-*

nopus and *Plantago*), Cu (*P.* subgen. *Coronopus*) and Pb (*P.* subgen. *Plantago*). For most metals we recorded more samples with low accumulation values (near the standard plant concentration) than high accumulation, except for Al where the median was above the hyperaccumulation level ($\tilde{X}_{ST}$= 1.37; $p[\tilde{X}=1]$= 0.001). On the other extreme, the median values for Mn were below the concentration values of the reference plant ($\tilde{X}_{ST}$= -0.018; $p[\tilde{X}=0]$= 0.000) and only one sample reached a high level of accumulation (max_{ST} = 0.36). We cannot escape from some biasing of the data, as the species surveyed are only 15 out of more than 200. Nevertheless, hyperaccumulation was detected not only in cryptic species (e.g. *P. almogravensis*) but as well in common ones (*P. coronopus*, *P. lanceolata*, *P. major*).

If we consider metal accumulation as a whole (not discriminating on the metal; Fig. 4), 9/15 species reached hyperaccumulation values and 2/15 reached *very high* metal accumulations, suggesting that the hyperaccumulation traits might be present in 11/15 (73%) species observed. The *P.* subgen. *Coronopus* is the most represented with 86% (6/7) of the species showing the exceptional hyperaccumulation trait; 67% (2/3) in *P.* subgen. *Plantago* and 60% (3/5) in *P.* subgen. *Psyllium* (including the *very high* accumulation values). All the subgenera's sections were also represented with hyperaccumulators, except for *P.* subgen. *Psyllium* where we detected only 1/3 sections with hyperaccumulation (sect. *Lanceifolia*, max_{ST} = 3.8), 1/3 with very high accumulation (sect. *Hymenopsyllium*, max_{ST} = 0.79) and 1/3 with high accumulation (sect. Psyllium, max_{ST} = 0.51). Only for one species, *P. australis*, the samples represented mostly hyperaccumulation ($\tilde{X}_{ST}$ = 3.10) though where extracted from one single study (Bech et al. 2012; Tab. 1).

The results showed that hyperaccumulation of Al were apparent (maximums or medians over the 67% line; Fig. 5) in the three subgenera sampled, being in 4/7 species of *P.* subgen. *Coronopus*, 3/4 species of *P.* subgen. *Psyllium* and in the only species of *P.* subgen.

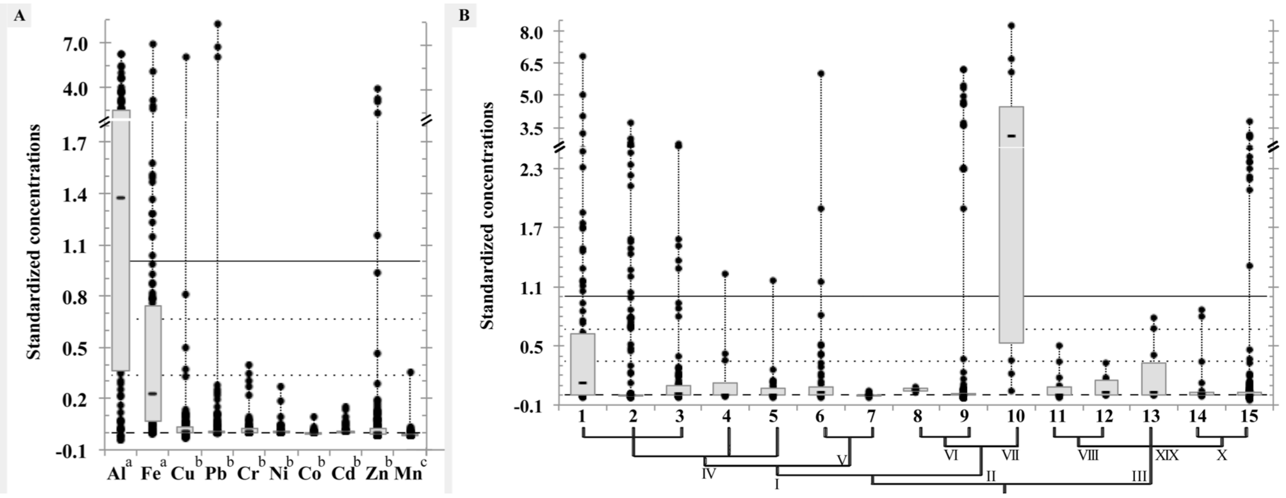


Figure 4. Shoot metal concentration in *Plantago* (A) and metal accumulation per species (B). Values standardized between (0) those of the reference plant and (1) the hyperaccumulation threshold. **Data** represented by median (–), 25–75 quartiles-box, and other values (•). **Horizontal lines:** filled – hyperaccumulation threshold; dashed – concentration of the reference plant; dotted – 33% and 67% of the range between hyperaccumulation and reference. **Subgenera:** I- *Coronopus*; II- *Plantago*; III- *Psyllium*. **Sections:** IV- *Maritima*; V- *Coronopus*; VI-*Plantago*; VII- *Virginica*; VIII- *Psyllium*; XIX- *Hymenopsyllium*; X- *Lanceifolia*. **Species:** 1- *P. algarbiensis*; 2- *P. almogravensis*; 3- *P. holosteum*; 4- *P. alpina*; 5- *P. maritima*; 6- *P. coronopus*; 7- *P. serraria*; 8- *P. asiatica*; 9- *P. major*; 10- *P. australis*; 11- *P. afra*; 12- *P. arenaria*; 13- *P. bellardii*; 14- *P. lagopus*; 15- *P. lanceolata.* For (A) the n = 92, 96, 101, 129, 71, 46, 42, 77, 163 and 89; for (B) n = 87, 160, 90, 12, 30, 57, 12, 2, 142, 12, 14, 11, 12, 20 and 245. Different letters (a–c) represent significant differences in the distribution (p ≤ 0.05, Independent-Samples Kruskal-Wallis Test).

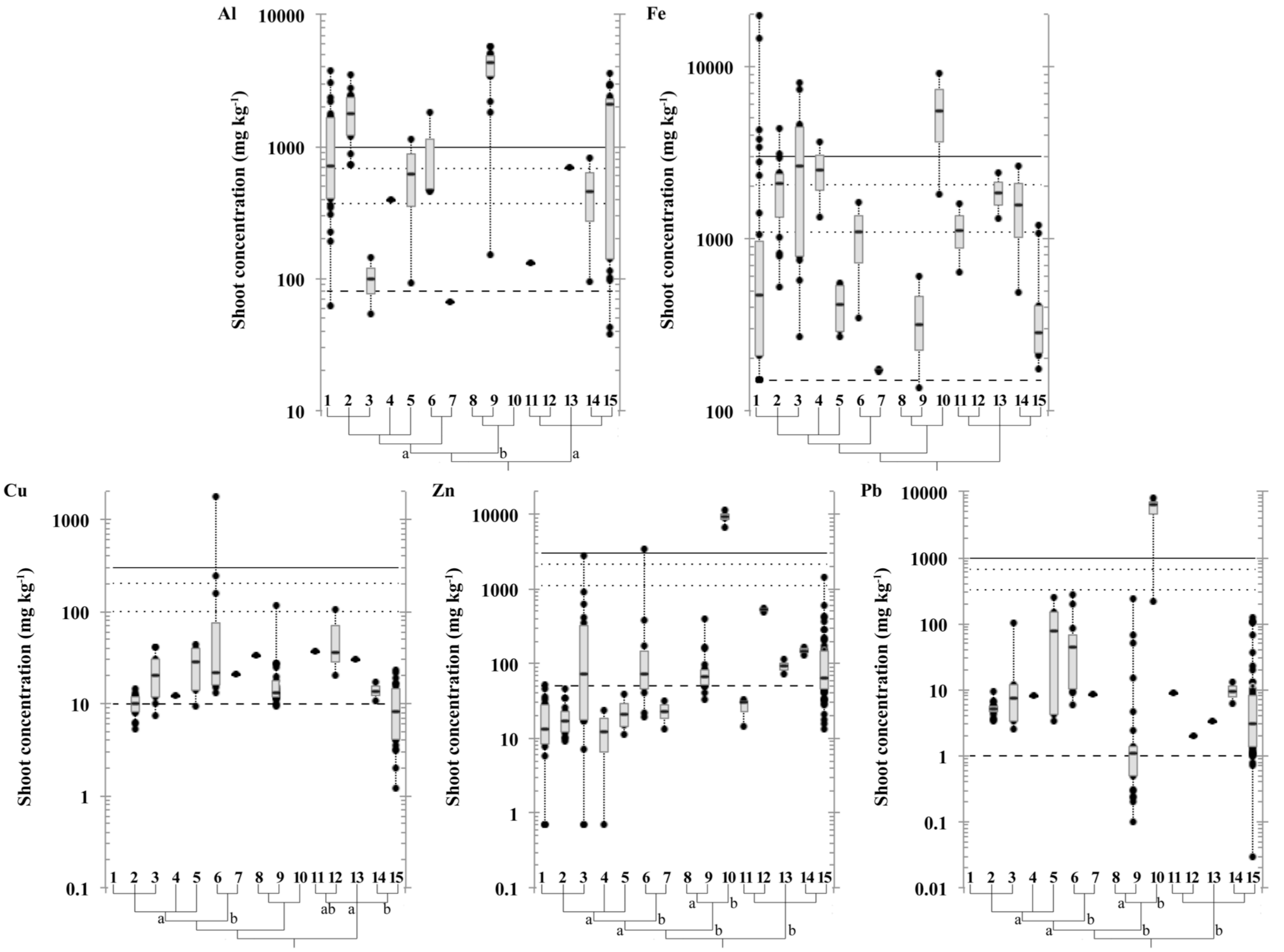


Figure 5. Metal hyperaccumulation in *Plantago* shoots I: high values. Species: 1- *P. algarbiensis*; 2- *P. almogravensis*; 3- *P. holosteum*; 4- *P. alpina*; 5- *P. maritima*; 6- *P. coronopus*; 7- *P. serraria*; 8- *P. asiatica*; 9- *P. major*; 10- *P. australis*; 11- *P. afra*; 12- *P. arenaria*; 13- *P. bellardii*; 14- *P. lagopus*; 15- *P. lanceolata*. Data represented by median (–), 25–75 quartiles-box, and other values (•). Horizontal lines: filled – hyperaccumulation threshold; dashed – concentration of the reference plant; dotted – 33% and 67% of the range between hyperaccumulation and reference. *n*(Al) = 28, 16, 2, 1, 2, 3, 1, 0, 14, 0, 1, 0, 1, 2 and 21; *n*(Fe) = 35, 16, 13, 2, 4, 3, 2, 0, 3, 2, 3, 0, 2, 2 and 9; *n*(Cu) = 0, 16, 9, 1, 5, 12, 1, 1, 25, 0, 1, 3, 1, 2 and 24; *n*(Zn) = 20, 16, 19, 2, 4, 12, 2, 0, 20, 4, 3, 2, 2, 2 and 55; , *n*(Pb) = 0, 16, 5, 1, 5, 12, 1, 0, 25, 4, 1, 1, 1, 2 and 55. Within the same rank level (subgenera or sections within a subgenus), different letters (a,b) represent significant differences in the distribution (p ≤ 0.05, Independent-Samples Kruskal-Wallis Test).

Plantago with Al results, in a total of 8/12 species (67%). The data distribution was similar between *P.* subgen. *Coronopus* and *Psyllium* and significantly different for *P.* subgen. *Plantago*, while there were no significant differences among the sections of the subgenera (among the three subgenera *p*= 0.000; Fig. 5). We have also determined that significant correlations were observed between the maximum accumulation values of Al and the Mitrakos stress indexes (SDS and WCD), at the *P.* subgen. *Coronopus* sect. *Maritima* level (*n*= 5; r_{SDS}= 0.94, *p*= 0.017; r_{WCS}= -0.98, *p*= 0.003); at the *P.* subgen. *Coronopus* level (*n*= 7; r_{SDS}= 0.62, *p*= 0.141; r_{WCS}= -0.72, *p*= 0.067) significant only when the results from the non-accumulator *P. serraria* are excluded (*n*= 6; r_{SDS}= 0.94, *p*= 0.006; r_{WCS}= -0.98, *p*= 0.001); but at the genus level there was no longer a significant correlation (*n*= 12; r_{SDS}= 0.06, *p*= 0.85; r_{WCS}= -0.22, *p*= 0.49).

The second metal more accumulated, Fe, evidenced the hyperaccumulation trait in the three subgenera also (Fig. 5), being 4/7 species in *P.* subgen. *Coronopus*, 1/2 species in *P.* subgen. *Plantago* and 2/4 species with very high accumulation in *P.* subgen. *Psyllium* (potentially hyperaccumulators), in a total of 7/13

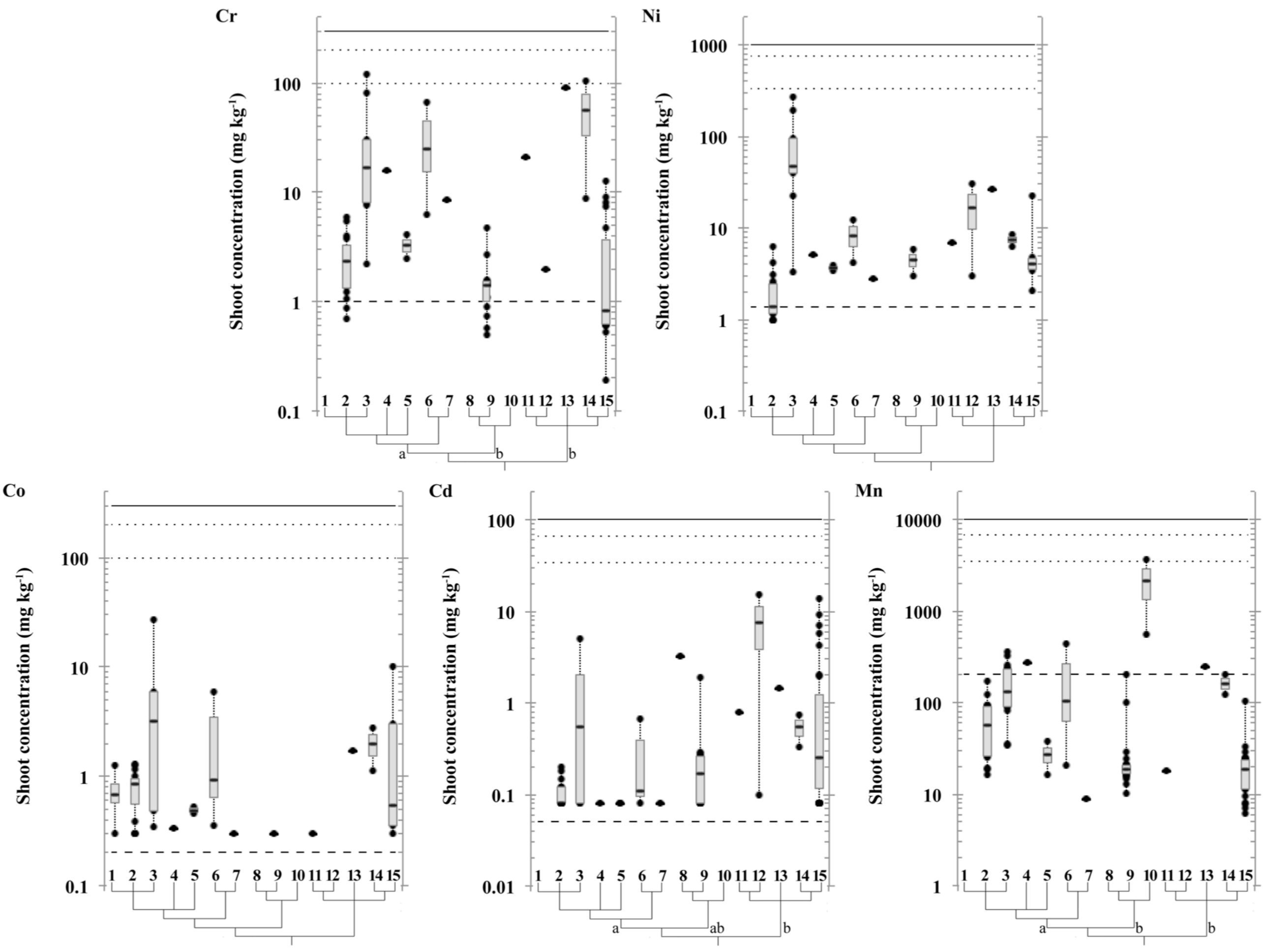


Figure 6. Metal concentration in *Plantago* shoots II: low values. Species: 1- *P. algarbiensis*; 2- *P. almogravensis*; 3- *P. holosteum*; 4- *P. alpina*; 5- *P. maritima*; 6- *P. coronopus*; 7- *P. serraria*; 8- *P. asiatica*; 9- *P. major*; 10- *P. australis*; 11- *P. afra*; 12- *P. arenaria*; 13- *P. bellardii*; 14- *P. lagopus*; 15- *P. lanceolata*. Data represented by median (–), 25–75 quartiles-box, and other values (•). Horizontal lines: filled – hyperaccumulation threshold; dashed – concentration of the reference plant; dotted – 33% and 67% of the range between hyperaccumulation and reference. *n*(Cr) = 0, 16, 9, 1, 2, 3, 1, 0, 15, 0, 1, 1, 1, 2 and 19; *n*(Ni) = 0, 16, 9, 1, 2, 3, 1, 0, 2, 0, 1, 2, 1, 2 and 6; *n*(Co) = 4, 16, 5, 1, 2, 3, 1, 0, 1, 0, 1, 0, 1, 2 and 5; *n*(Cd) = 0, 16, 4, 1, 2, 3, 1, 1, 15, 0, 1, 2, 1, 2 and 28; *n*(Mn) = 0, 16, 15, 1, 2, 3, 1, 0, 22, 2, 1, 0, 1, 2 and 23.

(54%) species. No significant differences were found either among the distribution of Fe in the subgenera (p= 0.508) or among their sections (p> 0.072).

The hyperaccumulation of Cu was recorded in 1/13 (8%) species (*P. coronopus*; Fig. 5). No significant differences were found among the distribution of Cu in the subgenera (p = 0.05) but there were differences among some of the sections within them ($p\leq$ 0.003).

For Zn, the hyperaccumulation trait was apparent in 2/7 species of *P.* subgen. *Coronopus* and 1/3 species of *P.* subgen. *Plantago*, in a total of 3/14 (21%) species (Fig. 5), however most species of *P.* subgen. *Coronopus* were often found in the non-accumulator level (below the reference plant values), having a different distribution than the other subgenera (p= 0.000). At the sections level, some significant differences were also noticed ($p\leq$ 0.002).

Finally, for Pb there is one species recorded as hyperaccumulator (*P. australis*) while most other records were within the low accumulation level (Fig. 5). The distribution of Pb values was significantly different for *P.* subgen. *Coronopus* than for the other subgenera (p= 0.000) as well as the distributions between some sections ($p\leq$ 0.002).

For the metals not hyper-accumulated in *Plantago* (Fig. 6), most were detected in low accumulation levels (Cr, Ni, Co and Cd) while, as mentioned, Mn showed mostly values below those of the reference plant. The *P.* subgen. *Coronopus* showed higher values in the distribution of Cr (p= 0.001) and Mn (p= 0.000) and lower values for Cd (p= 0.000), than the other subgenera. Among the subgenera, for Ni and Co, there were no significant differences in the distribution of concentrations (p> 0.33).

DISCUSSION

Metal hyperaccumulation trends within *Plantago* phylogeny

Considering the *reference plant* values (Markert 1992a), *Plantago* is an accumulator of several metals and probably an excluder of Mn. Metal hyperaccumulation in plants, especially for non-essential metals, is considered an abnormal trait (Brooks et al. 1977; Jaffré and Schmid 1974; and authors thereafter). Yet, within the *Plantago* genus we have detected *very high* to *hyper* accumulation of metals in 73% of the species surveyed (11/15), so *Plantago* species must have some special characteristics. Though hyperaccumulation of multiple metals has been observed (e.g. Baker and Brooks 1989), the hyperaccumulation trait should be distinguished according to the metals accumulated, as different physiological processes might be in action depending on the metal.

For the metal Al, our data suggest a phylogenetic origin for the ability to accumulate Al and consequently for the Al-hyperaccumulation trait. Considering the phylogenetic structure, hyperaccumulation was acknowledged in all three subgenera surveyed and in most of their sections, thus it seems more likely that the Al-hyperaccumulation trait has been lost 3–4 times in evolution, rather than been gained independently seven times. In fact, even in those species where it was not detected, it could be just a matter of low Al-availability in the soil, as in the case of the *P. holosteum*. The accumulation curve of *P. almogravensis* (Serrano et al. 2011[3]) depicts that situation, with the shoot concentration shown to be dependent of the soil available Al-concentrations, thus the low accumulation samples correspond to plants grown in soils with lower Al load. Within the order Lamiales (APG classification system) Al-hyperaccumulation has been acknowledged for the families Plantaginaceae (8/12 species in *Plantago*; Branquinho et al. 2007; Djingova and Kuleff 1999; Serrano et al. 2011[3]; and this work), Lentibulariaceae (2/8 *Genlisea* and 3/17 *Utricularia;* Jansen et al. 2002) and Lamiaceae (3/3 *Callicarpa*; Watanabe et al. 2007). Most reports of Al-hyperaccumulation are from tropical areas (e.g. Metali et al. 2012) while for the Mediterranean area, the reports of Al-hyperaccumulation have been scarce (e.g. Ericales/Ericaceae/*Erica*, in Turnau et al. 2007; Malvales/Cistaceae/*Cistus*, in Batista 2003). In the case of Al, our results show that especially the *P.* subgen. *Coronopus* sect. *Maritima* species, but also the accumulator species of sect. *Coronopus*, are subjected to high levels of SDS and WCD and correlate with the Al-accumulation levels, suggesting a connection of accumulation with environmental stress factors. The habit of section *Maritima* plants, is unusually woody for the genus, but not only it protects the plants from extreme environmental stresses (Baker et al. 2010; Brady et al. 2005; Cavieres et al. 2007; Serrano et al. 2015[4]; Zeppel et al. 2014), it is also commonly associated with Al-hyperaccumulation (Jansen et al. 2002).

The shoot concentrations of Fe reached *very high* to *hyper*-accumulation values in several species, according to our 3000 mg kg^{-1} hyperaccumulation criteria (see methods). This is in accordance to records of presence of these species in areas rich in Fe. In particular the *P.* subgen. *Coronopus* sect. *Maritima* species are present in in sandy-clay soils with stones or gravel, in saline soils and in metalliferous habitats, in particular, Fe-rich (rusty red soils, Fe-mine ores, exposed podzol B-horizon, and serpentine soils). The

---

[3] Chapter 4

[4] Chapter 3

accumulation curve from soil-available Fe-concentration for *P. almogravensis*, suggests a linear relationship rather than an accumulation curve (Serrano et al. 2011[3]) indicating that the species is probably an indicator of Fe ("unusual Fe accumulator"; Serrano et al. 2011[5]) and not a hyperaccumulator. Thus our proposed hyperaccumulation threshold for Fe is probably too low. Some plants can hyperaccumulate above 10 000 mg Fe kg^{-1} (*Imperata cylindrica*, in Rodriguez et al. 2005; *Eichhornia crassipes* in Streit and Stumm 1993).

Only one species reached Cu-hyperaccumulation values, *P. coronopus*, when it was sampled from areas rich in that metal (Chopin and Alloway 2007). Recently there have been doubts about the existence of true Cu-hyperaccumulators, as some reported cases seem to be due to deficient leaf decontamination (Faucon et al. 2007). In some cases, it seemed likely that even if true hyperaccumulation levels were reached, they represented the extremes of indicator behaviour, not the hyperaccumulation trait (Faucon et al. 2007). Nevertheless, some authors agree that being a micronutrient, Cu concentration is extremely controlled by the plant, and hardly deviates from ca. 10 mg kg^{-1} (Djingova et al. 2013; van der Ent et al. 2013) such that the previous 1000 mg kg^{-1} hyperaccumulation threshold was recently suggested to change to 300 mg kg^{-1} (Krämer 2010; van der Ent et al. 2013). As such, the high values obtained for *P. coronopus* represent an abnormal behaviour, whether it is bioindication or hyperaccumulation.

The Zn-hyperaccumulation trait seems to be only occasionally expressed in some species of *Plantago*. The Zn-hyperaccumulation trait was found in plants collected in metalliferous areas (Bech et al. 2012; Chopin and Alloway 2007; Güleryüz et al. 2002) and doesn't seem to have a phylogenetic link. Yet, White et al. (2012) has considered that *P. lanceolata* has a higher REML-estimated shoot Zn (RELM – REsidual Maximum Likelihood) than other angiosperm species, in accordance with our high accumulation results for this species. Like for Cu, the threshold concentrations have been under review (Broadley et al. 2007; Krämer 2010; Reeves and Baker 2000; van der Ent et al. 2013) considering that physiologically, plants that accumulate as much as 3000 mg Zn kg^{-1} are already expressing an abnormal behaviour.

For the metal Pb there is only one record of Pb-hyperaccumulation, for *P. australis* (Bech et al. 2012) from a metalliferous area. This species seem to be an extraordinary accumulator of multi-metals (Fe, Zn, Pb) including even Mn ($max_{ST} > 0.33$) generally excluded by the other *Plantago* species. Other authors have observed this characteristic of *Plantago* to exclude Mn, namely White et al. (2012) estimated REML Mn values of *P. lanceolata* to be below those of other angiosperm species.

Some species showed also multi-metal accumulation, and the *P.* subgen. *Coronopus* species in particular showed that characteristic: Al and Fe or Fe, Zn, Ni and Cr, in species from Sect. *Maritima*. In *P.* subgen. *Coronopus* sect. *Coronopus*, one species does not seem an accumulator (though the number of samples was low) while the other, *P. coronopus*, being a wide range species, was able to accumulate Al, Fe, Cu and Zn. This probably reflects the soil available concentrations (Al-Fe podzolic-origin areas, sesquioxide rich areas, Fe-Cr-Ni rich ultramafic serpentine areas), but maybe also some physiologic character of that particular phylogenetic group, favouring metal accumulation and tolerance. The mechanisms of tolerance can be specific or non-specific. The latter, are triggered by stress situations and respond with general protection mechanisms (cell detoxification, solute and osmoprotectant accumulation, solute transport and compartmentalisation), regardless of the stress origin (Conde et al. 2011). For instance, *P. arenaria* has been shown to display both adaptive and constitutive tolerance, depending on the metal considered (Remon et al.

---

[5] Chapter 4

2007). Also, authors (Bert et al. 2000; Remon et al. 2007) have suggested that constitutive tolerance might be related to other factors than just abundance of metals, like water stress factors. In the presence of metal tolerance mechanisms, drought resistance could be enhanced (Bhatia et al. 2005; Remon et al. 2007; Schat et al. 1997). However, xeromorphic characteristics and metal tolerance mechanisms seem to be inherited independently (Barceló and Poschenrieder 1990).

Going a step further, considering that the *P.* subgen. *Coronopus* is mostly constituted of Mediterranean species (Rahn 1996; Rønsted et al. 2002) and that there seems to be a coincidence of the onset of Mediterranean climate (3.2-2.3 Myr ago; Suc 1984) with the separation of the *Plantago* subgenera (3.5-2 Myr; Rønsted et al. 2002), maybe the *P.* subgen. *Coronopus* radiation in the Mediterranean climate was aided by its metal tolerance mechanisms enhancing drought tolerance. With the onset of climate change, plants either adapted to the new conditions or shifted from their previous niches. It could have been an advantage for plants adapting to Mediterranean climate under low nutrient availabilities to have already defences against stress, namely drought stress. This may also explain the maintenance of the accumulation trait, in plants from this region. The downside of adapting to severely stressful conditions could be the loss of competition advantages in milder habitats (Baker et al. 2010; Brady et al. 2005). Genetic trade-offs occur, rendering stress adapted plant species unable to recolonize their historical habitats (Anacker and Strauss 2014; Brady et al. 2005) and reducing their realized niche to areas of higher environmental stress. According to (Baker et al. 2010) after the last Quaternary Ice-Age, the only plants that were able to survive in soils with elevated concentrations of heavy metals and extreme climatic or edaphic conditions were shadow-sensitive xerophytes, using those areas as a refuge from competition (light) with trees unable to develop in those conditions. The studied taxa of *P.* subgen. *Coronopus* share that competition limitation in various degrees. In general, most *P.* subgen. *Coronopus* species are found in disturbed areas (e.g. seasonal flooding or trampling by animals or humans, that eliminate competitors) or in stressful environments like dry, polluted or compacted soils. The failure to compete outside metalliferous environments is more obvious in the *P. almogravensis* extant population. This rare species explores metalliferous outcrops, because it cannot compete with sclerophyllous vegetation in milder edaphic conditions (Branquinho et al. 2007; Serrano et al. 2011[6]; Anacker and Strauss 2014; Serrano et al. 2015[7]).

In summary, the trait of Al-hyperaccumulation is present in all the terrestrial subgenera of *Plantago*, and particularly is maintained in the *P.* subgen. *Coronopus*, suggesting a phylogenetic link. For other metals, though all subgenera studied have hyperaccumulators, their appearance seems more sporadic, thus either connected to facultative hyperaccumulation or to some other more general accumulation mechanism expressed in conditions of metal availability. Namely in *P.* subgen. *Coronopus*, prolific in Iberian/Mediterranean species, we suggest that the pre-existence of anti-stress physiological mechanisms (needed for metal accumulation), could have been an advantage for plants adapting to the stressful Mediterranean climate. It would be interesting to acknowledge if this hypothesis is typical of Mediterranean hyperaccumulator plants in general or more restricted to *Plantago*.

Phylogeny updated but unresolved

This work also involved the attempt to classify more thoroughly some Iberian taxa. Nonetheless, the ambiguities in classification of *P. algarbiensis* and *P. almogravensis* as species or subspecies (Franco 1984; Pedrol 2009) were not resolved. The same amount of

---

[6] Chapter 4

[7] Chapter 3

dissimilarity (one to three base pairs) in the ITS+*trnL-F* regions is found between species like *P. ovata* and *P. ciliata* (Rønsted et al. 2002), meaning it is not enough to accept or dismiss their taxonomic identity. In fact, we even added a new question, as *P. holosteum* was also included in the same clade as the other two species. Nevertheless, considering the taxa distribution and the environmental stress ranges (Mitrakos indexes) that do not overlap, it is unlikely that they represent the same species. Although for *P.* subgen. *Coronopus* the hybridization of species with the same basic chromosomal number (2n = 12, sect. *Maritima*), especially when they are in the same degree of polyploidy is known to happen (Chater and Cartier 1976; Franco 1984). For that to occur the species have to be sympatric, which at the present it is not the case, nor with each other, neither with other sect. *Maritima* species, except for *P. holosteum*. Differences in morphology (e.g. size and shape of leaves and reproductive structures, ramification) between these species where also accounted for in Chapter 2. Furthermore, the physiologic responses (e.g. differences in organic acid production, root growth) seem to be different between *P. algarbiensis* and *P. almogravensis*, when grown *in vitro* in the presence of Al (Martins et al. 2013), supporting the dissimilar species view.

A putative *P. holosteum* from the Pyrenees clustered in the same clade as the Portuguese taxa, denoting that the cluster has a wide geographical range (at least the width of the Iberian Peninsula) and is not limited to the Occidental region, contributing to explain the difficulties in the classification of these taxa: throughout Iberia their differences are minimal. For instance, *Flora Iberica* (Pedrol 2009) has combined several former taxa as *P. holosteum* (*sensu* Pedrol 2009, includes e.g. *P. radicata*, *P. acathophyla*, *P. subulata granatensis*, *P. holosteum* and some *P. alpina*) and as *P. algarbiensis* (*sensu* Pedrol 2009, includes e.g. *P. almogravensis, P. algarbiensis* and *P. acanthophylla* var. *bracteosa*). In a recent work, the analysis of *P. almogravensis* and *P. algarbiensis* genetic variation by Inter-Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers, was also not conclusive: although the populations clustered separately based on genetic similarity (Simple Matching similarity index, SM = 0.67), they showed a low level of genetic diferentiation of the population (Gst = 0.1873; ranges between 0 and 1; Ferreira et al. 2013). So far, for the genetic regions available for *Plantago* species, the sequence identity is still rather high, e.g. *matK* and *rbcL* regions used in DNA barcode investigation showed 93% and 97% identity, respectivelly, between *Plantago* species (NCBI 2014). Therefore, dedicated efforts will have to be done in the future using other molecular markers or more variable genomic regions, to provide a finner separation and identification of the taxonomic species.

With this work we have envisaged the existence of a distinct subgroup in section *Maritima* (*P. subulata* clade, 80 BP). This has been mentioned in morphological descriptions (e.g. *P. subulata* group has needle like leaves and the *P. maritima+alpina* species have broader linear leaves; Rahn 1996), but was not discernible in the original Rønsted et al. (2002) phylogeny.

ACKNOWLEDGEMENTS

Collection and identification of plant material: T Monteiro-Henriques (UL-ISA); C Antunes, C Costa, M Porto, S Chozas, S Tapia and T Mexia (UL-CBA/ESFE); C Garcia, J Magos Brehm, MJ Pinto and S Lobo Dias (MUHNAC). Processing plants and analysing metals: A Perez, C Antunes, and L Aires. Discussion and critical review: MA Martins-Loução, C Branquinho (*Investigador FCT*), H Cotrim (*Ciência* 2007), MJ Pinto, A Nunes and P Pinho. Information about the sequences and taxonomic records: N Rønsted and M Chase, and to other authors that made *Plantago* sequences available in GenBank. The authorization for collecting *P. almogravensis* and *P. algarbiensis* material was provided by *Parque Natural do Sudoeste Alentejano e Costa Vicentina* and *Instituto de Conservação da Natureza e das Florestas (ICNF)*. Logistic support by project *Estudo de referência de Plantago almogravensis* 2004 and financial support by *Fundação para a Ciência e a Tecnologia* HC Serrano grant, SFRH/BD/38289/2007 and projects POCTI/BIA-BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004.

REFERENCES

Albach DC, Chase MW. 2001. Paraphyly of *Veronica* (Veroniceae; Scrophulariaceae): Evidence from the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA. *Journal of Plant Research* 114: 9-18

Albach DC, Martinez-Ortega MM, Chase MW. 2004. *Veronica*: Parallel morphological evolution and phylogeography in the Mediterranean. *Plant Systematics and Evolution* 246: 177-94

Anacker BL, Strauss SY. 2014. The geography and ecology of plant speciation: range overlap and niche divergence in sister species. *Proc Biol Sci* 281: 20132980

Anthos. 2012. Sistema de información de las plantas de España. In *Real Jardín Botánico (CSIC) y Fundación Biodiversidad*. Published on the internet http://www.anthos.es, accessed at 01-2014

Araújo PV, Carapeto A, Lourenço J, al.) e. 2013. *Plantago (várias espécies) - mapa de distribuição*. In *Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica*. Published on the internet http://www.flora-on.pt/#wid21842186218721882189219021912192195 219821962200, accessed at 13-12-2013

Babalonas D, Karataglis S, Kabassakalis V. 1987. Zinc, Lead and Copper Concentrations in Plants and Soils from two Mines in Chalkidiki, North Greece. *Journal of Agronomy and Crop Science* 158: 87-95

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81–126

Baker AJM, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In *Ecology of Industrial Pollution*, ed. LC Batty, KB Hallberg, pp. 7-40. Cambridge, U. K.: Cambridge University Press

Barceló J, Poschenrieder C. 1990. Plant water relations as affected by heavy-metal stress - a review. *Journal of Plant Nutrition* 13: 1-37

Barrutia O, Artetxe U, Hernandez A, Olano JM, Garcia-Plazaola JI, et al. 2011. Native Plant Communities in an Abandoned Pb-Zn Mining Area of Northern Spain: Implications for Phytoremediation and Germplasm Preservation. *International Journal of Phytoremediation* 13: 256-70

Batista MJAF. 2003. *Comportamento de Elementos Químicos no Sistema Rocha-solo-sedimento-planta na Área Mineira de Neves Corvo: Implicações Ambientais*. Universidade de Aveiro, Portugal

Bech J, Duran P, Roca N, Poma W, Sánchez I, et al. 2012. Shoot accumulation of several trace elements in native plant species from contaminated soils in the Peruvian Andes. *Journal of Geochemical Exploration* 113: 106 - 11

Bello MA, Chase MW, Olmstead R, Rønsted N, Albach D. 2002. The paramo endemic *Aragoa* is the sister genus of *Plantago* (Plantaginaceae; Lamiales): evidence from plastid *rbcL* and nuclear ribosomal ITS sequence data. *Kew Bulletin* 57: 585-97

Bert V, MacNair MR, DeLaguerie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* 146: 225-33

Bhatia NP, Baker AJM, Walsh KB, Midmore DJ. 2005. A role for nickel in osmotic adjustment in drought-stressed plants of the nickel hyperaccumulator *Stackhousia tryonii* bailey. *Planta* 223: 134-39

Boyd RS. 2007. The defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. *Plant and Soil* 293: 153-76

Boyd RS, Martens SN. 1992. The *raison d'etre* for metal hyperaccumulation by plants In *The Vegetation of Ultramafic (Serpentine) Soils*, ed. AJM Baker, J Proctor, RD Reeves, pp. 279–89. Andover: Intercept Ltd.

Brady KU, Kruckeberg AR, Bradshaw HD. 2005. Evolutionary ecology of plant adaptation to serpentine soils In *Annual Review of Ecology Evolution and Systematics*, pp. 243-66. Palo Alto: Annual Reviews

Bramwell D. 1997. *Flora of the Canary Islands: pocket guide*. Madrid, Spain: Editorial Rueda SL.

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. *New Phytologist* 173: 677-702

Broadley MR, Willey NJ, Wilkins JC, Baker AJM, Mead A, White PJ. 2001. Phylogenetic variation in heavy metal accumulation in angiosperms. *New Phytologist* 152: 9-27

Brooks R, Lee J, Reeves R, Jaffre T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration* 7: 49-57

Cappa JJ, Pilon-Smits EAH. 2014. Evolutionary aspects of elemental hyperaccumulation. *Planta* 239: 267-75

Cavieres LA, Badano EI, Sierra-Almeida A, Molina-Montenegro MA. 2007. Microclimatic modifications of cushion plants and their consequences for seedling survival of native and non-native herbaceous species in the High Andes of Central Chile. *Arctic Antarctic and Alpine Research* 39: 229-36

Chater AO, Cartier D. 1976. *Plantago* L. In *Flora Europaea*, ed. T Tutin, V Heywood, A Burges, D Valentine, pp. 38–44. Cambridge, UK: Cambridge Univ. Press

Chenery EM. 1948. Aluminium in the Plant World - Part I. General Survey in Dicotyledons. *Kew Bulletin* 3: 173-83

Chopin EIB, Alloway BJ. 2007. Distribution and mobility of trace elements in soils and vegetation around the mining and smelting areas of Tharsis, Riotinto and Huelva, Iberian Pyrite Belt, SW Spain. *Water Air and Soil Pollution* 182: 245-61

Conde A, Chaves MM, Geros H. 2011. Membrane Transport, Sensing and Signaling in Plant Adaptation to Environmental Stress. *Plant and Cell Physiology* 52: 1583-602

Djingova R, Kuleff I. 1999. Seasonal Variations in the Metal Concentration of *Taraxacum officinale*, *Plantago major* and *Plantago lanceolata*. *Chemistry and Ecology* 16: 239-53

Djingova R, Mihaylova V, Lyubomirova V, Tsalev DL. 2013. Multielement Analytical Spectroscopy in Plant Ionomics Research. *Applied Spectroscopy Reviews* 48: 384-424

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15

Faucon MP, Shutcha MN, Meerts P. 2007. Revisiting copper and cobalt concentrations in supposed hyperaccumulators from SC Africa: influence of washing and metal concentrations in soil. *Plant and Soil* 301: 29-36

Ferreira V, Matos M, Correia S, Martins N, Goncalves S, et al. 2013. Genetic diversity of two endemic and endangered *Plantago* species. *Biochemical Systematics and Ecology* 51: 37-44

Fitch WM. 1971. Toward defining course of evolution - Minimum change for a specific tree topology. *Systematic Zoology* 20: 406-16

Fitzgerald EJ, Caffrey JM, Nesaratnam ST, McLoughlin P. 2003. Copper and lead concentrations in salt marsh plants on the Suir Estuary, Ireland. *Environmental Pollution* 123: 67-74

Franco JA. 1984. *Nova Flora de Portugal (Continente e Açores)*. Lisboa, Portugal.

Freitas H, Prasad MNV, Pratas J. 2004. Analysis of serpentinophytes from north-east of Portugal for trace metal accumulation - relevance to the management of mine environment. *Chemosphere* 54: 1625-42

Güleryüz G, Arslan H, Kirmizi S, Güçer S. 2002. Investigation of influence of tungsten mine wastes on the elemental composition of some alpine and subalpine plants on Mount Uludag, Bursa, Turkey. *Environmental Pollution* 120: 707-16

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98

Haridasan M, Araújo GM. 1988. Aluminium-accumulating species in 2 forest communities in the Cerrado region of central Brazil. *Forest Ecology and Management* 24: 15-26

Harrison CJ, Langdale JA. 2006. A step by step guide to phylogeny reconstruction. *Plant Journal* 45: 561-72

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25: 1965-78

Hoggard RK, Kores PJ, Molvray M, Hoggard GD, Broughton DA. 2003. Molecular systematics and biogeography of the amphibious genus *Littorella* (Plantaginaceae). *American Journal of Botany* 90: 429-35

Jaffré T, Schmid M. 1974. Accumulation du nickel par une Rubiacée de Nouvelle-Calédonie, *Psychotria douarrei* (G. Beauvisage) Däniker. *Comptes Rendus de l'Académie des Sciences.Série D : Sciences Naturelles* 278: 1727-30

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Jansen S, Watanabe T, Caris P, Geuten K, Lens F, et al. 2004. The distribution and phylogeny of aluminium accumulating plants in the Ericales. *Plant Biol (Stuttg)* 6: 498-505

Kabata-Pendias A. 2010. *Trace Elements in Soils and Plants*. CRC Press.

Krämer U. 2010. Metal hyperaccumulation in plants. *Annu Rev Plant Biol* 61: 517-34

Lazaro JD, Kidd PS, Martinez CM. 2006. A phytogeochernical study of the Tras-os-Montes region (NE Portugal): Possible species for plant-based soil rernediation technologies. *Science of the Total Environment* 354: 265-77

Lestan D, Grcman H, Zupan M, Bacac N. 2003. Relationship of soil properties to fractionation of Pb and Zn in soil and their uptake into *Plantago lanceolata*. *Soil & Sediment Contamination* 12: 507-22

Marhold K. 2011. Plantaginaceae. In *Euro+Med Plantbase - the information resource for Euro-Mediterranean plant diversity*. Published on the internet http://ww2.bgbm.org/EuroPlusMed/, accessed at 01-08-2014

Markert B. 1992a. Establishing of reference plant for inorganic characterization of different plant-species by chemical fingerprinting. *Water Air and Soil Pollution* 64: 533-38

Markert B. 1992b. Presence and significance of naturally-occurring chemical-elements of the periodic system in the plant organism and consequences for future investigations on inorganic environmental chemistry in ecosystems. *Vegetatio* 103: 1-30

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

McGrath S, Dunham S, Correll R. 2000. Potential for phytoextraction of zinc and cadmium from soils using hyperaccumulator plants In *Phytoremediation of contaminated soil and water*, ed. Norman Terry, G Bañuelos: Lewis Publishers: New York, NY, USA

Metali F, Salim KA, Burslem D. 2012. Evidence of foliar aluminium accumulation in local, regional and global datasets of wild plants. *New Phytologist* 193: 637-49

Mitrakos K. 1981. Plant life under Mediterranean climatic conditions In *Portugaliae Acta Biologica: Morfologia, fisiologia, genética e biologia geral*, pp. 33-44: Instituto Botânico de Lisboa

Mower JP, Stefanovic S, Hao W, Gummow JS, Jain K, et al. 2010. Horizontal acquisition of multiple mitochondrial genes from a parasitic plant followed by gene conversion with host mitochondrial genes. *Bmc Biology* 8

Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, et al. 2012. How to measure and test phylogenetic signal. *Methods in Ecology and Evolution* 3: 743-56

Mężyk Z, Więckowski SK. 1999. Studies of Trace Element Content in Selected Medical Herbs. *Polish Journal of Environmental Studies* 8: 129–30

Nadgórska-Socha A, Ptasiński B, Kita A. 2013. Heavy metal bioaccumulation and antioxidative responses in *Cardaminopsis arenosa* and *Plantago lanceolata* leaves from metalliferous and non-metalliferous sites: a field study. *Ecotoxicology* 22: 1422-34

NCBI. 2014. Nucleotide database. In *National Center for Biotechnology Information (NCBI), USA*. Published on the internet http://www.ncbi.nlm.nih.gov, accessed 06-2014

Orłowska E, Godzik B, Turnau K. 2012. Effect of different arbuscular mycorrhizal fungal isolates on growth and arsenic accumulation in *Plantago lanceolata* L. *Environ Pollut* 168: 121-30

Pedrol J. 2009. *Plantago* L. In *Flora Iberica*, ed. C Benedi, E Rico, J Güemes, A Herrero, pp. 4–38. Madrid, Spain: Real Jardín Botánico CSIC

Pina-Martins F, Paulo OS. 2008. concatenator: sequence data matrices handling made easy. *Mol Ecol Resour* 8: 1254-5

Pollard AJ, Reeves RD, Baker AJM. 2014. Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* 217–218: 8-17

Poschenrieder C, Cabot C, Tolra R, Llugany M, Martos S, et al. 2011. Metal defense against biotic stress; is it real? *Agrochimica* 55: 29-44

Rahn K. 1996. A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society* 120: 145-98

Reeves RD. 1992. The hyperaccumulation of Ni by serpentine plants. In *The Vegetation of Ultramafic (serpentine) Soils: Proceedings of the First International Conference on Serpentine Ecology, University of California, Davis, 19-22 June 1991*, ed. AJM Baker, J Proctor, RD Reeves: Intercept

Reeves RD. 2006. Hyperaccumulation of trace elements by plants In *Phytoremediation of Metal-Contaminated Soils*, ed. J-L Morel, G Echevarria, N Goncharova, pp. 25-52: Springer Netherlands

Reeves RD, Baker AJM. 2000. Metal-accumulating plants. In *Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment*, ed. I Raskin, BD Ensley, pp. 193–229. New York: Wiley

Remon E, Bouchardon JL, Faure O. 2007. Multi-tolerance to heavy metals in *Plantago arenaria* Waldst. & Kit.: Adaptative versus constitutive characters. *Chemosphere* 69: 41-47

Remon E, Bouchardon JL, Le Guedard M, Bessoule JJ, Conord C, Faure O. 2013. Are plants useful as accumulation indicators of metal bioavailability? *Environmental Pollution* 175: 1-7

Roccotiello E, Serrano HC, Mariotti MG, Branquinho C. 2015. Nickel phytoremediation potential of the Mediterranean *Alyssoides utriculata* (L.) Medik. *Chemosphere* 119: 1372-78

Rodriguez N, Menendez N, Tornero J, Amils R, de la Fuente V. 2005. Internal iron biomineralization in *Imperata cylindrica,* a perennial grass: chemical composition, speciation and plant localization. *New Phytologist* 165: 781-89

Rønsted N, Chase MW, Albach DC, Bello MA. 2002. Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid *trnL-F* sequence data. *Botanical Journal of the Linnean Society* 139: 323-38

Schat H, Sharma SS, Vooijs R. 1997. Heavy metal-induced accumulation of free proline in a metal-tolerant and a nontolerant ecotype of *Silene vulgaris. Physiologia Plantarum* 101: 477-82

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Shallari S, Schwartz C, Hasko A, Morel JL. 1998. Heavy metals in soils and plants of serpentine and industrial sites of Albania. *Science of the Total Environment* 209: 133-42

Streit B, Stumm W. 1993. Chemical properties of metals and the process of bioaccumulation in terrestrial plants In *Plants as biomonitors: indicators for heavy metals in the terrestrial environment*, ed. B Markert, pp. 31–60. Weinheim- Cambridge

Suc JP. 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature* 307: 429-32

Sun Y, Skinner DZ, Liang GH, Hulbert SH. 1994. Phylogenetic analysis of *Sorghum* and related taxa using Internal Transcribed Spacers of Nuclear Ribosomal DNA. *Theoretical and Applied Genetics* 89: 26-32

Swofford D. 2003. PAUP*: *Phylogenetic Analysis Using Parsimony (* and Other Methods)*, Version 4.0b 10. Sunderland, Massachusetts, US: Sinauer Associates

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of 3 noncoding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105-09

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-80

Turnau K, Henriques FS, Anielska T, Renker C, Buscot F. 2007. Metal uptake and detoxification mechanisms in *Erica andevalensis* growing in a pyrite mine tailing. *Environmental and Experimental Botany* 61: 117-23

van der Ent A, Baker AJM, Reeves RD, pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil* 362: 319-34

Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytol* 181: 759-76

Watanabe T, Broadley MR, Jansen S, White PJ, Takada J, et al. 2007. Evolutionary control of leaf element composition in plants. *New Phytologist* 174: 516-23

Weising K, Nybom H, Pfenninger M, Wolff K, Meyer W. 1994. *DNA Fingerprinting in Plants and Fungi.* Taylor & Francis.

White PJ. 2012. Heavy metal toxicity in plants In *Plant Stress Physiology*, ed. S Shabala, pp. 210-37. Wallingford, UK: CABI

White PJ, Broadley MR, Thompson JA, McNicol JW, Crawley MJ, et al. 2012. Testing the distinctness of shoot ionomes of angiosperm families using the Rothamsted Park Grass Continuous Hay Experiment. *New Phytologist* 196: 101-09

Ye M, Li JT, Tian SN, Hu M, Yi S, Liao B. 2009. Biogeochemical studies of metallophytes from four copper-enriched sites along the Yangtze River, China. *Environmental Geology* 56: 1313-22

Yoon J, Cao XD, Zhou QX, Ma LQ. 2006. Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. *Science of the Total Environment* 368: 456-64

Zeppel MJB, Harrison SP, Adams HD, Kelley DI, Li G, et al. 2014. Drought and resprouting plants. *New Phytologist* (early view)

Zhou Q, Wei S. *International Conference on the Investigation, Remediation and Management of Soil and Groundwater Contaminated Sites, Taipei, 2010.*

ANNEX I

Table T1. Taxa used in the phylogenetic analysis.

Species		Authority	ITS (GenBank Acc. No)	*trnL-F* (GenBank Acc. No)	References
Plantago	*afra*	L.	AY101892	AY101945	1
Plantago	*albicans*	L.	AY101905	AY101958	1
Plantago	*algarbiensis*	Samp.	KJ579143	KJ579148	this work
Plantago	*almogravensis*	Franco	KJ579144	KJ579149	this work
Plantago	*alpina*	L.	AY101877	AY101932	1
Plantago	*amplexicaulis*	Cav.	AY101900	AY101954	1
Plantago	*arborescens*	Poir.	KJ579140	KJ579145	this work
Plantago	*arenaria*	Waldst.	AY101891	AY101944	1
Plantago	*aristata*	Michx.	AY101911	AY101963	1
Plantago	*asiatica*	L.	AY101862	AY101918	1
Plantago	*atrata*	Hoppe.	AY101895	AY101949	1
Plantago	*australis*	Lam.	AY101874	AY101929	1
Plantago	*bellardii*	All.	AY101902	AY101956	1
Plantago	*ciliata*	Desf.	AY101906	-	1
Plantago	*cornuti*	Gouan.	AY101859	AY101915	1
Plantago	*coronopus*	L.	AY101882	AY101937	1
Plantago	*crassifolia*	Forssk.	AY101881	AY101936	1
Plantago	*cretica*	L.	AY101901	AY101955	1
Plantago	*debilis*	R.Br.	AY101868	AY101922	1
Plantago	*erecta*	Morris.	AY101909	AY101962	1
Plantago	*famarae*	Svent.	AY101888	-	1
Plantago	*hookeriana*	Fisch.	AY101913	AY101965	1
Plantago	*lagopus*	L.	AY101897	AY101951	1
Plantago	*lanceolata*	L.	AY101898	AY101952	1
Plantago	*leiopetela*	Lowe.	AY101899	AY101953	1
Plantago	*lundborgii*	Sparre.	AY101907	AY101959	1
Plantago	*macrorhiza*	Poir.	AY101883	AY101938	1
Plantago	*major*	L.	AY101861	AY101917	1
Plantago	*maritima*	L.	KJ579141	KJ579146	this work
Plantago	*mauritanica*	Boiss.	AY101890	AY101943	1
Plantago	*maxima*	Jacq.	AY101864	-	1
Plantago	*media*	L.	AY101865	AY101920	1
Plantago	*myosuros*	Lam.	AY101873	AY101928	1
Plantago	*nivalis*	Boiss.	AY101896	AY101950	1
Plantago	*nubicola*	(Decne.) Rahn	AJ548972	AY101948	1,2
Plantago	*ovata*	Forssk.	AY101903	AY101957	1
Plantago	*palmata*	Hook.f.	AY101860	AY101916	1
Plantago	*patagonica*	Jacq.	AY101912	AY101964	1
Plantago	*holosteum**	Scop.	KJ579142	KJ579147	this work
Plantago	*raoulii*	Decne.	AY101867	AY101923	1
Plantago	*reniformis*	Beck.	AY101858	AY101914	1
Plantago	*rigida*	Kunth.	AY101876	AY101931	1
Plantago	*rugelii*	Decne.	AY101863	AY101919	1
Plantago	*sarcophylla*	Zohary.	AY101893	AY101946	1
Plantago	*sempervirens*	Crantz.	AY101889	AY101942	1
Plantago	*sericea*	Ruiz.	AY101910	AY101960	1
Plantago	*serraria*	L.	AY101880	AY101935	1
Plantago	*spathulata*	Hook.f.	AY101869	AY101924	1
Plantago	*squarrosa*	Murray.	AY101894	AY101947	1
Plantago	*stauntonii*	Reichardt.	AY101870	AY101925	1
Plantago	*stocksii*	Boiss.	AY101904	-	1
Plantago	*subspathulata*	Pilg.	AY101884	AY101939	1
Plantago	*subulata*	L.	AY101878	AY101933	1
Plantago	*tandilensis*	Pilg.	AY101908	AY101961	1
Plantago	*tenuiflora*	Waldst.	AY101866	AY101921	1
Plantago	*tomentosa*	Lam.	AY101872	AY101927	1
Plantago	*trinitatis*	Rahn.	AY101871	AY101926	1
Plantago	*uniflora*	L.	AY101885	AY101940	1
Plantago	*uniglumis*	Walp.	AY101875	AY101930	1
Plantago	*webbii*	Barnéoud.	AY101887	AY101941	1
Veronica	*chamaedrys*	L.	AF313003	AF486377	3,4
Veronica	*fruticulosa*	L.	AF313004	AF486383	3,4
Veronica	*glandulosa*	Hochst ex Benth.	AF313008	AF486394	3,4
Aragoa	*abietina*	H.B.K.	AJ459404	HQ593823	5,6
Aragoa	*cupressina*	Kunth	AJ459402	-	5

* Synonym *P. radicata* Hoffms. & Link.

References: 1- (Rønsted et al. 2002); 2- (Hoggard et al. 2003); 3- (Albach and Chase 2001); 4- (Albach et al. 2004); 5- (Bello et al. 2002); 6- (Mower et al. 2010).

ANNEX II

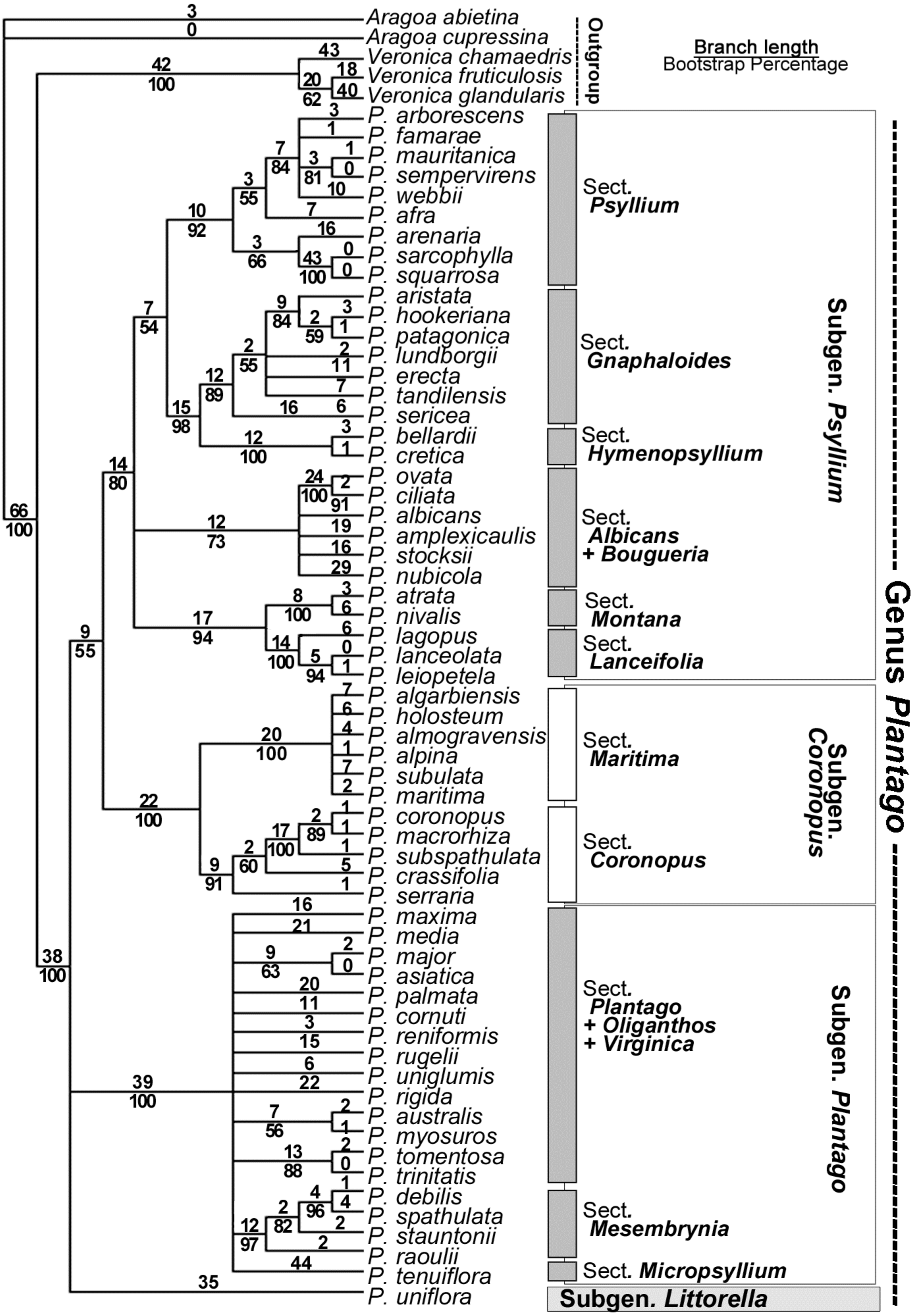


Figure F1. Bootstrap tree (strict consensus) from analysis of nuclear ribosomal ITS sequences. Branch length (bp) and bootstrap percentages (BP > 50%) are shown within and above the branches, respectively. The subgenera and sections from Rønsted et al. (2002) are indicated.

ANNEX III

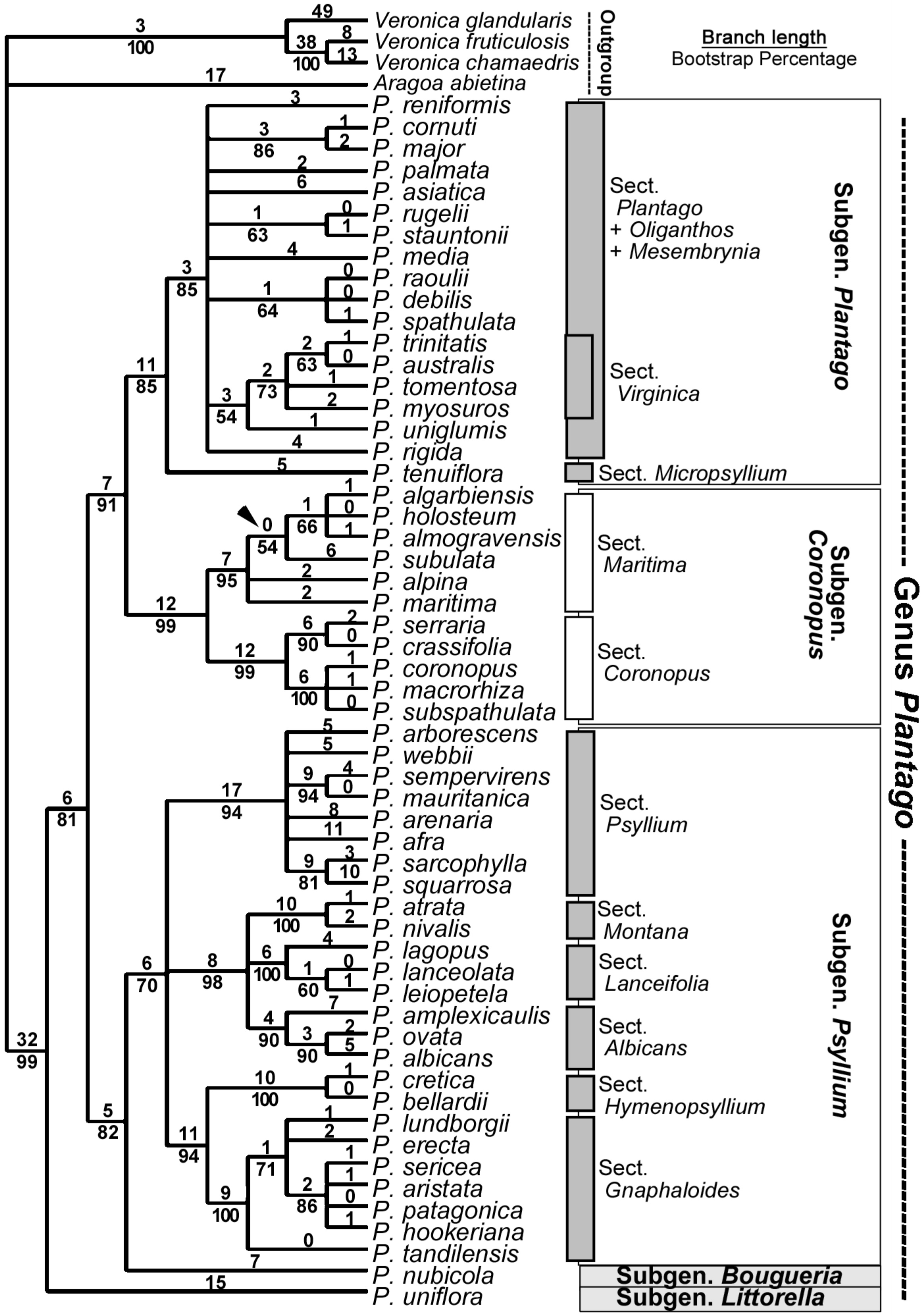
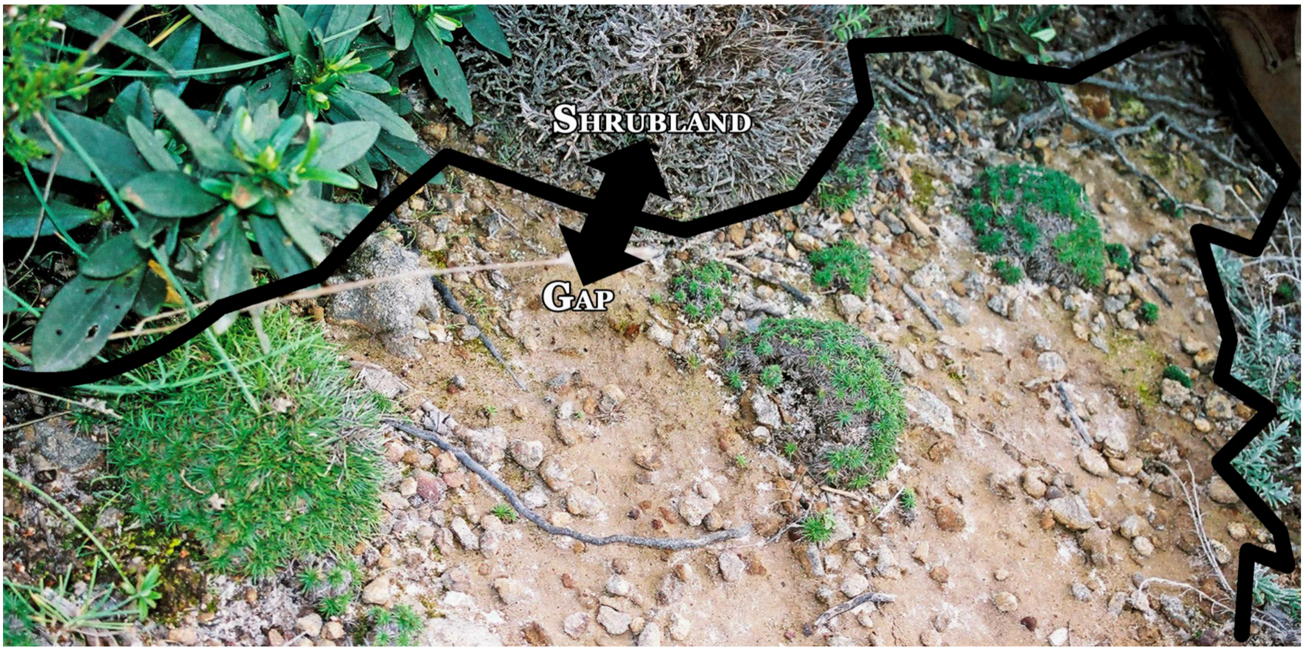


Figure F2. Bootstrap tree from analysis of plastid *trnL-F* sequences. Branch length (bp) and bootstrap percentages (BP > 50%) are shown within and above the branches, respectively. The subgenera and sections after Rønsted et al. (2002) are indicated. The branch marked with an arrow is found in 69% of the trees, therefore is not present in the strict consensus.

CHAPTER 6

SPATIAL STRUCTURE OF SOIL MICROBIAL-COMMUNITIES FROM AN AL-HYPERACCUMULATOR'S METALLIFEROUS HABITAT



Microhabitats at *Plantago almogravensis* realised niche.

CONTENTS

SPATIAL STRUCTURE OF SOIL MICROBIAL-COMMUNITIES FROM AN AL-HYPERACCUMULATOR'S METALLIFEROUS HABITAT

ABSTRACT

The soil microbial communities display heterogeneous spatial structures related to random soil composition, texture or biotic inputs. In the presence of strong stress drivers, plant-microorganism interactions must be affected and the spatiality of microbial communities might be enhanced and thus detected. Using a geostatistical approach, we sampled high stress areas related to metal toxicity, in particular metalliferous areas including geochemical islands rich in bioavailable Al and Fe. These geochemical islands are the ecological niches of a rare plant, the Al-hyperaccumulator species *Plantago almogravensis*. Using a community level physiological profiling method (CLPP) we determined overall activity and functional diversity indexes as well as responses of functional groups of microbial communities to biotic and edaphic factors present in the area, and constructed spatial maps of those microbial communities.

The shrubland and gap (geochemical island) microhabitats represented the range of biotic and edaphic factors where the microorganisms were distributed. Microbial abundance and diversity were associated with the shrubland. Conversely, the stressful gaps showed reduced abundance and diversity in the responses of the microbial communities. Spatial variability of the microbial communities was associated with vegetation abundance (namely shrubs), soil moisture and Al-toxicity (Al:Ca ratio). Functional groups diversity of microbial communities was associated with a predominant use of complex substrates in the shrubland (polymers, amines/amides or phenols) and more simple C-sources (carbohydrates and carboxylic acids) by the gap sampled communities. Since the Al-hyperaccumulator plant was associated mostly with the use of carboxylic acids, a possible involvement in Al-tolerance mechanisms is depicted.

INTRODUCTION

The ecology of a plant is dependent on the hidden bellow-ground, where plants have their roots, experience the edaphic environment and interact with microorganisms (Bardgett et al. 1998; Epelde et al. 2010; Kowalchuk et al. 2002; van der Heijden et al. 2008; Wardle et al. 1999; Zak et al. 2003). Plants contribute for soil organic matter adding litter as well as complex rhizospheric exudates (from simple ions to complex C and N compounds; Dakora and Phillips 2002). In turn, microbes decompose the organic matter and cycle organic (e.g. C, N) and mineral nutrients. Other microbes are responsible for closer interactions, either mutualistic or pathogenic (van der Heijden et al. 2008). Thus, the spatial distribution of microorganisms is more influenced by the presence of plant rhizospheres rather than by the bulk soil abiotic conditions (Ettema and Wardle 2002; Kirk et al. 2004; Klironomos et al. 1999). In nutrient poor ecosystems plant-microorganism interactions are more specific, tight and play a key role in the organization of ecological communities. These are evidenced in two ways: (i) soil microbes influence plant diversity and spatial distribution and (ii) plant abundance and richness influence soil microbial communities (van der Heijden et al. 2008). If besides the nutrient poor soils we also have a toxic environment due to metalliferous soils, the settings seem ideal for the existence of particular microbial communities responsible for the delivery of limiting nutrients (N, P) and/or protection (e.g. producing metal chelating compounds) to the few plant species found in those areas (van der Heijden et al. 2008).

Aluminium is the most abundant metal in the Earth's crust, nonetheless not only it has no obvious biological function as it is, in fact, extremely toxic when in a bioavailable form (Piña and Cervantes 1996; Poschenrieder et al. 2008). Aluminium toxicity is the 3rd major soil constrain to agriculture affecting 17% of the agricultural soils (Wood et al. 2000). Air pollution induced acidification of soil and water, and increased the problem of Al-toxicity (Piña and Cervantes 1996; Wood et al. 2000). Aluminium bioavailability is dependent on pH, when pH<5 the most toxic species are made available (Al^{3+}). Bioavailability in soil can be ameliorated in the presence of mineral surfaces, cations (Ca, Mg, K and Na) and organic matter, to which Al can be adsorbed (Boudot et al. 1994; Piña and Cervantes 1996; Poschenrieder et al. 2008).

The flora and microflora capable of thriving in Al rich zones must have certain tolerance to the high metal availabilities. One special type of plant capable of tolerating and uptake abnormal amounts of aluminium is called hyperaccumulator (Chenery and Sporne 1976; Jansen et al. 2002; Masunaga et al. 1998). In the rhizosphere of metal-hyperaccumulator plants, microorganisms often exhibit increased metal tolerance and may contribute to the high metal uptake to the plant (e.g. reviewed in Alford et al. 2010). The mechanisms of Al-hyperaccumulation are still not well understood; neither are the mechanisms of Al-toxicity regarding soil microorganisms (Piña and Cervantes 1996). In general, Al inhibits bacterial growth, though there are strains capable of greater resistance or even capable of using Al compounds as C-source (e.g. *Pseudomonas fluorescens* using citrate-Al; Appanna and Stpierre 1994).

Works involving the distribution of soil microorganisms in the rhizosphere of metal hyperaccumulator plants (e.g. Cd, Li et al. 2010; Ni, Aboudrar et al. 2007; Zn, Epelde et al. 2010); involving soil microorganisms and Al, in areas of acidic forest soils (e.g. Illmer et al. 2003); or microbial tolerance to soil Al (e.g. Kawai et al. 2000; Piña and Cervantes 1996), have already been reported. To our knowledge, there are no works concerning the spatial patterns of microbial communities and their putative functional diversity, in Al-rich areas (like geochemical islands) associated to Al-hyperaccumulator plant species.

Plantago almogravensis Franco is an Al-hyperaccumulator (> 3.0 mg kg^{-1}, Branquinho et al. 2007; Serrano et al. 2011[1]) inhabiting very heterogeneous soils with respect to nutrient composition, metal concentration and depth. This plant has its ecological niche in the shallow geochemical islands (rich in Al and Fe) scattered within a deeper podzolic soil, and it is not able to compete with the vegetation that thrives outside those areas (Serrano et al. 2015[2]). It was also found that its physiological performance was lower in areas with higher Al concentration and above a certain threshold no plant survived (Serrano et al. 2015[2]). It would be expected that given the stressful edaphic conditions described before and the presence of a Al-hyperaccumulator plant, the spatial pattern of the soil microbial communities and their functional diversity would be related to such biotic and abiotic factors.

The objective of this work was to evaluate the spatial distribution of soil microbial communities in a gradient area scattered with Al-rich geochemical islands, the niche of an Al-hyperaccumulator plant.

---

[1] Chapter 4

[2] Chapter 3

To evaluate the microbial communities, Community Level Physiological Profiles (CLPP) have been used (EcoPlates™, BIOLOG) both for the activity and functional diversity (overall use of substrates), and the functional guilds (use of six functional groups) of the communities. This method (Garland and Mills 1991; Insam and Goberna 2004) is based on the spectrophotometric measurement of the development of colour induced by NADH, reducing a tetrazolium dye. Microplates containing a set of different C-sources are inoculated with the soil bacteria, so that when in presence of an assimilable C-source, the production of NADH by cell respiration will form a deep blue colour. Though CLPP is a culture-biased method, when involving metal toxicity, those methods have been considered ecologically relevant (Ellis et al. 2003; Garland and Mills 1991; Insam 1997). The spatial analysis was approached using geostatistical methods that manage regionalized variables, providing the possibility to develop a spatial model of the variable (Soares 2000).

Specifically, we proposed to envisage spatial distribution patterns of overall activity and diversity, and of putative functional diversity (metabolic potential), of soil microbial communities. This will be done through the development of spatial models and determining the importance of edaphic and biotic factors on those microbial community patterns. We hypothesize that: (i) microbial communities diversity and abundance would decrease with the increment of stressful edaphic conditions (namely Al-toxicity) and (ii) the presence of a plant specialist (Al-hyperaccumulator), would be related to particular patterns of microbial functional diversity.

Table 1. General soil characterization.

Soil properties	*n*	Mean ± SD	Range
Sand content (%)	10	80 ± 5.0	69 - 88
Organic matter (%)	6	3.7 ± 0.7	2.4 – 4.4
Carbonate (%)	6	0.2 ± 0.1	0.1 – 0.4
Nitrate (mg kg^{-1})	17	19 ± 13	5.2 - 44
pH in water	67	5.82 ± 0.44	4.46 - 6.75
pH in KCl	10	4.78 ± 0.64	3.90 – 5.94
Total N (%)	10	0.03 ± 0.03	0 – 0.08
Total C (%)	10	1.21 ± 0.68	0.35 – 2.30
Total Al (%)	17	2.4 ± 1.0	1.4 – 5.6
Total Fe (%)	17	0.60 ± 0.31	0.2 – 1.3

MATERIALS & METHODS

Field characterization and sampling scheme

This study was conducted at *Sudoeste Alentejano e Costa Vicentina* Natural Park, in the southwest Atlantic coast of Portugal (Fig. 1; more details about the area in Serrano et al. 2015[3]). The soil matrix is a loamy sand podzol (Cardoso 1965) but some eroded areas outcrop and exposed the podzol B-horizon. This created hardpans with hardly any soil on top, rich in iron sesquioxides and aluminium (Tab. 1), like geochemical islands. Two of those geochemical islands were chosen as study areas being located less than 50 m from the sea and 70 m from each other (Fig. 1). The plots included different microhabitats: gap areas without shrubs (the geochemical island core) and at least, 1 m into the surrounding deeper podzol soil, covered with shrubs (shrubland). This shrubland was composed by a mosaic of dense dwarf sclerophyllous shrubs (*Calluna* spp., *Cistus* spp., *Erica* spp., *Ulex* spp.; Serrano et al. 2015[3]). In the gap areas, one could find (i) mostly *P. almogravensis* (a dwarf woody chamaephyte or hemicryptophyte; stems with terminal leaf rosettes and long woody main root with shorter laterals and thin absorbing superficial roots); (ii) other scattered herbaceous plants (mostly hemicryptophytes, e.g. *Eryngium dilatatum*, *Pulicaria odora*) and (iii) bare areas without any vegetation (Fig. 1). In the interface of the shrubland and gap areas (edge), remains of shrub leaves accumulated covering the soil and it is likely that the underground is still much influenced by the shrubs root systems.

---

[3] Chapter 3

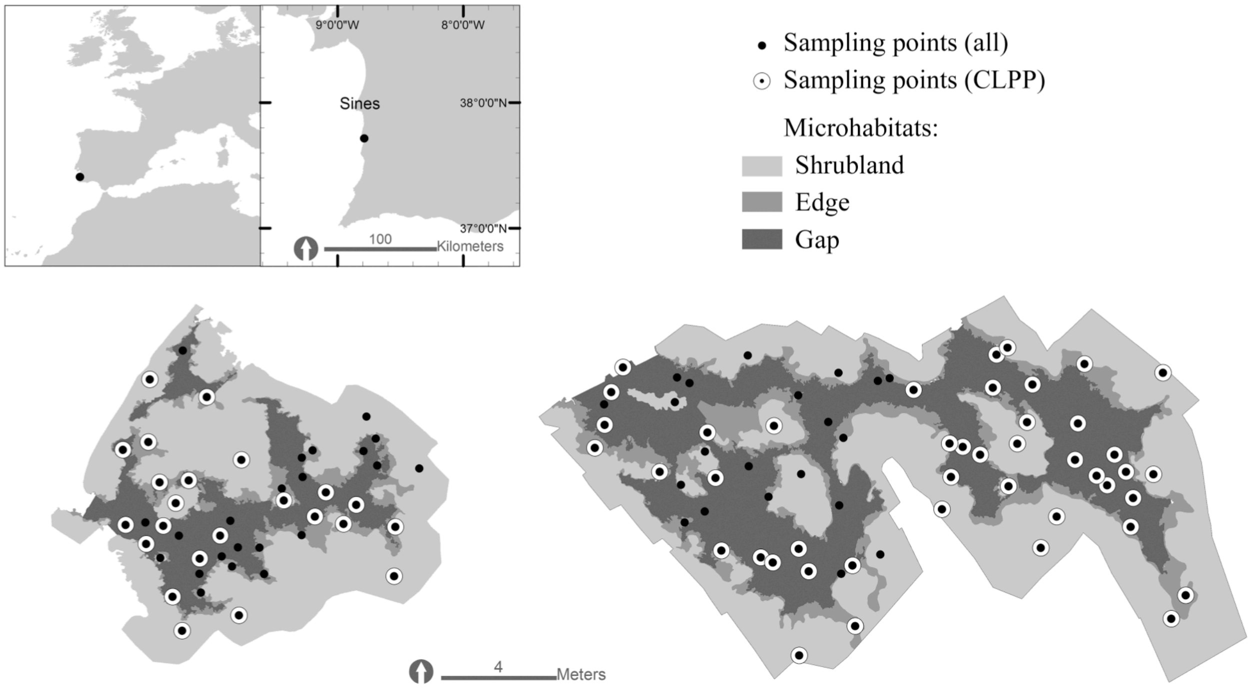


Figure 1. Location of the sampling site and plots with general microhabitats (gap, edge and shrubland) and sample points (total: n = 110; CLPP (microbiology): n = 67).

Ortho-photographic maps of the plots were created (see Serrano et al. 2015[4] for details on the photographic cartography) and a total of n= 110 sampling points, ≤ 30 cm apart, were randomly generated (stratified by microhabitats) (Random Points generator, ArcView 3.3; Serrano et al. 2015[4]). In each of the designed sample points (n = 110), we collected soil for physicochemical analyses. In 67 of those sample points (n = 67), the soil was also used for the microbial analysis (Community-Level Physiological Profiling – CLPP). Field sampling was carried out in March 2007. From the ortho-photographic maps (and *in situ* confirmation), we also characterized the **density** of *P. almogravensis* (individuals per m^2) and the soil **biotic cover**, calculated within a 30 cm radius buffer-area around the designed sample points. The biotic cover included the percentage of cover by **shrubs**, *P. almogravensis* (***Plantago***), other herbaceous plants and litter (**others**), or the absence of biotic cover (**bare soil cover** percentage), in each point.

Soil collection and physicochemical tests

Surface soil was collected at each point, ca. 0-5 cm deep (≥ 100 cm^3), stored in closed plastic bags and preserved from high temperatures inside a cool-box, until storage (4°C). Spatulas used to collect soil were cleaned between each sample with water and 98 % ethanol to avoid cross contamination. **Soil depth** was evaluated using a 0.50 m length probe (Serrano et al. 2015[4]). Soils with no resistance to the entire probe length (depth ≥50 cm) were scored as 50 cm. **Soil relative humidity** (RH; $g\ g_{soil}^{-1}$) was evaluated after drying at 60°C (Serrano et al. 2015[4]). **Soil pH** (1:10 w/v) was determined on air-dried sieved soil (2 mm), with a pH probe (CRISON, micropH2001; Serrano et al. 2015[4]).

The concentration of **soil bioavailable elements** ($mg\ kg^{-1}$ of dry soil) was calculated for nutrients Ca, Fe, K and Mg, and non-essential/toxic element Al. Two estimation methods were used, one that extracts the easily soluble ions from the soil (*soluble* Fe; 2 h water extraction 1:20 w/v; modified after Serrano et

---

[4] Chapter 3

Table 2. Substrates on the EcoPlates.

Well n. – substrate (functional group)		
1 – blank	12 – D- Mannitol (CH)	23 – α-Ketobutyric Acid (CA)
2 – Pyruvic Acid Methyl Ester (CH)	13 – N-Acetyl-D-Glucosamine (CH)	24 – D-Malic Acid (CA)
3 – Tween 40 (PL)	14 – D-Glucosaminic Acid (CA)	25 – L- Arginine (AA)
4 – Tween 80 (PL)	15 – Glucose-1-Phosphate (CH)	26 – L- Asparagine (AA)
5 – α-Cyclodextrin (PL)	16 – DL-α-Glycerol Phosphate (CH)	27 – L-Phenylalanine (AA)
6 – Glycogen (PL)	17 – D- Galactonic Acid γ-Lactone (CA)	28 – L- Serine (AA)
7 – D-Cellobiose (CH)	18 – D-Galacturonic Acid (CA)	29 – L-Threonine (AA)
8 – α-D-Lactose (CH)	19 – 2-Hydroxy Benzoic acid (PH)	30 – Glycyl-L-Glutamic Acid (AA)
9 – β-Methyl-D-Glucoside (CH)	20 – 4-Hydroxy Benzoic Acid (PH)	31 – Phenylethyl-amine (AM)
10 – D-Xylose (CH)	21 – γ-Hydroxybutyric Acid (CA)	32 – Putrescine (AM)
11 – i-Erythriol (CH)	22 – Itaconic Acid (CA)	

al. 2011[5]) and another that extracts ions more strongly adsorbed to the soil matrix (*bioavailable* Ca, K, Mg and Al; 5 mM Na_2EDTA, ethylenediaminetetraacetic acid elution; Serrano et al. 2015[6]). Flame Atomic Absorption Spectrometry (SpectrAA50, VARIAN) was used for measuring Ca, Fe, K and Mg; and Graphite Atomic Absorption Spectrometry (932 plus, GBC) for Al (Branquinho et al. 2007).

The variable *macronutrients* was calculated as the sum of Ca, K and Mg concentrations. We used the mass concentration ratio of Al/Ca (Al-Toxicity Index), for estimating Al-toxicity of the soil samples (Serrano et al. 2015[6]; modified after Cronan and Grigal 1995).

Community Level Physiological Profiling (CLPP)

Fresh soil was homogenised inside the bag and aliquots weighted and suspended in sterile water (1:5 w/v). After vortexing (30 sec.) the samples were centrifuged (10 min, 5000 x g) to remove soil particles. The supernatant was diluted in sterile water (3:25 v/v) to reduce its background colour and inoculated in the EcoPlates (adapted from Lindahl and Bakken 1995). Plates were incubated at 20° C in the dark and colour development was measured at 592 nm with a microplate multimode detector (ANTHOS, Zenyth 3100), twice a day (Insam and Goberna 2004). Each EcoPlate has three sets of 31 different substrates (C-sources; Tab. 2) and a blank well (3x32 wells). According to their chemical nature, the substrates can be grouped into six functional groups: the guilds amino acids (AA), amines/amides (AM), carboxylic acids (CA), carbohydrates (CH), phenols (PH) and polymers (PL) (Insam 1997).

Data analysis

The samples of the two plots were analysed together, because only small differences were found between them, considering the distribution of the environmental variables, namely in the amount of *Others* cover or in the bioavailable Al (Mann-Whitney U Test, $p = 0.034$ and $p = 0.0001$, respectively).

Microbial functional diversity: Absorbance values from the wells were blanked against their own first reading to correct for background (Insam and Goberna 2004) after which the overall colour development was calculated (OD – Optical Density). A *Microsoft visual basic 6.0* programming language was used for personal programming of an s-curve function (eq. 1) to model absorbance evolution along time and to calculate the area under the curve (ACD).

$$Y = K/\left(1 + e^{-r(t-s)}\right) \text{ [eq. 1]}$$

Where, Y = OD (corrected absorbance); K = represents the asymptote of the curve; r = is the exponential rate of change; t = time following inoculation; s = time when Y=K/2 (mid point of the exponential portion of the curve). The fitted model parameters had

---

[5] Chapter 4

[6] Chapter 3

an average coefficient of determination $R^2 = 0.98$ ($n = 1431$) for positive reactions and $R^2 = 0.70$ ($n = 713$) for negative reactions (see richness definition).

For each sample, Shannon's diversity index (H) for the substrates consumed, evenness (E) of the activity across the substrates and richness of substrates used (S) were calculated (Insam and Goberna 2004) to evaluate diversity in C-source consumption, from the substrates with an absorbance value >0.25 (positive reactions) at the end of the experiment (t = 15700 min = ca. 11 days).

$$p_i = Y_i / Y_{sample} \text{ [eq. 2]}$$

$$H = -\sum_1^{32} p_i \cdot \ln p_i \text{ [eq. 3]}$$

$$E = H / H_{max} = H / \ln S \text{ [eq. 4]}$$

The area under the curve (ACD – Area of Colour Development) calculated from the absorbance values overtime (15700 min) was also chosen to evaluate CLPP because it integrates both quantitative and functional results, being a measure of the overall activity and abundance of the microbial community (Insam and Goberna 2004; Lindstrom et al. 1998). For each sample, the average *ACD* of the 32 wells was calculated (eq. 5) as well as the relative ACD'_i (eq. 6) to account for inoculum differences (Preston-Mafham et al. 2002):

$$ACD_{sample} = \sum_1^{32} ACD_i / 32 \text{ [eq. 5]}$$

$$ACD'_i = ACD_i / \sum_1^{32} ACD_i \text{ [eq. 6]}$$

According to their chemical nature, the 31 substrates were grouped into six functional guilds (AA, AM, CA, CH, PH and PL) by summing their respective relative ACD' and dividing by the number (n) of substrates in the group (eq. 7) ($\text{ACD'}_{\text{guild}}$ – relative activity of the functional guild).

$$ACD'_{guild} = (\sum(ACD_i / \sum_2^{32} ACD_i))/n \text{ [eq. 7]}$$

Spatial modelling: The spatial analysis was approached using geostatistical methods that manage regionalized variables, providing the possibility to develop a spatial model of the variable (Soares 2000). These are variables distributed in space, where the set of observed measurements (collected data) represent a single realization of a spatial random process. Inferences about this random function are possible if the following assumptions are made: variables are correlated in space (spatial dependence exists) and the random function is second-order intrinsic stationary.

For each CLPP data set (H, S, E, $\text{ACD}_{\text{sample}}$, $\text{ACD'}_{\text{guilds}}$), a spatial model was built. In a first step, spatial correlations between samples were generalized, as a correlation function of distance between any two points (semivariogram), which summarized the main spatial continuity patterns of the CLPP variables. Isotropic spherical models were fitted to the experimental semivariograms. In a second step, a least-squares linear regression algorithm (ordinary kriging) was applied, to estimate maps for each CLPP variable, taking into account the model's spatial dependence, previously fitted (*ArcGIS*, ESRI).

Statistical analysis: To study the relation among the environmental (biotic and edaphic) variables with the CLPP variables, non-parametric tests were used (non-normal data, Shapiro-Wilk, $P < 0.05$). Namely, correlations were investigated using Spearman's Rank Order (ρ) and, to compare levels between groupings the Independent-Samples Mann-Whitney U Test (for two groups). Principal components analyses (PCA) where used to extract the joint contribution of the environmental variables to the distribution of the microbial communities, followed by analysis of variance (T-test) to compare groups of samples scores. The significance level for statistical tests was set at $p = 0.05$, except where stated otherwise (*SPSSstatistics*, IBM).

RESULTS

Characterization of the study area

The plot areas included as main microhabitats, the core of the geochemical island (gaps) and the surrounding shrubland areas (the edge areas were included in the shrubland). Significant differences were found between them (Fig. 2A–B). In the gaps, the concentrations of Fe, Al and Al-toxicity (A1, A2 and A3) were higher and showed a higher density and proportion of *Plantago* (B1, B2), as well as of bare soil (B3). In the shrubland dominated the shrub cover (B5), supported by higher soil depths, more humidity and nutrients (A6, A5 and A4). The cover by other biotic factors (B4) was not significantly different between the two microhabitats, neither was the soil pH

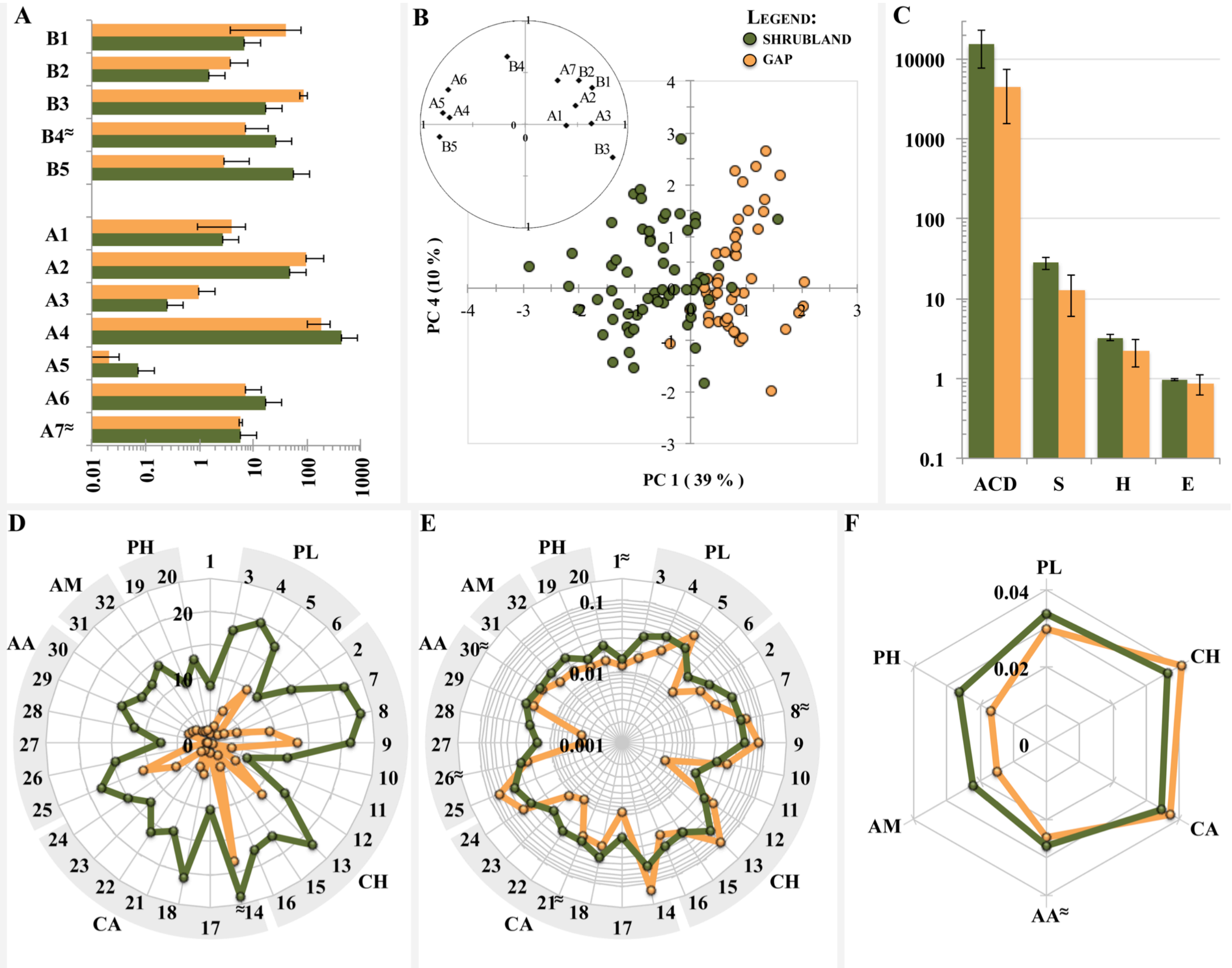


Figure 2. Comparison of the variables' means, between gap and shrubland samples. **(A-B) Environmental variables** in the gap ($n = 49$) and shrubland ($n = 61$; includes edge area); No significant differences ($p > 0.217$) between the variables means using $n = 110$ or $n = 67$, so the former are presented. **Variables:** B1- *Plantago* density (plants·m^{-2}); B2- cover percentage by *Plantago* (%); B3- bare soil cover (%); B4- herbaceous plants and litter (*Others*) cover (%); B5- shrub cover (%); soil bioavailable A1- iron (mg Fe kg^{-1}), A2- aluminium (mg Al kg^{-1}) and A4- macronutrients (mg kg^{-1}); A3- soil Al-toxicity index; A5- relative soil humidity (RH g g^{-1}); A6- soil depth (cm) and A7- soil pH in water. **(A) bio-edaphic variables** (bar chart, with SD); all pairs are significantly different except for (B4≈) when total $n = 67$, $p = 0.101$ ($n = 110$, $p = 0.005$); (A7≈) when total $n = 110$, $p = 0.302$ ($n = 67$, $p = 0.023$). **(B) PCA of environmental variables** (PCA_{Env} scores). **(C-F) CLPP variables** in the gap ($n = 30$) and shrubland ($n = 37$). **(C) H, S, E and $ACD_{samples}$** (bar chart, with SD); all pairs are significantly different. **(D) ACD** (abs·min x10^3, absolute values) of the 32 substrates; all pairs are significantly different except for (#14≈) $p = 0.068$. **(E) ACD'_i** (relative values, log scale); all pairs are significantly different except for (#1≈ natural C-sources) $p = 0.126$; (#8≈) $p = 0.082$; (#21≈) $p = 0.079$; (#26≈) $p = 0.162$; and (#30≈) $p = 0.830$. **(F) ACD'_{guild}** (relative values); all pairs are significantly different except for (AA≈) $p = 0.579$.

(A7). Because of the interconnections between biotic and edaphic variables, we used a PCA to extract the components (PCs, with eigenvalues >1) that best reflected all that variability.

The PCA extracted 4 components of these environmental variables explaining 74% of the variability (Fig. 2B). The first component ($PC1_{Env}$, 39%) had high (>0.3) scores for all variables except *Others* cover (B4), being positive for the gap associated features and negative for the shrubland features, as were described before. The first $PC1_{Env}$ separated significantly gap and shrubland samples ($p < 0.001$) (Fig. 2B). The second component ($PC2_{Env}$, 14%) was related to the presence of Al and Fe but also to nutrients and moisture, making its interpretation more difficult. The third ($PC3_{Env}$, 11%) and fourth components ($PC4_{Env}$, 10%) higher scores were associated to the presence of *Plantago* ($PC3_{Env}$) or *Other* biotic factors ($PC4_{Env}$), and like $PC1_{Env}$, separated gap and shrubland samples ($p = 0.044$ and $p = 0.028$, respectively).

In the studied plots, the CLPP activity (ACD_i) ranged from $1.0x10^2$ to $3.4x10^4$ abs·min, while the diversity variables ranged between 0–32 for richness (S) of substrates used; 0–1.00 for the evenness (E) of the activity in the different substrates; and the combination of these two, a Shannon's diversity index of 0–3.46. The microbial communities from shrubland samples were more abundant (active) than from gap samples (Fig. 2C-D), with correspondence to a higher richness of substrates consumed (S), evenness of the responses (E) and overall diversity (H). The microbial richness in the shrubland was rather stable (ca. 27-32 substrates used, not considering the edge areas) while that in the gap had a large variability (ca. 0-23 substrates used; Fig. 2C–D).

Notwithstanding the differences in activity and abundance, the two areas had also (putative) functional differences, observed at the patterns of substrate use (Fig. 2E-F). Namely, in the gaps we observed (Fig. 2E) a higher relative use of substrates #5 (PL); #9, #10, #12 and #13 (CH); #14 and #24 (CA); and #25 (AA); corresponding in average to an increased use of carbohydrates and carboxylic acids (Fig. 2F). In the shrubland samples, we found a higher relative use of substrates #3 and #6 (PL), #7 and #11 (CH), #17, #22 and #23 (CA), #27 and #28 (AA); or in average more amines/amides, phenols and polymers (Fig. 2E–F).

Spatial modelling of microbial communities

The spatial modelling was calculated for the CLPP activity and overall-diversity variables ($ACD_{samples}$, H, S, E) and functional groups (PL, CH, CA, AA, AM, PH). The variograms were calculated and fitted to the spherical functions summarized in Tab. 3. Those functions can be used to create interpolation maps for the variables in the study area (Fig. 3–4). No anisotropy was detected for the variograms and the spatial continuity of the CLPP variables was identical (range ≈ 2 m; Tab. 3).

Considering the quantity and diversity of the microbial communities, the models with higher spatial dependence (structural variance; Tab. 3) were those for activity ($ACD_{samples}$, 97%) and Shannon's H (94%) (Fig. 3). The nugget effect (discontinuity at the origin of the variogram, representing random errors and short-scale variability) is observable in the variables

Table 3. Spatial structures for the CLPP variables.

Variables	Range (m)	Structural variance (%)	Lag (m)
Activity ($ACD_{samples}$)	1.82	97%	0.4
Diversity (H)	1.9	94%	0.9
Richness (S)	1.9	78%	0.6
Evenness (E)	1.82	82%	0.6
Polymers (PL)	1.9	100%	0.6
Carbohydrates (CH)	1.9	76%	0.6
Carboxylic acids (CA)	1.9	56%	0.6
Amino-acids (AA)	1.9	22%	0.6
Amines/amides (AM)	1.9	95%	0.4
Phenols (PH)	1.9	89%	0.6

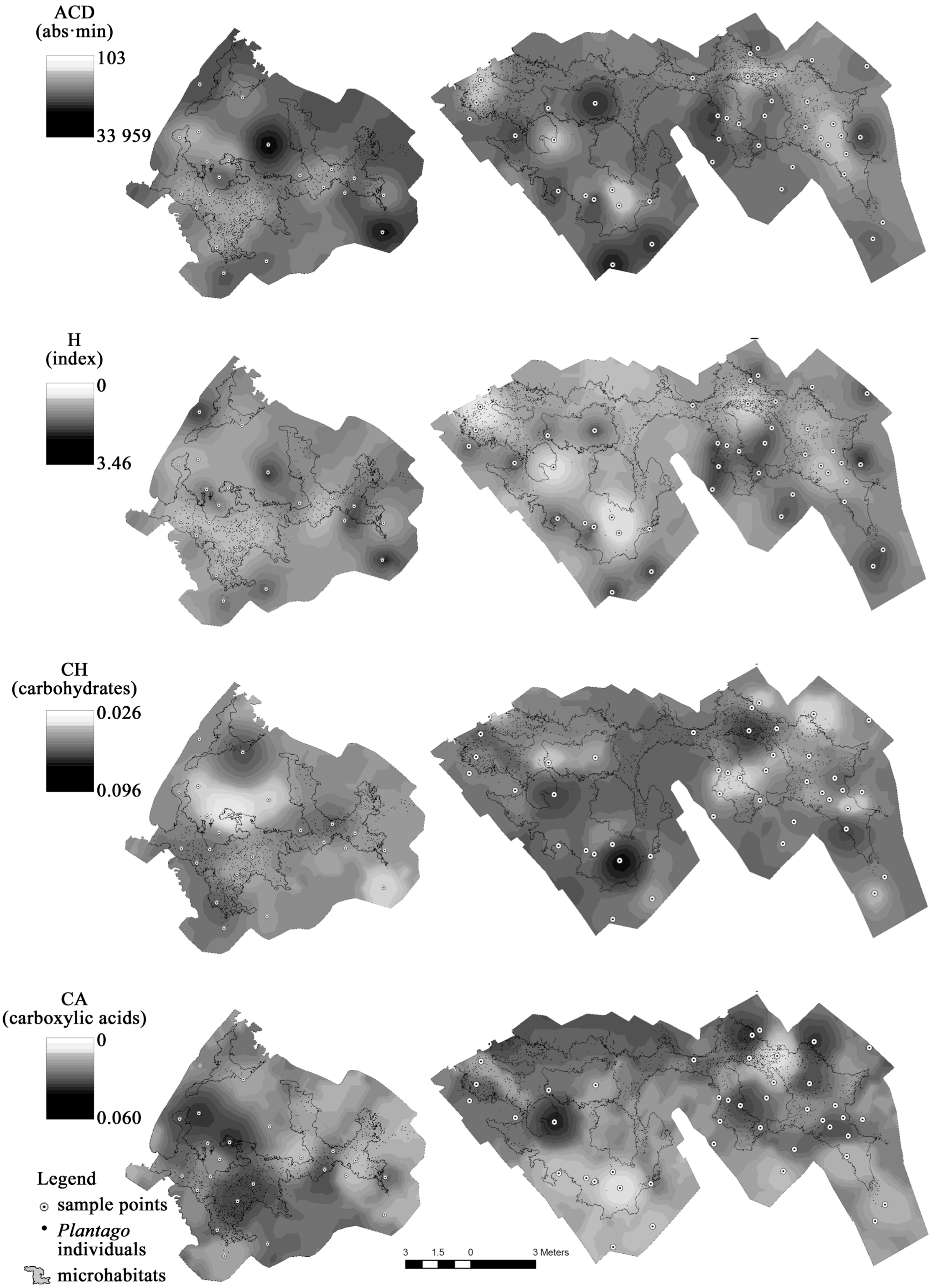


Figure 3. Spatial models of CLPP activity and overall-diversity (ACD, H) and functional groups (CH, CA). Microhabitats: contours limit the shrubland/gap areas (see Fig. 1); *Plantago* individuals represented by dots.

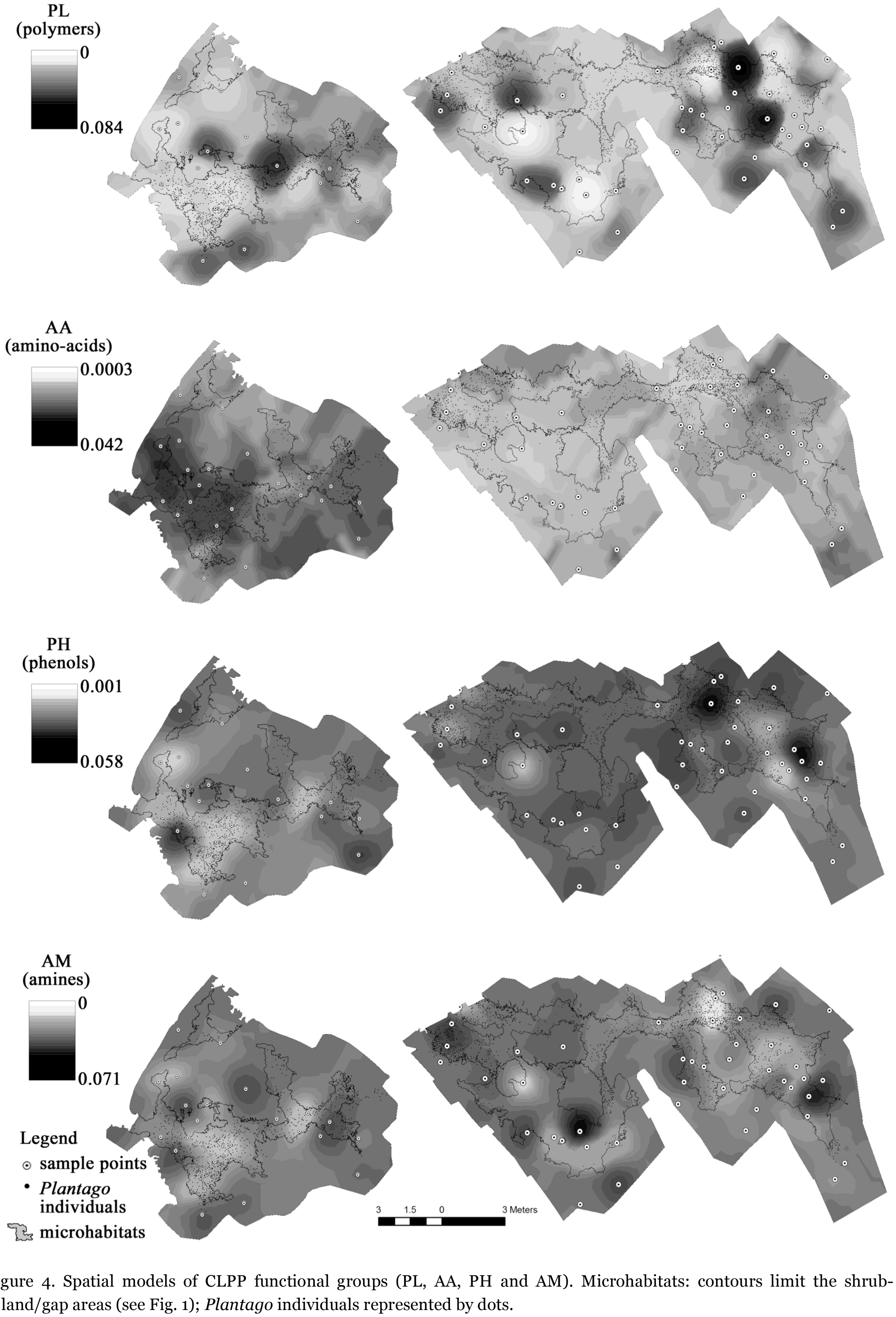


Figure 4. Spatial models of CLPP functional groups (PL, AA, PH and AM). Microhabitats: contours limit the shrubland/gap areas (see Fig. 1); *Plantago* individuals represented by dots.

with lower percentages of structural variance. The nugget effect reduced the richness model variance to 78% and evenness to 82% indicating probably particular short scale events affecting particular samples, which our sampling scheme was not able to capture.

Considering the CLPP functional groups, the group amino acids presented the lowest structural variance (22%; Fig. 4), thus the model would not account for most of the factors involved in its variability. On the other hand, polymers (100%), amines/amides (95%) and phenols (89%) were the better explained by the spatial patterns (Tab. 3; Fig. 4). Carbohydrates and carboxylic acids presented a higher proportion of unpredictable error (nugget) but still above 50% structural variance (Fig. 3).

Specific factors (edaphic and biotic) associated with the microbial communities

The correlation among environmental and CLPP variables is summarized in Tab. 4. It is notable that CLPP overall activity and diversity variables had stronger correlations with the environmental variables than the CLPP functional groups. The first component extracted from the environmental variables ($PC1_{Env}$) was a very good explanatory variable for most CLPP variables, while $PC2_{Env}$ to $PC4_{Env}$ showed none to a weak correlation with a CLPP factor (Tab. 4). Another general pattern is that the presence of *Plantago* showed weaker correlations than the presence of shrubs or the absence of plants (bare soil cover). The *Others* cover (herbaceous plants and litter) showed no significant correlations with any of the CLPP variables, neither did pH with the functional groups. The soil pH, had only weak to medium correlations with H and E, and did not show significant differences between the gap and shrubland areas (Fig. 2A).

The CLPP overall activity and diversity variables (S, H, E, ACD_{sample}) showed all similar patters of correlation with the environmental variables (Tab. 4). The strongest correlations were either with the biotic factors shrubs (positive) and the absence of plants (negative, for soil *Bare* cover), or the edaphic factors soil relative humidity (RH, positive correlation) and Al-Toxicity (negative). The soil depth or macronutrients were also positively related to the activity and diversi-

Table 4. Spearman's correlation coefficients (ρ) between environmental factors and CLPP activity and overall-diversity or functional diversity variables. Only variables with at least one correlation $p \leq 0.10$ are shown.

	Edaphic							**Biotic**				**Environmental**	
	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**B1**	**B2**	**B3**	**B5**		
Variables	Fe	Al	Al-Tox	Macro	RH	Depth	pH	²*Plantago*	¹*Plantago*	Bare soil	Shrubs	$PC1_{Env}$	$PC4_{Env}$
n	67	66	66	66	67	67	67	67	67	67	67	66	66
S	-0.443**	-0.283*	-0.682**	0.555**	0.731**	0.579**	NS	-0.376**	-0.315**	-0.724**	0.731**	-0.744**	NS
E	-0.384**	-0.246*	-0.622**	0.537**	0.670**	0.486**	-0.312*	-0.382**	-0.282*	-0.625**	0.623**	-0.650**	NS
H	-0.404**	-0.278*	-0.672**	0.551**	0.739**	0.577**	-0.221^{i}	-0.382**	-0.311*	-0.722**	0.738**	-0.747**	NS
ACD	-0.372**	-0.228^{i}	-0.665**	0.596**	0.713**	0.585**	NS	-0.353**	-0.292*	-0.731**	0.760**	-0.745**	NS
PL	-0.455**	NS	-0.330**	0.304*	0.303*	0.228^{i}	NS	NS	NS	-0.307*	0.206^{i}	-0.300*	0.225^{i}
CH	NS	NS	NS	-0.255*	-0.303*	-0.293*	NS	NS	NS	0.265*	-0.284*	0.291*	NS
CA	0.229^{i}	NS	0.272*	-0.207^{i}	NS	NS	NS	0.214^{i}	NS	NS	NS	0.218^{i}	NS
AA	NS	0.229^{i}	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AM	NS	NS	-0.279*	0.343**	0.370**	0.276*	NS	-0.236^{i}	NS	-0.301*	0.313*	-0.377**	NS
PH	NS	-0.216^{i}	-0.355**	NS	0.484**	0.262*	NS	-0.240^{i}	-0.219^{i}	-0.271*	0.385**	-0.337**	NS

P. almogravensis cover (¹) and density (²). RH – soil relative humidity; Macro – macronutrients; Al-Tox – Al-toxicity index; S – Richness; E – Evenness; H – Shannon's diversity index; ACD – Activity; PL – polymers; CH – carbohydrates; CA – carboxylic acids; AA – amino acids; AM – amines/amides; PH – phenols. Significance level: (NS) p > 0.10, (i) p ≤ 0.10, (*) p ≤ 0.05 and (**) p ≤ 0.01.

ty of the microbial communities, while Fe and Al were negatively correlated. In general, these results are well related to the traits found in the gap and shrubland, thus the strong negative correlation with the $PC1_{Env}$ of the environmental variables described before (Fig. 2B; Tab. 4).

The correlations between the abundance of *P. almogravensis* (cover and density) and CLPP overall activity and diversity variables were weak to medium and negative (Tab. 4). The CLPP variables of functional groups were also weakly correlated with *Plantago*, namely *Plantago*'s density was positively correlated with carboxylic acids, as there was a weak increase of the use of carboxylic acids in samples from higher *Plantago* density.

The increased use of carboxylic acids was related to samples with higher Al-toxicity and Fe and less macronutrients (Tab. 4). The microbial communities that use more polymers, amines/amides and phenols are associated with the conditions of the shrubland (Tab. 4 and Fig. 2A–F). While polymer users seem negatively affected by the presence of soluble Fe, the later two were more positively related to soil relative humidity (RH) (Tab. 4). The relative activity of microbial communities, using carbohydrates as substrates, was positively related with the absence of plants (bare soil), in connection with a decrease in shrub cover, nutrients, moisture and soil depth (Tab. 4).

DISCUSSION

Major drivers of microbial diversity and activity

This study shows the spatial distribution of microbial communities highly associated with the presence of shrub vegetation, thus all the CLPP overall activity and diversity variables increased in areas with more shrub vegetation and decreased in areas with more bare soil. This would be expected since plant biomass is a driver for microbial proliferation (Epelde et al. 2010): litter is decomposed and mineralised and live roots provide rhizospheric substrates and microenvironments for symbionts (Dakora and Phillips 2002; van der Heijden et al. 2008). Thus, with their large biomass, shrubs create a positive environment for an increase in abundance and diversity of microbial communities. On the other hand, for a smaller type of plant, *Plantago*, there were mostly negative correlations between its density and cover with the microbial community overall activity and diversity variables. This allows us to suggest that the strong driver effect of edaphic factors, responsible for the observed vegetation pattern (Serrano et al. 2015[7]), is so hard that affects both the tolerant plant species and its associated microbial communities. In fact, previous works have shown that the geochemical island does not have shrub vegetation mostly because Al-toxicity is too high for those plants to grow there (Serrano et al. 2015[7]). Al-toxicity gave space to other plants to thrive in this low competitive environment and *Plantago* with the Al-hyperaccumulator trait, is such a plant (Serrano et al. 2011[8], 2015[7]).

With this work, it was possible to model the spatial distribution of soil microbial communities in plots with Al-rich geochemical islands for overall activity and diversity and functional groupings. The variables $ACD_{samples}$ (related to the amount and activity of the culturable microorganisms) and Shannon's diversity index (combining the richness of positive results with their evenness) produced the best geostatistical models of soil's microbial communities, for the overall activity and diversity CLPP variables. Richness and Evenness are more sensitive to local variability, accounted by the nugget value, which may be associated with the random presence of vegetation (e.g. sporadic herbaceous plants) or soil's heterogeneity (like pH or particle size; e.g. urine patches, Ritz et al. 2004). All the CLPP activity and overall-diversity indicators

---

[7] Chapter 3

[8] Chapter 4

showed a spatial continuity (≈2 m) compatible with the changes among microhabitats (shrubland and gap), in fact producing strong correlations with the environmental variables.

In this work we also found that the soil microorganisms activity and diversity increased in samples from areas with high relative humidity in the soil (and depth) and macronutrients, and decrease in those with high Al-toxicity and thus from areas with high Fe or Al and low macronutrients. The spatial pattern of soil microorganisms was associated to that of the soil edaphic features that in turn determined the patterns of vegetation (Klironomos et al. 1999) on those metalliferous areas. The geochemical islands are not only vegetation-islands, but also microorganisms-islands. This is summarized by the strong negative correlations with $PC1_{Env}$, for which the scores separate fairly the geochemical island from the surrounding matrix. Some of the gap samples showed negative reactions to many substrates, which in extremis could represent a real loss of functional diversity and thus of resilience of the microbial community, with probable consequences for the resilience of the plant community (van der Heijden et al. 2008). In fact, in nutritional poor stressful environments, microorganisms can be responsible for an adequate deliver of nutrients to the plants present (Alford et al. 2010 and references therein), thus their loss may endanger the plant community.

This work confirms our initial hypothesis that stressful edaphic conditions (namely low RH and high Al-toxicity) reduce the activity and the diversity of the microbial communities. Yet, we are not able to completely separate the edaphic and biotic factors in their influence to the activity and diversity of the microbial communities, as all factors are extremely interlinked (Epelde et al. 2010; Klironomos 2002; Millard and Singh 2010; van der Heijden et al. 2008).

The functional groups' diversity in microbial communities

In this work we analysed the (putative) functional diversity of the soil's microbial communities, associated with substrate consumption, acknowledging six functional guilds (CH, PL, AM, CA, PH and AA). The spatial models obtained could be divided into three groups: (i) polymers, that showed the best spatial model without local variance; (ii) the groups carbohydrates, carboxylic acids, phenols and amines/amides showed some local variance but in overall they were explained by spatial patterns; (iii) amino-acids, that were not explained by spatial patterns but by local variance. The strength and direction of the correlations between these functional groups and the environmental variables were particular for each, unlike the results rather uniform (in value and direction) obtained for the overall activity and diversity CLPP variables. This specificity suggest that the spatial patterns of the relative use of substrates, namely the functional groups, could be better for the understanding of the spatial patterns of soil microorganism's ecology than overall measures of diversity. Nonetheless, within each functional group, the pattern of use for individual substrates may show different directions, but the CLPP method has no power to discriminate at that level (Preston-Mafham et al. 2002). The method is relevant for comparisons among communities, not so much for their characterization. This is because the substrates used do not reflect the natural environment (Preston-Mafham et al. 2002) despite being considered adequate to distinguish environmental samples (Insam 1997).

Some of the microorganisms were associated with the geochemical island being in proportion, dominating in the gaps, both in areas with bare soil or with *Plantago*. The use of carbohydrates as a substrate by microorganisms was dominant in samples from gap areas of bare soil, shallow, with low moisture and low in macronutrients. In the same way, carboxylic acids were proportionally more used by microorganisms in samples from gaps, but associated to higher *Plantago* densities and areas rich in soluble Fe, Al-toxicity and low in nutrients. The microbial communities present in an area of bare soil, without direct sources of plant biomass, must rely on C-sources originated elsewhere, often already decomposed by other processes. The carbohydrates are considered the simplest C-sources of the six types of substrates (Goberna et al. 2005), thus explaining that their use is predominant in the gap bare areas, as microorganisms should be more adapted to use the predominant types of C-sources available. Whereas in the shrubland, the use of more complex substrates (polymers, amines/amides or phenols) is favoured as there is a direct input of large amounts of biomass and exudates (Goberna et al. 2005). The microbial communities using polymers associated mainly to soil soluble Fe, being negatively affected by this metal. Since geochemical islands are made of Al and Fe sesquioxides, we expect that the use of polymers by microorganisms would be dominant in areas outside the gaps. The use of amines/amides and phenols substrates responded to almost the same environmental variables, though we could better spatially model amines/amides than phenols. Higher consumption was associated with the shrubland characters like higher soil moisture and depth, low Al-toxicity, low *Plantago* density and high shrub cover. They differ in that amines/amide's users are associated with samples with more macronutrients, while phenol's users were associated to low Al samples. The use of amino acids by microorganisms from our plots was not associated with the environmental variables studied, thus our spatial modelling was poor, only a weak correlation was determined (negative with soil Al) and there were no average differences between gap and shrubland microhabitats.

The stressful edaphic conditions of the gaps, that we have observed affecting negatively the activity and diversity of the microbial communities, seemed to affect differently the six functional groups of microorganisms. The predominant use of carboxylic acids or carbohydrates in the gaps suggests that the microorganisms present there were more tolerant to the stressful factors of the geochemical islands. In particular, carboxylic acids was the only group to which Al-toxicity (and Fe) correlated positively. Al-toxicity combines the effect of Al and Ca bioavailability (Cronan and Grigal 1995). Al has been mentioned to inhibit the growth of bacterial species but also of being ineffective to others (Amelung et al. 2001; Holzle and Neubert 1982; Piña and Cervantes 1996).

Functional diversity patterns related to an Al-hyperaccumulator

The gaps are the special geochemical niche where *P. almogravensis* (*Plantago*) is able to grow (Serrano et al. 2015[9]). Although with our sampling, we could not detect strong correlations between *Plantago* and the microbial communities, some patterns towards functional diversity were envisaged, as we hypothesized.

The presence of the Al-hyperaccumulator *P. almogravensis* had a negative relation to the soil microbial community, particularly when the species density is considered. The rhizosphere of *P. almogravensis,* especially when the plants are close together (viz. the combined effect of several rhizospheres) was a hostile environment to some microorganisms, diminishing the diversity and abundance of microbial communities. Those communities able to degrade amines/amides and phenols were particularly affected, but the microorganisms using carboxylic acids as C-source seemed more tolerant and increased their proportion. Considering that, metal resistant microorganisms are more often found in hyperaccumulator rhizospheres than in bulk soil (Aboudrar et al. 2007; Alford et al. 2010), if we had specifically sampled for rhizospheric soil it is plausible that the relations found were stronger. Our sampling scheme was spatially random, thus the samples may or not represent

---

[9] Chapter 3

Plantago rhizospheric soil, being more probable to be so in the samples from high *Plantago* densities.

In this study, the proportion of the microbial community degrading carboxylic acids was higher in areas with intense Al-toxicity, where *Plantago* density is higher. Though microbial degradation of carboxylic acids can be inhibited in the presence of Al or Fe hydroxides, due to the formation of a complex between them (Jones 1998), at the same time this process can increase microbial Al-tolerance because the complex CA-Al is less bioavailable than free Al (Dakora and Phillips 2002; Jones et al. 2004; Piña and Cervantes 1996). Thus, the microbial communities from the gap soil samples that can use carboxylic acids as substrate would proliferate as long as there is a source of carboxylic acids available. It is known that *P. almogravensis* has the ability to exude malic acid in the presence of Al, under controlled conditions (Martins et al. 2013). Similarly, this has been observed for other typical hyperaccumulators or plants tolerant to Al, that use organic acids to either immobilize or uptake the metal (Delhaize et al. 1993; Jansen et al. 2002; Ma et al. 2001). In fact, a previous work showed a decrease of Al in in the external root fraction of *P. almogravensis*, which was not compatible with the increase in bioavailable Al in soil (Serrano et al. 2011[10]). Several possible explanations were suggested namely the release of organic acid anions that complex Al and can protect the root apoplast from Al binding (Serrano et al. 2011[10]). Curiously, malic acid (#24) was one of the carboxylic acids more used in the gaps, though the CLPP method does not recommend direct relations (Preston-Mafham et al. 2002).

Conclusions

As we move from the shrubland to the stressful gap conditions the total abundance (activity) and diversity of microorganisms is reduced as well as the composition of the soil microbial communities changes. In the shrubland's samples dominated the users of polymers, amines/amides and phenols, while in the gaps increased the consumers of carbohydrates and carboxylic acids. The spatial distribution of the microbial communities is in close association with the geochemical island edaphic environment (low soil RH, high Al-toxicity) that strongly affects the biotic environment (absence of plants, namely shrubs). Al-toxicity proved to be an important driver, and not always in a negative direction. In particular, we show that the abundance of the rare species *P. almogravensis* is directly linked to specific microbial community activities and functional groups (particularly carboxylic acids), which in turn may affect the resilience of the plant population at the geochemical island.

ACKNOWLEDGMENTS

This research has been supported by the POA project "Estudo de referência de *Plantago almogravensis*" from ICNB /JB-MNHN/PNSACV (Portuguese Institute for Nature Conservation and Biodiversity/Botanical Garden at the National Natural History Museum/ Sudoeste Alentejano e Costa Vicentina Natural Park); project POCTI/BIA-BDE/60664/2004 & PPCDT/BIA-BDE/60664/2004 from FCT (Portuguese Foundation for Science and Technology); HC Serrano PhD grant SFRH/BD/38289/2007 from FCT. Critical discussion: MA Martins-Loução, C Branquinho (*Investigador* FCT contract), M Ribeiro, C Antunes, MJ Pereira, MJ Pinto. For help with the geostatistics and design of the spatial maps, MJ Pereira, M Ribeiro and P Pinho.

REFERENCES

Aboudrar W, Schwartz C, Benizri E, Morel JL, Boularbah A. 2007. Soil microbial diversity as affected by the rhizosphere of the hyperaccumulator *Thlaspi caerulescens* under natural conditions. *International Journal of Phytoremediation* 9: 41-52

Alford ER, Pilon-Smits EAH, Paschke MW. 2010. Metallophytes-a view from the rhizosphere. *Plant and Soil* 337: 33-50

Amelung W, Miltner A, Zhang X, Zech W. 2001. Fate of microbial residues during litter decomposition as affected by minerals. *Soil Science* 166: 598-606

Appanna VD, Stpierre M. 1994. Influence of phosphate on aluminum tolerance in *Pseudomonas fluorescens*. *Fems Microbiology Letters* 124: 327-32

Bardgett RD, Wardle DA, Yeates GW. 1998. Linking above-ground and below-ground interactions: How plant responses to foliar herbivory influence soil organisms. *Soil Biology & Biochemistry* 30: 1867-78

---

[10] Chapter 4

Boudot JP, Becquer T, Merlet D, Rouiller J. 1994. Aluminum toxicity in declining forests - a general overview with a seasonal assessment in a Silver Fir forest in the Vosges Mountains (France). *Annales Des Sciences Forestieres* 51: 27-51

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Cardoso JC. 1965. *Os solos de Portugal, sua classificação, caracterização e génese. 1 - A sul do Rio Tejo*. Portugal: Secretaria de Estado da Agricultura.

Chenery EM, Sporne KR. 1976. Note on evolutionary status of aluminum accumulators among dicotyledons. *New Phytologist* 76: 551-54

Cronan CS, Grigal DF. 1995. Use of calcium aluminum ratios as indicators of stress in forest ecosystems. *Journal of Environmental Quality* 24: 209-26

Dakora FD, Phillips DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* 245: 35-47

Delhaize E, Ryan P, Randall P. 1993. Aluminum tolerance in wheat (*Triticum aestivum* L).2. Aluminum-stimulated excretion of malic-acid from root apices. *Plant Physiology* 103: 695-702

Ellis RJ, Morgan P, Weightman AJ, Fry JC. 2003. Cultivation-Dependent and -Independent Approaches for Determining Bacterial Diversity in Heavy-Metal-Contaminated Soil. *Applied and Environmental Microbiology* 69: 3223-30

Epelde L, Becerril JM, Barrutia O, Gonzalez-Oreja JA, Garbisu C. 2010. Interactions between plant and rhizosphere microbial communities in a metalliferous soil. *Environmental Pollution* 158: 1576-83

Ettema C, Wardle D. 2002. Spatial soil ecology. *Trends in Ecology & Evolution* 17: 177-83

Garland J, Mills A. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57: 2351-59

Goberna M, Insam H, Pascual JA, Sanchez J. 2005. Storage effects on the community level physiological profiles of Mediterranean forest soils. *Soil Biology & Biochemistry* 37: 173-78

Holzle E, Neubert U. 1982. Antimicrobial effects of an antiperspirant formulation containing aqueous aluminum-chloride hexahydrate. *Archives of Dermatological Research* 272: 321-29

Illmer P, Obertegger U, Schinner F. 2003. Microbiological properties in acidic forest soils with special consideration of KCl extractable Al. *Water Air and Soil Pollution* 148: 3-14

Insam H. 1997. A new set of substrates proposed for community characterization in environmental samples In *Microbial Communities. Functional versus structural approaches*, ed. H Insam, A Rangger, pp. 260–61. Heidelberg: Springer Verlag

Insam H, Goberna M. 2004. Use of Biolog® for the community level physiological profiling (CLPP) of environmental samples In *Molecular microbial ecology manual. Volumes 1 and 2*, ed. G Kowalchuk, F de Bruijn, I Head, A Akkermans, J van Elsas, pp. 853-60

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Jones D. 1998. Organic acids in the rhizosphere - a critical review. *Plant and Soil* 205: 25-44

Jones D, Hodge A, Kuzyakov Y. 2004. Plant and mycorrhizal regulation of rhizodeposition. *New Phytologist* 163: 459-80

Kawai F, Zhang DM, Sugimoto M. 2000. Isolation and characterization of acid- and Al-tolerant microorganisms. *Fems Microbiology Letters* 189: 143-47

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Khironomos JN, et al. 2004. Methods of studying soil microbial diversity. *Journal of Microbiological Methods* 58: 169-88

Klironomos J. 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* 417: 67-70

Klironomos JN, Rillig MC, Allen MF. 1999. Designing belowground field experiments with the help of semi-variance and power analyses. *Applied Soil Ecology* 12: 227-38

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL, van Veen JA. 2002. Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 81: 509-20

Li WC, Ye ZH, Wong MH. 2010. Metal mobilization and production of short-chain organic acids by rhizosphere bacteria associated with a Cd/Zn hyperaccumulating plant, Sedum alfredii. *Plant and Soil* 326: 453-67

Lindahl V, Bakken L. 1995. Evaluation of methods for extraction of bacteria from soil. *Fems Microbiology Ecology* 16: 135-42

Lindstrom JE, P. Barry R, Braddock JF. 1998. Microbial community analysis: a kinetic approach to constructing potential C source utilization patterns. *Soil Biology and Biochemistry* 30: 231-39

Ma JF, Ryan PR, Delhaize E. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6: 273-78

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

Masunaga T, Kubota D, Hotta M, Wakatsuki T. 1998. Mineral composition of leaves and bark in aluminum accumulators in a tropical rain forest in Indonesia. *Soil Science and Plant Nutrition* 44: 347-58

Millard P, Singh B. 2010. Does grassland vegetation drive soil microbial diversity? *Nutrient Cycling in Agroecosystems* 88: 147-58

Piña RG, Cervantes C. 1996. Microbial interactions with aluminium. *Biometals* 9: 311-16

Poschenrieder C, Gunse B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* 400: 356-68

Preston-Mafham J, Boddy L, Randerson P. 2002. Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. *Fems Microbiology Ecology* 42: 1-14

Ritz K, McNicol W, Nunan N, Grayston S, Millard P, et al. 2004. Spatial structure in soil chemical and microbiological properties in an upland grassland. *Fems Microbiology Ecology* 49: 191-205

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Soares A. 2000. *Geoestatística para as ciências da terra e do ambiente*. Portugal: IST Press. 206 pp.

van der Heijden MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310

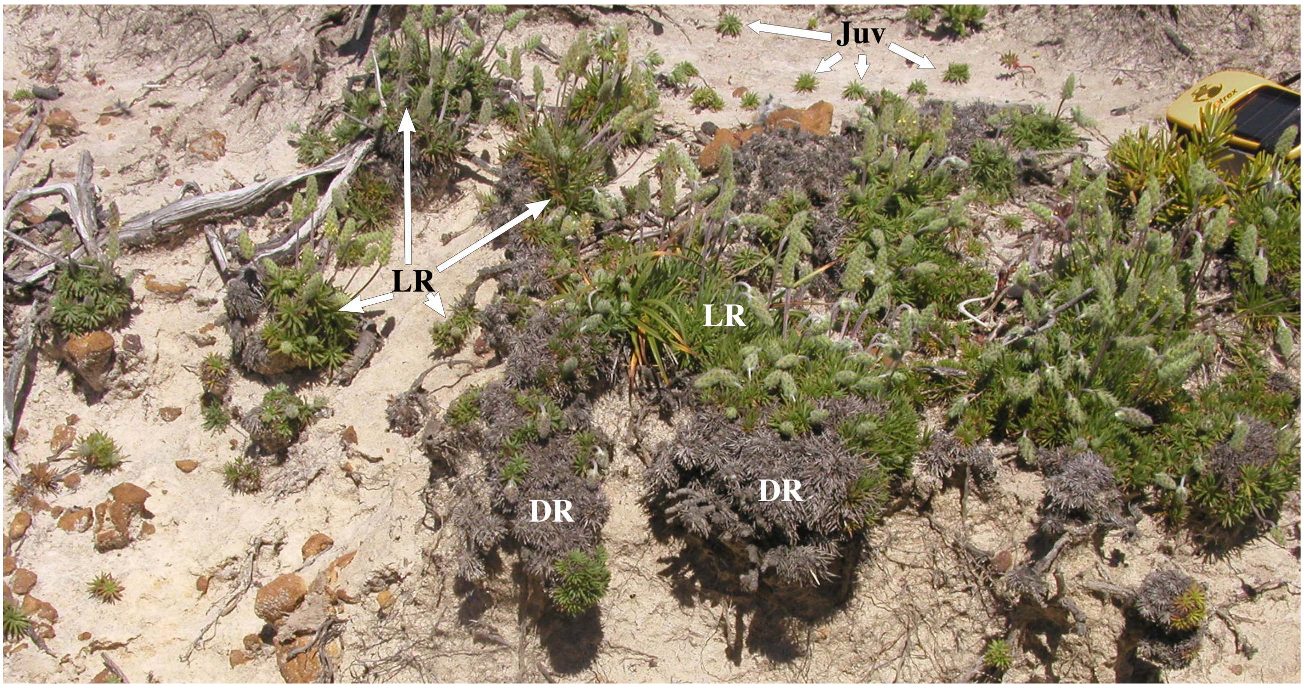
Wardle DA, Bonner KI, Barker GM, Yeates GW, Nicholson KS, et al. 1999. Plant removals in perennial grassland: Vegetation dynamics, decomposers, soil biodiversity, and ecosystem properties. *Ecological Monographs* 69: 535-68

Wood S, Sebastian KL, Scherr SJ, Institute WR. 2000. *Pilot analysis of global ecosystems: agroecosystems*. World Resources Institute.

Zak DR, Holmes WE, White DC, Peacock AD, Tilman D. 2003. Plant diversity, soil microbial communities, and ecosystem function: Are there any links? *Ecology* 84: 2042-50

CHAPTER 7

THE LIFE CYCLE OF *PLANTAGO* SPP.: IMPACT FACTORS & POPULATION DYNAMICS



Detail from a *Plantago almogravensis* cohort during flowering season. **Juv:** juveniles; **LR:** adults with live rosettes, many presenting flowers; **DR:** dry rosettes.

CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

Chapter 8

THE LIFE CYCLE OF *PLANTAGO* SPP.:
IMPACT FACTORS & POPULATION DYNAMICS

ABSTRACT

In this work we studied the extant population of a critically endangered species, *Plantago almogravensis*, to determine important constrains to its present and future conservation. To have a reference to compare its responses, we observed a sister species, with a slightly better conservation status (endangered, *P. algarbiensis*). To determine the status of a population/species one has to be aware of what is affecting the various phases of its life cycle and observe the dynamic of the individuals over time. Using laboratorial germination tests and following the development of the plants, we estimated the importance of possible limitation factors, namely light, Al-toxicity, seed bank persistence and soil microorganisms. From field visits, we observed the plant phenophases and accounted for a small negative budget for the population survival, which is in accordance to its critically endangered status.

INTRODUCTION

The status of a species is often difficult to determine due to its wide distribution. In the case of narrow endemics that task may seem facilitated but many factors concur to affect the life cycle and the population dynamics. Germination is the first step of a seed-plant life. To germinate successfully, the seed has to be dispersed to a suitable location, with determined light, temperature and humidity conditions, and contain the necessary amount of reserves to thrive before becoming autonomous. After the emergence of the radicle, germination proceeds with root growth and expansion of the cotyledons. Difficulties in anchorage, lack of nutrients or excess of toxic elements, lack or excess of water, excessive or insufficient light (photochemical input) may determine the faith of the seedlings (Blom 1992). As it develops, soil microorganisms (beneficial or detrimental), herbivores and other plants (from competitors to nursing plants), also influence the growth and survival of the plant (Baskin and Baskin 2014; Blom 1992). Moreover, the plant needs to adapt to climate changes to reproduce and thrive. In extreme situations they are subjected to summer drought and high temperatures, and to winter low temperatures or flooding, that may considerably damage or kill the plant. Nowadays, also the inability to adapt to the world fast climate changes may determine the faith of an entire species.

Plantago almogravensis Franco and *P. algarbiensis* Samp. are two rare endemics, sister species within the Iberian Peninsula. The first is considered Critically Endangered by IUCN (Bilz et al. 2011), as only one population is known (less than 3 ha in Portugal) while the second (IUCN Endangered) is found in Portugal and Spain. In previous works (Chapter 6; Serrano et al. 2011[1], 2015[2]) we have shown that *P. almogravensis* presents constrains due to competition and Al-toxicity, as well as differences in the soil microbial community. This species is mostly found within gap areas (Chapter 2), suggesting some light requirement (Blom 1992); the gaps were

---

[1] Chapter 4

[2] Chapter 3

observed to have an unusual chemical composition, rich in Fe and Al (Serrano et al. 2011[3]), that can be toxic to some plants (Barceló and Poschenrieder 2002; Ezaki et al. 2008; Poschenrieder et al. 2008). The severity of Al-toxicity is known to be influenced by the concentrations of Ca and other cations in the external solution or pH, among others (Kinraide and Parker 1987). The most visible effect of Al-toxicity is the stunt root growth together with other morphological changes that result ultimately in difficulties in water and nutrient uptake (Barceló and Poschenrieder 2002). Either directly or indirectly due to the root limitations, the effect of Al-toxicity, in the shoot can be seen in cell modifications, reduced photosynthesis, nutrient deficiency and reduced biomass (Barceló and Poschenrieder 2002; Vardar and Ünal 2007; Watanabe and Osaki 2002). The presence of mycorrhiza and particular functional groups of microorganisms in the nutritionally poor areas colonised by *P. almogravensis* (Chapter 6), suggests their close involvement (Allsopp and Stock 1995). In order to understand how those constrains affect the biology of this plant species along its life-cycle we proposed to investigate (i) germination and establishment in relation to those factors, namely: light, presence of Al and Ca and presence of soil microorganisms. Other important steps concerning the overall life cycle of the populations were also considered: (ii) the longevity of the seed bank and (iii) the population dynamics. When possible, comparisons were made to the life cycle of the sister species *P. algarbiensis*.

MATERIAL & METHODS

Locations and species studied

The main subject of this work is the taxa *Plantago almogravensis* Franco, with comparisons to the sister species *P. algarbiensis* Samp. (Franco 1984). Field samplings (Fig. 1) were conducted in the regions of Odemira (*P. almogravensis*); Silves-Albufeira, Faro and Huelva (*P. algarbiensis*). The experimental work (germinations) was conducted at the Botanical Garden of the University of Lisbon (MUHNAC).

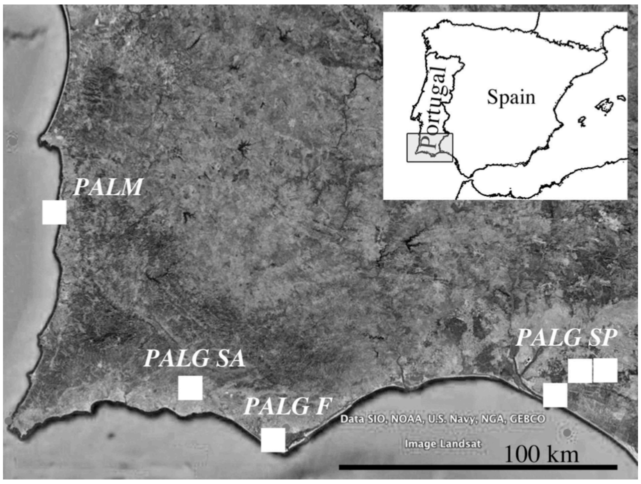


Figure 1. Map of the study areas. *Plantago almogravensis* (**PALM**) in Southwest Portugal (Odemira region) and *P. algarbiensis* (**PALG**) in Silves-Albufeira region (**SA**), Faro (**F**) and Huelva, Spain (**SP**). **White squares:** locations in a 10 km grid. Images: © Google Earth 2014.

Germination tests I: light and aluminium

Seeds of *Plantago almogravensis* Franco and *P. algarbiensis* Samp. were placed in 9 cm Petri dishes, filled with 30 ml of a 0.7% agar sterilized test medium. The test solutions (Tab. 1) were composed of $AlCl_3.6H_2O$ (0 mM, 0.05 mM or 0.5 mM) and $CaCl_2.2H_2O$ (0 mM, 15 mM); and one extra test with NH_4NO_3 (0.375 mM + 15 mM $CaCl_2$ + 0.5 mM $AlCl_3$). The pH of the solution was set at 4.2 before autoclaving with HCl and NaOH, 1 M (pH electrode micro pH 2002, CRISON), but the 0.5 mM Al solutions did not settle solid. For those test solutions a new batch was made, autoclaving separately the agar and the Al solution, and then mixing (Stein et al. 1973), thus not correcting the pH. Superficial pH was measured again with a flathead electrode (PH25, CRISON) at the end of the experiment, on blank plates (control) and on the used test plates, to evaluate medium pH changes due to the experiment.

---

[3] Chapter 4

Table 1. Summary of test mediums and number of seeds/seedlings for *Germination I*.

Tests:	Dark Al_0	Al_0	Al_0+Ca	Al_{low}	Al_{low}+Ca	Al_{high}	Al_{high}+Ca	Al_{high}+Ca+N
				Medium composition				
Al mM	0	0	0	0.05	0.05	0.5	0.5	0.5
Ca mM	0	0	15	0	15	0	15	15
N mM	0	0	0	0	0	0	0	0.75
				Number of seeds				
P. almogravensis								
plate 1	17	17	17	17	17	17	17	17
plate 2	17	17	17	17	17	14	17	17
plate 3	17	17	17	17	17	14	17	17
plate 4	17	18	18	18	18	14	18	17
P. algarbiensis								
plate 1	25	25	25	25	25	25	25	25
plate 2	25	25	25	25	25	25	25	25
plate 3	25	25	25	25	25	25	25	25
plate 4	25	25	25	25	25	25	25	25
				Number of seedlings evaluated for development				
P. almogravensis								
plate 1		6	6	11	8	7	9	
plate 2		8	10	6	5	5	3	
plate 3		7	6	6	8	9	3	
plate 4		7	9	7	6	5	5	

Legend: **dark:** test without light, all the other tests were subjected to the light regime; **Al_0:** control without Al source; **Al_{low}:** test with 0.05 mM Al; **Al_{high}:** test with 0.5 mM Al; **Ca:** test with 15 mM Ca.

In each test, four Petri dishes (n = 4) were used. Seeds collected in 2008, 2009 and 2011 were distributed proportionally among the plates, and disposed in a 5x5 layout (Tab. 1). The plates were sealed and germination (February 2012) proceeded in a chamber with a temperature regime of 16.5/8 °C day/night and 10 h day length (photon flux density = 69.6 µE m^{-2} s^{-1}), similar to field conditions in the germination season (average of monthly maximum and minimum means of November-February, 2009-2012; IMP 2008–2012). The control in the dark was sealed with aluminium foil and observed only at the end of the experiment (8 weeks), to avoid any light input. Germination was considered at radicle emergence.

Continuing the evaluation of the effects of Al and Ca after germination, seedlings of *P. almogravensis*, 44-55 days old at the end of the germination experiment (Tab. 1), were photographed to determine the presence of leaf primordia and digitalized to measure the root length (software Adobe Photoshop® and ESRI ArcGIS®).

The analysis of variance (ANOVA) was performed using general linear models in normal or log-transformed data, with plate nesting when appropriate, using $p \leq 0.05$ as the significance level. When differences were found, post-hoc tests were used (Tukey's Honestly Significant Differences — Tukey's HSD). The graphic data were presented as bars with mean ± SEM of n = 4 plates, or box-plots limited by hinges (25th and 75th percentiles), a dash (median, 50th percentile) and whiskers (minimum and maximum values), of the n replicates on Tab. 1.

Germination tests II: microorganisms

Native soil (for each species) was mixed (1:1 v/v) with washed river sand and divided for three batches: Natural, Bacterial and Sterilised soil. The latter two were subject to dry heat sterilization (at 120 ºC for 1 week with a brief break of 1 day for a 10% w/v rewet, to break spore dormancy); an identical amount of water was added to a 250 ml portion of native soil, filtered through a 8 µm pore (Whatman® n.40) with vacuum; this solution was mix with half the sterilised substrate, forming the *Bacterial* soil batch. Thus the *Sterilised* batch would lack most the fungal and bacterial flora; the *Bacterial* batch would have the bacterial microorganisms reintroduced, while lacking the fungal part, filtered at 8 µm; and the *Natural* batch would represent the entire native soil microbial community.

The soil batches were divided per pots and seeded (December) with three seeds each (*P. almogravensis,* n = 11 pots; *P. algarbiensis,* n = 21 pots). Germination, growth and survival of the plants were accompanied during about one year. Germination proceeded in open chambers; afterwards pots were transferred to open air (protected garden), at the Botanical Garden (BG).

The analyses of variance (ANOVA) performed considered $p \leq 0.05$ as the significance level. When differences were found, the Tukey's HSD post-hoc test was used. The error bars in graphic data represent the SEM of n pots.

Germination tests III: storage

To account for seed viability decline due to storage, the results from the germination experiments I and II, together with others (e.g. soil and wet cotton germinations) were gathered and analysed, regarding time from seed collection (crop year) until seeding. Considering that germination protocols and storage conditions were diverse and recurrently not reproducible (non-controlled environments), these results should be interpreted lightly.

Phenology

Boundaries for the phenological phases were determined roughly for *P. almogravensis* and *P. algarbiensis,* from field visits between 2004 and 2011. Microclimatic data (T °C: temperature; HR %: relative humidity) were recorded (2009-2010; at 2h intervals) with climatic loggers (DS1923 iButton Hygrochron). Sensors were scattered within the populations, in Odemira (n = 3), Silves-Albufeira (n = 4), Faro (n = 1) and Huelva (n = 2). Each was placed inside shrubs, to avoid detection. The data collected and the Climate Normals (1971-2000) are shown on Fig. 2.

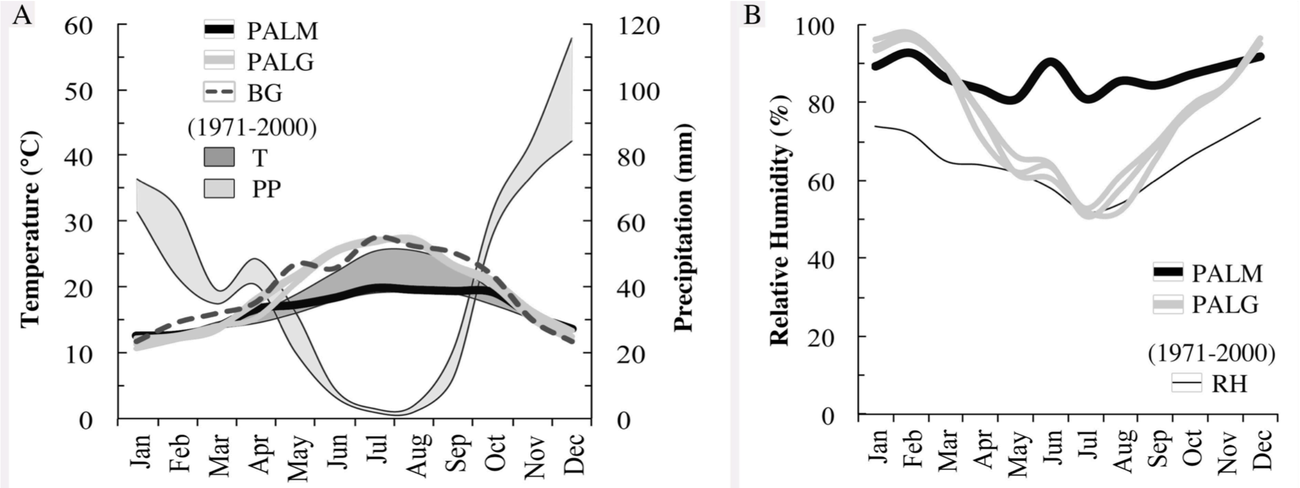


Figure 2. Ombrothermic curves of monthly mean (**A**) Temperature and Precipitation, and (**B**) curves for air Relative Humidity. **Climate Normals 1971-2000:** air temperature (**T**) and precipitation (**PP**), depicted as the range from stations Sines, Faro (www.ipma.pt) and Huelva (www.aemet.es); Air Relative Humidity (**RH**), with values just for Huelva. **Climatic loggers data:** 2006-2007 in the Botanical Garden (**BG**); 2009-2010 in the wild populations of *P. almogravensis* (**PALM**) and *P. algarbiensis* (**PALG**). The three lines for PALG account for the Silves-Albufeira, Faro and Huelva data, separately.

Population dynamics for *P. almogravensis*

An area of ca. 1 m diameter in a cohort of *P. almogravensis*, located in a gap area (subject to high environmental and edaphic stress conditions) was monitored photographically in a series of years (2008, 2009, and 2011). These photographs were used to compare the establishment of new recruits, mortality and flower production.

RESULTS & DISCUSSION

Germination and establishment

Requirement for light

Seeds placed in agar for germination with or without light (Fig. 3A) reached similar final germination percentages. The sister species *P. algarbiensis* showed a similar reaction to light (Fig. 3A). Germination and establishment of a light sensitive species is often limited at the shaded canopy of neighbouring plants, in competition for the available light. Our results suggest that competition for light would not be critical at germination stage for either *P. almogravensis* or *P. algarbiensis*, since the germination results with and without light were similar. This is consistent with results from other authors for the same species (Martins et al. 2012), but not for other *Plantago* species, where germination is often inhibited by darkness (Blom 1992; Zaady et al. 1997).

Eighth weeks after seeding, all seedlings in the dark had died, while those under a light regime kept on growing. Furthermore, 32% died after radicle formation without development of cotyledons (only 8% in *P. algarbiensis*), suggesting a delay in germination in dark conditions and/or the need of a photochemical stimulus to continue development. Thus, light seems to have an important role in the recruitment of *P. almogravensis* after germination, as it does for other *Plantago* species (Blom 1992; Rahn 1996).

In the field, the light competition could contribute to explain the distribution pattern of the *P. almogravensis* individuals (Chapter 2; Serrano et al. 2015[4]). Most individuals are found in gap areas, without any shrub cover, or in their semi-covered margins. Only less than 2% are found under the canopy of shrubs, in the shrubland (Chapter 2). For these,

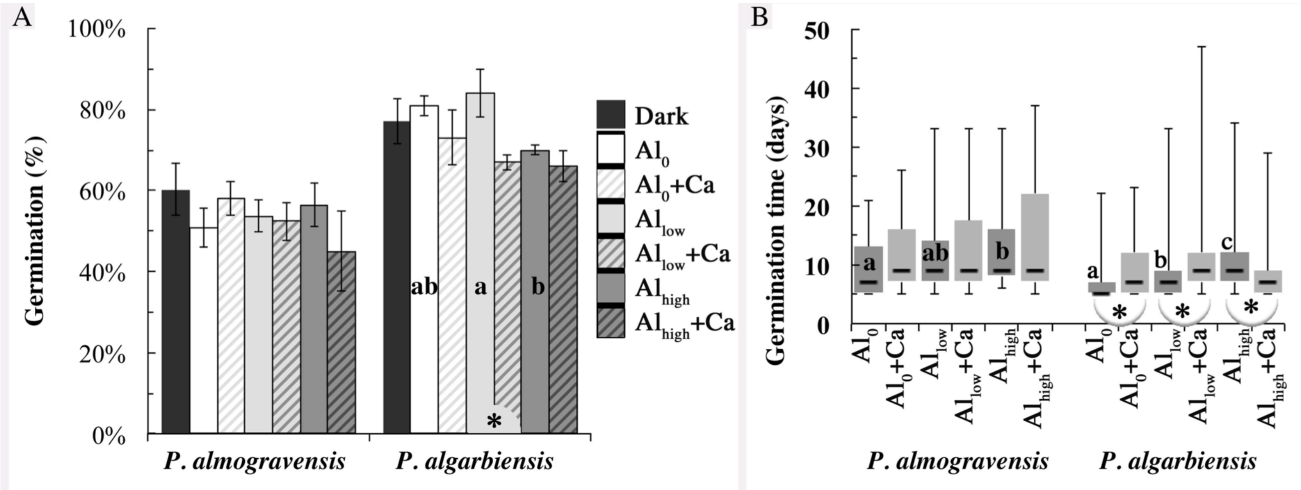


Figure 3. Seed germination in agar. **A:** Final mean germination (8 weeks after seeding); **B:** Time to germination. **Al_0**: control without Al source (blank); **Al_{low}**: test with 0.05 mM Al; **Al_{high}**: test with 0.5 mM Al; **Ca**: test with added Ca (15 mM); **Dark**: blank kept without light. Different letters for each species indicate significant differences among Al_0, Al_{low} and Al_{high} tests; (*) indicate significant differences between the tests with and without Ca.

---

[4] Chapter 3

though adult individuals seem physiologically fit, new recruits are hardly found suggesting that the (current) adult plants managed to establish without competition (Brokaw and Busing 2000). Considering that in a natural succession, when a spatial niche is left unoccupied by an individual, another can take its place (Baskin and Baskin 2014; Lavorel et al. 1994), maybe those occasional *P. almogravensis* individuals occupied temporary gaps formed from shrub senescence, fire, herbivory or plant collection, natural for those sites. Because the sclerophyllous shrubs have a much quicker growth rate and size, than *P. almogravensis*, the temporary gap closed rapidly, blocking the light and any further recruitment (Brokaw and Busing 2000).

Influence of aluminium and calcium

To understand the influence of Al for germination and the possible alleviation of Al-toxicity by Ca, *Plantago* spp. seeds were germinated in agar supplemented with $AlCl_3$ and/or $CaCl_2$. In the doses used, Al had no significant effect on germination comparing with the control (Fig. 3A). Nevertheless, *P. almogravensis* seemed less affected by Al_{high} (Al_0 vs. Al_{high}, $p = 0.556$) than *P. algarbiensis* (Al_0 vs. Al_{high}, $p = 0.072$), though neither species reached a significant Al effect on germination percentage (Fig. 3A). Contrary to what was expected, Ca might have had an inhibitory effect on germination, especially for *P. algarbiensis* (Fig. 3A), where it reached a significant difference in the Al_{low} test (Fig. 3A). Similarly, the addition of $CaCl_2$ and of NH_4NO_3 to the germination medium, which is expected to lower Al-toxicity and to give a nitrogen source, respectively, had no significant effect on final germination percentage (results not shown).

The presence of increasing doses of $AlCl_3$ increased the mean germination time (Fig. 3B). For *P. almogravensis*, that increase was irrespective of the presence or absence of Ca, though apparently the trend is for $CaCl_2$ delay germination. Considering that Ca has water retention properties, perhaps its presence is perceived by the plants as an increased probability of flooded conditions. In these conditions, the mucilaginous coatings are known to restrict oxygenation to the seed, delaying germination in some species (Baskin and Baskin 2014; Zaady et al. 1997). Similarly for *P. algarbiensis*, increasing doses of $AlCl_3$ also delayed germination (Fig. 3B), but the addition of $CaCl_2$ had contrasting effects depending on the Al concentration (Fig. 3B). Only in the Al_{high} tests of *P. algarbiensis* Ca seemed to have a positive effect, decreasing significantly the germination time, though it did not correspond to a higher germination percentage (Fig. 3A). Another possibility is that instead of Ca we perceived an effect of excess Cl ($CaCl_2$ + $AlCl_3$), but the experimental design would not distinguish this effect.

After emergence of the radicle, germination proceeds with root growth and expansion of the cotyledons. In the presence of high levels of Al (Al_{high}), the seedlings of *P. almogravensis* (Fig. 4A, Fig. 5C zoom) and *P. algarbiensis* (not shown) showed a tendency to stunt root growth. Yet, the aerial part seemed to develop normally, not delaying significantly the appearance of leaf primordia (Fig. 4B). At this stage, the seedlings should be still using the reserves from the cotyledons, thus the lack of an immediate effect on the appearance of leaf primordia.

The presence of Ca has significantly ameliorated the root growth in the Al_{high} doses both in length (Fig. 4A) and in reducing the tip deformations (Fig. 5C zoom, Fig. 5D). In this case, seems unlikely that low pH alone is responsible for the stunt root growth (Barceló and Poschenrieder 2002; Kidd and Proctor 2001) since in the Ca-ameliorated medium, the pH is less or equal to the pH of the non-ameliorated test, where the root length was significantly smaller (Fig. 4 A, C). The role of Ca in alleviating Al-toxicity, thus allowing normal root growth, has been also observed for other species (Barceló and Poschenrieder 2002; Cronan

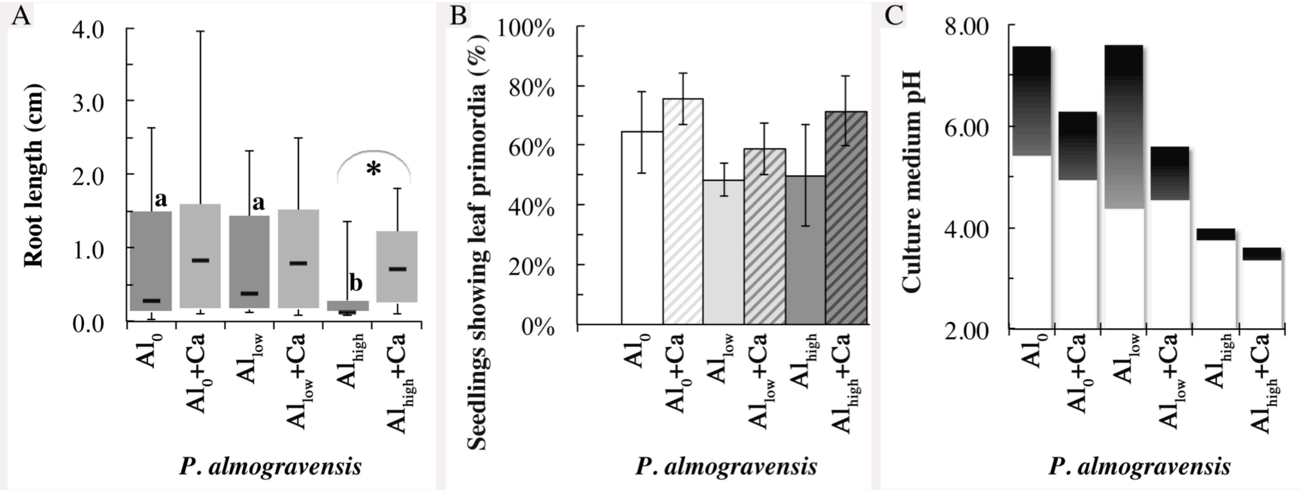


Figure 4. Seedling growth (44-55 days old). **A**: length of the root; **B**: percentage of seedlings showing leaf primordia; **C**: final culture medium pH, in unused plates (control, white bars) or plates with grown seedlings (black bars). **Al_0**: control without Al source (blank); **Al_{low}**: test with 0.05 mM Al; **Al_{high}**: test with 0.5 mM Al; **Ca**: test with added Ca (15 mM). Different letters indicate significant differences among Al_0, Al_{low} and Al_{high} tests; (*) indicate significant differences between the tests with and without Ca.

and Grigal 1995). In a previous work (Serrano et al. 2011[5]) an unusually high Ca saturation of the external fraction of the root (52% Ca vs. 23% Ca in soil and 22% inside the root) was observed in the field-plants hyper-accumulating Al, which may be associated with Ca protection to the root, from Al-toxicity.

Notably, these two species seem to have adapted to the survival in such hostile conditions. Despite the initial poorer root development, the *P. almogravensis* plantlets grown in Al_{high} managed to have a 30% survival after transplant to soil (Fig. 6) (survival considered as live plantlets in soil per seedling transplanted). For *P. almogravensis* the total survival (plantlets in soil per germinated seeds) was best in

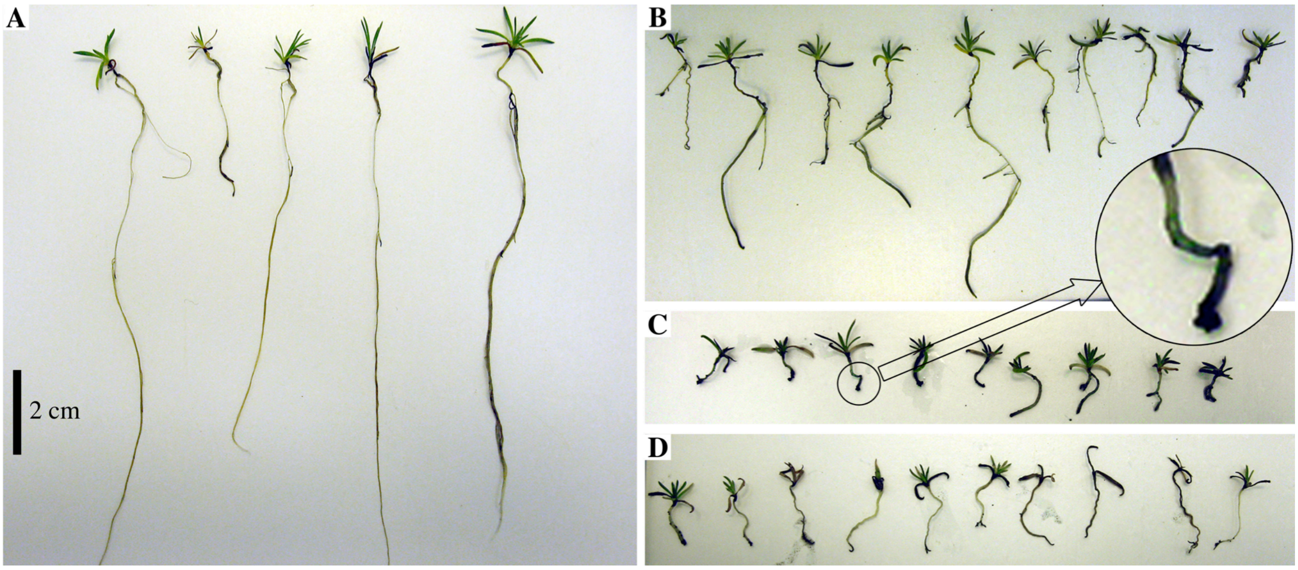


Figure 5. *P. almogravensis* plantlets (ca. 10 weeks after beginning of germination tests). **A:** control without Al source (blank, **Al_0**); **B:** test with 0.05 mM Al (**Al_{low}**); **C:** test with 0.5 mM Al (**Al_{high}**), zoom showing the stunt root growth; **D:** test with 0.5 mM Al +Ca (**Al_{high}+Ca**). The scale is the same for all images.

---

[5] Chapter 4

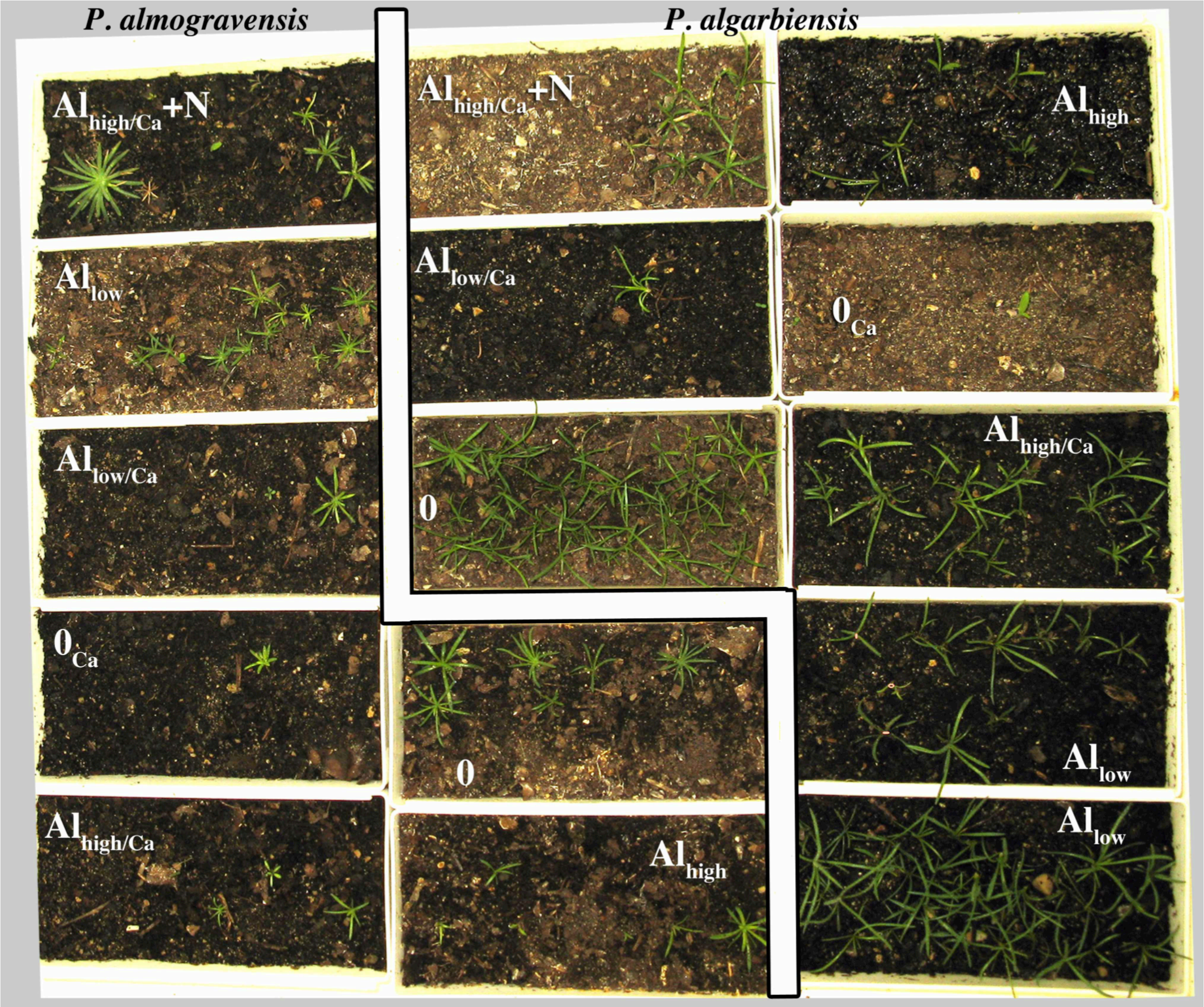


Figure 6. Survival of the plantlets germinated in agar, after 5 weeks in soil (16 weeks after beginning of germination tests). **O:** control without Al source (blank, **Al$_0$**); **Al$_{low}$:** test with 0.05 mM Al; **Al$_{high}$:** test with 0.5 mM Al; **Ca:** tests with 15 mM Ca.

Al$_{low}$ (33%) > Al$_0$(16%) ≥ Al$_{high}$(16%) ≥ Al$_{high}$+Ca+N (14%) > Al$_{high}$+Ca (9%) > Al$_{low}$+Ca (3%) > Al$_0$+Ca (2%) (Fig. 6). This sequence suggests a positive role for Al and N, but an overall negative role for Ca, in survival. For *P. algarbiensis* the sequence is similar, Al$_{low}$ (50%) > Al$_0$ (46%) > Al$_{high}$+Ca (27%) > Al$_{high}$+Ca+N (10%) ≥ Al$_{high}$ (9%) ≥ Al$_{low}$+Ca (1%) > Al$_0$+Ca (0%), though suggesting an alleviating effect for Ca, in the higher Al dosage (Fig. 6). Considering the high survival rate of the plantlets with smaller root systems (Fig. 5), the potential for a successful establishment, for these species, cannot be determined solely on biomass measurements, because it does not reflect their capacity to thrive in hostile conditions and their resilience to overcome them (Serrano et al. 2015[6]). The two species were well adapted to high levels of soluble Al and, even despite slower root growth, seem to have higher success rates in its presence. The presumed alleviating effect of Ca was only envisaged in the impairment of stunt root growth, at higher Al dosages, and much more effective in *P. algarbiensis* than *P. almogravensis*.

---

[6] Chapter 3

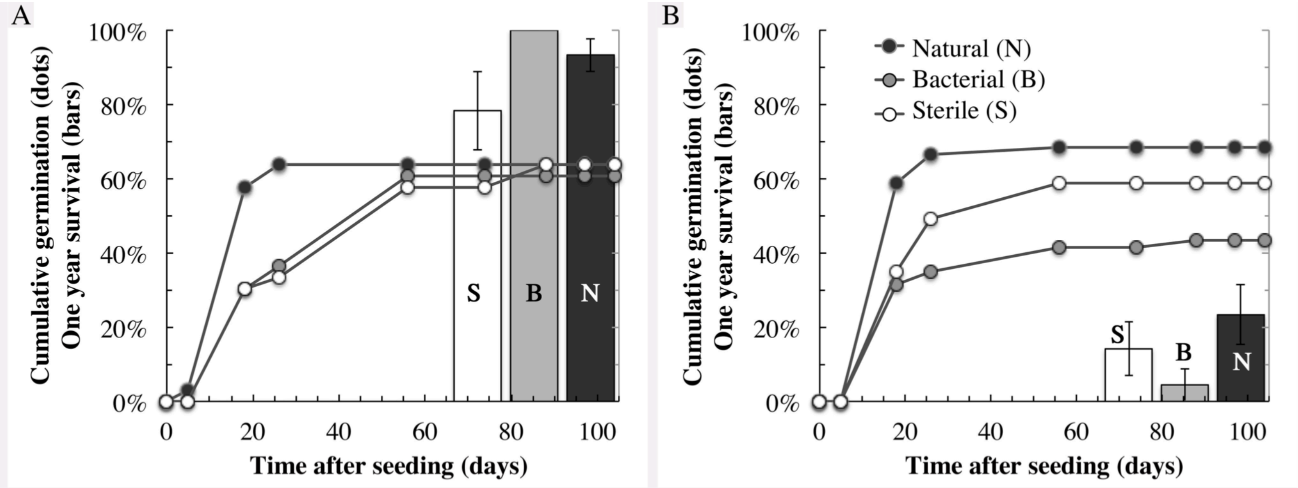


Figure 7. Germination and survival of *P. almogravensis* (**A**) and *P. algarbiensis* (**B**) in indigenous soil. **Natural:** non-treated soil; **Sterile:** sterilized soil; **Bacterial:** sterilized soil reintroduced with native bacteria. Seeding was made in December.

Role of soil microorganisms

The role of microorganisms in the first steps of plant life cycle was tested for germination and establishment (survival). This work has shown faster germination of *P. almogravensis* seeds, in soil (podzol) with a native microbial community present (*Natural*; Fig. 7A), than in soils totally or partially lacking those microorganisms (*Sterile* and *Bacterial*; Fig. 7A). Microorganisms have been described to degrade the seed coat, facilitating germination (Fenner and Thompson 2005; Long et al. 2015). The speed to react and occupy a vacant niche, as a window of opportunity under favourable life conditions such as the first winter rains, could determine the success or failure for the entrance of new seedlings in the field. Thus, microorganisms could be important partners to capture that window. Although the final germination percentage was not affected in our tests, the presence of the "right" soil microorganism community is known to help seedlings to cope with stressful conditions, particularly when seeds are barely rich in nutrients to support further seedling growth, e.g. providing phosphorous (Allsopp and Stock 1995). In the case of *P. algarbiensis* (Fig. 7B), the final germination in *Bacterial* soil was significantly lower than that in *Natural* native soil (p = 0.037; 43% vs. 68%), suggesting a negative feedback from the reintroduced bacteria, specifically when in the putative absence of fungal partners.

Establishment is also a critical step in life cycle. Considering the survival of the new plantlets in the first year, *P. almogravensis* in *Sterile* soil (Fig. 7A) survived slightly less than in soil with microorganisms, namely reintroduced bacteria (p = 0.057), again suggesting that the absence of soil microorganisms could be a limiting factor of plant survival and full establishment. Plantlet mortality (not shown) was higher after leaves started to dry (Fig. 8) in the late spring/summer, when average air temperatures reached over ca. 20°C (Fig. 2A, BG data). In these conditions, in the absence of microorganisms, namely fungus, *P. almogravensis* invested more in ramification (Fig. 8A). More rosettes imply more leaves, but also that some rosettes can be later sacrificed, if needed, for the organism to survive (Serrano et al. 2015[7]). Thus a reduction in the number of live rosettes was observed after the summer (Fig. 8A). Since the main component present in the *Natural* soil and absent from the *Bacterial* & *Sterile* batches are fungi,

---

[7] Chapter 3

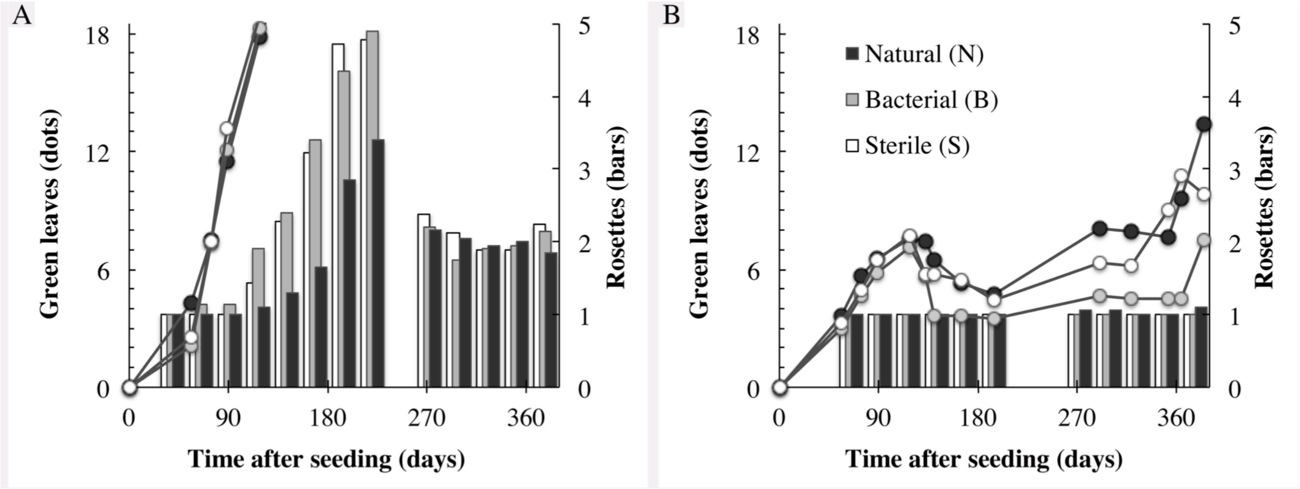


Figure 8. Number of leaves and rosettes of *P. almogravensis* (**A**) and *P. algarbiensis* (**B**) seedlings grown in indigenous soil. **Natural:** non-treated soil; **Sterile:** sterilized soil; **Bacterial:** sterilized soil reintroduced with native bacteria. Leaves > 20 were not counted. Seeding was made in December.

it is possible that symbiosis with fungus (mycorrhiza; Chapter 2) helps to reduce the need to invest in more ramifications, providing more nutrients or water to survive the summer and support resprouting (Zeppel et al. 2014). The strategy of *P. algarbiensis* is different (Fig. 8B). There was no investment in rosettes, and soil bacteria were detrimental to the number of leaves per plant. The few plants that survived managed to keep a few green leaves (Fig. 8B). Survival was disastrous, in the first year for *P. algarbiensis* (Fig. 7B), with no significant differences between the soil treatments (p = 0.198), though those from the *Natural* soil looked more vigorous (personal observations). Surprisingly, higher survival rates were observed for the highly endangered *P. almogravensis* than for the rare *P. algarbiensis* (Fig. 7), indicating more resilience, in the garden conditions, more close to that of *P. algarbiensis*, with respect to air temperatures (Fig. 2A). Unlike the plants in the field (and that of *P. algarbiensis*), these *P. almogravensis* plants did not lose too many leaves during the summer (always had more than 20 green leaves, Fig. 8A).

In general, a positive feedback was noted, between *P. almogravensis* and soil microorganisms, in germination and survival. For *P. algarbiensis* a negative feedback with the reintroduced soil bacteria was observed, though inconspicuous when within the whole native soil microbial community.

Seed age: effects for storage and persistence of the seed-bank

To approach the influence of seed age on seed germination, we used the time from seed collection to seeding in the laboratory, the seed storage time, as a surrogate of seed-bank longevity. Considering *P. almogravensis*, the results show a decline in germination with storage time (R^2 = 0.43, Fig. 9A) that suggests the seed bank does not have a great longevity. This is not unusual in perennial plants that invest in vegetative growth to withstand the unfavourable seasons, and it is within what was observed for other perennial *Plantago* spp. (less than 5 years; Blom 1992). *Plantago algarbiensis'* seed viability seems to be less affected by storage (R^2 = 0.13, Fig. 9B) than *P. almogravensis*, suggesting it might have a more longstanding seed bank. These results are just estimations that should be further investigated, preferably under field conditions. Laboratory results are confused by different variables (e.g. substrate used or storage conditions) that ultimately do not represent field conditions.

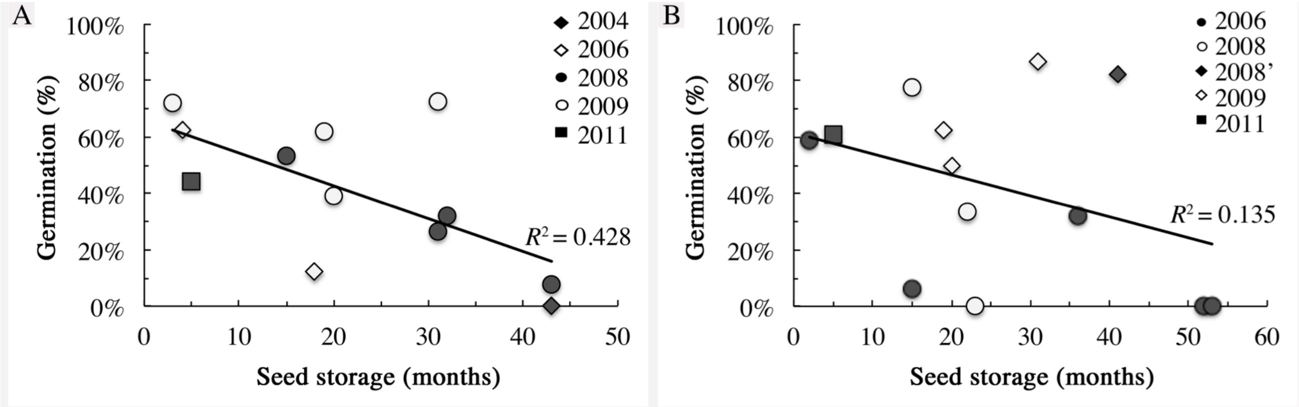


Figure 9. Germination in relation to seed age at seeding, for *P. almogravensis* (**A**) and *P. algarbiensis* (**B**). Different symbols represent crop year.

Phenophases in the life cycle of *Plantago* spp.

Most of the life cycle's phenophases for both *Plantago* spp. studied were similar (Tab. 2), except for the flowering season that started usually earlier in *P. algarbiensis* and could last also longer. The air temperatures and humidity (Fig. 2) are quite different in the two species locations at the start of flowering, thus it could help explain the different onset of flowering. Considering the seashore location of the *P. almogravensis* population, its average air humidity is always high, even when precipitation is at its lowest (summer) due to the sea mists (Fig. 2).

There is no ejection mechanism for the seed, thus it can remain on the decomposing spike for a long time. Seed dispersion can occur either during the dry season helped by wind or mechanically by animals, and in the rainy season aided by the rainwater droplets. When wet, the seed mucilage can have diverse effects; help anchor the seeds to the soil after a rain event (Zaady et al. 1997); obstruct transport by ants by adhering to the soil (Baskin and Baskin 2014; Zaady et al. 1997), or to a small extent, help in long distance dispersal by sticking to large mammals (epizoochory) and birds (Manzano and Malo 2006; Rahn 1996; Zaady et al. 1997). Germination occurs after the rainy season has started, and may last until late winter. A recruit, the juvenile plant that has established successfully from seedling, can become a reproductive adult, generally in the following year.

For the adults, during the summer, most of the leaves dry. After the first rains, the new green leaves start to develop and grow until the spring (Fig. 10), in a resprouting strategy (Zeppel et al. 2014). Some of the rosettes may not recover and the plants show dry dead patches, though then axillary buds may develop instead; in extremis the whole plant may dye if its carbon reserves were not sufficient (Zeppel et al. 2014). Older plants have been observed to die in the summer, namely after a year of exceptional flower production (personal observations), suggesting an inability to withstand the drought after that high investment in reproduction (carbon allocation) (Zeppel et al. 2014).

Table 2. Phenophases in *Plantago* spp. life cycle.

	P. almogravensis	*P. algarbiensis*
Flowering	May – June	April – July
Fructification	July	July - August
Dispersion	July – October*	
Germination	November - February	
Vegetative growth	September - June	
Dormancy	July - September	

(*) spikes may stand upright until next flowering season.

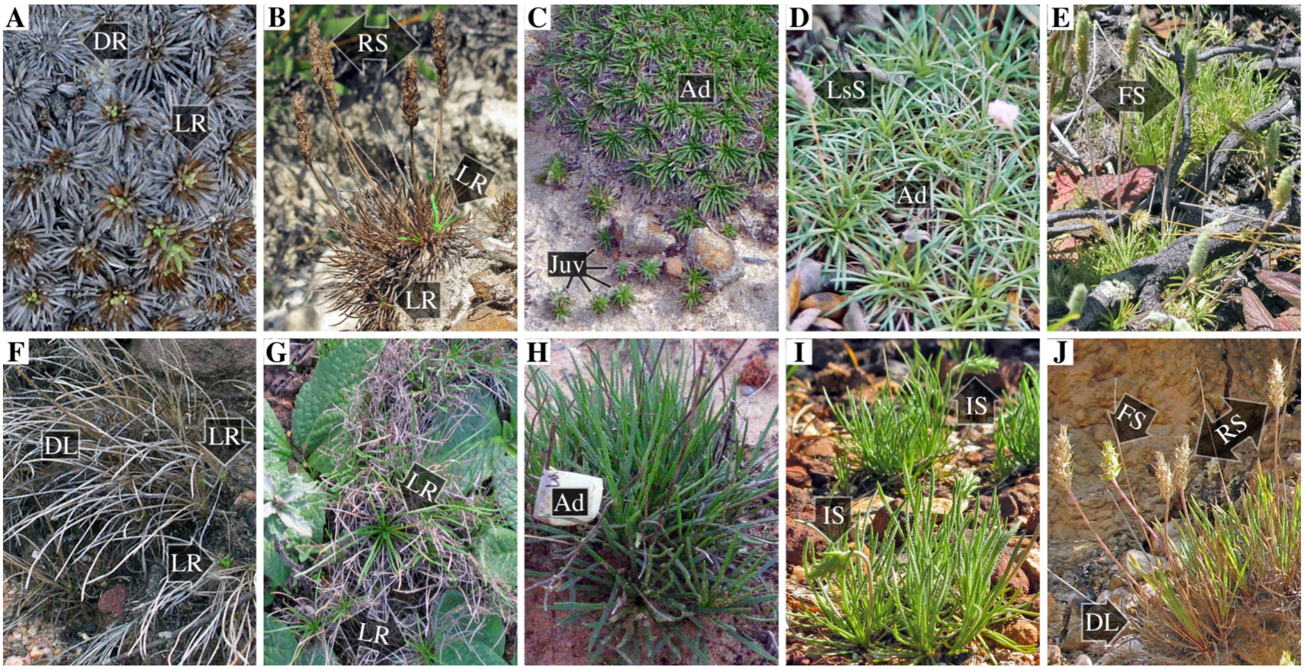


Figure 10. *Plantago almogravensis* (**A–E**) and *P. algarbiensis* (**F–J**) at different times of the year. **A–B:** September, green leaves on live rosettes (**LR**); **C:** February, adults (**Ad**) and juveniles (**Juv**); **D:** March, plants fully developed; **E:** May, flowering spikes (**FS**); **F:** October, new leaves growing over pre-summer dried leaves (**DL**); **G:** December, growing of leaves; **H:** February, plants with developed leaves; **I:** April, immature spike formation (**IS**); **J:** July, flowering and spike ripening (**RS**), and drying of leaves. **DR:** dead rosettes; **LsS:** last season spike.

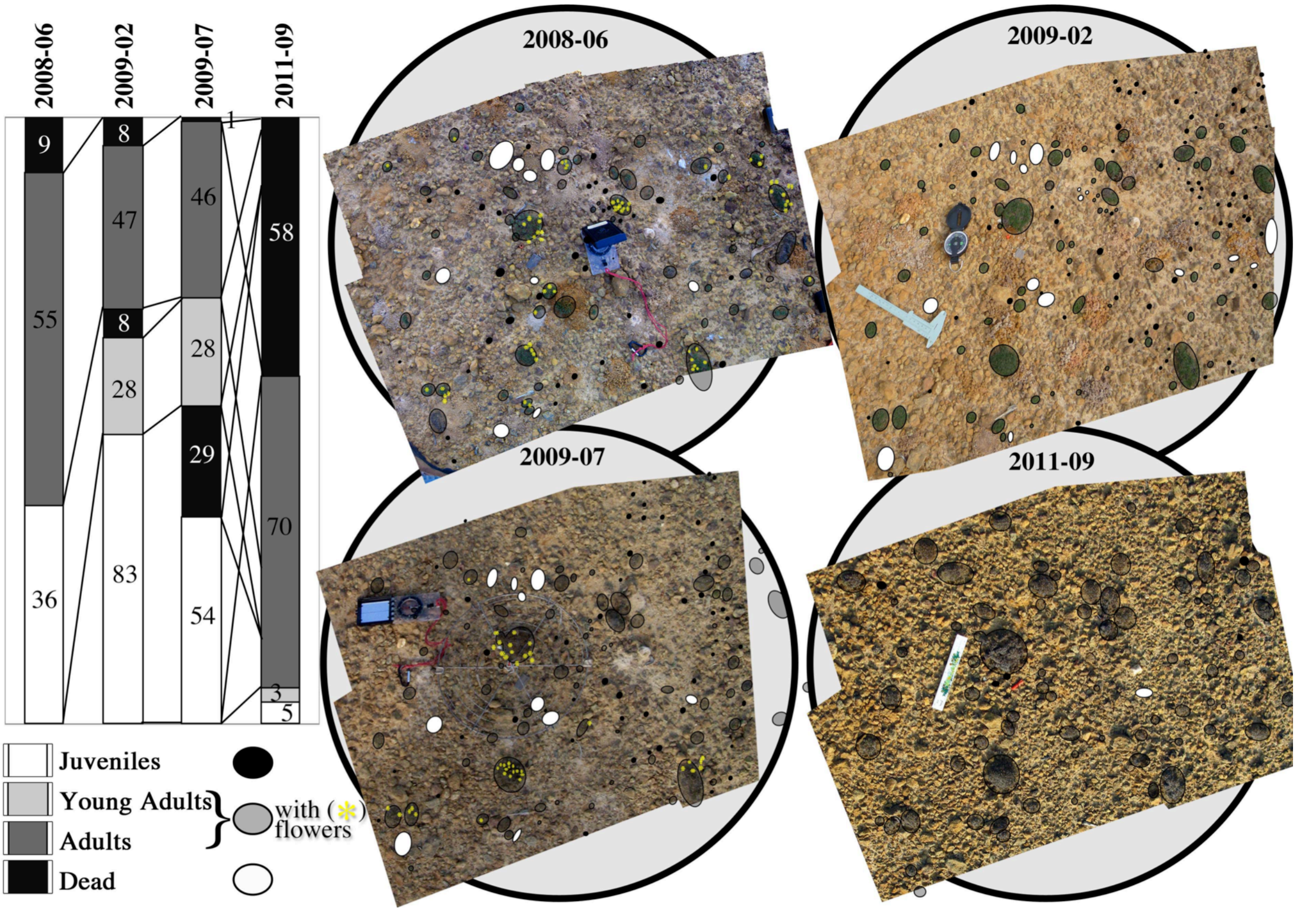


Figure 11. Population dynamics in a gap area (June 2008 to September 2011). **Juveniles:** germinated seedlings; **Young adult:** last season juvenile; **Adult:** plant over one year old; **Dead:** dry or missing plants. Circle diameter ca. 1 m. Photographs: C Branquinho; HC Serrano; MJ Pinto.

Population dynamic

The analysis of *P. almogravensis* population's dynamic in the gap cohort (Fig. 11) showed that the population balance (number of plants in 2011 per plants present in 2008) was negative (86%). Thus, in average, the cohort decreased 5% each year. There were less live plants in the area analysed. Nevertheless the results are very variable.

The balance was positive in the first year (141% between June 2008 and July 2009), but on the following years there was a large decline in the number of individuals (balance of 78% per year, between July 2009 and September 2011). The results attributed that variation to both high mortality (67% per year) and to low establishment of new individuals (32% per year, defined as number of young adults and juveniles per total plants present in 2011). We considered winter-germinated plants as seedlings (until before the Spring); afterwards, until the next winter, as juveniles, and in the next spring they fully become young adults capable of sexual reproduction thus effective recruits (Fig. 12).

Considering precipitation and mean air temperature (IMP 2008–2012), the years 2008 and 2009 were within normal values but after November 2009, the winter and spring were abnormally wet (over 200% the normal precipitation values seen on Fig. 2) while the dry months were dryer than normal (average 35% of Fig. 2 values). Considering the mortality associated with this normal (2008-2009) and abnormal (2009-2011) weather (Fig. 11), we found that is linked to the age of the plants. For the normal precipitation conditions, in average, the mortality was 35% per year, for juveniles germinated in 2008-2009; 22% per year, for juveniles detected in 2008 (maybe some mixed-up with small young adults); and just 16% per year, for the older adults detected since 2008. Those values changed to 89%, 65% and 26% per year, respectively, during the years of abnormal weather. The average mortality values between 2008 and 2011 (Fig. 11) were respectively for juveniles, young adults and adults of (62±38)%, (44±31)% and (21±6)% per year (Fig. 12). These results indicate that establishment is a critical step of *P. almogravensis* life cycle and the individuals get more resilient with aging. Not only older plants survived more they also showed a smaller increase in mortality during the abnormal weather conditions. Younger plants having less developed root systems and biomass would be more sensitive to flooding and drought stresses. Also, the dispersion pattern and germination of seeds would have been tampered, but the outcome could have been either positive or negative depending on other factors (e.g. suitability of the area of dispersion or delay of germination due to flooding).

Mortality was also linked to seasonality. Between February 2009 and July 2009 (establishment and flowering or vegetative growth) adult mortality was residual (2.1%), contrasting with 35% for the seedlings. From June 2008 to February 2009 (summer dormancy and vegetative growth seasons), 15% of the adults and 22% of the juveniles died, not recovering from the summer drought or winter floods.

From the analysis of the images in Fig. 11 we could identify flowering in an average of 21%±14% adults, which produced 4.2±4.1 spikes each, with an average 5.4 viable seeds per spike (16% to 27% seed viability, Chapter 2). If we assume that all the germinated seeds came from the analysed area, then just 1.3 seedlings germinated from each spike, which is very low. In the laboratory germination would easily reach 60% while here we detected only ca. 24% germination. Considering these values, plus a juvenile mortality of 62% and a young adults mortality of 44% (see above), for every flowering adult only 1.2 plants would be alive the year after germination (1 x 4.2 x 5.4 x 24% x 62% x 44% = 1.2). The production of viable seeds and their establishment should then be considered important limiting factors for the population increase,

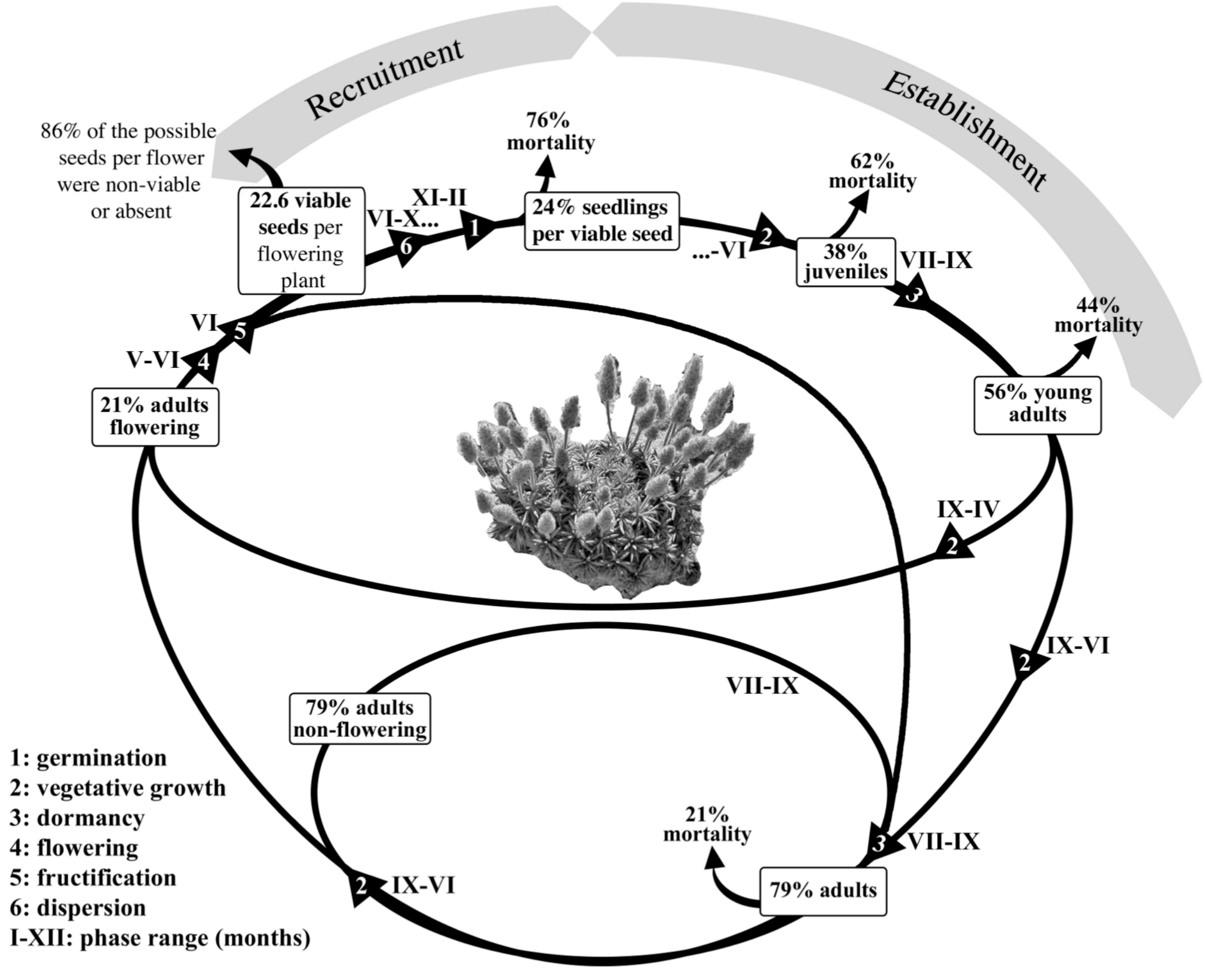


Figure 12. Basic life cycle of *P. almogravensis*. During the wet season, the individuals may grow vegetatively and/or invest in reproductive structures, after which there is period of some inactivity (dormancy) that depends on the magnitude of the summer drought stress.

but in the origin of all that, the low production of flowers should be indicative of low physiological fitness.

Though the results obtained are extremely dependent on the meteorological conditions for the given years, they seem to be representative of an endangered population's dynamic (Fig. 12), namely the average 5% per year, decrease, in an already depleted population. The most limiting factors for *P. almogravensis* seem to be related to flower and seed production (21% flowering plants, 16–27% seed viability (per flower) in the field but 70% in garden conditions, see Fig. 15 in Chapter 2), germination (24–68%) and juvenile (38%; Fig. 12) survival. Germination depends on seed viability and dispersion to a suitable location; survival of juveniles and adults varies with drought or flooding, Al-toxicity and light availability. Adults are more resilient (79% survival; Fig. 12). Flowering and number of spikes relies on water and nutrients, thus many of the adults are probably under nutritional or water stress and unable to induce flowering. In garden conditions (Chapter 2), recruits can produce flowers in the following spring, and start reproducing immediately. These results have implications for conservation and were validated by a field experiment in 2006 (ICN 2007). In an attempt to create a new *P. almogravensis* population in a suitable natural habitat, two methods were experimented: (i) direct seeding and (ii) transplant of eight juveniles germinated in garden conditions. Seven years after, in the follow-up of that experiment (Pinto et al. 2013, and personal observations), the results were (i) no new plants from direct germination, confirming the difficulty in the first stages of establishment; and (ii) the survival of three transplanted plants as well as the appearance of, at least, three new adults, thus a decrease of 4% per year (similar to the 5% in our results). This also confirms the higher resilience of older plants, even when subjected to transplants.

ACKNOWLEDGEMENTS

This work was supported by *Fundação para a Ciência e a Tecnologia* [grant SFRH/BD/38289/2007 to HC Serrano] by projects [POCTI/BIA- BDE/60664/2004 and PPCDT/BIA-BDE/60664/2004]; To *Parque Natural do Sudoeste Alentejano e Costa Vicentina* and *Instituto de Conservação da Natureza e das Florestas* (ICNF, Portugal) and *Parque Natural Donãna* (Spain) for the authorizations regarding *P. almogravensis* and *P. algarbiensis* populations. To Prof. M Carolino with the agar test mediums; C Antunes, D Brocas, L Aires and M Lo Cascio, with the experimental set up; A Clemente with environmentally controlled germination; MJ Pinto and S Chozas with the field ecology; and MA Martins-Loução and C Branquinho with the critical discussion.

REFERENCES

Allsopp N, Stock WD. 1995. Relationships between seed reserves, seedling growth and mycorrhizal responses in 14 related shrubs (Rosidae) from a low-nutrient environment. *Functional Ecology* 9: 248-54

Barceló J, Poschenrieder C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environmental and Experimental Botany* 48: 75-92

Baskin CC, Baskin JM. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Elsevier Science.

Bilz M, Kell SP, Maxted N, Lansdown RV. 2011. European Red List of Vascular Plants. pp. 130. Luxembourg: Publications Office of the European Union

Blom CWPM. 1992. Germination and Establishment. In *Plantago: A Multidisciplinary Study*, ed. PJC Kuiper, M Bos, pp. 88-98. Berlin, Germany; New York, New York, USA: Springer-Verlag

Brokaw N, Busing RT. 2000. Niche versus chance and tree diversity in forest gaps. *Trends in Ecology & Evolution* 15: 183-88

Cronan CS, Grigal DF. 1995. Use of calcium aluminum ratios as indicators of stress in forest ecosystems. *Journal of Environmental Quality* 24: 209-26

Ezaki B, Nagao E, Yamamoto Y, Nakashima S, Enomoto T. 2008. Wild plants, *Andropogon virginicus* L. and *Miscanthus sinensis* Anders, are tolerant to multiple stresses including aluminum, heavy metals and oxidative stresses. *Plant Cell Reports* 27: 951-61

Fenner M, Thompson K. 2005. *The Ecology of Seeds*. Cambridge University Press.

Franco JA. 1984. *Nova Flora de Portugal (Continente e Açores)*. Lisboa, Portugal.

ICN. 2007. Plano Nacional de Conservação da Flora em Perigo (1.a Fase). Relatório final do projecto LIFE – Natureza IIIP\8480. Relatório não publicado, Instituto da Conservação da Natureza (ICN), Lisboa, Portugal, http://www.icnf.pt/portal/naturaclas/patrinatur/conserv-flora-perigo/conse-flora-perigo, accessed 03-2015

IMP. 2008–2012. Boletim climatológico mensal. Instituto de Meteorologia de Portugal. www.meteo.pt, accessed 01-2012: Ministério da Educação e da Ciência, Instituto de Meteorologia, I.P.,Lisboa, Portugal

Kidd PS, Proctor J. 2001. Why plants grow poorly on very acid soils: are ecologists missing the obvious? *Journal of Experimental Botany* 52: 791-99

Kinraide TB, Parker DR. 1987. Cation amelioration of aluminum toxicity in wheat. *Plant Physiology* 83: 546-51

Lavorel S, Lepart J, Debussche M, Lebreton JD, Beffy JL. 1994. Small-scale disturbances and the maintenance of species-diversity in Mediterranean old fields. *Oikos* 70: 455-73

Long RL, Gorecki MJ, Renton M, Scott JK, Colville L, et al. 2015. The ecophysiology of seed persistence: a mechanistic view of the journey to germination or demise. *Biological Reviews* 90: 31-59

Manzano P, Malo JE. 2006. Extreme long-distance seed dispersal via sheep. *Frontiers in Ecology and the Environment* 4: 244-48

Martins N, Goncalves 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: 144-49

Pinto MJ, Serrano HC, Branquinho C, Martins-Loução MA. 2013. Éxito en la translocación de una planta con restricciones dispersivas. *Conservation Vegetal* 17: 8-9

Poschenrieder C, Gunse B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* 400: 356-68

Rahn K. 1996. A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society* 120: 145-98

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Stein JR, Hellebust JA, Craigie JS. 1973. *Handbook of Phycological Methods: Physiological And Biochemical Methods*. Cambridge University Press.

Vardar F, Ünal M. 2007. Aluminum toxicity and resistance in higher plants.

Watanabe T, Osaki M. 2002. Mechanisms of adaptation to high aluminum condition in native plant species growing in acid soils: A review. *Communications in Soil Science and Plant Analysis* 33: 1247-60

Zaady E, Gutterman Y, Boeken B. 1997. The germination of mucilaginous seeds of *Plantago coronopus*, *Reboudia pinnata,* and *Carrichtera annua* on cyanobacterial soil crust from the Negev Desert. *Plant and Soil* 190: 247-52

Zeppel MJB, Harrison SP, Adams HD, Kelley DI, Li G, et al. 2014. Drought and resprouting plants. *New Phytologist* (early view)

CHAPTER 8

GENERAL DISCUSSION

Plantago almogravensis' habitat.

CONTENTS

CHAPTER 8

GENERAL DISCUSSION

OBJECTIVES AND OVERVIEW

Throughout this work regarding the study of *Plantago almogravensis'* ecology, we proposed to recognize limiting factors contributing for the species endemicity and rarity. Furthermore, by acknowledging limitations, we expected to be able to contribute for the improvement of conservation measures. We focused on: (i) determining major factors behind *P. almogravensis'* ecological niche; (ii) diagnosing problematic phases in the species' life cycle through studying the dynamics of its population; (iii) comparing and integrating, whenever possible, sister species, to better understand its phylogeny and taxonomic status. We aimed to contribute to the theoretical study of rarity, the ecological role of *P. almogravensis* in its ecosystem and its evolutionary position among the other members of the genus, while suggesting also directions towards conservation measures.

PLANTAGO ALMOGRAVENSIS AND ITS NICHE

This work shows that *P. almogravensis* inhabits mostly stable vegetation gaps, which are areas devoid of the typical native vegetation which is dominated by shrubs (Chapters 2 and 6; Serrano et al. 2015[1]). Soil analysis confirmed that those gaps are not randomly distributed or occasional; the long lasting gaps correspond to geochemical islands, with a particular geological origin and composition, namely richness in Al and Fe and poorness of Ca and Mg (Branquinho et al. 2007; Serrano et al. 2011[2], 2015[1]). *Plantago almogravensis* is an obligatory metallophyte (Serrano et al. 2015[1]) that uses the hyperaccumulation strategy to cope with high Al-availability and toxicity (Serrano et al. 2011[2]). In accordance to the inclusive niches theory (Colwell and Fuentes 1975), we observed that the trade-off of having a wider tolerance to a stressful environment was to lose competitive ability towards the other vegetation (Serrano et al. 2015[1]). Although the geochemical islands provided refuge from stronger competitors, there *P. almogravensis* is not at its best physiological performance (Serrano et al. 2015[1]). Thus, the realized niche of *P. almogravensis* is limited by both abiotic and biotic factors (Fig. 1).

This work indicated that competition with shrub species for areas with optimal conditions limits the expansion of *P. almogravensis* populations outside the geochemical islands. Inside the geochemical island, the plant distribution is determined by chemo-edaphic-climatic (abiotic) restrictions (Fig. 1; Serrano et al. 2015[1]) and biotic interactions with soil microorganisms (Chapters 6 and 7).

Abiotic limitations

The geochemical island

Embedded in the podzol soil matrix, geochemical islands (gaps) are eroded areas exposing the iron hardpan (B-horizon) at the soil surface. These gaps represent the core of the realised niche of *P. almogravensis* (Fig. 1). Those areas are enriched in Al and Fe, thus considered metalliferous areas (Branquinho et al. 2007; Serrano et al. 2011[2], 2015[1]). This realized niche is limited by factors common in metalliferous

---

[1] Chapter 3

[2] Chapter 4

[3] Chapter 3

[4] **PSRN2000**: Plano Sectorial da Rede Natura 2000. RCM n°115-A/2008 de 21 de Julho.

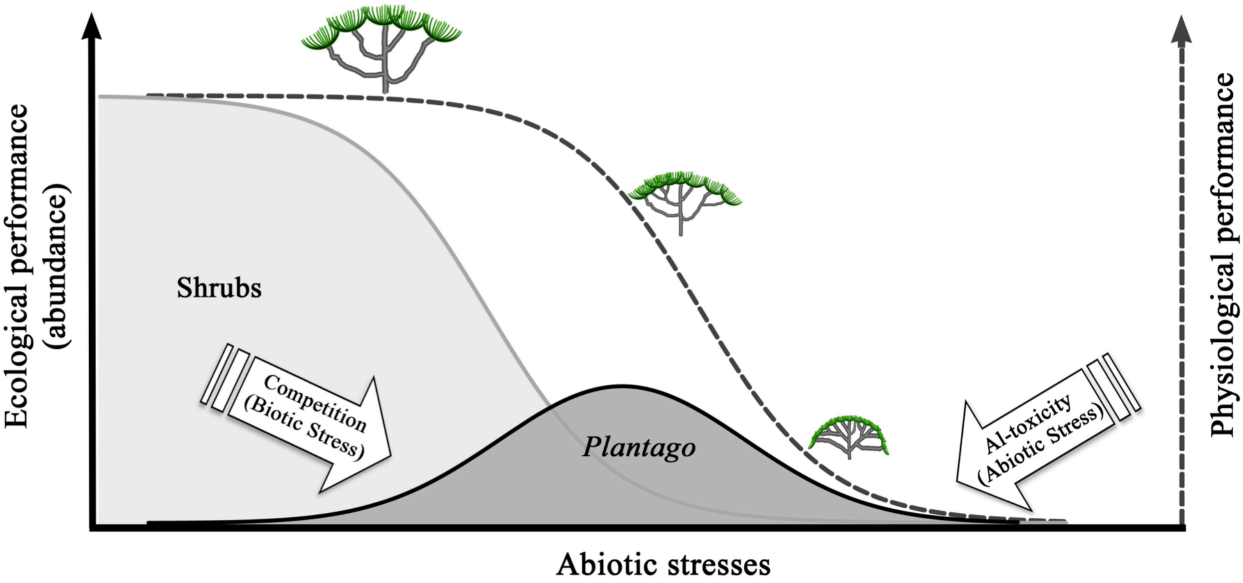


Figure 1. Hypothetical realized niche for *P. almogravensis* (*Plantago*). **Filled lines:** ecological performance; **dashed line:** physiological performance. The plant schemes represent *P. almogravensis* morphotypes, from general fitness (left) to increased ramification and nanism in the areas of more abiotic stress (right).

areas: chemical (high bioavailability of metals, namely Al), nutritional (low content of macronutrients), hydric (thin soils that don't retain much water and wind exposure, inducing drought) and photochemical (excessive light input in areas scarce in vegetation, where the sunlight reflects on the ground) (Serrano et al. 2015[3]). The growth is altered to increase ramification with formation of the cushion-like habit, nanism, shortening of the leaves and increased sclerophylly (Fig. 1; Serrano et al. 2015[3]). The combination of stress factors is responsible for the lower physiological performance in the gaps: enriched $\partial^{13}C$ values suggest closure of stomata and photosynthetic limitation (Fig. 1; Serrano et al. 2015[3]).

This work showed that one of the most important factors for *P. almogravensis* realized niche was soil Al-toxicity (ratio of Al to Ca or macronutrients). The presence of plants in the gaps is affected negatively by Al-toxicity, but *P. almogravensis* seems the most tolerant of them (Fig. 2, in Serrano et al. 2015[3]). Conversely, shrubs were the most sensitive to Al-toxicity and also showed a negative relation to the other gap limiting factors (Fig. 2–3 and Tab. 1–2 in Serrano et al. 2015[3]). Thus, these factors prevent shrubs from colonizing the stable gaps (Fig. 1), which can then become a refuge for *P. almogravensis*.

Still, not only some gap areas are too stressful, even for *P. almogravensis* to tolerate, but also not all of its area enables seed fixation and plant establishment (e.g. hard soil or rock, slope and slippery soil surface), thus limiting the colonization of the entire gap area (Chapters 2 and 6; Serrano et al. 2015[3]). Hence, the ecological island niche acts both as a refuge and as a cause for the rarity status of *P. almogravensis*. Though the plants find there a refuge from competition, those areas are somewhat isolated and restricted, limiting new colonisations or the arrival of migrants. The stressful conditions of the gap led plants to lower physiological performance, and in extreme conditions (Fig. 1) even the ecological fitness (abundance) is reduced. Further isolation could have been driven by agricultural practices, destroying nearby suitable habitats, promoting fragmentation and altering the native ecosystem (Pinto-Cruz 2010). The range of abiotic soil conditions of rare species' habi-

---

[3] Chapter 3

tats is often narrower than that of common species (Wamelink et al. 2014). Thus the conservation of those conditions and its gradient within ecosystems is particularly important for preserving rare species (Wamelink et al. 2014). Nowadays, the Natural Park status (PSRN2000[4]) is ought to prevent further destruction of the extant population habitat, though its limited size does not favour a natural expansion of the population (POPNSACV[5]).

Hyperaccumulation strategy

Plantago almogravensis is an Al-hyperaccumulator plant (Branquinho et al. 2007; Serrano et al. 2011[6]). The soil Al-gradient corresponded to a saturation curve of Al-accumulation in the plant, whose assymptote represents the physiological limit for the amount of metal accumulated by the species (Fig. 2, ca. 3 mg Al g^{-1}; see also Fig. 2 in Serrano et al. 2011[6]). As that limit is reached, the plants' physiological performance would decline, as more resources are diverted to cope with the metal (Serrano et al. 2015[7]).

Our work suggests that leaf turnover together with branch growth, would help *P. almogravensis* in diluting the Al-toxicity. New leaves could replace those saturated by Al (Serrano et al. 2015[7]). In other species, higher leaf and root turnover have been observed as attenuation strategies when saturation of Al has been reached (Brunner and Sperisen 2013; Jansen et al. 2002). In this work, we were able to detect a decline in physiological performance in the higher levels of soil Al-toxicity (Serrano et al. 2015[7]). In extreme cases of soil toxicity, it is expected that plants can no longer tolerate the metal and fade. In the case of natural populations, this is observed by the rarefication and absence of colonisation (Fig. 1). In the laboratory we observed higher plant and rosette mortality after forced exposure to high Al concentrations (Chapter 2).

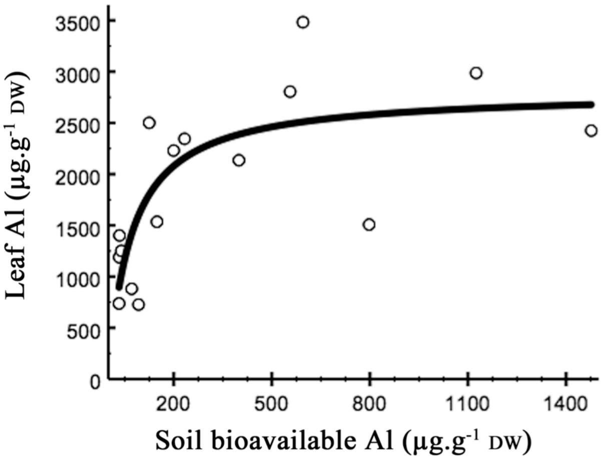


Figure 2. Accumulation of Al in *P. almogravensis* leaves, related to soil bioavailable Al. Fitted model: $y = (2804*x)/(69.7+x)$; $R^2 = 0.576$ (observed vs. fitted values); data from Serrano et al. (2011[6]).

The ability to maintain a stable population (with survival and recruitment of new individuals) in otherwise toxic environments is, therefore, characteristic of tolerant populations (Baker et al. 2010). Thus, *P. almogravensis* is able to develop two levels of tolerance: to soil Al-toxicity (presence of populations in toxic environments) and to internal physiological toxicity (the hyperaccumulation trait).

We identified Al-hyperaccumulation in various species of *Plantago* and also high- to hyper-accumulation of Fe and other metals (Chapter 5). Considering: (i) the large proportion of metal accumulators within *Plantago* phylogeny, (ii) the variety of metals accumulated and (iii) the facultative nature of *Plantago* hyperaccumulation, dependent on soil metal-availability (Chapter 5); it seems likely that the accumulation traits could have been inherited from a common ancestor (phylogenetic origin) and would rest upon general stress tolerances (Chapter 5;

---

[4] **PSRN2000**: Plano Sectorial da Rede Natura 2000. RCM n°115-A/2008 de 21 de Julho. [http://www.icnf.pt/portal/icnf/legisl/conv-dir-ac, accessed 24-07-2014].

[5] **POPNSACV**: Plano de Ordenamento do Parque Natural do Sudoeste Alentejano e Costa Vicentina. RCM n°11-B/2011 de 4 de Fevereiro. [http://www.icnf.pt/portal/icnf/legisl/legislacao/2011, accessed 02-02-2015].

[6] Chapter 4

[7] Chapter 3

Antonovics et al. 1971; Baker and Proctor 1990; Boyd and Martens 1998; Meharg 1994, 2003). The subgen. *Coronopus* species (Mediterranean) are common in Fe-based environments (e.g. pyrites, rusty red soils, Fe-mine ores, exposed podzol B-horizon, serpentine soils), where extreme drought and temperature stresses coexist with metal stresses (Chapter 5). The ability of *Plantago* to occupy these niches rich in metals, while accumulating those metals, seems to have been taken to an extreme in *P. almogravensis*. Ultimately, the trade-off between environmental resistance and tolerance to competition (Serrano et al. 2015[8]), favourable to the first, could have limited the expansion of the population and progressively reduced its distribution width, contributing to increase its rarity status.

Both Portugal and Spain are rich in metalliferous areas and old mines (e.g. along the Iberian pyritic belt). Men-aided introductions of species in similar metalliferous areas (*ex-situ* conservation), could help prevent a catastrophic extinction of the extant population of *P. almogravensis*. Eventually, the species could find in those areas of introduction, an alternative suitable habitat.

Biotic limitations

Competition with shrubs

This work shows that to thrive in the geochemical islands *P. almogravensis* had to increase ramification with formation of the cushion-like habit, shorten the leaves (nanism) and increase sclerophylly, while dealing with high Al-toxicity levels (Chapter 2; Serrano et al. 2015[8]). All these processes have a cost. For *P. almogravensis* that cost would be reflected in the loss of competitive ability towards the shrubs (Fig. 1). The Inclusive Niche Hypothesis states that, though both competitors would prefer the optimal space, only the stronger competitor thrives in it. It reduces the weaker species' fundamental niche to its realized space, where physiologically it does not perform so well, in a trade-off between competitive ability and environmental tolerance for both partners (Anacker et al. 2011; Colwell and Fuentes 1975). Competition involves the relative ability of an organism to exploit shared resources in limited supply, inter alia, water, nutrients and light (Begon et al. 1996; Serrano et al. 2015[8]). At the margin of the gaps and within the shrubland, the weaker competitor (*P. almogravensis*) would struggle for some of those resources, competing with the shrubs.

We showed that *P. almogravensis* could survive with low nutrient resources (e.g. in the gaps). Yet, it seems that microorganisms could be responsible for aiding *P. almogravensis* with nutrient acquisition, thus facilitating its survival in the nutritionally poor areas (Chapters 6 and 7). In the shrubland, not only the microbial communities are different (Chapter 6), but also the shrubs' nutrient uptake should be highly efficient to feed their larger size and faster growth rate, exerting a strong competition.

Competition for water, namely at germination and establishment, could be also important. Germination occurs in the rainy winter season (Chapter 7), when water is available very near the surface in the gaps, but at lower depths in the shrubland (see *Soil Depth* in Chapter 6 and in Serrano et al. 2015[8]). It is possible that *P. almogravensis* seedlings and juveniles cannot get enough water when in competition with shrubs (in the shrubland), when their root systems are still undeveloped.

Competition for light was not important at germination stage, as shown by identical laboratory germination results, with and without light (Fig. 2 in Chapter 7). However, light does seem to have an important role in the establishment of *P. almogravensis*, after germination, as it does for other *Plantago* species (Chapter 7; Blom 1992; Rahn 1996). In the shaded shrubland, the absence of juveniles near the sporadic

---

[8] Chapter 3

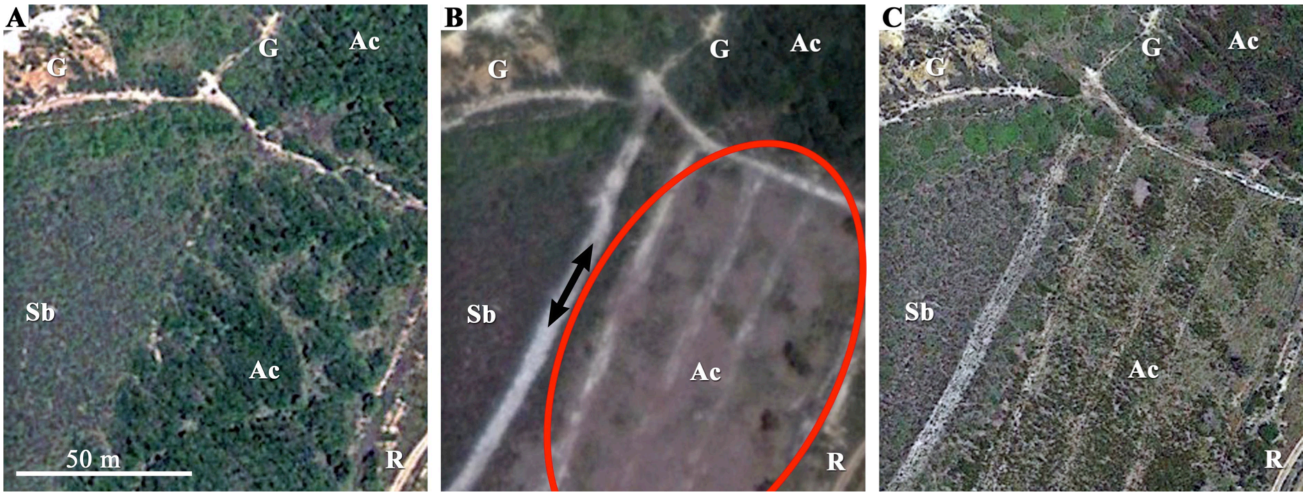


Figure 3. Aerial view of the habitat invaded by *Acacia* sp. with chronological sequence of its partial removal. [A] **May 2010:** the invasive species (**Ac**) has occupied a large (>50 m) ribbon, next to a dirt-road (**R**) parallel to the coast; the native shrubland (**Sb**) as well as large gaps (**G**), where *P. almogravensis* is ought to be found, are visible on the coast side (left); [**B**] **April 2011:** an area of c. 50x100 m of the *Acacia* sp. stand was cleared (**oval**) and *P. almogravensis* was latter observed flowering on the large cleared machine tracks on the left (**black arrows**, see text); [**C**] **May 2013:** the cleared area is still visible but either *Acacia* sp. or other plants, rather then the native shrubland, are developing. Another image (not shown) from June 2014 shows a second clearing of the *Acacia* sp. area. Images: © DigitalGlobe/Google Earth 2015.

P. almogravensis adult plants further reinforces that idea (Chapter 7). Therefore, competition for nutrients, water and light seem to limit the expansion of the realized niche of *P. almogravensis* towards the wider fundamental niche of the species (Fig. 1; Serrano et al. 2015[9]).

A new major threat for *P. almogravensis* population is the growth of invasive species in the Natural Park (e.g. *Acacia* spp. and *Carpobrotus edulis*; ICNF 2014[10]). The geochemical islands provided a refuge from competition with the native shrub species, but it is uncertain if it will be enough to detain the colonization by invasive exotic species. Though it is very difficult to clear an area from invasive species, if that is not done methodically (Fig. 3), represents a major threat for *P. almogravensis* survival, increasing the chances for its prompt disappearance.

The importance of clearing was shown in 2011, when it was observed (personal observations) an expansion of the *P. almogravensis* population in an area that *Acacia* sp. was starting to invade, next to an older *Acacia* sp. stand recently cut (Fig. 3). In the marginal area, cleared to make a path for the machines, some individuals of *P. almogravensis* had the opportunity to colonize the path area without competition. This showed the ability of the *P. almogravensis* plants to occupy areas cleared of competitors, though not of expanding to more distant areas (e.g. the old stand area). The same behaviour was observed for the populations of *P. algarbiensis*, in the roadsides of Huelva region. The vegetation is methodically cleared every year, to prevent fire along the roadsides, and *P. algarbiensis* is able to flourish in that cleared strip along the road. Similar examples are known for some serpentine endemic species (Anacker et al. 2011) capable of occupying areas cleared of competitors, but not to thrive when in their presence.

Interaction with soil microorganisms

This work showed that the abundance *P. almogravensis* is directly linked to the microbial commu-

---

[9] Chapter 3

[10] Leaflet **ICNF**: *Flora Invasora PNSACV*. [http://www.icnf.pt/portal/ap/p-nat/pnsacv/flora, edition 23-04-2014].

nity activity and to specific functional groups (Chapter 6). Not only the microbial community's abundance and diversity are reduced in gap areas, but also the proportion between functional groups is different (Chapter 6). Only one particular functional group of microorganisms (adapted to use organic acids) was found positively related to the distribution of *P. almogravensis* plants (Chapter 6). This result was compatible with descriptions of organic acid exudation by *P. almogravensis* in response to $AlCl_3$ (Martins et al. 2013), which suggests that the same type of exudates might be produced under field conditions (although not yet checked). In fact, organic acid exudates, as well as the activity of soil microorganisms, have been suggested (Serrano et al. 2011[11]) as explanations for the decrease observed in the exterior-root Al saturation (5%), when compared to Al saturation in soil (51%) or inside the root (37%). This feature could help in Al-tolerance for *P. almogravensis* by maintaining a low Al activity in the exterior of the root, more sensitive to Al-toxicity (Serrano et al. 2011[11]). If this is confirmed, Al induced reduction of root growth (Chapter 7) could be much less severe in the presence of the native microbial community than without it.

The presence of an Al-hyperaccumulator plant, mainly its abundance, affect the quality and quantity of the associated microbial community, which in turn may affect the resilience of the plant population at the geochemical island (Chapter 6). Our laboratorial tests confirmed an increased resilience for juveniles in contact with native soil microorganisms (Chapter 7), not only bacteria but also fungi, possibly mycorrhiza (Chapters 2 and 7). Knowing the mycorrhizal role on plant nutrition, such as in P and N acquisition (Cruz et al. 2004, 2007), as well as on different stress conditions of plant tolerance (Carvalho et al. 2003; Neto et al. 2006; Seguel et al. 2013; Turnau et al. 2007), it is expected that even under low N availability (Correa et al. 2008) the cost/benefit of such association should favour the plant (Correa et al. 2012). The wide range of leaf $\partial^{15}N$ observed in *P. almogravensis* (-3.17 to 1.43‰ by Serrano et al. 2015[12]) suggested that microorganisms may be involved in the process of N-acquisition facilitation, and, thus, involved on plant ecological performance and survival. Moreover, microorganisms are known for having an important role in taking up nutrients (namely phosphorus, manganese or zinc) while preventing the uptake of toxic elements by plants (van der Heijden et al. 2008). Also, the presence or absence of soil microorganisms seems more critical for *P. almogravensis* than for *P. algarbiensis* either in germination (speed) as in growth (lower need to invest in biomass) (Chapter 7). In the nutritional poor and Al-toxic gap environment, microorganisms adapted to those conditions could then have a very important role in plant survival, attenuating the negative effect of limited nutrients' availability and protecting the root from Al-toxicity.

The loss of particular microbial associations is considered an important factor for the rarity of a plant (Klironomos 2002). It seems *P. almogravensis* might be more dependant on interactions with particular soil microorganisms than *P. algarbiensis*, thus limiting its capacity to expand when those microorganism are absent and contributing to its higher endangered status. This works shows that particular groups of soil microorganisms interacting with *P. almogravensis* apparently improve its resilience and seedling's survival, during the summer months (Chapter 7). From a conservation perspective, it seems helpful to use native soil when doing *ex-situ* germinations and transplants, to provide *P. almogravensis* with native inoculum of microorganisms that could help in its establishment.

---

[11] Chapter 4

[12] Chapter 3

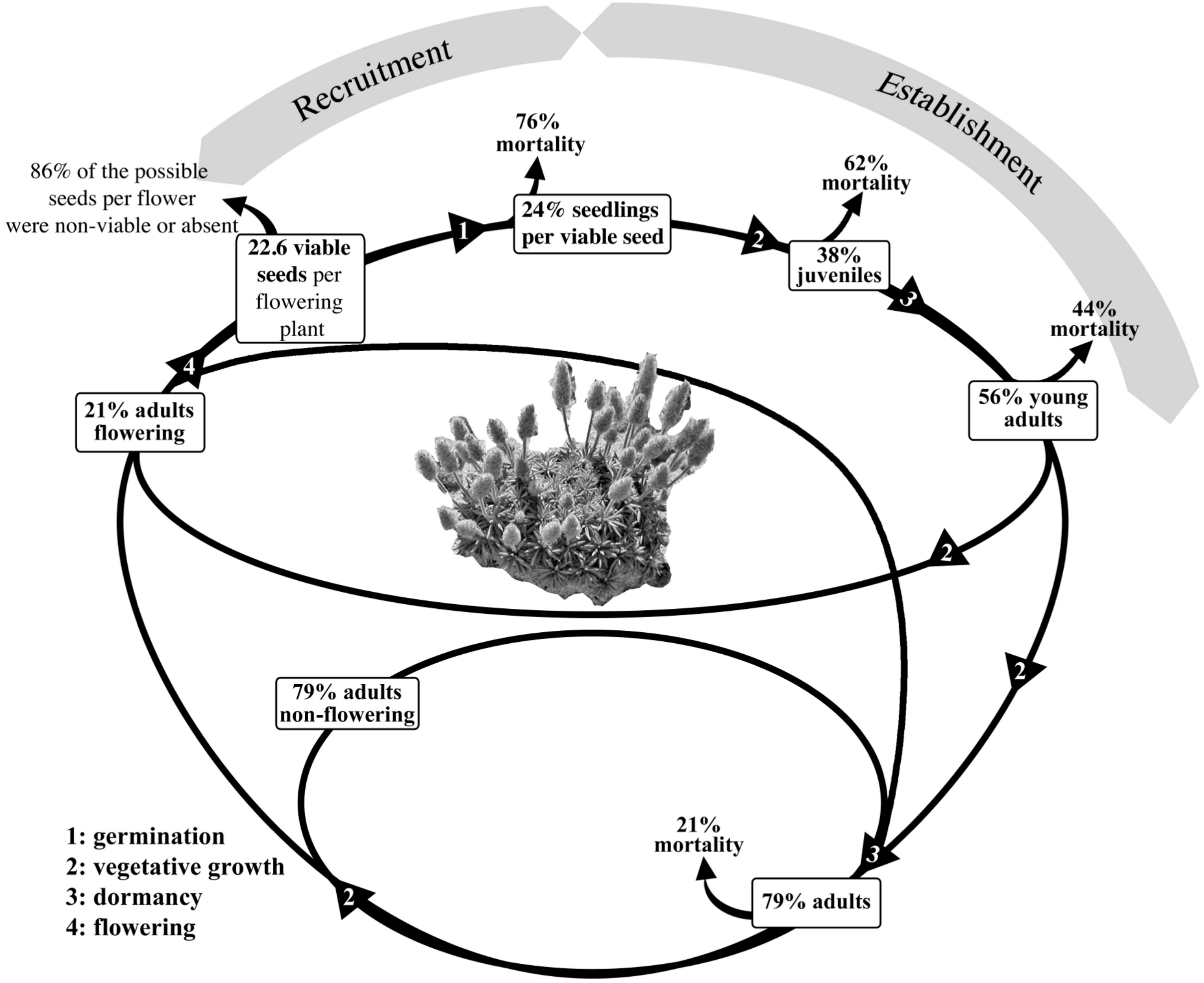


Figure 4. Life cycle for *P. almogravensis* (adapted from Chapter 7).

PHASES IN THE LIFE CYCLE

The low number of viable seeds collected from the plants (13.5% seeds per flower; Fig. 4; Chapters 2 and 7) suggests that pollination or the development of the seed, are impaired in *P. almogravensis* population, though we have not studied pollination directly. This may have genetic causes, such as an inbreeding depression caused by the small population size (Ouborg et al. 2006). Nevertheless, a low viable seed number is also recorded in the wider *P. algarbiensis* population (Chapter 2), suggesting that environmental causes might be also important rather than only genetic. This goes in accordance with the larger number of viable seeds (35%) obtained in *P. almogravensis* grown in botanic garden. The majority of *P. almogravensis* population is located in vegetation gaps (Chapter 2; Serrano et al. 2015[12]), where geochemical and environmental conditions are physiologically stressful and drive plants to nanism, including in the reproductive structures and seed size (Fig. 1; Fig. 12, 15 in Chapter 2; Serrano et al. 2015[12]). The reduction in seed size (Chapter 2) may represent fewer reserves for germination and seedling establishment. Lower peduncles and fewer flowers per spike reduce the distance to where anemophilous pollination is effective, limiting reproduction (Martin et al. 2009; Okubo and Levin 1989). In stressful situations, instead of investing in energy-expensive sexual structures, *P. almogravensis* has the possibility to invest in biomass or vegetative reproduction (Chapter 2), while only 21% of the plants were observed to produce flowers (Fig. 4

and Chapter 7). Unfortunately, vegetative reproduction limits the genetic pool, and it is also a rather slow method to expand the population. The investment needed for resprouting after the summer drought (dormancy phase in Fig. 4) is also a known cause for smaller seed production (Zeppel et al. 2014). Outside the gaps, where plants are under lower environmental stress, the dense sclerophyllous shrubland may act as a physical barrier to a broader dispersion of pollen, contributing to the low number of seeds produced. Assisted pollination could be a viable option to overcome this limitation, though the fitness of plants seems fundamental to predict the formation of viable seeds.

Seed dispersion to a suitable germination ground is very important for germination success. The same dispersion principles apply to pollen as to seeds' dispersal (Okubo and Levin 1989): physical barriers and shorter seed-bearing structures maintain the seeds in a close distance from their origin, if water, wind or animals do not disperse them. In this way, the large size of shrubs might limit seed dispersion (e.g. Fig. 5 in Chapter 2). In the gaps we found the higher abundances and densities of *P. almogravensis* associated with water runoff areas or gap margins (Fig. 6, 7 in Chapter 2; Serrano et al. 2015[13]) suggesting that wind or water-flow have a major role on dispersion, by accumulating the seeds in basin and marginal areas (Baskin and Baskin 2014). Apparently, seed dispersion is limited within a short distance (see previous example of the areas cleared of *Acacia* sp.). In the field only 24% of the viable seeds estimated was observed germinating, so 76% could have been lost (Fig. 4 and Chapter 7). Hence, the first step of plant life, seed recruitment, is very limited due to environmental and physical barriers, affecting both pollination and seed development and dispersion. So, assisted dispersion is recommended.

Germination, *per se*, does not seem a critical step for *P. almogravensis* reproductive success, as the viable seeds can germinate over 60% in a wide range of conditions: in soil, agar or cotton fibbers; from October to February; from light and temperature tightly controlled conditions to loose room temperature; and without any pre-treatment (Chapter 7). Germination was also not impaired by Al, though it may have a delaying effect on the onset of germination (Chapter 7). At germination stage, Ca was also suggested as a possible delaying factor (Chapter 7), perhaps due to its water retention properties, mimicking flooded conditions to which the seed can react by delaying germination, as seen in other species with similar mucilaginous seed coating (Baskin and Baskin 2014; Zaady et al. 1997). Light was not considered a requirement for germination (Chapter 7) and microorganisms have a limited role in germination, as discussed before (Chapter 7). The conservation programmes for *P. almogravensis* should consider these findings when preparing assisted germinations. The main problem envisaged is to preserve correctly the seeds to maintain their viability before seeding, as it is lost rapidly (less than 5 years) when in non-controlled laboratorial conditions (Chapter 7). Those observations favour the suggestion that the soil seed bank has no special role in population expansion.

The establishment of the seedlings of *P. almogravensis* is a highly risky phase, and only ca. 38% of the juveniles were observed surviving in field conditions (Chapter 7). A successful colonization implies the seedling's pre-adaptation to the edaphic stresses (O'Dell and Rajakaruna 2011), and the seedlings grown in high Al contents proved very resilient in comparison to others without that stress (Chapter 7). Yet, only ca. 3% of the gap's total area is colonized by *P. almogravensis* (Chapter 2); the remaining 97% is probably either too toxic (Al), has other environmental stresses (Serrano et al. 2015[13]), or has physical barriers to establishment (e.g. slope, rock, hard crust) (Chapter 2; Baskin and Baskin 2014; Zaady et al.

---

[13] Chapter 3

1997). Difficulties in anchorage to the substrate due to Al-induced reduction in root growth (Chapter 7) as well as water and nutrients limitation, may explain the failure to colonize large areas of the gap rich in Al. Further work is necessary to understand the role of Ca in alleviating Al-toxicity, as those seedlings grown in low Al media and more Ca (more alike the shrubland conditions), showed lower survival (Chapter 7; Serrano et al. 2011[14]). Assisted germinations in controlled environments and native soil have provided a better survival rate (over 90%) for the seedlings (Chapter 7) and thus proved to be a method to consider in future conservation programmes.

Many plants, mostly the juveniles, don't survive the summer (Chapter 7). The analysis of the ^{13}C isotopes (Serrano et al. 2015[13]) indicated that gap plants experience water stress in the summer. This would affect both the survival of the adults and the juveniles, especially if their root system is underdeveloped. Apparently, older plants may not be able of resprouting after a year of exceptional flower production (personal observations). It might have occurred that the carbon and nutrient reserves are allocated to sexual reproduction, not supporting the regrowth after the summer drought (Zeppel et al. 2014), and the plants die. However, the first year is probably the most critical as the juveniles have an undeveloped root system and no reserves to spare. At least 62% of the juveniles and 44% of the young adults have been recorded to die in the first year in field conditions (Chapter 7). Thus, only 21% of the germinated seeds would be effectively established (become young adults capable of reproduction). For the older adults the observed mortality was just 21%. Overall, juveniles and adults of *P. almogravensis* seem to be mostly limited by competition, Al-toxicity (causing water and nutrient deficiencies) and drought (leading to unrecoverable necrosis). Establishment is therefore a critical stage for *P. almogravensis* so it is proposed that assisted transplants in conservation programmes should be delayed until the young plants have gained enough biomass to withstand those stresses, that is to say, about one year.

Plantago almogravensis realised niche presents many justifications to the rarity of this species. The geochemical islands are scarce and isolated challenging expansion and migration, thus lowering the chances of genetic exchanges that would strengthen the population. The edapho-climatic stressful nature of the niche had led to nanism (among others), with impacts on fitness (lower physiological performance) and reproduction (e.g. less flowers, lower diffusion of pollen, smaller seeds, lower distance of seed dispersion). The dispersion to areas where seedlings can establish and adults can thrive seems to be a strong limiting factor for the growth of the population. To overcome this step, men-assisted nursery germinations followed by transplanting one year-old adult plants to suitable areas seem promising.

PHYLOGENY: GENETIC RELATIONS WITHIN *PLANTAGO*

In this work, the evaluation of *Plantago* molecular phylogeny was done using two genomic regions, ITS and *trnF-L*. As a result, it included *P. almogravensis* within the *P. subulata* aggregate, of sect. *Maritima* in subgen. *Coronopus* (Chapter 5). This classification is in agreement with phylogenies derived from morphological and chemical characters (*Euro+Med Plantbase* 2014[15]; Franco 1984; Rahn 1996). The results from the two genomic regions positioned *P. algarbiensis* (including data from Portuguese and Spanish populations) and *P. holosteum* (sensu Pedrol 2009) in the same monophyletic branch as *P. almogravensis*, and therefore, all were considered sister species (Fig. 5) within *P. subulata* aggregate.

---

[14] Chapter 4

[15] **Euro+Med Plantbase:** the information resource for Euro-Mediterranean plant diversity. [http://ww2.bgbm.org/EuroPlusMed/query.asp, accessed 01-08-2014].

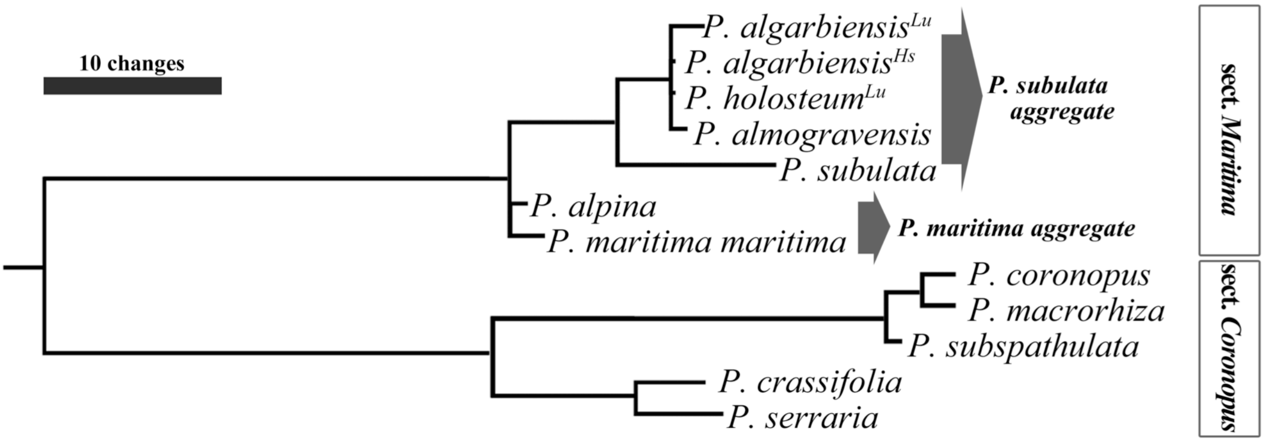


Figure 5. Phylogram of *Plantago* subgen. *Coronopus*. Detail of the ITS+*trnL-F* strict consensus tree in Chapter 5 (Fig. 2), modified to include data from *P. algarbiensis* Spanish populations. ***P. algarbiensis*^{Lu}**: Portuguese populations; ***P. algarbiensis*^{Hs}**: Spanish populations; ***P. holosteum*^{Lu}**: syn. *P. radicata*. The sequences of the sister species *P. almogravensis*, *P. algarbiensis* (Lu & Hs) and *P. holosteum* analysed, share 99.56% to 99.81% identity. Their 1864 bp aligned matrix differs in eight base positions, six uncertain due to unresolved ambiguities and three with different bases or gaps. In comparison, *P. subulata* shares 98.45% to 98.51% identity with those paraphyletic taxa in the same aggregate.

These results (Fig. 5) confirm the difficulties and ambiguities for taxonomists when classifying *Plantago* sect. *Maritima* species (e.g. see page 301 of Garrido et al. 2002, or the synonyms listed in Pedrol 2009). For instance, *Flora Iberica* (Pedrol 2009) has combined several former taxa as *P. holosteum* (sensu Pedrol includes e.g. *P. radicata*, *P. acathophyla*, *P. subulata granatensis*, *P. holosteum* and some *P. alpina*) and as *P. algarbiensis* (sensu Pedrol 2009 includes e.g. *P. almogravensis, P. algarbiensis* and *P. acanthophylla* var. *bracteosa*). In fact, the sampled *P. holosteum* population (North of Portugal, Tab. 1 in Chapter 5) was formerly considered as *P. radicata* (Franco 1984). Nevertheless, we analysed a plant collected distantly, in the Pyrenees (putatively *P. alpina*), that clustered in the same clade (results not shown), suggesting it could be considered a *P. holosteum*. Apparently this observation suggests that the cluster is not exclusive from the west of the Iberian Peninsula. This is an important question to address in future molecular works, because *ITS+trnLF* analysis did not resolve the *P. almogravensis* clade (Fig. 5), complicating even further the question of species definition. The analysis of *P. almogravensis* and *P. algarbiensis* genetic variation by ISSR and RAPD[16] markers was also not conclusive (Ferreira et al. 2013), neither the analysis with genomic regions used in barcode investigation (*matK* and *rbcL*), that between *Plantago* species have a rather high sequence identity (93% and 97%, respectivelly) (NCBI 2014[17]). Other genetic regions or methods should be used in the future to provide a finner separation and identification of the taxonomic species. Our own results regarding morphlogy (Chapter 2), have shown several differences between *P. almogravensis* and *P. algarbiensis* or *P. holosteum*, that other authors have not noticed, probably because most comparisons are made from limited herbarium material, not from observation of the taxa in their natural habitat. For instance, in Pedrol (2009) it is mentioned that mature pixydia and seeds were not observed for *P. algarbiensis* (page 12), limiting the strenght of their conclusions.

---

[16] Inter-Simple Sequence Repeats (**ISSR**) and Random Amplified Polymorphic DNA (**RAPD**) markers.

[17] National Center for Biotechnology Information (**NCBI**), USA. [http://www.ncbi.nlm.nih.gov, accessed 06-2014].

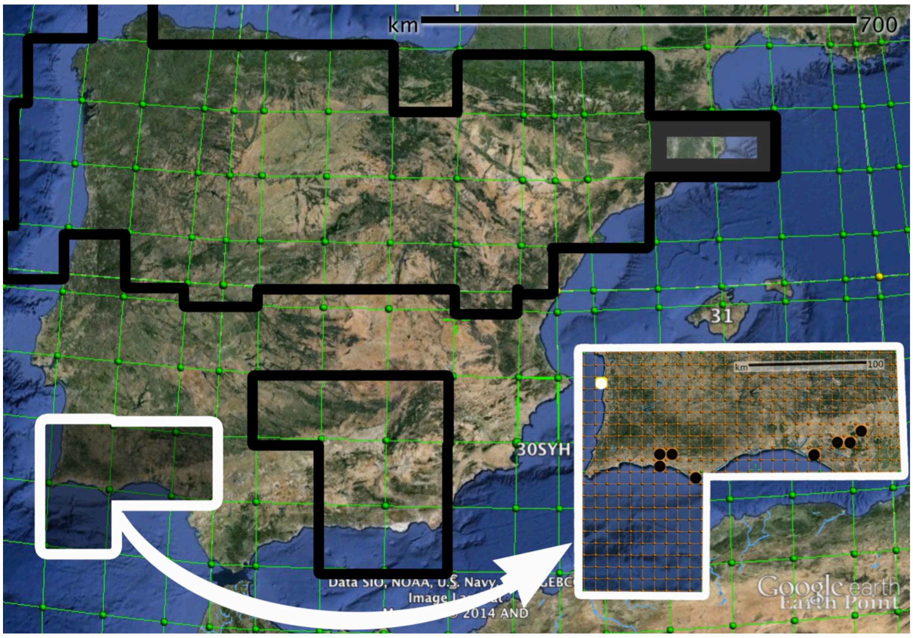


Figure 6. Relative locations of four *Plantago* species (*P. subulata* aggregate) in the Iberian Peninsula. MGRS grid (100 km): *P. almogravensis* & *P. algarbiensis* (**white**); *P. holosteum* (**black**); *P. subulata* (**grey**). Detail (10 km grid): *P. almogravensis* (**white dot**); *P. algarbiensis* (**black dots**). Images: © Google Earth 2015; gridlines: © www.earthpoint.us; data on locations: Chapter 5.

Rare endemic species can be considered paleo- or neo- endemics regarding their evolutive age and history (Anacker 2014; Kay et al. 2011; Kruckeberg 1954; Stebbins 1980). **Paleoendemics** are populations derived from former widespread species, after biotype depletion, occupying a small part of their former range. **Neoendemics** (also known as insular endemics) are younger lineages, originated after adaptive evolution. With this work we can not determine the "age" of *P. almogravensis* extant population, though there are open options interesting to explore.

Arguments favouring the neoendemic hypothesis:

i) Woodiness**:** One of the distinguishable features of *P. subulata* aggregate, from the other subgen. *Coronopus* species, its their woody character. On the one hand, woodiness is considered primitive for plants in general, and is also found in the more primitive sister genus *Aragoa* (Fig. 3 in Chapter 1; Albach et al. 2005). On the other hand, the *Plantago* ancestor is considered to be herbaceous (Rahn 1996). The ITS+*trnL-F* phylogeny indicates *P. subulata* aggreg. (woodier) to be more derived from their common ancestor than *P. maritima* aggreg. (Fig. 5). This suggests that in *P. subulata* aggreg. woodiness could be a derived character, known as secondary woodiness. Secondary woodiness, has been described in other endemic *Plantago* spp., arising associated to habitat shifts (Carlquist and Cole 1974; Dulin 2008; Dunbar-Co et al. 2008) and regarded as young species (neoendemics) (Rønsted et al. 2002 after Carlquist 1970).

ii) Peninsular distribution: The taxa in *P. subulata* aggregate are distributed throughout the Iberian Peninsula (Fig. 6). If *P. holosteum* is indeed a single species (see previous paragraphs) with a wide distribution, it is possible that *P. algarbiensis* and *P. almogravensis* became rare endemics due to fragmentation, from the main *P. holosteum* populations, at the extreme southwest-end of the Iberian Peninsula. A progressive isolation, together with particular climate and soils of the region, namely those metalliferous, would be strong drivers to ecotypic differentiation, followed eventually by especiation (O'Dell and Rajakaruna 2011). Isolation and geochemical pressures have differentiated other taxa as short as in 150 years (Macnair 1989 and thereafter). In particular for *P. almogravensis*, the conditionings of its special soil geochemistry and isolation, and morphology (Chapter 2), aggree with the neoendemic hypothesis.

Favouring the paleoendemic hypothesis:

i) The fact that *P. almogravensis* has weak competitive ability and may have been pushed to the geochemical islands. This suggests that the species could have lost adaptive ability to new competitors and environmental changes.

ii) If *P. almogravensis* woodiness is found to be an ancestral character state (Mabberley 1974), the extant population of *P. almogravensis* could then represent the biotypes that persisted from a formerly widely distributed species, and even the other sister species could have evolved from it. This would have similarities to some Macaronesian taxa derived from continental ancestors extinguished in Europe after the Pleistocene glaciation or in Africa due to desertification (Dulin and Kirchoff 2010).

In either case, the species is at risk of extinction due to its low numbers and restricted distribution. As far as we have approached, *P. almogravensis* phylogeny is still unresolved. It can likewise be considered a recent species (adapted to the geochemical islands), an old species (biotype depleted) or just an isolated population of another species. Our morphologic and ecological results indicate differences from the other sister taxa, suggesting it should be considered a species on its own right.

CONCLUSIONS & CONSERVATION REMARKS

There are many causes for the rarity of *P. almogravensis*; while some were discernable, others were not. The combination of several factors, in a spiral of causes, consequences and time, led to the current critically endangered status and the need of conservation. This study on the ecological niche of the rare endemic *P. almogravensis* has shown that altogether biotic and abiotic stress limit its ecological performance and expansion; abiotic stress mainly impacts on physiological traits while competition with shrub species limits mostly the dispersion of *P. almogravensis* populations to nearby soils with better growth conditions. If we want to preserve the extant population of *P. almogravensis* we should maintain the balance of the ecosystem to which the species is adapted. Disturbances in the water availability and nutrient input (e.g. agriculture) or excessive trampling (e.g. human activities) may affect the niche boundaries and the survival of the adult reproducing plants. The presence of invasive species, already seen in the area (e.g. *Carpobrotus* spp., *Acacia* spp.), with undetermined responses to Al-toxicity, may compromise the refuge of the plants in the gaps, that were safe from the natural shrubland colonization.

However, if we want to go further and increase the chances of survival of *P. almogravensis*, more efforts have to be made:

i) **Reduce fragmentation**: To increase the effectiveness of the pollination and reduce the isolation of the cohorts, we suggest the creation of corridors, clear of competing vegetation, uniting different cohorts that now are somewhat isolated. By connecting different cohorts, it would diminish the need for assisted dispersion or pollination, and potentiate genetic diversity (crossed pollination) and density (abundance), strengthening the fitness of the population (Smith et al. 2014). As Collwell and Fuentes (1975) stated: "*Broadening of the realized niche of a population with increased density, … can alter the trophic structure or spatial pattern of the communities, with secondary effects on the niches of other species*". Thus, increasing artificially the proportion of the rare species in the community might be the first step into further ecosystem changes, that favour its natural recover.

ii) **Identify new suitable habitats**: Sustainable conservation efforts should identify other suitable areas free of competitors, including those metalliferous, where *P. almogravensis* could establish. A location that balanced (less) competition and (lower) environmental stress-load, could improve the overall physiological performance of the population, including reproductive and survival fitness. Assisted colonization in similar edaphic areas in the southwest coast has proven difficult, but possible (Chapter 7; Pinto et al. 2013; Serrano et al. 2014). More populations imply a reduced risk of catastrophic extinction.

iii) **Assisted reproduction**: Due to the difficulties in the production of viable seeds and dispersion to suitable areas, assisted pollination, germination and transplants, are options to consider. The number of viable seeds might be improved by investment in assisted pollination to increase the effectiveness of reproduction. The germination and survival tests advise the preferential use of native soil, in assisted germinations and transplants, as it is a source of inoculum for what were considered beneficial microorganisms. After successful establishment, adults should be more resilient to summer drought and are even able of vegetative reproduction. Previous experiments (Chapter 7; Pinto et al. 2013) have confirmed that it is more successful to transplant young adults (one year-old) than to do field assisted-seeding, due to its lower survival rates.

Conservation efforts could be primarily directed to continue the assisted reintroduction and monitoring, on the PNSACV southwest coast, creating new subpopulations beyond, of course, maintaining or improving the ecological conditions of the current population. Important is to consider that any conservation measure or project may also contemplate ecological and taxonomic studies by incorporating species evolutionary uniqueness and plant physiological abilities.

REFERENCES

Albach DC, Meudt HM, Oxelman B. 2005. Piecing together the "new" Plantaginaceae. *American Journal of Botany* 92: 297-315

Anacker BL. 2014. The nature of serpentine endemism. *American Journal of Botany* 101: 219-24

Anacker BL, Whittall JB, Goldberg EE, Harrison SP. 2011. Origins and consequences of serpentine endemism in the California flora. *Evolution* 65: 365-76

Antonovics J, Bradshaw AD, Turner RG, Cragg JB. 1971. Heavy Metal Tolerance in Plants. In *Advances in Ecological Research*, pp. 1-85: Academic Press

Baker AJM, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In *Ecology of Industrial Pollution*, ed. LC Batty, KB Hallberg, pp. 7-40. Cambridge, U. K.: Cambridge University Press

Baker AJM, Proctor J. 1990. The influence of cadmium, copper, lead, and zinc on the distribution and evolution of metallophytes in the British-Isles. *Plant Systematics and Evolution* 173: 91-108

Baskin CC, Baskin JM. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Elsevier Science.

Begon M, Harper JL, Townsend CR. 1996. *Ecology: Individuals, Populations and Communities*. Blackwell Science.

Blom CWPM. 1992. Germination and Establishment. In *Plantago: A Multidisciplinary Study*, ed. PJC Kuiper, M Bos, pp. 88-98. Berlin, Germany; New York, New York, USA: Springer-Verlag

Boyd RS, Martens SN. 1998. The significance of metal hyperaccumulation for biotic interactions. *Chemoecology* 8: 1-7

Branquinho C, Serrano HC, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. *Environmental Pollution* 146: 437-43

Brunner I, Sperisen C. 2013. Aluminum exclusion and aluminum tolerance in woody plants. *Frontiers in Plant Science* 4: 172

Carlquist SJ. 1970. Wood anatomy of insular species of *Plantago* and problem of raylessness. *Bulletin of the Torrey Botanical Club* 97: 353-61

Carlquist SJ, Cole MJ. 1974. *Island Biology*. New York: Columbia University Press.

Carvalho LM, Correia PM, Caçador I, Martins-Loução MA. 2003. Effects of salinity and flooding on the infectivity of salt marsh arbuscular mycorrhizal fungi in *Aster tripolium* L. *Biology and Fertility of Soils* 38: 137-43

Colwell RK, Fuentes ER. 1975. Experimental studies of niche. *Annual Review of Ecology and Systematics* 6: 281-310

Correa A, Gurevitch J, Martins-Loução MA, Cruz C. 2012. C allocation to the fungus is not a cost to the plant in ectomycorrhizae. *Oikos* 121: 449-63

Correa A, Strasser RJ, Martins-Loução MA. 2008. Response of plants to ectomycorrhizae in N-limited conditions: which factors determine its variation? *Mycorrhiza* 18: 413-27

Cruz C, Egsgaard H, Trujillo C, Ambus P, Requena N, et al. 2007. Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhizal fungi. *Plant Physiology* 144: 782-92

Cruz C, Green JJ, Watson CA, Wilson F, Martins-Loução MA. 2004. Functional aspects of root architecture and mycorrhizal inoculation with respect to nutrient uptake capacity. *Mycorrhiza* 14: 177-84

Dulin MW. 2008. *An Investigation of Paedomorphic Secondary Xylem and Secondary Woodiness in Xanthorhiza Simplicissima, Coreopsis Gigantea, and Mahonia Bealei.* The University of North Carolina at Greensboro, ProQuest. 236 pp.

Dulin MW, Kirchoff BK. 2010. Paedomorphosis, Secondary Woodiness, and Insular Woodiness in Plants. *Botanical Review* 76: 405-90

Dunbar-Co S, Wieczorek AM, Morden CW. 2008. Molecular phylogeny and adaptive radiation of the endemic Hawaiian *Plantago* species (Plantaginaceae). *American Journal of Botany* 95: 1177-88

Ferreira V, Matos M, Correia S, Martins N, Goncalves S, et al. 2013. Genetic diversity of two endemic and endangered *Plantago* species. *Biochemical Systematics and Ecology* 51: 37-44

Franco JA. 1984. *Nova Flora de Portugal (Continente e Açores).* Lisboa, Portugal.

Garrido B, Aparicio A, Pérez Porras C, Aparicio J, García Martín F, et al. 2002. Contribuciones a la Flora de Andalucía (España). No 80: Flora de interés en bosquesisla de Andalucía Occidental. In *Acta Botánica Malacitana*, pp. 295-332

Jansen S, Broadley MR, Robbrecht E, Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. *Botanical Review* 68: 235-69

Kay KM, Ward KL, Watt LR, Schemske DW. 2011. Plant Speciation. In *Serpentine: The Evolution and Ecology of a Model System*, ed. SP Harrison, N Rajakaruna, pp. 71-95. Berkeley, CA: Univ. of California Press

Klironomos J. 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* 417: 67-70

Kruckeberg AR. 1954. The Ecology of Serpentine Soils: III. Plant species in relation to serpentine soils. *Ecology* 35: 267-74

Mabberley DJ. 1974. Pachycauly, Vessel-elements, Islands and Evolution of arborescence in herbaceous families. *New Phytologist* 73: 977-84

Macnair MR. 1989. A new species of *Mimulus* endemic to copper mines in California. *Botanical Journal of the Linnean Society* 100: 1-14

Martin MD, Chamecki M, Brush GS, Meneveau C, Parlange MB. 2009. Pollen clumping and wind dispersal in an invasive angiosperm. *American Journal of Botany* 96: 1703-11

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

Meharg AA. 1994. Integrated tolerance mechanisms - constitutive and adaptive plant-responses to elevated metal concentrations in the environment. *Plant Cell and Environment* 17: 989-93

Meharg AA. 2003. The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycological Research* 107: 1253-65

Neto D, Carvalho LM, Cruz C, Martins-Loução MA. 2006. How do mycorrhizas affect C and N relationships in flooded *Aster tripolium* plants? *Plant and Soil* 279: 51-63

Okubo A, Levin SA. 1989. A theoretical framework for data-analysis of wind dispersal of seeds and pollen. *Ecology* 70: 329-38

Ouborg N, Vergeer P, Mix C. 2006. The rough edges of the conservation genetics paradigm for plants. *Journal of Ecology* 94: 1233-48

O'Dell RE, Rajakaruna N. 2011. Intraspecific variation, adaptation, and evolution. In *Serpentine: Evolution and ecology in a model system*, ed. SP Harrison, N Rajakaruna, pp. 97-137. Berkeley, CA: Univ. of California Press

Pedrol J. 2009. *Plantago* L. In *Flora Iberica*, ed. C Benedi, E Rico, J Güemes, A Herrero, pp. 4–38. Madrid, Spain: Real Jardín Botánico CSIC

Pinto MJ, Serrano HC, Branquinho C, Martins-Loução MA. 2013. Éxito en la translocación de una planta con restricciones dispersivas. *Conservation Vegetal* 17: 8-9

Pinto-Cruz C. 2010. *Temporary ponds vegetation and dynamics: SW Portugal.* Universidade Técnica De Lisboa, Lisboa, Portugal. 118 pp.

Rahn K. 1996. A phylogenetic study of the Plantaginaceae. *Botanical Journal of the Linnean Society* 120: 145-98

Rønsted N, Chase MW, Albach DC, Bello MA. 2002. Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid *trnL-F* sequence data. *Botanical Journal of the Linnean Society* 139: 323-38

Seguel A, Cumming JR, Klugh-Stewart K, Cornejo P, Borie F. 2013. The role of arbuscular mycorrhizas in decreasing aluminium phytotoxicity in acidic soils: a review. *Mycorrhiza* 23: 167-83

Serrano HC, Antunes C, Pinto MJ, Máguas C, Martins-Loução MA, Branquinho C. 2015. The ecological performance of metallophyte plants thriving in geochemical islands is explained by the Inclusive Niche Hypothesis. *Journal of Plant Ecology* 8: 41-50

Serrano HC, Pinto MJ, Cotrim H, Branquinho C, Martins-Loução MA. 2014. A investigação ecológica como base de estratégias de conservação. In *O/El Botanico, Revista anual da/de la AIMJB*, pp. 37-39

Serrano HC, Pinto MJ, Martins-Loução MA, Branquinho C. 2011. How does an Al-hyperaccumulator plant respond to a natural field gradient of soil phytoavailable Al? *Science of the Total Environment* 409: 3749-56

Smith TB, Kinnison MT, Strauss SY, Fuller TL, Carroll SP. 2014. Prescriptive Evolution to Conserve and Manage Biodiversity. *Annual Review of Ecology, Evolution, and Systematics* 45: 1-22

Stebbins GL. 1980. Rarity of plant-species - synthetic viewpoint. *Rhodora* 82: 77-86

Turnau K, Henriques FS, Anielska T, Renker C, Buscot F. 2007. Metal uptake and detoxification mechanisms in *Erica andevalensis* growing in a pyrite mine tailing. *Environmental and Experimental Botany* 61: 117-23

van der Heijden MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310

Wamelink GWW, Goedhart PW, Frissel JY. 2014. Why Some Plant Species Are Rare. *Plos One* 9: e102674

Zaady E, Gutterman Y, Boeken B. 1997. The germination of mucilaginous seeds of *Plantago coronopus*, *Reboudia pinnata,* and *Carrichtera annua* on cyanobacterial soil crust from the Negev Desert. *Plant and Soil* 190: 247-52

Zeppel MJB, Harrison SP, Adams HD, Kelley DI, Li G, et al. 2014. Drought and resprouting plants. *New Phytologist* (doi: 10.1111/nph.13205)